# Characterisation of *Salmonella* serotype-specific interactions with bovine intestines *in vivo*.

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## ABSTRACT

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**Characterisation of *Salmonella* serotype-specific interactions with bovine intestines *in vivo*.**

Factors influencing *Salmonella* serotype-host specificity were assessed by characterising the interactions of five serotypes with the bovine host *in vivo*. *S. dublin* and *S. typhimurium* were highly virulent in calves infected intravenously whereas *S. choleraesuis* was only moderately virulent. *S. dublin* and *S. choleraesuis* were also virulent in calves infected orally. However, *S. gallinarum* and *S. abortusovis* were avirulent by either route of infection, demonstrating that genetic differences exist between serotypes, which contribute to host specificity in calves. Additionally, *S. dublin* was consistently recovered from systemic tissues 24 hours after oral inoculation of calves whereas *S. gallinarum* and *S. abortusovis* were recovered only intermittently. The ability of different serotypes to invade the intestinal mucosa and induce enteropathogenic responses was assessed. *S. typhimurium*, *S. dublin* and *S. gallinarum* invaded bovine intestines *in vivo* in greater numbers and induced greater enteropathogenic responses than either *S. choleraesuis* or *S. abortusovis*. A bovine intestinal cannulation model was developed to assess systemic translocation of *S. dublin* and *S. gallinarum*, from the intestinal lumen, and host responses to infection. Both serotypes were present within the mucosa and efferent lymph in a predominantly cell-free niche. In addition, *S. gallinarum* was primarily cell-free within the mesenteric lymph nodes (MLN), although there was some association of both serotypes with $CD14^+$ leucocytes in this tissue. Significantly, *S. dublin* was able to pass through the MLN in higher numbers than *S. gallinarum*, a factor that may influence the outcome of *Salmonella* infection in calves. However, host cell death did not appear to influence the pathogenesis of either serotype. Taken together, these results suggest that while initial interactions with the intestinal mucosa do not directly correlate with host specificity, the ability to survive within host tissues appears to be crucial for the induction of both systemic and enteric disease in orally infected calves.

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**TABLE OF CONTENTS:**

## LIST OF FIGURES:

**Chapter 1:**



**Chapter 2:**

**Chapter 3:**



**Chapter 4:**



**Chapter 5:**

**Chapter 6:**



**Chapter 7:**

## LIST OF TABLES:

## APPENDICES:

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AE | absorptive epithelium |
| ANOVA | analysis of variance |
| APC | antigen presenting cell |
| BGA | brilliant green agar |
| BSA | bovine serum albumin |
| BT | bacto-tryptose |
| CD | cluster differentiation |
| CEMEM | complete eagles minimal essential medium |
| cfu | colony forming units |
| CLSM | confocal laser scanning microscopy |
| cm | centimetre |
| DC | dendritic cell |
| diln | dilution |
| dpi | days *post* inoculation |
| DNA | deoxyribonucleic acid |
| ECACC | European collection of animal cell cultures |
| FACS | fluorescence activated cell sorter |
| FA-PBS | fluorescent antibody phosphate buffered saline |
| FAE | follicle-associated epithelium |
| FCS | foetal calf serum |
| GALT | gut-associated lymphoid tissue |
| *g* | gravity |
| g | gram |
| GFP | green fluorescent protein |
| GLM | general linear model |
| h | hours |
| HLN | hepatic lymph node |
| H and E | haematoxylin and Eosin |
| I.A.H. | Institute for Animal Health |
| IEL | intra epithelial lymphocyte |
| IL | interleukin |
| Ig | immunoglobulin |
| IFN-$\gamma$ | interferon gamma |
| iNOS | inducible nitric oxide synthase |
| kb | kilobase |
| l | litres |
| LB | luria bertani |
| LDH | lactate dehydrogenase |
| LPS | lipopolysaccharide |
| M | molar |
| mAb | monoclonal |
| MDCK | Madin Darby canine kidney |
| MDLN | mediastinal lymph node |
| MEM | minimal essential medium |
| MHC | major histocompatibility complex |
| min | minute |
| ml | milliliter |
| MLN | mesenteric lymph node |
| moi | multiplicity of infection |
| MSHA | mannose-sensitive haemagglutination |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| nm | nanometer |

| | |
|---|---|
| NO | nitric oxide |
| NBF | neutral buffered formalin |
| NK | natural killer |
| Nramp1 | natural resistance-associated macrophage protein |
| OD | optical density |
| PBS | phosphate buffered saline |
| PE | phycoerythrin |
| PFA | paraformaldehyde |
| PI | Propidium iodide |
| PMN | polymorphonuclear (leucocyte) |
| PP | Peyer's patch |
| psi | pounds *per* square inch |
| rpm | rotations *per* minute |
| RPMI | Roswell Park Memorial Institute |
| SCV | *Salmonella*-containing vacuole |
| SEM | standard error of the mean |
| SPI | *Salmonella* pathogenicity island |
| SQW | super Q water |
| TCR | T cell receptor |
| TNF-$\alpha$ | tumor necrosis factor alpha |
| TTSS | type three secretion system |
| TUNEL | terminal deoxynucleotidyl transferase d Uridine triphosphate nick end labelling |
| uv | ultraviolet |
| v | volt |
| VLA | Veterinary Laboratories Agency |
| v/v | volume/volume |
| w/v | weight/volume |

## NOMENCLATURE OF MAJOR GENETIC LOCI REFERRED TO IN THIS STUDY

| | |
|---|---|
| *hil* | hyper-invasive locus |
| *inv* | invasion |
| *pag* | PhoP activated gene |
| *prg* | PhoP repressed gene |
| *sip* | *Salmonella* invasion protein |
| *sop* | *Salmonella* outer protein |
| *spv* | *Salmonella* plasmid virulence |
| *spt* | secreted protein tyrosine phosphatase |

# CHAPTER 1
# INTRODUCTION

## 1.1 General introduction

Salmonellosis is a major cause of both enteric and systemic disease in humans and animals worldwide. The host and/or bacterial factors that determine whether serotypes remain restricted to the gastro-intestinal tract or are able to penetrate beyond the mucosa and cause severe systemic disease in a given host, typically defined as *Salmonella* serotype-host specificity, remain largely undefined.

Within the United Kingdom, *Salmonella*-induced enteritis is the second most common disease in humans associated with consumption of contaminated food and as such is responsible for more than 20,000 incidents annually (http://www.phls.co.uk/facts/Gastro/Salmonella/salmHum.Ann.htm). As many of the enteric and systemic symptoms observed during bovine salmonellosis are similar to those of non-typhoidal *Salmonella*-induced human infection, the bovine model is particularly suitable for studying the disease in humans. Salmonellosis represents a disease of significant economic importance in the U.K. cattle industry in terms of animal mortality and loss of productivity. Furthermore, sub-clinical *Salmonella* infections in cattle can lead to carcass contamination potentiating introduction of the pathogen into the human food chain. In 1999, there were 3,912 incidents of salmonellosis reported in cattle, sheep, pigs and poultry, 32% of which were associated with adult cattle or calves (Veterinary Laboratories Agency [VLA] report, 1999).

The focus of this thesis will be to contribute to a better understanding of *Salmonella* host specificity in calves by characterising the precise interactions of five *Salmonella* serotypes, of diverse host prevalence, with the bovine host and in particular the bovine intestines *in vivo*. This introduction will therefore review the current literature on *Salmonella* pathogenesis with particular reference to aspects of the disease process that potentially influence serotype-host specificity.

## 1.2 Introduction to *Salmonella*

### *1.2.1 Classification*

Members of the genus *Salmonella* are typically motile, gram-negative, rod-shaped bacteria belonging to the family *Enterobacteriaceae*. In the early stages of *Salmonella* classification, the Kauffmann-White Scheme (White, 1926; Kauffmann, 1941) designated isolates as individual species (e.g. *S. dublin*) on the basis of possession of 'O' (somatic), 'H' (flagella) and in some species 'Vi' (capsular) antigens. The use of DNA:DNA hybridisation and multi-locus enzyme electrophoresis has more recently defined only two species in the

genus *Salmonella*, which contain many serotypes (serovars), namely *S. enterica* and *S. bongori* (Crosa *et al.*, 1973; Le Minor and Popoff, 1987; Reeves *et al.*, 1989). *S. enterica* comprises seven subspecies designated I (*enterica*), II (*salamae*), IIIa (*arizonae*), IIIb (*diarizonae*), IV (*houtenae*), VI (*indica*) and VII (Le-Minor and Popoff, 1987; Boyd *et al.*, 1996). Most of the 2,463 *Salmonella* serotypes (Popoff *et al.*, 2000) belong to *S. enterica* subspecies I (Popoff and Le Minor, 1997). However, only about 50 of these are isolated in any significant numbers as human or veterinary pathogens (Miller *et al.*, 1995) and are the causation of more than 99% of all clinical disease (Popoff and Le Minor, 1997). Current nomenclature proposes that each serotype within subspecies I retains the original 'species' name as the common name (e.g. *S. enterica* subspecies *enterica* serotype Dublin) while serotypes belonging to *S. bongori* and the other subspecies of *S. enterica* would be defined by antigenic formulae. While this terminology is widely accepted, it has not been agreed by the Judicial Commission of the International Committee of Systematic Bacteriology, and a request has recently been made to retain the Latin genus followed by the specific epithets (e.g. *S. dublin*) (Ezaki *et al.*, 2000). As this matter remains unresolved, and for continuity with previous literature, *Salmonella* serotypes discussed in this thesis will be referred to by their species names (e.g. *S. dublin*).

In addition to serological classification, strains within serotypes may be identified by several techniques including phage typing (Cordano *et al.*, 1971; Anderson *et al.*, 1977), biotyping (Descamps *et al.*, 1982), plasmid profiling (Threlfall *et al.*, 1989), ribotyping (Grimont and Grimont, 1986) and IS200 typing (Stanley *et al.*, 1991). Polymerase chain reaction (PCR) based techniques such as rapid amplification of polymorphic DNA (RAPD) (Hilton *et al.*, 1996) and enzyme-linked immunosorbent assay (ELISA) (Luk *et al.*, 1997) have also recently been designed to identify *Salmonella* from clinical samples.

### *1.2.2 Definitions of host specificity*

While the majority of serotypes within *S. enterica* subspecies I result in mild enteritis in many hosts, a small number also cause severe systemic disease and/or abortion in a limited number of host species (reviewed by Uzzau *et al.*, 2000a), a feature commonly known as *Salmonella* serotype-host specificity. At present the genetic and phenotypic characteristics that mediate limitation of certain serotypes to particular hosts are not fully understood.

The terms host restricted, host adapted and host specific have been used interchangeably by different researchers (Selander *et al.*, 1990; Barrow *et al.*, 1994; Bäumler *et al.*, 1998; Uzzau *et al.*, 2000a). However, current knowledge regarding a role for acquisition or loss of genetic information by different serotypes in determining host range, remains to be fully elucidated. Therefore, for the purpose of this thesis, the following literal

terms will be used to group *Salmonella* serotypes for their prevalence within a particular host. First, host-restricted serotypes typically infect a limited number of phylogenetically related species, resulting in enteritis, septicaemia and often abortion. For example, *S. dublin* and *S. choleraesuis* are predominantly associated with disease in cattle (Gibson, 1961) and pigs (Sojka *et al.*, 1977) respectively, but may also infrequently cause disease in other mammalian hosts including humans (Buxton, 1957; Allison *et al.*, 1969; Sojka and Field, 1970; Sojka *et al.*, 1977; Fang and Fierer, 1991). Second, host-specific serotypes are almost exclusively associated with systemic disease in a single host. For example, *S. typhi* in humans and primates (Edsall *et al.*, 1960; Hornick *et al.*, 1970a, Hornick *et al.*, 1970b), *S. gallinarum* in fowl (Barrow, *et al.*, 1994; Shivaprased, 2000) and *S. abortusovis*, which is a common cause of abortion in adult sheep (Jack, 1968; Jack, 1971). Animals infected with host-restricted or host-specific serotypes, if recovered, often become symptomless carriers of *Salmonella* and as such frequently excrete bacteria into the environment potentiating an increase in the number of infected animals (Sojka *et al.*, 1974; Kovota *et al.*, 1988). Third, non-host specific or ubiquitous serotypes for example *S. typhimurium* and *S. enteritidis*, although capable of causing systemic disease in some hosts, including mice, typically cause intestinal infections in a wide range of phylogenetically unrelated host species (Lax *et al.*, 1995). However in particular individuals, for example the young, elderly or immunocompromised, the resulting infection may be more severe.

### *1.2.3 Aetiology, epidemiology and clinical signs of salmonellosis*

*Salmonella* infections vary in severity depending on the infecting serotype and the infected host. For example, in young adult humans *S. typhimurium* typically results in self-limiting enteric infection whereas in susceptible mice, the same serotype results in severe systemic disease (reviewed by Tsolis *et al.*, 1999b). The incidence of disease associated with host-specific, host-restricted or ubiquitous serotypes constantly changes over time. The reasons for this are largely unknown but may depend, in part, on the presence of antibiotic resistant strains, changes in farming practices or association with concurrent diseases. The recorded incidents of *S. dublin*, *S. choleraesuis*, *S. gallinarum*, *S. abortusovis* and *S. typhimurium* in cattle, pigs, fowl and sheep respectively between the years 1958-1999 are shown in figure 1.1. Additionally, the nature of disease caused by these serotypes in both adult and young animals is shown in table 1.1.

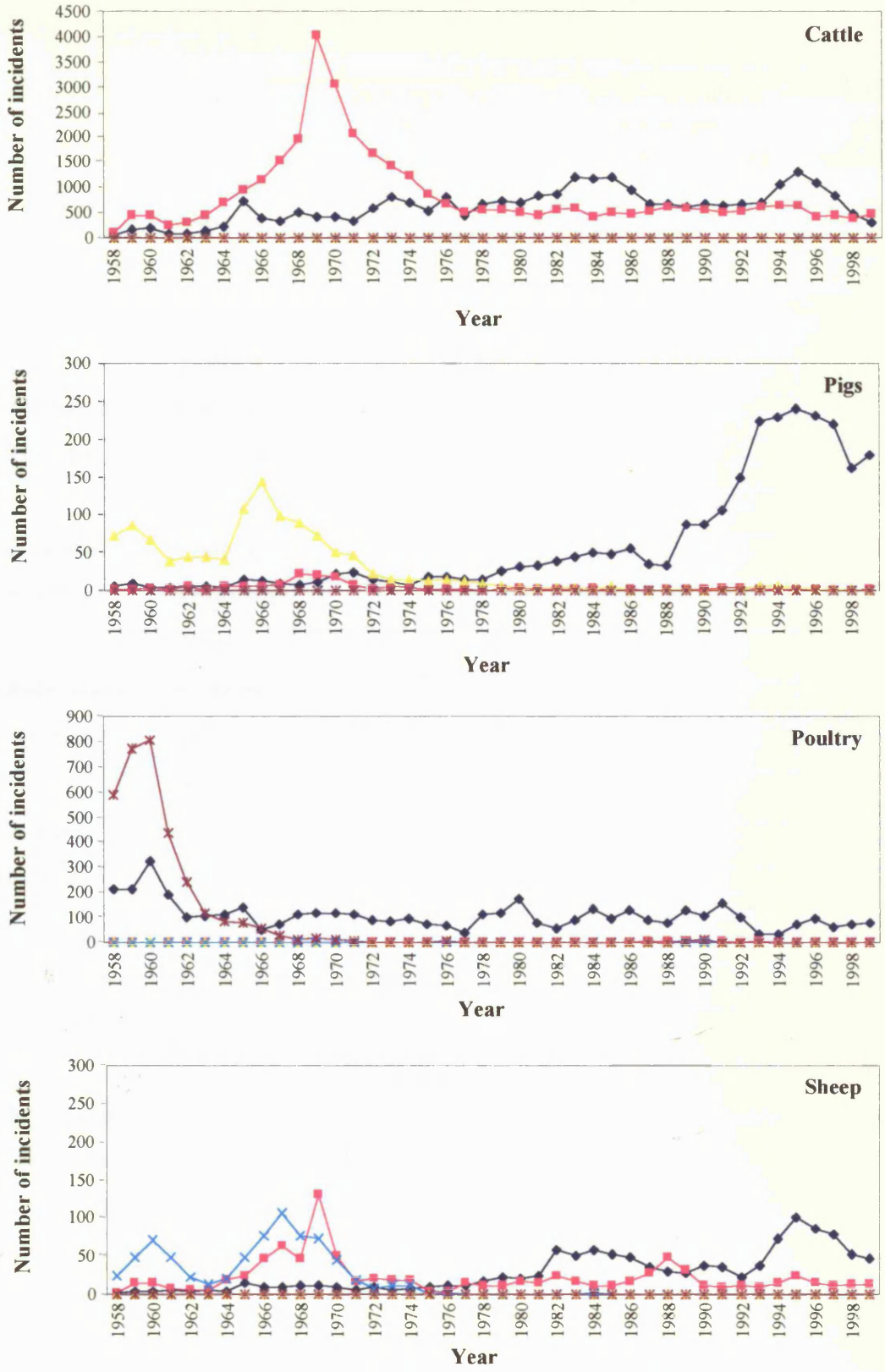



**Figure 1.1 Number of incidents of *S. dublin* (■), *S. typhimurium* (◆), *S. choleraesuis* (▲), *S. gallinarum* (✱) or *S. abortusovis* (×) in England and Wales (1958-1999) associated with cattle, pigs, poultry and sheep.**

Reference sources: Sojka and Field, 1970; Sojka *et al.*, 1977; MAFF and VLA annual reports, 1975-1999.

| | | Host-specific serotypes | | Host-restricted serotypes | | Ubiquitous serotypes | |
|---|---|---|---|---|---|---|---|
| ***Salmonella* serotype** | **Nature of disease** | **Adults** | **Young** | **Adults** | **Young** | **Adults** | **Young** |
| ***S. gallinarum* (chickens)** | **Enteric** | + | +++ | - | - | – | – |
| | **Systemic** | +++ | +++ | - | - | – | – |
| | **Abortion** | – | – | - | - | – | – |
| ***S. abortusovis* (sheep)** | **Enteric** | – | ++ | – | – | – | – |
| | **Systemic** | – | ++ | – | – | – | – |
| | **Abortion** | +++ | – | – | – | – | – |
| ***S. dublin* (cattle)** | **Enteric** | – | – | +++ | +++ | – | – |
| | **Systemic** | – | – | +++ | ++ | – | – |
| | **Abortion** | – | – | +++ | – | – | – |
| ***S. choleraesuis* (pigs)** | **Enteric** | – | – | + | ++ | – | – |
| | **Systemic** | – | – | + | +++ | – | – |
| | **Abortion** | – | – | +++ | – | – | – |
| ***S. typhi* (humans)** | **Enteric** | + | + | – | – | – | – |
| | **Systemic** | +++ | +++ | – | – | – | – |
| | **Abortion** | ? | – | – | – | – | – |
| ***S. typhimurium* (several hosts)** | **Enteric** | – | – | – | – | +++ | +++ |
| | **Systemic** | – | – | – | – | – | ++ |
| | **Abortion** | – | – | – | – | +/- | |

**Table 1.1 Nature of salmonellosis in young and adult animals resulting from infection with different host-specific, host-restricted or ubiquitous serotypes.**

- not applicable or posing no clinical threat

+ mild infection

++ moderate infection

+++ severe infection

Reference source: Uzzau *et al.*, 2000a

### *1.2.3.1 Cattle*

The main aetiological agents of salmonellosis in cattle are *S. dublin* and *S. typhimurium* which account for nearly 90% of bovine incidents in the U.K. Currently about 450 incidents of *S. dublin* and 300 incidents of *S. typhimurium* are reported annually and typically about 97% of all isolates of the host-restricted serotype *S. dublin* are associated with cattle (VLA report, 1999).

Salmonellosis in calves usually occurs between two to six weeks after birth. The peak incidence of disease and mortality following infection with *S. dublin* and *S. typhimurium* occurs at 4 and 3 weeks of age respectively (Wray *et al.*, 1991; Wray and Davies, 2000). Following infection with either serotype, fever, dullness and loss of appetite are typically observed together with the presence of foul smelling brown, pasty or liquid diarrhoea that may contain blood, mucus or sloughed intestinal mucosa. The calves become weak and dehydrated and death usually occurs in untreated animals 5-7 days after the onset of illness (Gibson, 1961).

In adult cattle acute or subacute forms of salmonellosis are recognised. Following acute disease, the symptoms are similar to those described above, in addition to a reduced milk yield. If left untreated, up to 75% of animals may die 4-7 days after the onset of clinical signs. During subacute disease, the symptoms are milder, and are characterised by diarrhoea from which the animals usually recover. With both forms of the disease abortion is common, and currently *S. dublin* infection is the second most commonly diagnosed cause of bovine abortion in the U.K. (Wray and Davies, 2000). In both adults and calves, systemic infection may lead to complications such as pneumonia, meningitis or bone lesions (Wray *et al.*, 1991). Furthermore survivors of *S. dublin* infection often remain as carriers and may excrete the organism either continuously or intermittently in their faeces for many years (Sojka *et al.*, 1974; Kovota *et al.*, 1988; Wray and Davies, 2000). A similar disease is produced following infection with other serotypes including *S. typhimurium* although in adults abortion and systemic disease are less frequent (Jones, 1992).

### *1.2.3.2 Pigs*

*S. choleraesuis var kunzendorf* and *S. typhimurium* are the two most important aetiological agents of salmonellosis in pigs worldwide (Fedorka-Cray *et al.*, 2000). Prior to the elimination of *S. choleraesuis* from the U.K. in the early 1970s, 99% of all incidents of this serotype were isolated from pigs while approximately 2% of *S. typhimurium* incidents were associated with this host (Sojka and Field, 1970; Sojka *et al.*, 1977). Currently *S. typhimurium* represents the major serotype isolated from pigs in the U.K. accounting for 68% of incidents (VLA report, 1999).

Clinical porcine salmonellosis can be separated into the enterocolitic and systemic or septicaemic forms of the disease depending on the infecting serotype (Lawson and Dow, 1966; Wilcock and Schwartz, 1992; Fedorka-Cray *et al.*, 2000). *S. choleraesuis* typically causes systemic disease that occurs mainly in intensely reared weaned pigs less than 5 months of age. However, the disease may occasionally be seen in adults as a cause of sudden death or abortion. Common symptoms include pyrexia, lethargy and a shallow cough. Diarrhoea is often not present until the third or fourth day of disease when watery, yellow faeces may be seen. Mortality is typically high and the first evidence of disease in a herd is often the finding of dead or dying pigs with purple extremities and abdomens. Complications of systemic disease include pneumonia, hepatitis or meningoencephalitis and recovered pigs often remain as carriers or faecal shedders. The enterocolitic form of the disease is most commonly seen in pigs from weaning to four months of age. Disease may be acute or chronic and is usually due to infection with *S. typhimurium* rather than *S. choleraesuis*. Typically, infected animals develop watery, yellow diarrhoea, which rarely contains blood or mucus. Affected pigs are pyrexic, dehydrated and have a reduced appetite. Mortality is low however, recovered animals may remain as carriers or shedders for several months.

#### *1.2.3.3 Fowl*

Prior to the successful elimination of *S. gallinarum* and *S. pullorum* from U.K. and European poultry flocks during the 1960s, more than 99% of incidents caused by these serotypes were associated with fowl. Furthermore, these two serotypes accounted for approximately 70% of all *Salmonella* incidents in birds (Sojka and Field, 1970). Currently more than 65 different *Salmonella* serotypes are isolated annually from U.K. poultry. *S. typhimurium* accounts for 6.4% of incidents while *S. enteritidis*, which has declined in prevalence rapidly in recent years, accounts for only 3.5% (VLA report, 1999).

Infection of birds with *S. gallinarum*, or the related biotype *S. pullorum*, results in two different disease syndromes namely fowl typhoid and pullorum disease respectively (Pomeroy and Nagaraja, 1991; Snoeyenbos, 1991). Fowl typhoid typically results in a systemic disease affecting birds of all ages, although mainly those over three months, whereas pullorum disease tends to be restricted to an enteric infection in birds under six weeks of age. Clinical signs observed include lethargy, anorexia, drooping wings, dehydration, diarrhoea, pyrexia and laboured breathing. In older birds, similar symptoms are observed in addition to decreased egg production and infertility. Complications of the disease can include blindness and joint swelling. Mortality is typically high in chicks, peaking during the second week after hatching (reviewed by Shivaprased, 2000).

Clinical signs resulting from infection with non-typhoidal serotypes, for example, *S. typhimurium* or *S. enteritidis* include anorexia, depression, diarrhoea, drowsiness and ruffled feathers (Barrow, 2000). With the exception of very young chicks, *S. enteritidis* and *S. typhimurium* rarely cause clinical disease but are able to colonise the gut (Barrow *et al.*, 1987; Barrow and Lovell, 1990).

#### *1.2.3.4 Sheep*

Prior to the successful elimination of *S. abortusovis* from U.K. sheep flocks during the early 1970s, this serotype was exclusively isolated from sheep and accounted for approximately 60% of all *Salmonella* incidents in this host (Sojka and field, 1970; Sojka *et al.*, 1977). *S. dublin* was the second most commonly isolated serotype accounting for 26-60% of incidents and reaching a peak that corresponded with an increase in bovine incidents of this serotype during the late 1960s. *S. typhimurium* and *S. dublin* infections in U.K. sheep have declined in prevalence in recent years and currently account for 25 and 7% of incidents respectively (VLA report, 1999).

*S. abortusovis* continues to represent a major problem in countries with a sheep based economy, including southern Europe and Western Asia (Wray and Linklater, 2000) where it is one of the main causes of ovine abortion during the last 4-6 weeks of pregnancy. Adult sheep infected with *S. abortusovis* are typically asymptomatic however, the disease manifests in abortion by pregnant ewes, which often display transient pyrexia, with the production of weak or stillborn lambs. Lambs that are born alive typically develop bacteraemia and die within a few days or they may be born strong and die within the first two weeks of birth showing signs of pneumonia, diarrhoea or septicaemia (Jack, 1968; Jack, 1971).

*S. dublin* and *S. typhimurium* infections of adult ewes typically result in enteric or systemic symptoms and sometimes abortion (Saunders *et al.*, 1966; Jack, 1971; McCaughley *et al.*, 1971; Wray and Linklater, 2000). Newborn lambs may also develop diarrhoea and mortality is typically high.

#### *1.2.3.5 Humans and mice*

*S. typhi*, the host-specific serotype isolated exclusively from humans and primates (Edsall *et al.*, 1960; Hornick *et al.*, 1970a, Hornick *et al.*, 1970b), is the causative agent of typhoid fever. This systemic, prolonged, febrile illness continues to be a major health problem especially in developing countries with poor sanitation and a lack of personal hygiene. The worldwide annual incidence of typhoid fever is estimated to be approximately 16 million cases resulting in more than 600,000 deaths a year (Merican, 1997).

Murine systemic salmonellosis, resulting from infection with *S. typhimurium*, is commonly used as an experimental model of human typhoid fever. However the murine model has several fundamental pitfalls which need to be considered when extrapolating data from other hosts (Hormaeche *et al.*, 1995; Tsolis *et al.*, 1999b). For example, *S. typhimurium* does not cause typhoid fever in humans and conversely, *S. typhi* will not result in typhoid-like disease in susceptible mice, suggesting that virulence mechanisms influencing pathogenesis differ between the two serotypes. The use of different inbred mouse strains has resulted in a range of experimental models of murine typhoid fever exhibiting low, medium and high natural resistance (Hormaeche, 1979). In addition, *S. typhimurium* infection of innately susceptible mice does not model all aspects of typhoid fever pathogenesis (reviewed by Tsolis *et al.*, 1999b). For example, the incubation period during clinical cases of typhoid fever is typically 7-14 days, which is in contrast to 3-7 days for murine typhoid fever. The mechanisms leading to death in the murine typhoid fever model result from tissue damage in the liver and spleen (Khan *et al.*, 1998), while in human patients most deaths result from perforation of the small intestine (Bitar and Tarpley, 1985). Furthermore, susceptible mice are not able to control bacterial growth within the liver and spleen. As a result, the mice typically die 6-10 days *post* infection, making it difficult to study virulence mechanisms that contribute to the later stages of human typhoid fever. The murine typhoid fever model is also unsuitable for the investigation of virulence factors associated with enteritis in other species due to the difficulties in quantifying diarrhoea in mice. For example, mutation of *slyA* in *S. typhimurium* results in an attenuated phenotype in mice (Libby *et al.*, 1994; Buchmeier *et al.*, 1997), yet causes only a minor reduction in bovine virulence and modest reductions in enteropathogenic responses in bovine ligated ileal loops (Watson *et al.*, 1999).

## 1.3 Functional anatomy and physiology of the mammalian gastrointestinal tract and lymphatic system

An understanding of the mammalian gastrointestinal anatomy and physiology is essential for a comprehensive interpretation of data relating to bacterial interactions with the intestinal mucosa and systemic tissues. The description that follows will concentrate on anatomical regions that have particular relevance to *Salmonella* pathogenesis, and due to the *in vivo* nature of this project, where possible special reference will be made to the bovine systems.

### *1.3.1 General anatomy of the gastrointestinal tract*

The digestive tract runs from the mouth, via the oesophagus, to the anus and in cattle comprises the four **stomachs**, the **small intestine** (duodenum, jejunum and ileum) and the **large intestine** (caecum, appendix, colon and rectum). The anatomy and function of the mammalian gastrointestinal tract has been reviewed extensively by Junqueira and Carneiro (1980), Neutra (1988), Carola *et al*, (1992), and Kato and Owen, (1994) and that of the bovine gastrointestinal tract by Getty, (1984).

In short, the structure of the entire gastrointestinal tract is made up of four concentric layers (figure 1.2a). From the lumen outwards these are:

1. The **mucosa**, which is composed of the epithelium, lamina propria and muscularis mucosae. This layer differs, according to the area of the alimentary canal, a feature that reflects the changes in functional activity (discussed in the context of the small intestine in section 1.3.2.2).

2. The **submucosa,** which is a collagenous connective tissue layer that contains large lymph and blood vessels together with nerves that connect with the mucosa and muscularis.

3. The **muscularis** consists of at least two layers of muscle, the inner circular layer and the outer longitudinal layer, which are responsible for moving the luminal contents along the digestive tract. This process is known as peristalsis.

4. The **adventitia or serosa**, which is the outermost layer of the gastrointestinal tract and is composed of several layers of collagenous and elastic loose connective tissue. This region has major circulatory, lymphatic and nervous association.

### *1.3.2 Regions of the gastrointestinal tract*

#### *1.3.2.1 The stomach(s)*

The bovine stomachs consist of the forestomach (rumen, reticulum and omasum) and the abomasum. The first stomach, or rumen, is where partially chewed food is stored and churned until rumination when small amounts of food are regurgitated and chewed prior to

passage into the other stomachs. In the abomasum, glands within the mucosa secrete acidic gastric juices to further mix and digest food before it is passed into the small intestine. In newborn calves, the rumen and reticulum remain functionless while the diet is restricted to milk. The four stomachs do not reach maximum capacity until the animal is approximately 18 months of age.

#### *1.3.2.2 The small intestine*

This area of the gastrointestinal tract, along with the proximal colon, is a major site of *Salmonella* interaction with the host following oral infection. The function of the small intestine is both secretory, in the form of enzymes to complete the digestive process, and absorptive for products and fluid produced by digestion. The absorptive function is aided by modifications to the mucosa and submucosa, which increase the surface area. These include folds or plicae, which are arranged circularly around the lumen and are prominent in the jejunum. In addition, the mammalian small intestine contains numerous finger, or leaf like, evaginations of the mucosa termed absorptive villi (figure 1.2b). When the villi are present over Peyer's patches they are termed dome villi (section 1.4.3.2). The surface area is further increased by invaginations, or intestinal glands called crypts of Lieberkuhn, that extend into the muscularis mucosae but do not penetrate it.

##### *1.3.2.2.1 The luminal surface*

The presence of a mucus coat lining the intestine, together with a population of resident gut microflora, provides a barrier to the mucosal invasion of pathogens (reviewed by Kato and Owen, 1994).

The mucus coat is composed of a suspension of glycoproteins (mucin), the composition of which varies depending on the particular species and region of the gastrointestinal tract (Rhodes, 1990). Release of mucus by goblet cells, induced by bacterial infection or other environmental stimuli, facilitates intestinal movement and may help in the elimination of pathogenic microorganisms. Mucus also contains immunoglobulin (Ig) (mainly secretory IgA), lysozyme and lactoferrin, which provide antibacterial activity. The sticky nature of mucus gives protection to the epithelial surface by trapping foreign antigens and covering binding sites with mucin and secretory IgA (Magnusson and Stjernström, 1982; Sajjan and Forstner, 1990).

The resident gut microflora is composed of at least 400 species of bacteria (Kato and Owen, 1994). Resident microflora coexist within the gastrointestinal tract and aid in the prevention of pathogen attachment by producing antimicrobial substances, for example colicins and volatile fatty acids (Byrne and Dankert, 1979), by competing for nutrients and by stimulating the growth of mucosal epithelium (Thompson and Trexler, 1971). The resident

bacterial concentration increases distally along the length of the ileum and is regulated by many factors including the flow rate of luminal contents and the amount of mucus present.

#### *1.3.2.2.2 Epithelial layer*

The mucosal epithelial layer (villi, crypts and the neck zone where crypts and villi merge) forms an interface between the external and internal environments of the gastrointestinal tract. It functions as both a site of absorption of nutrients and as a barrier preventing the entry of pathogens, by the presence of physiological and immunological defences.

The intestinal villi and crypts are covered with a continuous layer of absorptive columnar epithelial cells (enterocytes) (figure 1.2c). Their luminal surface contains numerous tightly packed microvilli (brush border) that increase the cell surface area. Additionally, microvilli are coated with glycoprotein, called the glycocalyx, which contains several enzymes and proteins that are important in the digestion and transport of nutrients. The core of each microvillus contains a bundle of longitudinal filaments that merge just beneath the microvillus border with the terminal web, which is composed of interlocking actin filaments (figure 1.2d).

Enterocytes are connected with adjacent epithelial cells at their apices by junctional complexes formed between the plasma membranes. These consist of tight and anchoring junctions, which are present just below the brush border region. Tight junctions are formed by intramembrane proteins and prevent the diffusion of molecules between the cells. Anchoring junctions, which provide mechanical stability to the monolayer, may be formed either by linkage of the actin filament network (adherent junction) or by connection of the intermediate filament network (desmosome junction).

Most crypt epithelial cells (undifferentiated epithelial cells) migrate upwards and mature to become villous enterocytes where they are eventually shed at desquamation zones on the villus tip or on the dome apex in gut associated lymphoid tissue (Schmidt *et al.*, 1985). The migration rate is rapid (typically 2-4 days) and is thought to depend on the presence of hormones, growth factors, cytokines and luminal factors such as nutritional availability, and the presence of gut microflora (Levine, 1991). Crypt cells differentiate into at least three different cell types (Cheng and Leblond, 1974) including goblet cells which secrete mucus, Paneth cells which produce tumour necrosis factor-α (TNF-α) and lysozyme, suggesting an antibacterial role, and endocrine cells which release hormones in response to changes in the external environment.

In addition specialised membranous (M) cells can be found in dome villi. These cells have several unique features including sparse microvilli, a thin glycocalyx and a poorly defined terminal web. M cells are important in the initiation of mucosal immune responses

functioning to transport antigenic substances from the gut lumen to lymphoid cells (Neutra *et al.*, 1996; Savidge, 1996; Siebers and Finlay, 1996; Jepson and Clark, 1998). While it was believed that M cells develop from crypt enterocytes (Smith and Peacock, 1980; Bye, 1984), recent evidence suggests that they represent a separate cell type induced in the dome-associated crypts by unknown lymphoid factors (Gerbert *et al.*, 1999).

In addition to their function as a selective barrier, epithelial cells have several unique phenotypic and functional characteristics. These include the ability to take up luminal antigen and present peptides to lymphocytes, the ability to express major histocompatibility complex (MHC) class I and II molecules (Kaiserlain, 1999) and the ability to secrete pro-inflammatory cytokines that recruit inflammatory cells to the site of infection (Eckmann *et al.*, 1993). These cells can also produce nitric oxide (Witthoft *et al.*, 1998) and prostaglandins (Eckmann *et al.*, 1997b) thereby participating in the innate immune response to infection.

#### *1.3.2.2.3 Lamina propria*

The lamina propria forms the core of the villi and is composed of glycogen, reticulin fibres, fibroblasts and glycosaminoglycan matrix through which blood capillaries, lymphatics (lacteals), nerves and smooth muscle fibres run. The area just below the epithelium is rich in phagocytic cells and lymphocytes. Additionally the lamina propria contains accumulations of lymphoid nodules (section 1.4.3.2), making it a major site of immunological response (Mowat and Viney, 1997).

#### *1.3.2.2.4 Basement membrane*

The epithelial cells of the small intestine are anchored to underlying connective tissue by a basement membrane which is composed of collagen, laminin, fibronectin and glycosaminoglycans (Ohtsuka *et al.*, 1992). The basement membrane also acts as a partial barrier for diffusion and filtration and functions to guide migration of enterocytes from the crypts to the villus tips.

### *1.3.2.3 The large intestine*

The primary function of the large intestine is the reabsorption of any remaining water and ions ($Na^+$, $K^+$, and $Cl^-$) from the liquid matter, and the excretion of undigested substances as faeces aided by the secretion of mucus from goblet cells. The mucosa of the large intestine does not contain villi or plicae circularis therefore the absorptive surface is much lower than in the small intestine.

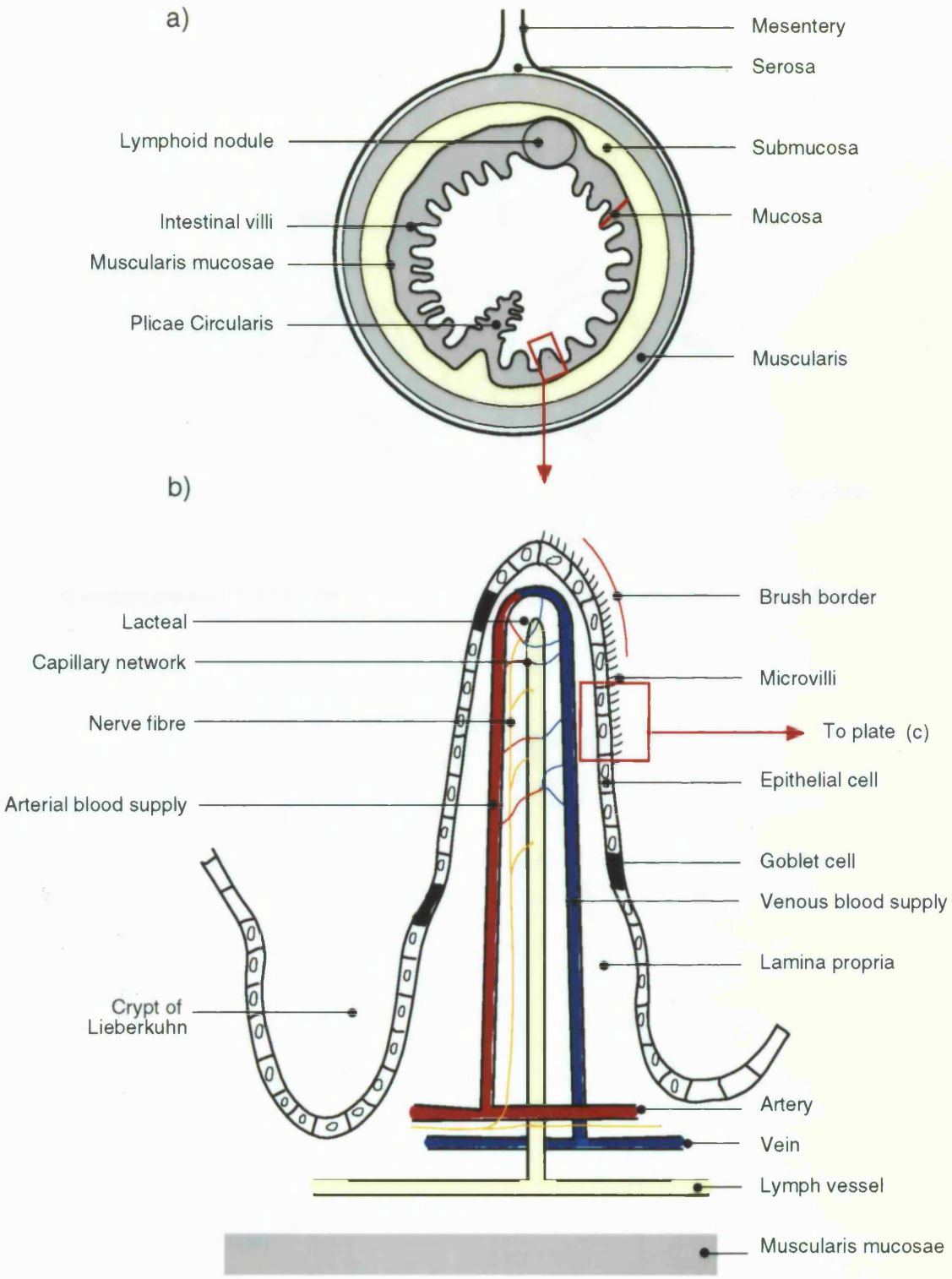



**Figure 1.2 Schematic representation of a cross section showing the structural organisation of the gastrointestinal tract (a) and the internal structure of a villus (b).**
Red boxes indicate enlarged region in next plate. (Not drawn to scale)

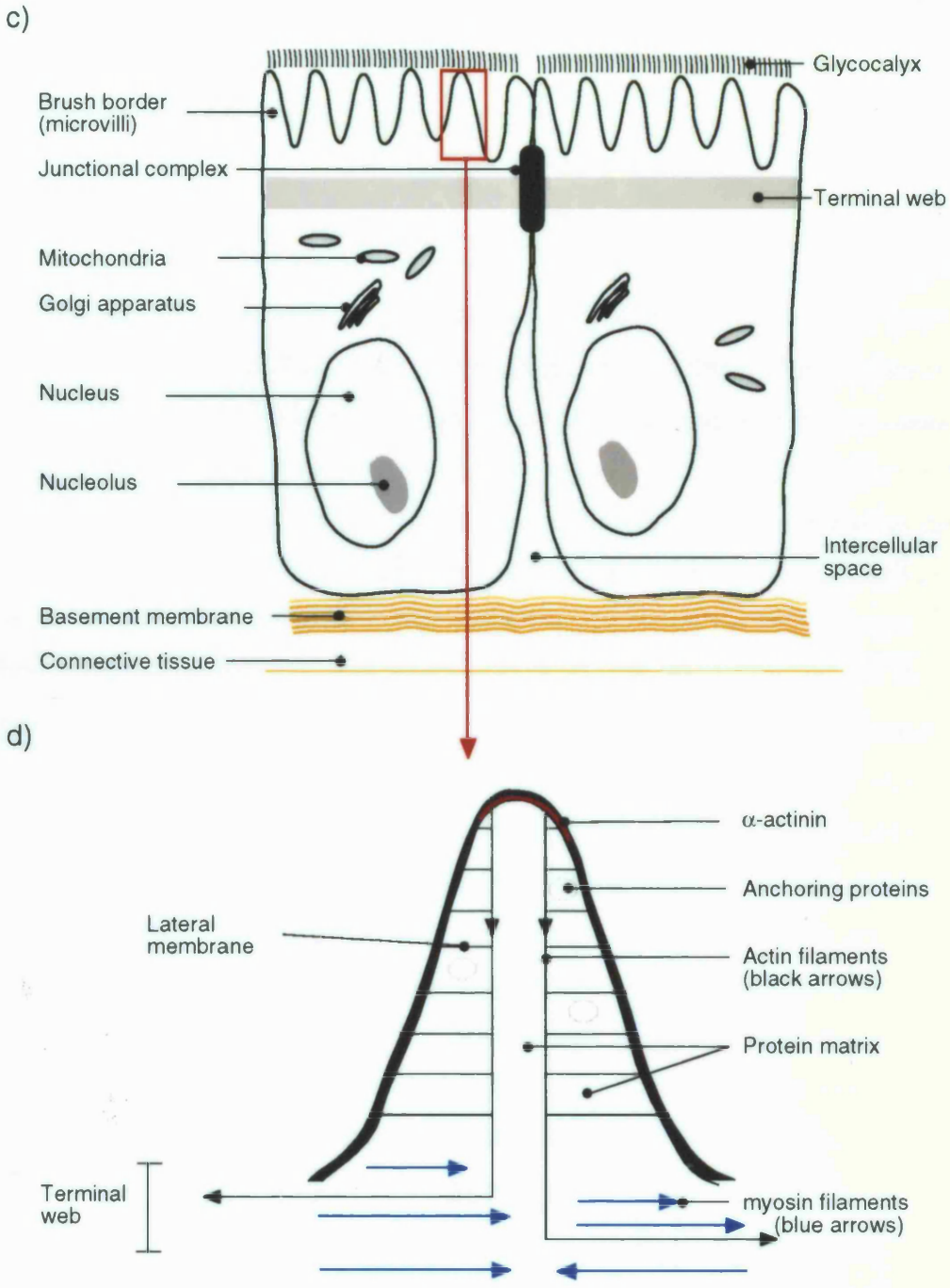



**Figure 1.2 (continued) Schematic representation of a villus enterocyte (c) and intestinal microvillus (d).**
Red box indicates enlarged region in next plate. (Not drawn to scale)

### *1.4 Functional systems of the gastrointestinal tract*

The major functional systems of the gastrointestinal tract that have direct relevance to this project are discussed in the following sections and include the electrolyte and water transport system (section 1.4.1), the intestinal immune system (section 1.4.2) and the lymphatic system (section 1.4.3).

#### *1.4.1 Physiology of electrolyte transport and water movement in the ileum*

The main function of the gastrointestinal tract is the active inward transport of ions and nutrient solutes, followed by the passive absorption of water (reviewed by Donowitz and Welsh, 1987; Stephen and Pietrowski, 1986; Mims *et al.*, 1995). Fluid transport is a bi-directional process and typically equilibrium occurs along the length of the intestines with net absorption in healthy animals and net secretion associated with diarrhoea (figure 1.3a). Both fluid absorption at villus tips and fluid secretion at villus crypts are dependent on the $Na^+/K^+$ ATPase ($Na^+$ pump), which is situated in the basolateral membrane of enterocytes. It is the location of the diffusion system that determines the path taken by ion movement. In villus cells these are found in brush borders whereas in crypt cells they are located in the basement membrane. For fluid absorption the $Na^+/K^+$ pump maintains a low intracellular $Na^+$ concentration, a high $Na^+$ concentration in the intercellular spaces, a high intracellular $K^+$ concentration and a negative cell voltage. This creates a large electrochemical gradient promoting the movement of $Na^+$ through channels on the apical membrane, followed by the inward diffusion of $Cl^-$ and the osmotic internalisation of water. The sodium pump also drives fluid secretion, which occurs due to effects of coupled inward movement of $Na^+$ and $Cl^-$ across the crypt basolateral membrane combined with the active recycling of $Na^+$ by the $Na^+/K^+$ ATPase pump. $Cl^-$ ions exit by diffusing down an electrochemical gradient and across the undifferentiated crypt apical membrane into the lumen creating an osmotic gradient into which $Na^+$ follows the $Cl^-$ and water moves passively.

In addition the rate of microcirculation within the villus contributes to the regulation of fluid transport in the gut. Villus enterocytes create gradients, with huge osmotic forces generated at the villus tips, where the circulating blood acts as a countercurrent multiplier amplifying the gradient. The movement of $Na^+$ into the venous blood supply creates a concentration gradient between the arterial and venous vessels that result in the absorption of water from the arterioles and surrounding tissue. Consequently, there is an increase in the osmolarity of incoming blood moving into the tip region, so it is the villus unit as a whole rather than the enterocytes which are responsible for fluid uptake (figure 1.3b).

Net intestinal fluid secretion caused by a dysfunction in the regulation of water and ion transport can be the result of individual or a combination of intracellular and/or

extracellular mediators. An increase in intracellular secondary messengers, including cAMP, cGMP and $Ca^{2+}$ may result from the presence of bacterial toxins or prostaglandins which have the effect of interfering with $Na^{+}$ and $Cl^{-}$ transport pathways (Donowitz and Welsh, 1987). Extracellular mediators include the release of chemicals for example, prostaglandins, neurotransmitters, cytokines, leukotreines, histamine, serotonin or bradykinin (Banwell, 1990; Ciancio and Chang, 1992). Increased fluid secretion can also result from the alteration of blood flow, an increase in mucosal permeability, activation of enteric neurones, direct stimulation of enteric secretions, increased removal and replacement of villus tip enterocytes, shortening of villi and increased crypt cell mitosis (Stephen and Pietrowski, 1986).

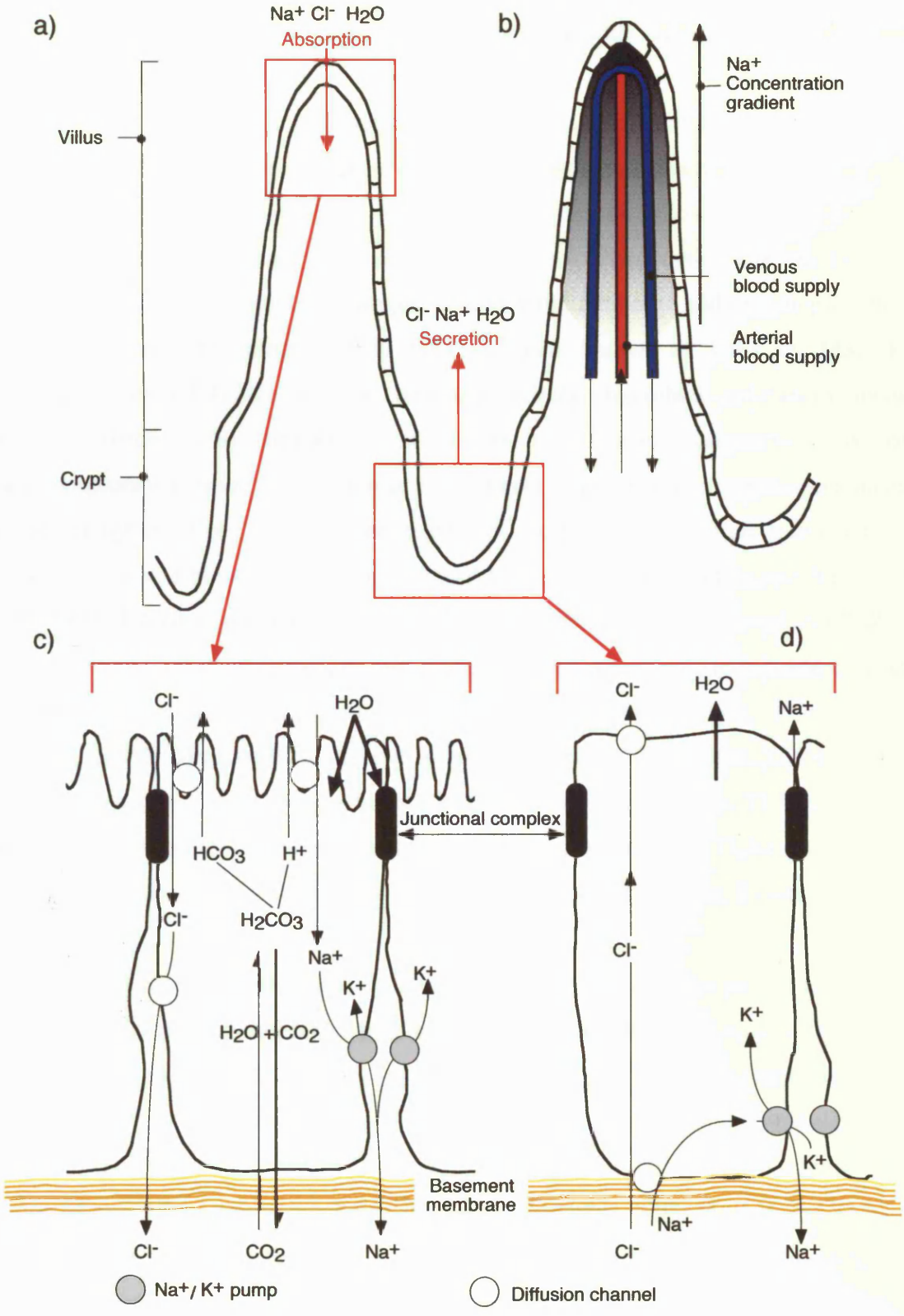



**Figure 1.3 Schematic representation of villus and crypt fluid absorption and secretion mechanisms (a) and counter current multipier with the grey shading indicating a $Na^+$ concentration gradient moving into the villus tip region (b). Electrolyte transport in intestinal mucosa representing absorption in villus enterocytes (c) and secretion in crypt cells (d).** Modified from Mims pathogenesis of infectious disease (1995). (Not drawn to scale)

### *1.4.2 Intestinal immune system*

The continuous exposure of the gastrointestinal tract to a wide array of bacteria means that the presence of phagocytic cells, antigen-presenting cells (APC) and lymphocytes are essential for immune surveillance.

The major phagocytic cell populations present in the lamina propria are resident macrophages and dendritic cells (DC). In addition polymorphonuclear leucocytes (PMNs) typically present in blood, and only found in normal tissues following infection, are also important for the active phagocytosis of foreign material. Phagocytic cells can engulf both pathogens directly and opsonised antigen coated with antibody and/or complement. For example the leucocyte integrins CD11b/CD18 (also known as CR3 or Mac-1) and CD11c/CD18 (or CR4) are able to recognise several microbial substances including lipopolysaccharide (LPS) (Janeway *et al.*, 1999). Additionally, phagocytic cells contain surface receptors that are able to recognise components common to many pathogens including the macrophage mannose receptor, the scavenger receptor, which binds many sialic acid ligands, and CD14, a molecule that binds LPS (Wright *et al.*, 1990; Kielian and Blecha, 1995; Wright, 1995; Ulevitch and Tobias, 1999). Recently, several Tol-like receptors (TLR) have been identified in blood macrophages and monocytes that act as transmembrane coreceptors to CD14 in the cellular response to LPS (Yang *et al.*, 1998; Wright, 1999; Kopp and Medzhitov, 1999). More specifically two of these receptors, TLR2 and TLR4, have been shown to be essential for the recognition of distinct bacterial components. TLR2 discriminates peptidoglycan, lipoprotein, lipoarabinomannan and zymosam, whereas TLR4 recognises LPS, lipoteichoic acid and taxol (Takeuchi *et al.*, 1999; Tapping *et al.*, 2000; Takeuchi and Akira, 2001).

Resident tissue macrophages are efficient phagocytic cells that play a crucial role in establishing inflammation and presenting antigen within the intestinal mucosa. The exposure of macrophages to bacterial antigens, such as LPS, triggers cellular responses aimed at destroying the phagocytosed pathogen. These include, among others, the up-regulation of co-stimulatory molecules, including CD80 and CD86, MHC-I and MHC-II expression, the secretion of pro-inflammatory cytokines, including interleukin-1 (IL-1), IL-6 and TNF-α, the generation of reactive compounds that can kill the phagocytosed pathogen and acidification of the phagolysosome (Pastoret *et al.*, 1998; Seljelid and Eskeland, 1993).

Intestinal DC play a vital role in the initiation and modulation of mucosal immune responses by their ability to capture and process antigen and readily migrate to lymphoid organs (Cyster, 1999; Sousa *et al.*, 1997). Furthermore, DC are potent APC, capable of priming naïve T-lymphocytes (Banchereau and Steinman, 1998). The function of DC appears to be linked to their differentiation state for example, immature DC that have not encountered

antigen are efficient phagocytic cells. While DC that have encountered antigenic stimuli during migration undergo a process of maturation in which they develop into competent APC. This process is facilitated by enhancing cytokine secretion (Winzler *et al.*, 1997), increasing costimulatory molecules and MHC expression (Rescigno *et al.*, 1998) and down regulating their ability to capture and present antigen (Sallusto and Lanzavecchia, 1994; Sallusto *et al.*, 1995).

PMNs (heterophils in avian species) are short-lived cells that are recruited to enter sites of infection, following antigenic stimulation, where they play a crucial role in the early stages of the acute intestinal inflammatory response by ingesting and destroying invading microorganisms (Lehrer and Ganz, 1990). PMNs produce bacteriostatic and toxic products to rapidly kill microorganisms. They are also able to produce a range of cytokines including IL-1, IL-6 and TNF-$\alpha$ that contribute to host defence (Janeway *et al.*, 1999). To reach an area of infection or tissue damage PMNs leave the circulation (termed extravasation or diapedesis) by adhering to endothelial cells. This is facilitated by expression of adhesion molecules, called selectins, in response to local secretion of cytokines. Interactions between leucocyte integrins (CD11a/CD18 and CD11b/CD18) and adhesion molecules like ICAM-1, on endothelial cells, permit firm attachment to the endothelium prior to leucocyte extravasation (Janeway *et al.*, 1999).

The lamina propria also contains large numbers of T ($CD4^+$ and $CD8^+$) and B-lymphocytes, found in clearly defined areas (section 1.4.3.2 and figure 1.4a). These cells become activated and proliferate in response to antigen displayed by APC. Lymphocytes migrate through the lymphoid tissue before returning to the bloodstream via the lymph. T and B-lymphocytes that have been activated in the Peyer's patches and mesenteric lymph nodes will preferentially recirculate back to mucosal tissue (Phillips-Quagliata and Lamm, 1988; Janeway *et al.*, 1999). This is due to their ability to decrease the expression of L-selectin, which facilitates interaction with high endothelial venules in peripheral lymph nodes, and to up regulate the $\alpha_4\beta_7$ integrin. The ligand of this integrin, MAdCAM-1, is expressed by blood vessels in mucosal tissues (Berlin *et al.*, 1993) and is upregulated during intestinal inflammation (Viney *et al.*, 1996). T and B-lymphocytes that home back to the intestines participate in immunity against specific antigens or microorganisms mainly by the $CD4^+$ regulation and differentiation of IgA- producing B-cells (Mowat and Viney, 1997).

### *1.4.3 The lymphatic system*

The lymphatic system is a network of diffuse lymphatic tissue, solitary nodules, lymph vessels and nodes (reviewed by Brandtzaeg, 1989; Carola *et al.*, 1992).

### *1.4.3.1 Diffuse lymphoid tissue*

The mammalian gastrointestinal tract contains diffuse lymphatic tissue in the form of large numbers of scattered lymphocytes both in the epithelium and lamina propria. These intra-epithelial lymphocytes (IEL) provide a means for generating disseminated immune responses throughout the length of the intestine (Brandtzaeg *et al.*, 1989). Virtually all IEL are T-lymphocytes, B-lymphocytes or plasma cells that secrete IgA. In the intestinal epithelium, the T-lymphocytes are predominantly of the $CD8^+$ (cytotoxic/suppresser) phenotype, while those in the lamina propria are mainly the $CD4^+$ (helper/inducer) subset. T-lymphocytes can be either γδ or αβ as determined by their T cell receptor (TCR) (Guy-Grand and Vassalli, 1993), although the percentage of each type can vary depending on the species. For example, in cattle γδ $TCR^+$ T-lymphocytes can account for up to 50% of IEL (Mackay and Hein, 1989), while in humans, the proportion of these cells is typically low (< 10%) (Viney *et al.*, 1990). The precise function of IEL are not well defined. It has been speculated that these cells may represent a primitive defence mechanism recognising non-polymorphic antigens derived from pathogens or the products of damaged enterocytes Alternatively, IEL may be fully differentiated effector cells required to kill infected epithelial cells *in vivo*. IEL may stimulate epithelial renewal or, due to their location, they may play a role in maintaining tolerance to intestinal antigens (reviewed by James and Klapproth, 1996; Mowat and Viney, 1997).

### *1.4.3.2 Solitary lymphoid nodules*

In cattle, the gut associated lymphoid tissue (GALT) is organised in aggregations of unencapsulated lymphoid follicles in the intestinal mucosa and submucosa which are referred to as Peyer's patches (PP), and in isolated nodules within the lamina propria (Parsons *et al.*, 1991). The newborn calf has discrete PP in the duodenum and jejunum and a single continuous PP in the terminal ileum, which extends in to the caecum. In the large intestine discrete PP are found in the caecum, proximal colon and terminal rectum (Liebler *et al.*, 1988a Liebler *et al.*, 1988b; Parsons *et al.*, 1989). By approximately 18 months of age, the continuous PP becomes involuted and the discrete PP begins to disappear (Parsons *et al.*, 1989). Identical changes occur in sheep where the continuous PP are believed to be a site of primary generation of B cells analogous to the bursa of Fabricius in birds (Reynolds and Morris, 1983). In contrast, the discrete PP and lamina propria nodules appear to function as secondary lymphoid organs and develop germinal centres upon antigen stimulation.

Peyer's patches are composed of follicles which are B-lymphocyte dependent, and the parafollicular areas which are T-lymphocyte dependent (figure 1.4a). In the small intestine each follicle extends into a domed villus that is covered by the follicle-associated epithelium (FAE) (Siebers and Finlay, 1996) and is present between normal absorptive villi. The FAE of

the bovine continuous PP consists of a homogenous population of specialised antigen sampling M cells (Landsverk, 1987) (section 1.3.2.2.2), while in the discrete PP, these cells are scattered amongst absorptive enterocytes (Liebler *et al.*, 1988b). The Peyer's patch mucosa is considered to be a major site for antigen presentation to T-lymphocytes assisted by the M cell transport of luminal antigens through invaginations in the basolateral cell membrane (reviewed by Savidge, 1996) (figure 1.4b).

#### *1.4.3.3 Peripheral lymph nodes*

Lymph nodes are highly active organised structures, found along the course of lymphatic vessels, that act as filters for foreign antigen entering the node (Morrison *et al.*, 1986; Pastoret *et al.*, 1998) (figure 1.5). Afferent lymphatics enter the node through the capsule and empty lymph into the subcapsular sinus. Lymph then passes in sinuses through the cortex and paracortex, which are segmented due to the presence of connective tissue traberculae, into the medulla of the node. Antigen removal, by phagocytic and reticular cells, is facilitated by a slowing of lymph flow and creation of turbulence as lymph moves through the tissue. The medullary sinuses join at the hilus where lymph is able to leave the node via the efferent lymphatic vessels.

The lymph node cortex incorporates the B-lymphocyte dependent follicular areas or germinal centres, which contain surface immunoglobulin (sIg)-positive lymphocytes expressing MHC II. The paracortical areas are T-lymphocyte dependent and contain sIg and MHC II negative lymphocytes. This arrangement of lymph nodes means that different lymphocyte populations entering the node have distinct preferences for where they localise and as such facilitates humoral or cell-mediated immune responses involving different compartments. In addition, each of these areas has its own specialised accessory cell namely follicular dendritic cells, important in antigen trapping and stimulation of germinal centre formation, and interdigitating cells, important in the stimulation of T-lymphocyte mediated immune responses, respectively (Morrison *et al.*, 1986) (table 1.2a).

#### *1.4.3.4 Lymph and lymphatic vessels*

The lymphatic capillaries, which originate in tissue spaces as blind end tubes, are the most peripheral vessels of the lymphatic system. Specialised lymphatic capillaries called lacteals extend into the intestinal villi where they absorb fat from the small intestine and transport it into the blood for distribution throughout the body. Lymphatic capillaries join to form afferent lymphatics, which transport lymph directly into nodes. Lymph exits the nodes via larger efferent lymphatics that eventually join together to form two ducts, the right lymphatic and the thoracic. The right lymphatic duct drains the upper right quadrant of the

body and empties into the right subclavian vein while the thoracic duct drains the remainder of the body, including the abdominal area, and empties its contents into the left subclavian vein. The lymph that flows through these vessels originates as interstitial fluid drained from body tissues and contains predominantly lymphocytes. In addition, phagoctyic cells within lymph help trap and transport antigenic material to local lymph nodes where an immune response is mounted and the foreign material removed (reviewed by Carola *et al.*, 1992). The phenotypic distribution of bovine circulating leucoctye populations in afferent and efferent lymphatic vessels is given in table 1.2b.

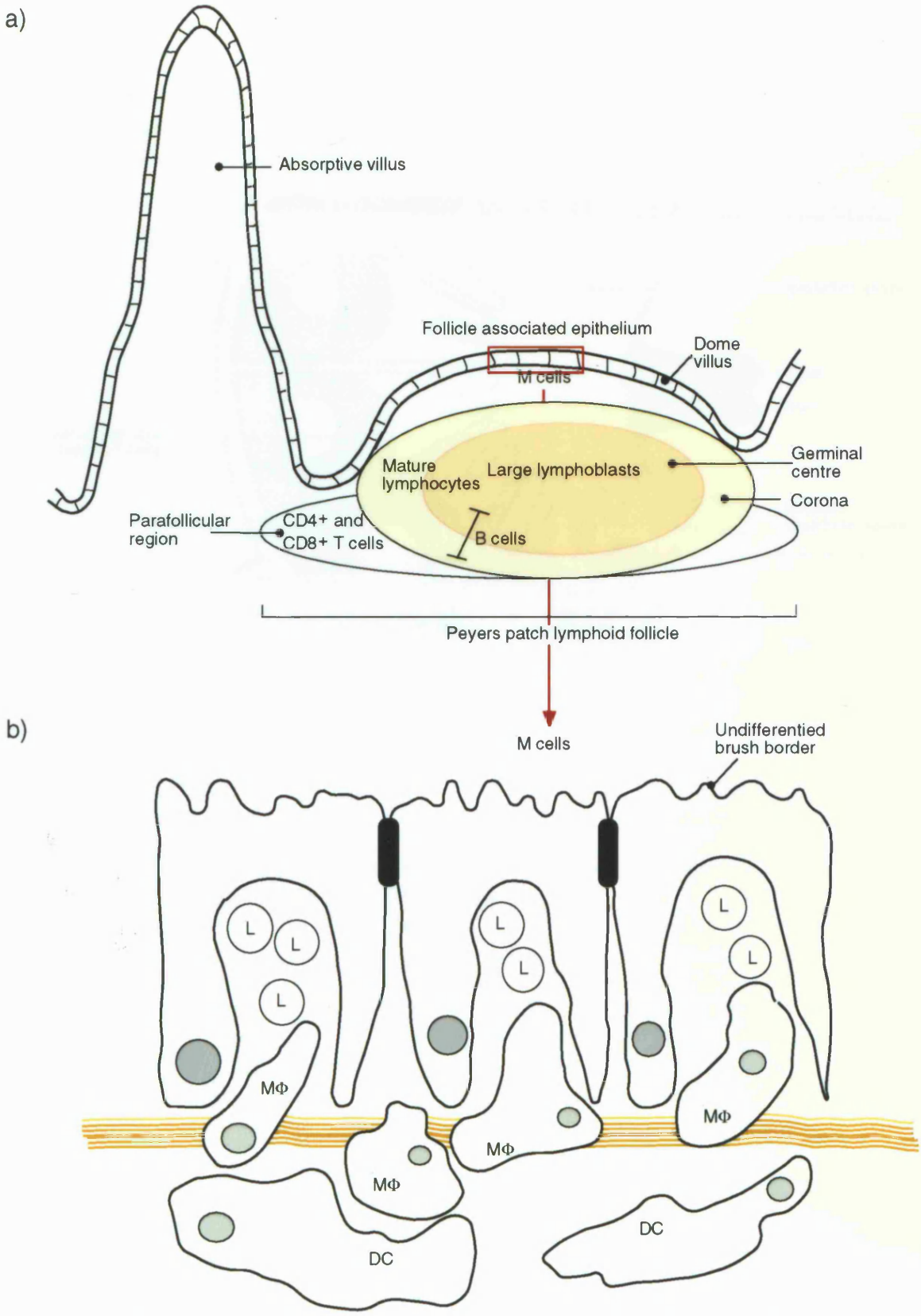



**Figure 1.4 Schematic representation of absorptive and dome villi (a) and homogenous M cell population typical of the bovine distal ileal follicle associated epithelium (b).** DC - dendritic cell; Mϕ - macrophage; L - Lymphocyte. Red box indicates enlarged region in next plate (not drawn to scale).

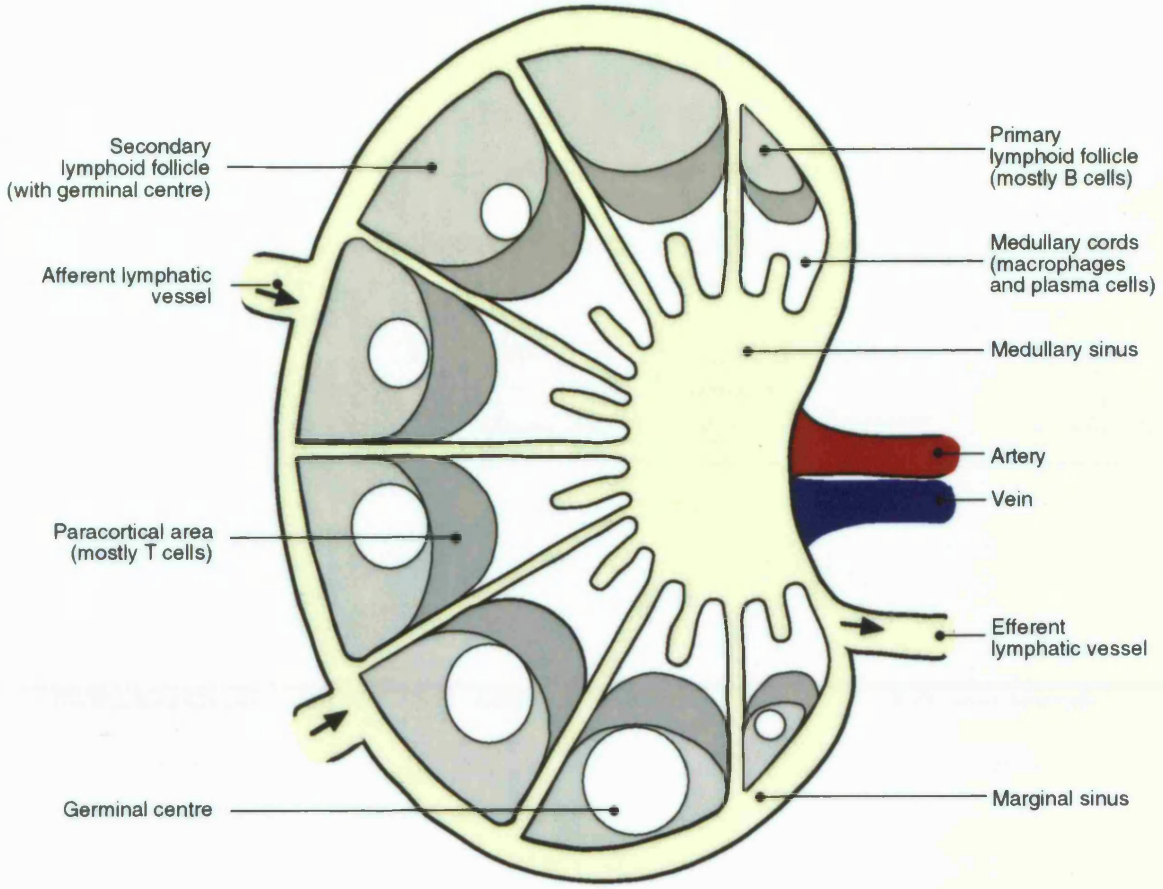



**Figure 1.5 Schematic representation of a mesenteric lymph node.**
(Adapted from Janeway *et al.*, 1999 4th edition).

(a)

| Leucocyte phenotype | Location within node | Composition |
|---|---|---|
| **$CD4^+$ T cells** | Paracortex and scattered in follicles | 16% |
| **$CD8^+$ T cells** | Paracortex and few in follicles | 9% |
| **B cells** | Follicles and germinal centres | 60% |
| **Macrophages** | Medulla, germinal centres, some in paracortex | 4% |
| **Follicular dendritic cells** | Germinal centres | Unknown |
| **Interdigitating dendritic cells** | Paracortex | Unknown |
| **γδT cells** | Paracortex and cortex adjacent to sinuses; some in medulla | 5% |
| **Reticulin cells** | Throughout node except germinal centres | Unknown |

(b)

| Cell phenotype | Afferent lymph | Efferent lymph |
|---|---|---|
| **$CD4^+$ T cells** | 25-30% | 50-60% |
| **$CD8^+$ T cells** | 10-15% | 20-30% |
| **B cells** | 10-30% | 10-20% |
| **Macrophages** | 0 | 0 |
| **Dendritic cells** | 5-20% | 0 |
| **γδ$TCR^+$ cells** | 10-30% | 5-15% |
| **$WC1^+$ γδ T cells** | 5-20% | 5-15% |

**Table 1.2 Phenotypic and histological distribution of host cells in bovine lymph nodes (a) or bovine lymph (b) (adapted from Pastoret *et al.*, 1998; Morrison *et al.*, 1986).**

## 1.5 Evolution of *Salmonella* serotypes

Bacterial survival within the host results from a balance of many gene products acting at the correct time in the correct location (Bowe *et al.*, 1998). These genes can be found within the chromosome as units of one or a few virulence genes, termed pathogenicity islets (Marcus *et al.*, 2000), or on plasmids (Rotger and Casadesús, 1999). In addition, they may be components of active bacteriophages for example SopEΦ, the lysogenic bacteriophage encoding SopE (Mirold *et al.*, 1999), or as large cassettes composed of a series of genes and operons termed pathogenicity islands (PI) (Hacker *et al.*, 1997; Bäumler, 1998; Hacker and Kaper, 2000).

Acquisition of pathogenicity islands, required for different stages of pathogenesis, is believed to have contributed to the divergence of *Salmonella* and *Escherichia coli* from a common ancestor 120 to 160 million years ago (Ochman and Wilson, 1987; Doolittle *et al.*, 1996). Expansion in host range by *S. enterica* subspecies I, to include mammals and birds, may have constituted a further refinement in the evolution of the genus as *S. bongori* and *S. enterica* subspecies II to VII are predominantly associated with cold blooded vertebrates (Popoff *et al.*, 2000). Furthermore, it has been established that clones of the host-specific or host-restricted serotypes are fewer in number and less diverse than those of the ubiquitous serotypes (Selander *et al.*, 1990) suggesting that they arose more recently.

*S. enterica* contains at least ten putative pathogenicity islands. The best characterised of these being *Salmonella* pathogenicity island 1 (SPI-1), which was acquired early on in the evolution of this pathogen and is necessary for the intestinal phase of pathogenesis (Mills *et al.*, 1995; Galán, 1996; Marcus *et al.*, 2000; Wallis and Galyov, 2000) (sections 1.7.2 and figure 1.6). Acquisition of a second pathogenicity island (SPI-2) is predominantly required for systemic virulence in mice and calves (Ochman *et al.*, 1996; Shea *et al.*, 1996; Bispham *et al.*, 2001) (section 1.9.4 and figure 1.9). SPI-1 and SPI-2 encode structurally similar but functionally distinct type three secretion systems (TTSS), namely TTSS-1 and TTSS-2, which translocate effector proteins, required for virulence, from bacteria into host cells (reviewed by Hueck, 1998; Cornelis and van Gijsegam, 2000) (figure 1.7). Two other pathogenicity islands, SPI-3 (Blanc-Potard and Groisman, 1997; Blanc-Potard *et al.*, 1999) and SPI-4 (Wong *et al.*, 1998) are primarily required for the systemic phase of the disease although their contribution to pathogenesis is at present poorly defined. SPI-5-encodes at least one effector protein (SopB) secreted via TTSS-1 and a cluster of genes coding for Pathogenicity Island Proteins (PipA,B,C,D) (Galyov *et al.*, 1997; Wood *et al.*, 1998) that are required for the induction of intestinal secretory and inflammatory responses (section 1.8.3). In addition, recent sequencing of the *S. typhi* genome suggests that this serotype contains five other PI not found in *E. coli* (Parkhill *et al.*, 2000). These encode fimbrial operons SPI-6 (Townsend *et al.*, 2001)

and SPI-10, Vi biosynthetic genes (Hashimoto *et al.*, 1993), the SopE prophage (Mirold *et al.*, 1999) and a type IV pilus operon SPI-7 (Zhang *et al.*, 2000), bacteriocin pseudogenes SPI-8 and a type 1 secretory apparatus SPI-9.

## 1.6 *Salmonella* pathogenesis: intestinal phase of infection

A common feature of *Salmonella* serotypes capable of causing gastro-enteritis and/or systemic disease is their ability to penetrate the intestinal mucosa. As such, the host and bacterial factors important for intestinal colonisation, invasion and induction of the enteropathogenic responses (*Salmonella*-induced intestinal fluid secretion, influx of inflammatory cells and mucosal damage) will be discussed in the following sections.

### *1.6.1 Initiation of Salmonella infection*

The primary route of infection is believed to be faecal-oral through the ingestion of contaminated food or water. Studies in cattle have demonstrated that experimental infection by this route results in symptoms similar to those observed following natural infection (Smith and Jones, 1967; Wray and Sojka, 1977; Wallis *et al.*, 1995; Watson *et al.*, 1998). In chickens, the vertical transmission of bacteria to developing eggs in an infected oviduct, or when follicles in the ovary are infected, is also known to occur naturally (Poppe, 2000). Experimental infection of several animal species including cattle (Wray and Sojka, 1977; Wathes *et al.*, 1988), pigs (Fedorka-Cray *et al.*, 1995) and sheep (Tannock and Smith, 1971; Hannam *et al.*, 1986) by the intra-nasal route has also been reported. While the significance of this route following infection is unknown, *Salmonella* has been isolated from the nostrils of naturally infected sheep (Meehan *et al.*, 1992) and pneumonia associated with *S. choleraesuis*-infected pigs is not uncommon (Turk *et al.*, 1992). It could therefore be speculated that airborne transmission contributes to pathogenesis in intensive rearing units where ventilation might be poor. In addition to the routes mentioned above, experimental infection of mice and other laboratory animals by the intravenous, intraperitoneal, subcutaneous or intramuscular routes are commonly used to initiate clinical salmonellosis.

The infectious dose required to cause disease varies depending on many factors including the infecting strain, breed, genetic and immune status of the animal, but typically oral doses in excess of 8 $\log_{10}$ cfu $ml^{-1}$ are used to initiate infection in cattle. It is possible that animals will become infected naturally with smaller doses as concurrent disease, dietary changes, pregnancy and other stress factors may lead to increased susceptibility (Jones, 1992).

Following oral infection, *Salmonella* must be able to withstand initial physical host barriers, including gastric acidity, the presence of intestinal mucus and the presence of resident gut microflora, in order to colonise the intestinal mucosa (reviewed by Foster and

Spector, 1995). Studies using susceptible mice have shown that less that 1% of the initial inoculum is viable in the intestinal lumen one hour after oral inoculation (Carter and Collins, 1974). However, in experimental oral infections the administration of sodium bicarbonate, together with the inoculum, can help to neutralise stomach acidity (Jones, 1988).

### *1.6.2 Gross pathological changes induced by Salmonella serotypes in vivo*

Following oral infection, *Salmonella* preferentially invade the intestinal mucosa at the terminal ileum and/or proximal colon (Carter and Collins, 1974). Depending on many factors, including the serotype-host combination, pathology may be predominantly restricted to the intestines (e.g. *S. typhimurium* in adult animals), predominantly confined to systemic sites (e.g. *S. gallinarum* in chickens), present in both systemic and enteric tissues (e.g. *S. dublin* in calves and *S. choleraesuis* in pigs) or virtually absent (e.g. *S. abortusovis* in adult sheep) (reviewed by Uzzau *et al.*, 2000a). However, the gross pathological changes associated with systemic or enteric salmonellosis are typically similar in pigs (Reed *et al.*, 1986; Wilcock and Schwartz, 1992), chickens (Henderson *et al.*, 1999; Poppe, 2000; Shivaprased, 2000), sheep (Wray and Linklater, 2000) and calves (Wray and Davies, 2000) and will be described in brief.

Intestinal pathology includes the presence of reddened, enlarged mucosae, particularly in the lower small intestine and caecum, often with small haemorrhagic spots or button shaped ulcers. The intestinal contents are typically muco-purulent and contain sloughed intestinal mucosa and the mesenteric lymph nodes are often oedematous, enlarged and congested. Systemic pathology typically includes enlargement of the liver, spleen and heart with these tissues often containing small white necrotic foci. The lungs, particularly in *S. choleraesuis* infected pigs (Wilcock and Schwartz, 1992), are often deep purple in colour and contain necrotic lesions. In birds infected with *S. gallinarum* or *S. pullorum*, bursal necrosis often occurs and the contents of the yolk sac may be caseous or coagulated (Henderson *et al.*, 1999; Shivaprased, 2000). Adult ewes that have aborted due to *S. abortusovis* infection typically display placental damage and necrotic uterine lesions, while the foetuses often have excess fluid in the thoracic and abdominal cavities together with haemorrhages and oedema in many organs (Wray and Linklater, 2000). Ewes that die of salmonellosis following infection with *S. dublin* or *S. typhimurium* exhibit more typical enteric and/or systemic pathology as described for other species.

### *1.6.3 Intestinal morphological changes induced by Salmonella serotypes in vivo*

The detailed morphological changes that take place prior to and during invasion of the intestinal mucosa have been studied in detail in *S. typhimurium*-infected hosts following oral

challenge or using the ligated ileal loop model. The earliest studies investigated the initial interactions of *S. typhimurium* with the intestinal epithelial mucosa of starved, opium pre-treated (to slow intestinal movement), orally infected guinea pigs by electron microscopy (Takeuchi, 1967; Takeuchi and Sprinz, 1967). Results of these studies demonstrated that when *Salmonella* comes within a critical distance of the enterocyte brush border, the localised microvilli and apical cytoplasm begin to degenerate and distort forming what was subsequently referred to as membrane 'ruffles' (Francis *et al.*, 1993; Jones *et al.*, 1994). Bacteria enter the villus epithelial cells predominantly through the brush border, but also via the intercellular junctional complex, where they typically become enclosed in a membrane-bound vacuole that decreases in size as it descends from the apical to the basal cytoplasm. The damaged microvilli and apical cytoplasm gradually repair as the bacteria traverse deeper into the cell. Up to 12 hours after infection, many salmonellas still penetrate the epithelium, but by 24 hours bacteria are predominant in the lamina propria, Peyer's patches and submucosa. An intense *Salmonella*-induced inflammatory response is evident in the lamina propria and bacteria are commonly found within macrophages or neutrophils. By 48 hours after infection, there is extensive inflammation that extends to the submucosa, the villi are shortened and blunted and there is elongation of crypt glands, emptying of goblet cells and an increased extrusion of enterocytes (Takeuchi and Sprinz, 1967).

The *Salmonella*-induced intestinal morphological changes described in guinea pigs appear to be similar in a wide range of host species including monkeys (Kent *et al.*, 1966; Rout *et al.*, 1974), calves (Frost *et al.*, 1997), rabbits (Giannella *et al.*, 1973a; Wallis *et al.*, 1986b), chicks (Turnbull and Richard, 1978; Popiel and Turnbull, 1985), pigs (Reed *et al.*, 1986; Wilcock and Schwartz, 1992) and mice (Carter and Collins, 1974; Jones and Falkow, 1994).

### *1.6.4 Routes of intestinal invasion*

The mechanism of intestinal invasion by salmonellas appears to be both host and serotype dependent, and as such this process could potentially influence host specificity. Infection studies in mice have shown that *S. typhimurium* is associated almost exclusively with the Peyer's patch mucosa as early as thirty minutes after oral infection (Carter and Collins, 1974; Clark *et al.*, 1994; Jones *et al.*, 1994). M cells are subsequently destroyed, forming a gap in the follicle associated epithelium, allowing organisms to invade enterocytes adjacent to the dead cell (Kohbata *et al.*, 1986; Jones *et al.*, 1994). *Salmonella* invasion has been intensively studied in calves and the findings demonstrate that the outcome of host-pathogen interactions in cattle and mice are very different. In calves *S. typhimurium* interacts with both M cells and enterocytes within fifteen minutes of ligated loop infection (Frost *et al.*,

1997). Furthermore similar bacterial recoveries of *S. typhimurium* are obtained from both bovine Peyer's patch mucosa and absorptive epithelium three hours after ligated ileal loop infection (Watson *et al.*, 1995). Consequently, it can be concluded that the foci of infection appear to develop within murine but not bovine Peyer's patches. It is likely that these observations reflect the contrasting pathogenesis of *S. typhimurium* in different hosts. For example, M cell targeting in mice could promote uptake and dissemination by phagocytic cells, such as macrophages and DCs (Pascopella *et al.*, 1995), while interaction with both enterocytes and M cells in the bovine intestine typically results in enteric salmonellosis (Frost *et al.*, 1997; Watson *et al.*, 1995; Watson *et al.*, 1998).

There is some evidence suggesting that the route of invasion correlates with the host specific phenotype in a particular animal species. For example, in contrast to *S. typhimurium*, which results in murine typhoid fever, the mouse avirulent serotypes *S. typhi* and *S. gallinarum*, either invade but do not destroy or are simply unable to invade M cells respectively (Pascopella *et al.*, 1995). Similarly, microscopic analysis of porcine mucosa has shown that following oral inoculation with *S. choleraesuis*, but not *S. typhimurium*, foci of infection develop in Peyer's patches (Pospischil *et al.*, 1990). Additionally, following oral inoculation of chickens, microscopy and viable count analysis has shown that *S. gallinarum*, rather than *S. typhimurium* has a preference for lymphoid bursal tissue (Henderson *et al.*, 1999).

### *1.6.5 Use of in vitro and in vivo models to study intestinal invasion*

A great deal has been learnt about the genetic, cell biological and biochemical analysis of the infection process through the use of different cultured epithelial and macrophage-like cell lines. This technique was first described as a model to determine the invasiveness of *S. typhimurium* into HeLa cells (Giannella *et al.*, 1973b). Subsequent morphological analysis has demonstrated that *Salmonella* appear to enter cultured epithelial cells in a manner similar to that described previously from *in vivo* studies (section 1.6.3). In addition, invasion of epithelial cells is regulated by specific environmental conditions, which would appear to mimic the *in vivo* situation within the intestine. For example, bacteria invade most efficiently in oxygen-limiting conditions (Ernst *et al.*, 1990; Schiemann and Shope, 1991), in media with high osmolarity (Tartera and Metcalf, 1993) and when the bacteria are grown to early log, rather than stationary phase (Ernst *et al.*, 1990; Lee and Falkow, 1990). These observations have led to the conclusion that cell lines provide a suitable model for quantitative and qualitative analysis of the mechanisms involved in intestinal invasion (Finlay and Falkow, 1989). However, it is clear that they are no substitute for the use of suitable animal models. The isolated nature of cultured cells does not allow modelling of the complex interactions

between bacteria and host cells that occur in the multicellular intestinal mucosa *in vivo*. Tissue culture cells are often immortalised therefore many normal host cell functions cannot be studied and some such cells lose important characteristics upon culturing. For instance, some epithelial cell lines lack polarity and some macrophage lines fail to elicit an oxidative burst following *Salmonella* infection. In terms of host specificity, cultured cell lines are of limited use due to the lack of appropriate epithelial cell lines from different animals and the difficulty in culturing specific intestinal cell types such as M cells, which potentially play a pivotal role in the infection process.

The use of an asymmetric organ culture system has enabled the quantitative analysis of *S. typhimurium* invasion of rabbit intestinal mucosa to be performed on freshly isolated functioning gut biopsies (Worton *et al.*, 1989; Amin *et al.*, 1994). This system has the advantage of assessing invasion in a whole tissue environment where interactions between bacteria and the host are less compromised. The morphological changes observed within this system are similar to those reported after the use of the same strains in both *in vivo* and *in vitro* assays (Wallis *et al.*, 1986b; Worton *et al.*, 1989). However, the structure of the tissue can only be kept intact for about two hours, most likely due to the absence of a functioning blood supply and lymphatic drainage, and as such limits the time period that bacterial-host interactions can be studied. Furthermore, disruption to the vascularity does not permit the complex interactions between epithelial cells and bacteria, such as diapedesis and migration of inflammatory cells into the intestinal mucosa, to occur beyond the invasive process.

The *in vivo* ligated ileal loop model has successfully been used to study the mechanisms of intestinal invasion in different host species including calves (Watson *et al.*, 1995), pigs (Bolton *et al.*, 1999b), sheep (Uzzau *et al.*, 2001), chickens (Chadfield *et al.*, 2001), mice (Jones *et al.*, 1994) and rabbits (Giannella *et al.*, 1973a; Wallis *et al.*, 1986b). The use of this model facilitates the testing of multiple strains within the same animal thereby eliminating intra-animal variation and reducing the number of animals used. Furthermore, construction of loops in specific parts of the gastrointestinal tract enables invasion to be quantified from different regions of the gut and facilitates a direct comparison to be made between invasion of Peyer's patch mucosa and absorptive epithelia within the same ligated ileal loop (Watson *et al.*, 1995).

### *1.6.6 Factors mediating bacterial attachment to intestinal mucosa*

In order to invade intestinal epithelial cells, bacteria must first attach or adhere to the cell surface of the gastrointestinal tract. It is likely that factors mediating attachment are present on the bacterial surface and as such the importance of potential adhesins including fimbriae, flagella and LPS will be discussed in the following sections. Although little is

known about the role of *Salmonella* adhesins in their respective hosts, it is possible that the specific nature of the host-pathogen interaction may contribute to the host specific phenotype. For example, it has been demonstrated that *S. typhi*, but not *S. typhimurium*, uses the cystic fibrosis transmembrane conductance regulator (CFTR) for entry into epithelial cells (Pier *et al.*, 1998).

The expression of receptors on eukaryotic cells may result in bacterial tropism for a specific cell type. M cells and enterocytes within the follicle associated epithelium express characteristic glycoconjugates on their apical surfaces which may vary depending on the host species and region of the gut (Finzi *et al.*, 1993; Giannasca *et al.*, 1994; Jepson *et al.*, 1995; Lelaurd *et al.*, 1999). Several pathogens, including *Yersinia* Spp. utilise binding to specific integrins on the surface of eukaryotic cells or to ligands such as collagen, laminin and fibronectin (Isberg and Tran van Nhieu, 1994). However, the precise mechanism by which *Salmonella* colonise the gastro-intestinal tract is not fully understood.

#### *1.6.6.1 Role of fimbriae*

Several fimbrial operons have been identified in *Salmonella* (reviewed by Thorns and Woodward, 2000), the most important characteristics of those that have a potential role in serotype-host specificity will be discussed in brief.

Type 1 fimbriae are associated with several members of the family *Enterobacteriaceae* and are found on most *Salmonella* serotypes. They are characterised by their ability to mediate the mannose-sensitive haemagglutination (MSHA) of red blood cells *in vitro* (Duguid *et al.*, 1966; Buchanan *et al.*, 1985). The role of type 1 fimbriae in pathogenesis is unclear due in part to the use, in some studies, of bacterial cultures enriched for either fimbriated or non-fimbriated cells or *S. typhimurium* strains that were not stably non-fimbriated (Duguid *et al.*, 1966; Tavendale *et al.*, 1983; Lindquist *et al.*, 1987; Horiuchi *et al.*, 1992; Isaacson and Kinsel, 1992). Despite the use of defined Δ*fim* mutants, contradictory results have also been obtained regarding the role of these fimbriae both *in vitro* and *in vivo*. For example, a *S. typhimurium* Δ*fim* mutant showed a decreased ability to adhere to and invade HeLa, but not Hep-2, MDCK, Int 407 or T84 cells (Bäumler *et al.*, 1996b). Contrastingly, a Δ*fimH* (fimbrial adhesin gene) mutant appears to be required for adhesion to Hep-2 cells (Hancox *et al.*, 1998). Furthermore, the *fimH* gene has been implicated in binding to murine enterocytes but not bladder epithelial cells (Thankhavel *et al.*, 1999). Studies comparing intestinal colonisation of mice and chickens using isogenic *fim*$^-$ insertion mutants of *S. typhimurium* or *S. enteritidis* or the wild type parent strain have shown that type 1 fimbriae are not necessary for intestinal colonisation or virulence (Lockman and Curtiss III, 1992; Allen-Vercoe *et al.*, 1999). However use of a *fimD*$^-$ insertion mutant has demonstrated

that type 1 fimbriae are apparently necessary for the ability of *S. enteritidis* to colonise the rat intestine (Naughton *et al.*, 2001). These reputed differences could be explained by the use of different culture conditions, defined mutations, strains, serotypes, and cell lines or animal models. Furthermore, the ability of bacteria, under certain conditions, to utilise potentially 'redundant' fimbrial operons might account for apparent differences in a requirement for these appendages.

In contrast to other serotypes within *S. enterica,* in *S. gallinarum* and *S. pullorum*, the *fim* operon encodes surface appendages termed type 2 fimbriae. Although these are morphologically and antigenically similar to type 1 fimbriae (Crichton *et al.*, 1989; Crichton and Old, 1990; Sojka *et al.*, 1998), they do not express MSHA and as such are unable to agglutinate erythrocytes (Duguid *et al.*, 1966). This observation suggests that birds may select against type 1 fimbriation which may have contributed to the expansion or restriction in host range of *S. gallinarum* and *S. pullorum*.

As discussed in section 1.6.6, one possible factor that may determine the host range of fimbriae is the variation in glycosylation patterns found between the epithelial surfaces of different host species. As such, the specific repertoire of fimbriae expressed by particular *Salmonella* serotypes may contribute to tissue tropism in particular hosts. Two fimbrial operons, plasmid encoded fimbriae (*pef*) and long polar fimbriae (*lpf*) (Friedrich *et al.*, 1993; Bäumler and Heffron, 1995; Bäumler *et al.*, 1996a; Bäumler *et al.*, 1996c; Bäumler, 1997a; Bäumler, 1997b), have been characterised for their ability to mediate attachment of *Salmonella* to different murine intestinal epithelial cells. PEF display tropism for the villous epithelium (Bäumler *et al.*, 1996a) while LPF appear to mediate adherence to M cells in the FAE (Bäumler *et al.*, 1996c). In addition, PEF are required for the accumulation of fluid in the suckling infant mouse model (Bäumler *et al.*, 1996a). The relevance of this observation to the induction of enteritis in other species is unknown as mice are typically used as a model for typhoid fever and PEF do not contribute to fluid accumulation in rabbit ligated ileal loops (Horiuchi *et al.*, 1991). Disruption of one or more fimbrial genes does not significantly reduce the virulence of *S. typhimurium* in mice (Bäumler *et al.*, 1997b; Van der Velden *et al.*, 1998) which suggests that additional factors mediate adhesion to epithelial surfaces *in vivo*.

In *S. typhimurium* a mutation in a novel fimbrial operon, termed bovine colonisation factor (*bcf*), has been shown to reduce the ability of *S. typhimurium* to colonise bovine, but not murine Peyer's patches (Tsolis *et al.*, 1999c). However, with the recent complete genome sequencing of *S. typhi* (Parkhill *et al.*, 2001; http://www.sanger.ac.uk/Projects- /S_typhi/), it has been shown that a correlation between host range and the presence of a single fimbrial operon is unlikely. This is because many fimbrial operons including the recently identified *typhi* colonisation factor (*tcf*) (Folkesson *et al.*, 1999) and *bcf* are not specific to *S. typhi* or

*S. typhimurium* respectively (Townsend *et al.*, 2001). The *S. typhi* genome does appear to contain a unique combination of putative fimbrial operons (Zhang *et al.*, 2000; Townsend *et al.*, 2001) which may contribute to the colonisation and entry of this serotype in humans. It remains to be determined whether unique adhesins are present in other serotypes that might contribute to host specificity.

Recently, it has been proposed that the *S. enteritidis* fimbriae 14 (SEF14) may play a role in systemic infections in mice. These results indicate that surface appendages may be important for pathogenesis beyond initial colonisation of epithelial cells (Edwards *et al.*, 2000). SEF14 is restricted to *S. enteritidis* and other closely related serotypes (Clouthier *et al.*, 1993), however, a role for this fimbrial operon during initial intestinal interactions remains unclear. It has been shown that SEF14 are not required for invasion of cultured epithelial cells (Dibb-Fuller *et al.*, 1999) or virulence following oral inoculation of mice (Ogunniyi *et al.*, 1997). However, immunisation of mice with a purified structural subunit protein of SEF14, namely SefA, has demonstrated that the protein can elicit delayed-type hypersensitivity in susceptible mice. In addition, SefA is able to stimulate *in vitro* proliferation of, and cytokine release from, T-lymphocytes obtained from these animals to levels comparable to those induced by the whole organism (Ogunniyi *et al.*, 1994) suggesting that these fimbriae are able to stimulate cell-mediated immunity (Ogunniyi *et al.*, 1994). More recently, the use of defined *sef* mutants has demonstrated that *sefD*, encoding the putative adhesion subunit of SEF14, results in attenuation of virulence in mice following intraperitoneal inoculation. Furthermore, *sefD* is required for uptake or survival of *S. enteritidis* in murine macrophages (Edwards *et al.*, 2000). As such, it has been suggested that SEF14 may be involved in either attachment to the macrophage surface or survival after uptake.

#### *1.6.6.2 Role of flagella and motility*

The possession of flagella by *Salmonella* Spp. potentially contributes to pathogenesis *in vivo* by conferring upon the bacteria the ability to respond to chemoattractants and either move towards favourable environments or away from adverse stimuli (Suerbaum, 1995). Expression of flagella genes may help bacterial penetration of the intestinal mucus layer in order to reach sites for colonisation and invasion (Freter *et al.*, 1981; Carsiotis *et al.*, 1984; Walker *et al.*, 1999). Additionally, flagella may function as virulence factors mediating the adherence to, or penetration into, eukaryotic cells (Finlay and Falkow, 1989; Harshey and Toguchi, 1996). At present the role of motility in attachment and invasion is not well defined. However, it is clear that motility is not essential for virulence as the avian-specific serotypes *S. gallinarum* and *S. pullorum* are non-flagellate, non-motile and poorly invasive *in vitro* (Barrow and Lovell, 1989; Henderson *et al.*, 1999) yet result in severe systemic disease in

chickens (Shivaprased, 2000). These serotypes posses the *fliC* gene, which encodes the phase 1 structural flagella protein (Li *et al.*, 1993). However, it is unknown whether this gene is expressed *in vivo* and if so whether it provides a serotype-specific advantage in the appropriate host species.

### *1.6.6.3 Role of LPS in intestinal colonisation*

Lipopolysaccharide (LPS), a component of the outer membrane of Gram negative bacteria has three structural and functional domains including the lipid A, core region and O antigen. LPS is a potent immunostimulatory molecule, which activates innate host defence mechanisms resulting in inflammation and the pathophysiologic alterations observed in septic shock (reviewed by Reeves, 1995). It has recently been demonstrated that toxicity of the lipid A domain is required for death of mice following infection with *S. typhimurium* (Khan *et al.*, 1998). Circulatory LPS has been demonstrated to result in increased translocation of intestinal bacteria (Deitch *et al.*, 1987) which could influence the invasiveness of *Salmonella* due to loss of epithelial integrity. Despite a considerable amount of work on the subject, the function of LPS following interactions with the intestinal mucosa remains unclear. Rough mutants of *S. typhimurium* are less invasive in the murine ileum or colon compared with the corresponding smooth strains (Tannock *et al.*, 1975; Nevola *et al.*, 1985) suggesting that the properties of LPS might influence invasion. However, following infection of cultured cells some studies have demonstrated that rough LPS strains have a reduced ability to invade HeLa cells (Finlay *et al.*, 1988; Mroczenski-Wildey *et al.*, 1989) while others have shown such mutants to be as invasive as the parent strains both *in vitro* and *in vivo* (Giannella *et al.*, 1973b; Gahring *et al.*, 1990).

Studies using mutations that result in a rough phenotype have to be interpreted with caution. The use of transposon mutagenesis might lead to selection for spontaneously arising rough mutants that may serve as better recipients for conjugation during the generation of the mutant. Mutations altering the structure of LPS could influence invasion by a secondary, rather than a primary mechanism (Martin *et al.*, 2000). For example, alteration of the structure of LPS could have pleiotrophic effects by influencing the function of TTSS-1, fimbriae or flagella expression. Supporting this suggestion, it has recently been shown that mutation of *waaN*, a gene involved in acylation of lipid A (Somerville *et al.*, 1996; Khan *et al.*, 1998) reduced *in vitro* invasion of *S. typhimurium* and enteropathogenic responses in bovine ligated ileal loops (Watson *et al.*, 2000a). However the secretion of at least two effector proteins, SipA and SipC, required for invasion and enteropathogenesis was greater in the wild type strain as compared with the *waaN* mutant. These results suggest that the observed phenotype was not solely attributed to the function of *waaN*.

## 1.7 Mechanisms involved in *Salmonella* epithelial cell invasion

### *1.7.1 Salmonella uptake and cell signalling*

Interactions between bacteria and host cells have been largely studied using cultured epithelial cell lines, although the invasion events observed using such cells appear to be very similar to those described in animal infection models (section 1.6.3).

Shortly after bacteria adhere to the apical epithelial surface, cytoskeletal rearrangements occur in the epithelial cell inducing the formation of membrane ruffles that enclose adherent bacteria in large vesicles (Finlay and Falkow, 1990; Francis *et* al., 1992; Francis *et al.*, 1993). This process, termed bacterial-mediated endocytosis, requires the co-ordinated secretion of many bacterial proteins (Francis *et al.*, 1992). Formation of membrane ruffles is dependent on the aggregation of cytoskeletal elements such as actin, α-actinin, talin, ezrin and tropomyosin (Finlay *et al.*, 1991). This has been demonstrated by the observation that specific inhibitors of microfilament formation (cytochalasins) prevent membrane rearrangements and block *S. typhimurium* entry (Kihlström and Nilsson, 1977; Finlay and Falkow, 1989; Finlay *et al.*, 1991; Ginocchio *et al.*, 1992).

The signal transduction pathways that lead to the formation of membrane ruffles have been studied extensively. It has been suggested that *S. typhimurium* invasion of Int 407 epithelial cells stimulates the epidermal growth factor receptor (EGRF) resulting in activation of the mitogen activated protein (MAP) kinase. This is followed by activation of a cascade of signalling molecules culminating in an influx of $Ca^{2+}$ and the subsequent reorganisation of the host cell cytoskeleton (Galán *et al.*, 1992b; Pace *et al.*, 1993). However, *S. typhimurium* can enter cell lines which do not express EGRF (Galán *et al.*, 1992b; Francis *et al.*, 1993; Rosenshine *et al.*, 1994) and this serotype is competent for invasion in mice with a defective EGFR (McNeil *et al.*, 1995). These results suggest that *Salmonella* may stimulate other signal transduction pathways that promote bacterial entry. Recent observations indicate that members of the Rho family, including Cdc42, rac and rho, play a pivotal role in regulating the actin cytoskeleton (Hall, 1998). In addition, Rho-family G proteins regulate gene transcription mainly via their ability to stimulate signalling through MAP kinases (Hall, 1998). $Ca^{2+}$ appears to be a common second messenger important in different signalling pathways and appears to be necessary for *S. typhimurium* entry into epithelial cells. For example, the chelation of intracellular, but not extracellular, $Ca^{2+}$ blocks bacterial uptake into HeLa cells (Ruschowski *et al.*, 1992) and mutations in the SPI-1 gene *invE* render bacteria unable to trigger $Ca^{2+}$ release (Ginocchio *et al.*, 1992).

### *1.7.2 Molecular mechanisms of Salmonella epithelial cell invasion*

A 40 kb segment of the *Salmonella* chromosome called *Salmonella* pathogenicity island 1 (SPI-1), mapping at centisome 63 (Mills *et al.*, 1995), contains many of the genes required for entry into non-phagocytic cells. SPI-1 encodes more than thirty genes, which include structural and accessory components of the TTSS-1, regulatory proteins, secreted effector proteins and their chaperones together with an iron transport system (Galán, 1996; Darwin and Miller, 1999; Sukhan *et al.*, 2001) (figure 1.6).

#### *1.7.2.1 Genetic regulation of SPI-1*

*Salmonella* has evolved intricate, complex signalling pathways to regulate the local and global expression of genes required for both invasion and entropathogenesis (section 1.8) (reviewed by Lucas and Lee, 2000). At least four putative proteins encoded by SPI-1 are required for the regulation of invasion genes. These include the central regulator HilA (Bajaj *et al.*, 1995; Bajaj *et al.*, 1996; Ahmer *et al.*, 1999; Darwin and Miller, 1999; Lostroh *et al.*, 2000), the HilA repressors *hilC* and *hilD* (Eichelberg *et al.*, 1999; Rakeman *et al.*, 1999; Schechter *et al.*, 1999), InvF and the co-factor SicA (Darwin and Miller, 1999; Eichelberg and Galán, 1999; Darwin and Miller, 2000).

Transcription of invasion genes is also affected by different chromosomal loci located outside of SPI-1 (Pegues *et al.*, 1995) that regulate bacterial gene expression in response to environmental cues (Perraud *et al.*, 1999). For example, HilA is regulated *in vitro* by several two-component signal transduction systems including PhoP/PhoQ (Groisman *et al.*, 1989; Miller *et al.*, 1989). PhoQ is the outer membrane sensor that responds to low $Ca^{2+}$ and $Mg^{2+}$ levels and activates a cytoplasmic component PhoP (Garcia Vescovi *et al.*, 1996). PhoQ is inactive when these cations are present, for example in extracellular spaces, which induces expression of PhoP-repressed genes (*prg*) thereby facilitating expression of components of the SPI-1 TTSS-1 (Pegues *et al.*, 1995; Cotter, 2000). In addition, PhoR/PhoB (Lucas *et al.*, 2000), EnvZ/OmpR (Lucas and Lee, 2000) and BarA/SirA (Johnston *et al.*, 1996; Ahmer *et al.*, 1999; Altier *et al.*, 2000) regulate SPI-1 gene expression in response to $P_i$ levels, osmotic conditions and other environmental stimuli respectively.

#### *1.7.2.2 Structure and function of type three secretion systems (TTSS)*

The proteins that comprise the *Salmonella* TTSS-1 share particular homology with the *Shigella* and *Yersinia* type three secretion systems and components of the flagella assembly apparatus (Galán, 1996; Hueck, 1998). Functional homology with other pathogens has been demonstrated by the observations that mutations in *S. typhimurium invA* (Ginocchio and Galán, 1995) and *spaP* (Groisman and Ochman, 1993) can be complemented by the related

*S. flexneri* genes *mxiA* and *spa24* respectively. Additionally, *S. typhimurium* is able to secrete and translocate the *Yersinia* YopE protein when expressed with its own chaperone (Rosquist *et al.*, 1995).

TTSS can be considered as molecular syringes that span the bacterial inner and outer membranes facilitating the translocation of secreted proteins from the bacterial cytoplasm into the cytoplasm of target eukaryotic cells by an ATP-dependent mechanism (Kubori *et al.*, 1998) (figure 1.7). Injection of bacterial effector proteins into host cells results in the modification or subversion of signalling pathways leading to a variety of responses that are discussed in the following sections (reviewed by Hueck, 1998; Cornelis and van Gijsegam, 2000; Sukhan *et al.*, 2001).


**Figure 1.6 Genetic organisation of SPI-1**

Genes encoding structural components of the needle complex (grey); accessory proteins involved in apparatus assembly or function (blue); effector proteins (black); chaperones (green); regulators (yellow); proteins involved in regulation of secretion (red); iron transport system (pink); uncharacterised proteins (white); striped boxes indicate genes with two putative functions.

Gene abbreviations: *sit*, *Salmonella* iron transporter; *avr*, avirulence; *spr*, *Salmonella* pathogenicity island-1 encoded regulator; *hil*, hyper-invasion locus; *org*, oxygen-regulated gene; *prg*, PhoP repressed gene; *iag*, invasion-associated gene; *spt*, secreted protein tyrosine phosphatase; *sic* *Salmonella* invasion chaperone; *iac*, invasion-associated acyl carrier protein; *sip*, *Salmonella* invasion protein; *spa*, surface presentation of antigens; *inv*, invasion.

Reference sources: Hueck, 1998; Darwin and Miller, 1999; Galán and Zhou, 2000; Klein, 2000; Sukhan, 2000; Mirold, 2001; Sukhan, 2001

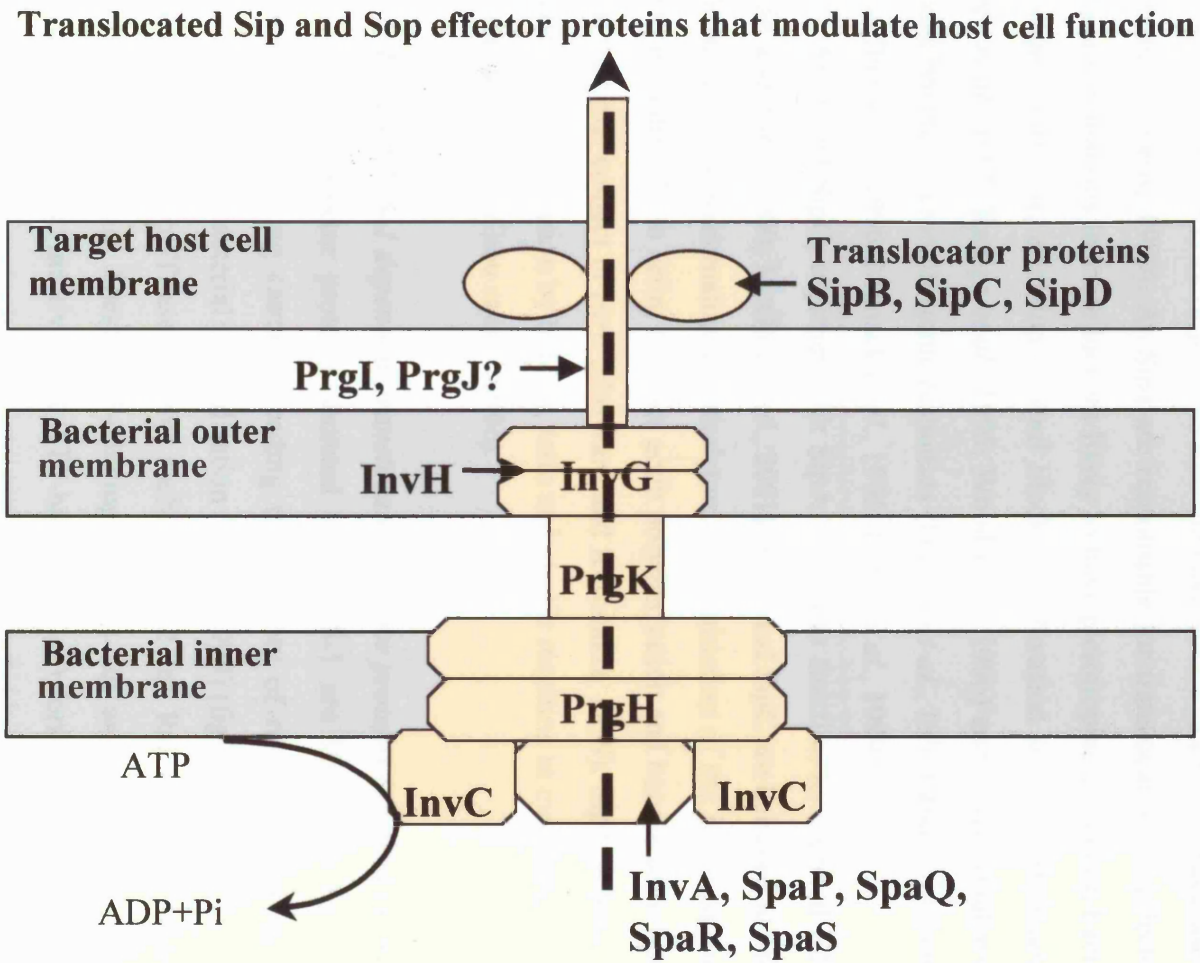



**Figure 1.7 Structure and organisation of the SPI-1 encoded TTSS-1 (showing the presence of translocator and effector proteins).** The precise location of some of the needle complex apparatus proteins are as yet unknown. Reference sources: Gálan and Zhou, 2000; Sukhan *et al.*, 2001.

### *1.7.2.3 SPI-1 encoded translocator proteins*

Translocation of effector proteins through the eukaryotic cell membrane requires the function of a group of translocators, namely *Salmonella* invasion protein (Sip) B, SipC and SipD (Kaniga *et al.*, 1996; Wood *et al.*, 1996; Darwin and Miller, 1999). The mechanisms by which Sip proteins mediate the translocation process is not well understood. However, a comparison with functionally similar proteins in *Yersinia* and *Shigella* Spp. has determined that they contain a hydrophobic domain that shares homology with a family of pore forming toxins (Sukhan, 2000). This suggests that Sips may form pores in the membrane of host cells allowing the entry of effector proteins into the eukaryotic cytoplasm (Hakansson *et al.*, 1996; Neyt and Cornelis, 1999). As Sips are responsible for translocation of effectors into target host cells, mutations in these loci are likely to have pleiotrophic effects on bacterial function. Consistent with this, mutation of *sipB* blocks cell invasion *in vitro* (Hermant *et al.*, 1995; Hueck *et al.*, 1995; Kaniga *et al.*, 1995; Wood *et al.*, 1996) and *in vivo* (Galyov *et al.*, 1997), reduces bovine enteropathogenic responses (Galyov *et al.*, 1997) and influences macrophage lysis (Chen *et al.*, 1996; Monack *et al.*, 1996; Hersh *et al.*, 1999).

SipB and SipC, together with SipA, may also function as secreted effector proteins (Galán and Zhou, 2000; McGhie *et al.*, 2001). SipA and SipC are believed to contribute to the modulation of the mammalian cytoskeleton at the initiation of the invasion process. SipC is able to bind directly to actin, nucleate actin polymerisation and bundle actin into cables at the site of bacterial contact *in vitro* (Hayward and Koronakis, 1999). SipA is believed to enhance the efficiency of invasion by binding actin and plastin resulting in cytoskeletal rearrangements (Zhou *et al.*, 1999b; Zhou *et al.*, 1999c).

### *1.7.2.4 TTSS-1 dependent translocated effector proteins involved in invasion*

Several effector proteins secreted via TTSS-1 are known to have a role in the activation of signalling cascades leading to a variety of responses including cytoskeletal rearrangements and bacterial internalisation (Galán, 1999) (figure 1.8).

Activation of RhoGTPase proteins such as Cdc42 and Rac play a key role in regulating the actin cytoskeleton. These GTP-binding proteins act as molecular switches and cycle between GDP-bound (inactive) and GTP-bound (active) forms. Only the active GTP-bound RhoGTPases can interact with downstream elements of signal transduction cascades thereby mediating specific cellular functions (Hall, 1998). The secreted effector proteins *Salmonella* outer protein E (SopE) (Wood *et al.*, 1996), a SopE homologue SopE2 (Bakshi *et al.*, 2000) and SopB, an inositol phosphate polyphosphate (Galyov *et al.*, 1997; Norris *et al.*, 1998), are efficient guanine nucleotide exchange factors for Cdc42 (Chen *et al.*, 1996; Hardt *et al.*, 1998; Galán and Zhou, 2000; Stender *et al.*, 2000; Zhou *et al.*, 2001). Recent evidence suggests that

while SopE efficiently activates both Cdc42 and Rac1 *in vitro* and *in vivo*, SopE2 and SopB efficiently activate Cdc42 alone (Galán and Zhou, 2000; Friebel *et al.*, 2001). These results demonstrate that expression of effector proteins enables *S. typhimurium* to differentially activate specific signalling pathways within host cells. Furthermore, evidence suggests that these proteins work in concert to mediate the invasion process (Mirold *et al.*, 2001). The effector protein *Salmonella* protein tyrosine phosphatase (SptP) functions as a GTPase activating protein that serves to downregulate or oppose the activity of SopE/SopE2-induced activation of Cdc42 and Rac and aids recovery of the eukaryotic brush border morphology (Fu and Galán, 1998a; Fu and Galán, 1998b).

Relatively little is currently known about the expression of these effector proteins *in vivo* and to date, few comparisons have been drawn between effects in different hosts and different serotypes. For example in *S. typhimurium*, SipA is required for bovine (Tsolis *et al.*, 2000), but not murine, virulence (Kaniga *et al.*, 1995) and *S. typhi* expresses the poorly conserved RhoGTPase activating protein SopE, but not the more common protein SopE2 (Mirold *et al.*, 1999; Mirold *et al.*, 2001).

In addition, the role of at least one other effector protein, AvrA, remains to be determined (Hardt and Galán, 1997). Future work may therefore discover a role for this and other *Salmonella* secreted effector proteins in pathogenesis.

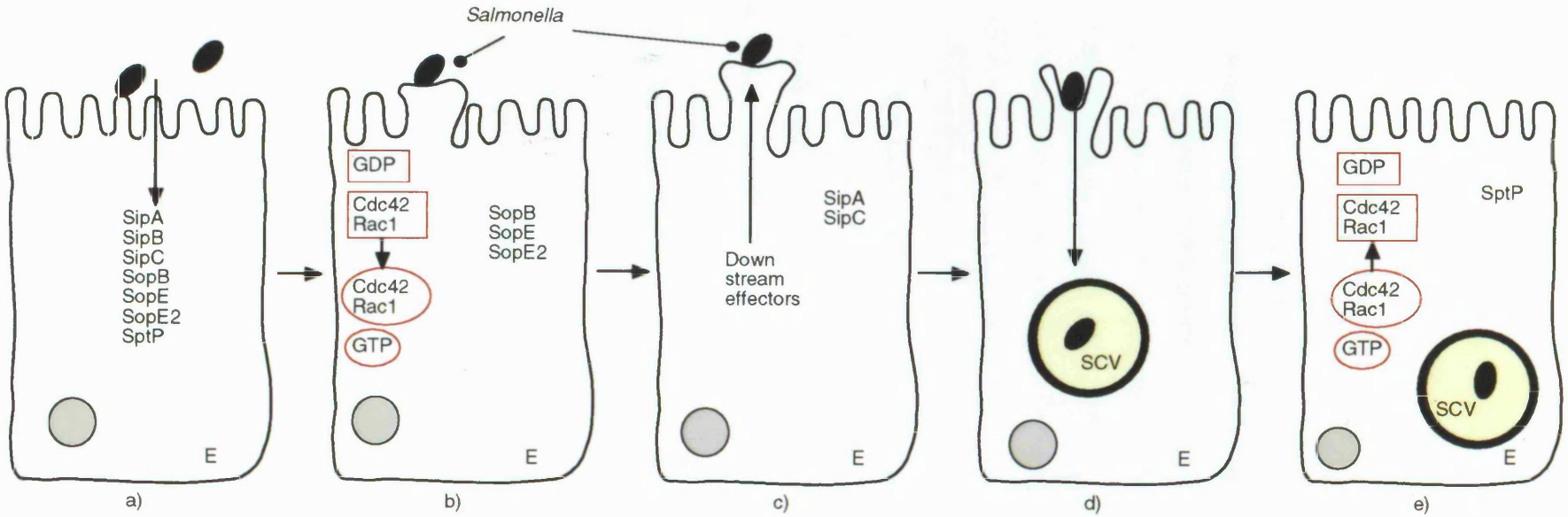



**Figure 1.8 Schematic model for *Salmonella* entry into host cells *in vitro*.** Contact with host cells results in the delivery of effector proteins into target eukaryotic cells via the SPI-1 encoded TTSS-1 (a). Translocation of SopB, SopE and SopE2 results in activation of Cdc42 and Rac1 (b). SipA and SipC contribute to the rearrangement of the actin cytoskeleton (c). Bacteria become internalised within membrane-bound vacuoles (d). Delivery of SptP reverses the action of SopB, SopE and SopE2 by stimulating their GTPase activity thereby facilitating cell recovery (e). E- epithelial cell; SCV - *Salmonella*-containing vacuole. (Adapted from Galán and Zhou, 2000). (Not drawn to scale)

## 1.8 *Salmonella*-induced enteropathogenesis

*Salmonella*-induced enteritis results in an influx of inflammatory cells into the intestinal mucosa and lumen (Turnbull and Richard, 1978; Wallis *et al.*, 1989), disruption of electrolyte transport (Rout *et al.*, 1974) and damage to mucosal integrity (Giannella *et al.*, 1973a; Clarke and Gyles, 1987; Wallis *et al.*, 1986b) (collectively termed the enteropathogenic response) (reviewed by Wallis and Galyov, 2000).

### *1.8.1 Use of in vitro and in vivo models to study enteropathogenesis*

The use of biologically relevant model systems is imperative for the study of enteropathogenic responses *in vivo*. While the murine model of typhoid fever is useful for elucidating virulence mechanisms relating to the systemic phase of pathogenesis, it is unsuitable for studying enteropathogenic responses as mice do not suffer from overt quantifiable diarrhoea (Tsolis *et al.*, 1999b). In contrast, calves represent a biologically relevant host for studies on enteritis as they are a natural target species for the enteric form of the disease (Wray and Davies, 2000).

Oral challenge of relevant hosts has been widely used to study enteric responses to *Salmonella* infection (Wallis *et al.*, 1995; Watson *et al.*, 1998; Uzzau *et al.*, 2001). The use of this natural infection route has clear advantages over other models but, can prove expensive as only a single strain can be tested *per* animal. As with all infection models, it is important to optimise the infection dose. Two recent investigations have monitored enteritis in calves, following infection with wild type strains and their corresponding mutants, using high inocula. In these studies, animals typically died between 1 and 3 days after inoculation, which is atypical of a natural infection. As such, it is likely that any effects of a mutation could be masked by the severity of this infection model (Tsolis *et al.*, 1999a; Tsolis *et al.*, 2000).

The use of the ligated ileal loop model has been widely exploited to study inflammatory and secretory responses to *Salmonella* infection in different animal species including calves and rabbits (Giannella *et al.*, 1973a; Wallis *et al.*, 1995). In the rabbit ligated ileal loop model, only strains that caused enteritis in primates resulted in enteropathogenic responses in ligated loops (Giannella *et al.*, 1973a; Rout *et al.*, 1974; Wallis *et al.*, 1986b) making this a good substitute for the very expensive primate model. It has been shown in cattle that mutant strains with reduced enteropathogenicity in ligated ileal loops were also attenuated following oral challenge (Watson *et al.*, 1998). In contrast, mutants that induced enteropathogenesis in ligated loops also induced enteritis in orally challenged calves (Wallis *et al.*, 1995; Watson *et al.*, 1999). The use of the ligated ileal loop model enables many strains to be compared in a single animal thereby eliminating inter-animal variation and reducing the number of animals required. As with any model system it is important to optimise inoculation

conditions in order to maximise responses. For example, it is now known that log phase cultures induce more potent secretory and inflammatory responses than stationary phase cultures in both calves (P. Watson, personal communication) and rabbits (Wallis *et al.*, 1989). This observation is of particular relevance in the rabbit model as stationary phase bacteria result in a potent inflammatory response in the absence of fluid secretion (Wallis *et al.*, 1989), implicating log phase–expressed bacterial factor(s) in the induction of fluid secretion.

Several studies have used *in vitro* cell culture models to assay the magnitude of transepithelial PMN migration elicited by *Salmonella*-infected human intestinal epithelium (McCormick *et al.*, 1993; McCormick *et al.*, 1995a; McCormick *et al.*, 1995b; McCormick *et al.*, 1998). Evidence suggests that transepithelial signalling to neutrophils, by different serotypes, correlates with *Salmonella*-induced enteritis in humans (McCormick *et al.*, 1995b). While this is a useful model for studying specific aspects of the inflammatory response, such an *in vitro* system cannot represent fully the complex interactions that occur *in vivo*.

### *1.8.2 Factors contributing to Salmonella-induced enteropathogenesis*

The mechanisms whereby salmonellas disrupt the normal function of the intestine leading to the induction of enteritis are at present not fully understood.

Structural damage to mucosal integrity, as a consequence of bacterial invasion (Giannella *et al.*, 1973a; Clarke and Gyles, 1987; Wallis *et al.*, 1986b; Everest *et al.*, 1999), can result in the loss of absorptive surfaces which may contribute to enteritis by altering the absorption/secretion ratio within the intestinal mucosa. In rabbits however, it has been demonstrated that intestinal damage does not appear to be either necessary for the onset or directly responsible for fluid secretion (Giannella *et al.*, 1973a; Rout *et al.*, 1974; Wallis *et al.*, 1986b).

*In vitro* studies using *Salmonella*-infected human intestinal epithelial cells have shown that bacterial-host interactions stimulate epithelial cells to release chemoattractants important in the induction of inflammatory responses (Eckmann *et al.*, 1993; McCormick *et al.*, 1993; Jung *et al.*, 1995; McCormick *et al.*, 1995a; McCormick *et al.*, 1995b; McCormick *et al.*, 1998). For example, release of IL-8 plays a role in the recruitment of PMNs through the lamina propria to the subepithelial space (McCormick *et al.*, 1993; McCormick *et al.*, 1995a). In addition, the apical epithelial release of pathogen-elicited epithelial chemoattractant (PEEC) is believed to direct PMNs through the tight junctions into the gut lumen (McCormick *et al.*, 1998). Distinct signalling pathways have been shown to mediate induction of these two PMN chemoattractants *in vitro* (Gewirtz *et al.*, 1999). Furthermore, production of inflammatory mediators may also contribute to *Salmonella*-induced intestinal pathology.

The contribution of an inflammatory influx in the induction of fluid secretion has been investigated in several studies. In bovine ligated ileal loops, the magnitude of fluid secretion induced by a particular serotype correlates to the magnitude of the inflammatory influx, suggesting that the two parameters are related (Wallis *et al.*, 1995; Galyov *et al.*, 1997; Watson *et al.*, 1998). In addition, the transepithelial migration of PMNs in an *in vitro* model has been correlated to the induction of enteritis in humans (McCormick *et al.*, 1995b). However, in rabbit ligated ileal loops, inflammation has been detected in the absence of fluid secretion when bacteria grown to stationary phase are used as the inoculum. As previously stated, this observation implicates a log phase-specific factor in the induction of fluid secretion (Wallis *et al.*, 1989).

Evidence suggests that prostaglandin synthesis by PMNs, recruited to the site of infection, contribute to the induction of fluid secretion (Giannella *et al.*, 1975; Giannella, 1979). Release of prostaglandins results in an increase of adenylate cyclase activity *in vitro* causing an inhibition of sodium absorption and an increase in chloride secretion (Kimberg *et al.*, 1971). More recent reports agree with a role for prostaglandin synthesis in this process but suggest that it is mediated by epithelial cells rather than PMNs (Wallis *et al.*, 1990; Eckmann *et al.*, 1997b). Furthermore, it remains unclear at present whether the increased chemokine and prostaglandin release by eukaryotic cells in response to *Salmonella* infection results from activation of signalling pathways by effector proteins secreted via TTSS-1.

Although intestinal invasion appears to be a pre-requisite for the induction of secretory and inflammatory responses *in vivo* (Giannella, 1979; Watson *et al.*, 1995; Watson *et al.*, 1998), the precise requirement of this process remains unclear. For example, in calves *S. typhimurium* induces greater secretory and inflammatory responses than *S. dublin* (Watson *et* al., 1998) despite the fact that both of these serotypes are similarly as invasive *in vivo* (Watson *et al.*, 1995). This result implicates a role for serotype-specific factors in mediating these responses in calves. Furthermore *Salmonella* serotypes that are similarly as invasive *in vitro* elicit differences in PMN transmigration and induction of enteritis in humans (McCormick *et al.*, 1995b; Gerwitz *et al.*, 1999). In addition, the *S. dublin* effector protein SopB is translocated into HeLa cells treated with cytochalasin demonstrating that bacteria do not need to be internalised to deliver effectors of enteropathogenesis (Galyov *et al.*, 1997). To date, it has not been possible to study the effects of invasion on the induction of enteropathogenesis as mutation of proteins encoded by TTSS-1 results in pleiotrophic effects *in vivo* (Galyov *et al.*, 1997; Watson *et al.*, 1998).

### *1.8.3 Molecular contribution to Salmonella-induced enteropathogenesis*

*Salmonella*-induced enteritis is largely dependent on effector proteins secreted via TTSS-1 (Wallis and Galyov, 2000). While a role for at least one effector protein has been attributed directly to the induction of fluid secretion and recruitment of PMNs *in vivo*, the precise functions of other effectors remain to be determined.

SopB is as an inositol polyphosphate 4-phosphatase (Norris *et al.*, 1998) which influences enteropathogenesis by subverting inositol phosphate signalling pathways. *Salmonella* infection of cultured epithelial cells results in an increase in the intracellular concentration of the inositol polyphosphate D-myo-inositol 1,4,5,6 tetrakisphosphate [Ins(1,4,5,6)$P_4$] (Eckmann *et al.*, 1997a) which in turn leads to an increase in intracellular chloride secretion and subsequent fluid loss into the intestinal lumen. A point mutation in *sopB* abolishes inositol phosphatase activity *in vitro* and a strain carrying such a mutation does not induce increased levels of Ins (1,4,5,6)$P_4$ levels following infection of HeLa cells (Norris *et al.*, 1998). Mutation of *sopB* has the effect of reducing, but not abolishing, *S. dublin*-induced enteropathogenic responses in bovine ligated ileal loops (Galyov *et al.*, 1997). However, mutation of both *sopB* and *sopD* has a more profound effect on these responses suggesting that effector proteins act together to elicit enteritis (Jones *et al.*, 1998). Recently, another effector protein SopA has been shown to attenuate, but not abolish, enteropathogenesis *in vivo*. Unlike SopB or SopD, this effector protein failed to elicit transepithelial signalling to PMNs suggesting that Sops may have different roles in controlling the influx of inflammatory cells (Wood *et al.*, 2000).

At least one putative effector protein (SseD), encoded on SPI-2 and secreted via TTSS-2, has been shown to attenuate *S. dublin*-induced enteropathogenic responses in calves (Bispham *et al.*, 2001). While the precise mechanisms involved in this process have not been elucidated, it can be speculated that effector proteins secreted via TTSS-2 may influence enteropathogenic responses by directly modulating host cell function. Alternatively this attenuation may be the result of reduced net intracellular growth which may indirectly affect the delivery of virulence factors to host cells (Bispham *et al.*, 2001).

## 1.9 Systemic salmonellosis

The ability to survive within the systemic tissues of a particular host is characteristic of the majority of host-specific or host-restricted, but not ubiquitous, *Salmonella* serotypes (Barrow *et al.*, 1994; Wallis *et al.*, 1997; Watson *et al.*, 2000c; Uzzau *et al.*, 2001). Several factors and virulence mechanisms, which will be discussed in the following sections, contribute to this stage of pathogenesis.

### *1.9.1 Route and mechanism of bacterial dissemination to systemic sites*

The precise route and mechanism by which pathogenic salmonellas disseminate to extraintestinal sites or target tissues has remained largely unstudied, primarily due to logistical difficulties in determining the exact nature of this process *in vivo*. Translocating salmonellas can be found in high numbers within mesenteric lymph nodes, following infection of ligated ileal loops (Wells *et al.*, 1988) and after oral inoculation of different hosts including calves (Wallis *et al.*, 1995; Watson *et al.*, 1998; Villarreal-Ramos *et al.*, 2000). As such, it is generally believed that bacteria disseminate from the intestinal mucosa via the draining lymphatics. This is supported by the observations of a detailed study using Chicago blue dye to delineate the lymphatic drainage pathways for sequential segments of the gastro-intestinal tract in mice orally infected with *S. enteritidis* (Carter and Collins, 1974). Bacteria were recovered from the ileum and caecum 6 hours after infection and were present in the lymph nodes draining these areas, but not in lymph nodes draining uninfected parts of the intestine, 48 hours after infection.

The circulatory system represents the other possible route of dissemination as bacteria may gain access to blood vessels if the damage to the intestinal mucosa is extensive. Indeed, 24 hours after oral inoculation of pre-conditioned guinea pigs with *S. typhimurium*, Takeuchi and Sprinz, (1967) noted that the endothelium of intestinal blood vessels displayed obvious signs of injury, although they saw no bacteria associated with these vessels. It is feasible that both the circulatory and the lymphatic systems are important in bacterial dissemination as the thoracic duct, which carries lymph from the intestine, empties directly into the left subclavian vein.

*In vitro* and *in vivo* studies have demonstrated that salmonellas can interact with host cells in both the intestinal mucosa and at systemic sites (reviewed by Finlay and Brummell, 2000). As such, it is largely accepted that salmonellas translocate in an intracellular niche, which provides an environment for bacterial replication and potentiates a mechanism for dissemination that avoids evoking a primary immune response. However, as many serotypes appear to be resistant to the bactericidal properties of serum (Uzzau *et al.*, 2000a), it can also be speculated that salmonellas may translocate in an extracellular niche.

Due to their location and the observation that salmonellas can interact with resident macrophages in the lamina propria, these cells have been proposed to represent an important target cell contributing to systemic dissemination (Popiel and Turnbull, 1985; Watson *et al.*, 1995; Frost *et al.*, 1997). Furthermore, salmonellas are able to survive and replicate within macrophages *in vitro* and mutants that are unable to survive within these cells are avirulent in mice (Fields *et al.*, 1986). Access to alveolar macrophages, potentiated by foraging or grazing of livestock, may represent an alternative route by which bacteria cross epithelial barriers

(Fedorka-Cray *et al.*, 1995). Interestingly, pneumonia and respiratory disease are common symptoms accompanying *S. dublin* infection in cattle or sheep and *S. choleraesuis* infection in pigs (Jack, 1971; Turk *et al.*, 1992; Jones, 1992) suggesting that the host specific phenotype might be the result of specific mechanisms involving both intestinal and pulmonary dissemination.

A recent study proposed that *S. typhimurium* translocates from the murine intestinal mucosa via the circulatory system in $CD18^+$ leucocytes that are possibly macrophages (Vazques-Torres *et al.*, 1999). However as the authors point out, migration of macrophages from peripheral sites to the bloodstream is not conventionally believed to occur. The authors used a highly mutated strain, which was unable to invade, adhere to M cells or replicate *in vivo*. Furthermore, the authors presented both fluorescence activated cell sorter (FACS) data showing that less than 0.1% of the $CD18^+$ cells in peripheral blood were associated with *Salmonella* and Giemsa-stained microscopy pictures depicting *Salmonella* within unconventional looking monocytes. Together, this data would seem to require confirmation, as it appears to be of questionable relevance to what occurs following infection with naturally virulent salmonellas.

A role for PMNs in the translocation of salmonellas cannot be ruled out as these cells have been shown to harbour *S. typhimurium* at systemic sites during the early stages of murine salmonellosis (Dunlap *et al.*, 1994; Richter-Dahlfors *et al.*, 1997). PMNs represent the first line of defence and are actively recruited to the bovine intestinal mucosa following infection with *S. typhimurium* (Frost *et al.*, 1997; Watson *et al.*, 1998). However depletion of chicken heterophils results in systemic salmonellosis following infection of birds with the ubiquitous serotype *S. enteritidis* (Kogut *et al.*, 1994). In addition, the host specific avian pathogen *S. pullorum*, in contrast to *S. typhimurium*, appears to be able to invade chicken epithelium without recruiting a heterophil infiltration (Henderson *et al.*, 1999). These results suggest that rather than having a role in the translocation process, PMNs or heterophils may serve to limit the infection to the gastrointestinal tract with some combinations of serotype and host.

The ability of dendritic cells to migrate to lymph nodes upon stimulation has been frequently reported (Kelsall and Strober, 1996; Reudl *et al.*, 1996). Accordingly, it can be speculated that these cells may play a crucial role in translocating *Salmonella* from the intestinal mucosa (Ylrid *et al.*, 2000). Recently, *in vivo* and *in vitro* studies have reported that DC are able to open the tight junctions between epithelial cells, send dendrites outside the epithelium and directly sample salmonellas within the intestinal lumen prior to lymphatic dissemination (Rescigno *et al.*, 2001). Furthermore *Salmonella* are able to survive within DC both within the murine lamina propria (Hopkins and Kraehenbuhl, 1997) and *in vitro* using

DC derived from human blood (Schoppet *et al.*, 2000), from flt ligand-treated mice (Marriott *et al.*, 1999) or from a murine DC line (Garcia-Del Portillo *et al.*, 2000).

In addition to phagocytic cells, *in vitro* experiments have demonstrated that *S. typhimurium* is able to invade and survive within immortalised B and T-lymphocytes (Verjans *et al.*, 1994). Although this observation has not been confirmed *in vivo* it is possible, due to the location of lymphocytes within the intestinal mucosa and their ability to translocate to regional lymph nodes, that salmonellas may associate with gut lymphocytes.

### *1.9.2 Survival of Salmonella at systemic sites*

While the survival and persistence of salmonellas within extraintestinal tissues has received a lot of study, particularly in the murine typhoid fever model of infection, the precise location where bacteria reside and proliferate is still a contentious issue. The involvement of different cellular and acellular niches have been suggested including neutrophils (Dunlap *et al.*, 1994) and macrophages or Kupffer cells (Buchmeier and Heffron, 1989; Nnalue *et al.*, 1992; Matsui *et al.*, 2000a). However, host cell death is known to result following *Salmonella* infection of both Kupffer cells isolated from susceptible mice (Harrington and Hormaeche, 1986) and macrophages. The relevance of these observations to *Salmonella* pathogenesis is discussed in section 1.9.7. In addition, non-phagocytic cells such as hepatocytes (Lin *et al.*, 1987; Conlan and North, 1992) and even the extracellular space (Hsu, 1989) have also been proposed to harbour *Salmonella* at systemic sites.

The majority of the inoculum is killed within the first two hours following intravenous administration of *S. typhimurium* to mice (Hormaeche *et al.*, 1993). Consequently, the early stages of systemic salmonellosis have been difficult to study by electron or light microscopy, as the bacterial load within the tissues, following relevant infectious doses, is too low for detection. Several studies have therefore used artificially high inocula to visualise bacteria in the liver and spleen (Lin *et al.*, 1987; Hsu, 1989; Conlan and North, 1992; Nnalue *et al.*, 1992). These results are potentially complicated due to the presence of artefacts resulting from the onset of septic shock. The recent use of confocal laser scanning microscopy (CLSM) to examine immunostained tissue sections from infected murine liver (Richter-Dahlfors *et al.*, 1997) has negated the need for a high inoculum and allows a much larger area of sample to be studied as compared with conventional electron microscopy. Such an approach has led to the conclusion that *S. typhimurium* is likely to reside within murine splenic or hepatic PMNs for up to three days after intravenous inoculation and in macrophages during the later stages of the infectious process.

### *1.9.2.1 Contribution of intra-macrophage survival to host specificity*

Survival within cells of the reticuloendothelial system is believed to be a key stage in *Salmonella* pathogenesis (O'Brien *et al.*, 1979; Fields *et al.*, 1986; Libby *et al.*, 1994; Hensel *et al.*, 1998). Furthermore, there is some evidence that the ability to survive within reticuloendothelial cells contributes to the host specific phenotype *in vivo* (Barrow *et al.*, 1994). As such, several *in vitro* studies have correlated survival and persistence in macrophages with the outcome of infection. For example, *S. typhimurium* is able to persist in higher numbers than *S. typhi* in primary murine macrophages (Lissner *et al.*, 1985; Vladoianu *et al.*, 1990; Alpuche-Aranda *et al.*, 1995; Ishibashi and Arai, 1996; Schwan *et al.*, 2000). Similarly, the virulence of *S. typhi* for humans can be correlated to an ability to persist better in human, compared to murine macrophages (Alpuche-Aranda *et al.*, 1995; Ishibashi and Arai, 1996; Schwan *et al.*, 2000). It has been reported that differences in the recognition of complement receptors may contribute to the intracellular survival of different serotypes (Ishibashi and Arai, 1990; Ishibashi and Arai, 1996). For example, in the presence of opsonin, both *S. typhi* and *S. typhimurium* appear to be selectively recognised via complement receptor 1 on human or murine macrophages respectively.

The relative persistence of *S. typhi* and *S. typhimurium* in human macrophages does however appear to vary between different studies, with *S. typhi* reported to persist in both higher (Alpuche-Aranda *et al.*, 1995; Ishibashi and Arai, 1996) and lower (Vladoianu *et al.*, 1990) numbers than *S. typhimurium*. This variation may be explained by the use of only one strain *per* serotype and/or a failure to quantify macrophage lysis, which can seriously affect interpretation of results from persistence studies (Guilloteau *et al.*, 1996b). In addition, macrophage-derived cell lines are known to be more permissive to *Salmonella* than primary macrophages (Buchmeier and Heffron, 1989; Vladoianu *et al.*, 1990). Consequently studies using these cells to quantify bacterial uptake and persistence have reported conflicting results (Vladoianu *et al.*, 1990; Pascopella *et al.*, 1995; Schwan *et al.*, 1997; Schwan *et al.*, 2000) and should therefore be interpreted with caution. Furthermore, in studies using primary murine macrophages the genetic background of the host needs to be considered carefully. The use of macrophages derived from inherently susceptible mice may differ in their response to infection compared with those obtained from naturally resistant animals (Harrington and Hormaeche, 1986).

Recently, the use of primary porcine alveolar macrophages has demonstrated that there appears to be no correlation between host specificity and persistence of two *S. dublin*, *S. typhimurium* or *S. choleraesuis* strains of defined virulence (Watson *et al.*, 2000c). Similar experiments using bovine alveolar macrophages have shown that *S. typhimurium* and *S. dublin*, but not *S. choleraesuis*, induce significant damage to these macrophages as early as

3 hours after infection (Wallis *et al.*, 1997; Watson *et al.*, 2000b) making it difficult to quantify intra-macrophage persistence in cells derived from this host.

From these experiments it can be suggested that while host specificity does not appear to be determined by the ability to persist within macrophages *in vitro*, variations in experimental technique and source of cells used often make such studies difficult to compare.

### *1.9.3 Bacterial virulence factors associated with survival of salmonellas within the host*

During pathogenesis, *Salmonella* Spp. encounters different niches. As such, bacteria must be able to acquire nutrients, survive within intracellular and extracellular locations and be able to resist a variety of host defence mechanisms aimed at destroying the pathogen. The virulence factors that contribute to the survival of *Salmonella in vivo* and *in vitro* are described in the following sections.

#### *1.9.3.1 Acquisition of iron*

Many pathogenic bacterial species including *Salmonella* require iron for growth and replication. Available iron, within vertebrate hosts, is maintained at low concentrations by combining with a number of high affinity iron-binding proteins, including transferrin in serum or lactoferrin at mucosal surfaces (reviewed by Ratledge and Dover, 2000).

Enteric pathogens can scavenge iron from the host-binding proteins by the production of high affinity iron chelators called siderophores (Finkelstein *et al.*, 1983; Payne, 1988; Litwin and Calderwood, 1993). In some pathogens the production of transferrin-binding proteins appears to be highly specific. For example, *Neisseria* Spp. and *Pasteurella* Spp. produce highly conserved iron-binding proteins that specifically bind the transferrin of only their particular natural host (Morton and Williams, 1989; Fuller *et al.*, 1998). The main siderophore secreted by *Salmonella* is enterobactin (also known as enterochelin) although other iron-supplying mechanisms may be involved in pathogenesis (Kingsley *et al.*, 1995; Reissbrodt *et al.*, 1997). To date, the precise role of siderophores in virulence remains unclear. For example, mutants preventing the synthesis of enterobactin in *S. typhimurium* have been reported to both reduce (Yancey *et al.*, 1979) and to have no effect on virulence (Benjamin *et al.*, 1985) following intraperitoneal infection of mice. More recently it has been proposed that the ability to synthesise enterobactin is not required for full virulence in the murine model, although it is required for growth within serum (Tsolis *et al.*, 1996). It is likely that the extent of extracellular growth that occurs *in vivo* may determine the importance of iron chelating systems in pathogenesis. For example, it has been suggested that once in an intracellular environment *Salmonella* may not require siderophores for iron acquisition because of the relatively high intracellular iron concentrations (Kingsley *et al.*, 1995). This may have direct

relevance to host specificity, as it is known that *Salmonella* encounter both intracellular and extracellular environments during the pathogenic cycle.

*Salmonella* Spp. utilise specialised transport systems that mediate the uptake of siderophore-iron complexes. Most of these require the function of iron-regulated outer membrane proteins as a receptor, the bacterial outer membrane protein TonB which couples the energised state of the inner membrane with the outer membrane (Kingsley *et al.*, 1996; Tsolis *et al.*, 1996), a transporter complex to take iron across the inner membrane and soluble enzymes for the release of iron from the ferrisiderophore complex (Kingsley, 1997). In iron deficient media the growth of many bacterial species, including *Salmonella*, triggers the expression of iron regulated genes. Among enteric bacteria, this process is controlled at the transcriptional level by the regulatory protein feric uptake regulation (Fur) (Hantke *et al.*, 1984; Bagg and Neilands, 1987). All genes involved in the synthesis of siderophores and transport of ferrisiderophore complexes in *E. coli* are negatively regulated by the Fur protein. In the absence of iron, Fur cannot bind and transcription is derepressed (Litwin and Calderwood, 1993).

#### *1.9.3.2 Salmonella stress and nutritional requirements*

In order to survive frequently encountered nutrient limitation and environmental stresses, enteric pathogens such as *Salmonella* must respond to and withstand potentially lethal extremes of natural environments (reviewed by Spector, 1998).

Analysis of host restricted/specific or ubiquitous *Salmonella* serotypes has determined a difference in their nutritional requirements. For example, while *S. typhimurium* and *S. enteritidis* are able to grow on relatively simple defined media *in vitro*, specific amino acids or vitamins must be added to support the growth of serotypes with a restricted host range. *S. abortusovis* and *S. gallinarum* require the amino acid cysteine and in addition *S. gallinarum* requires leucine, aspartic acid and the vitamin thiamine while *S. abortusovis* also requires nicotinic acid and thiamine (Stokes and Bayne, 1958). *S. typhi*, *S. choleraesuis* and *S. dublin* require tryptophan, cysteine or nicotinic acid respectively (Virgilo and Cordano, 1981; Fierer and Fleming, 1983). The relevance of these requirements to bacterial virulence is not fully understood, but two explanations have been suggested. First, it is possible that the evolution of certain *Salmonella* serotypes towards a limited host range may have involved genetic rearrangements that induced specific nutritional requirements. Alternatively, the high content of specific amino acids or vitamins in a host may have supported the growth of particular serotypes and as such restricted their host range (Fierer and Fleming, 1983).

*Salmonella* serotypes with mutations in stress genes, for example, *htrA*, regulatory genes, for example *phoP* and *cya/crp* or nutritional genes, for example aromatic (*aro*) or

purine (*pur*) biosynthesis, exhibit reduced virulence in different hosts and as such are potential candidates for vaccine strains (reviewed by Mastroeni *et al.*, 2001). However, not all mutations that attenuate a particular serotype in one species fully attenuate virulence in other serotype-host combinations (Hone *et al.*, 1988; Tacket *et al.*, 2000; Villarreal-Ramos *et al.*, 2000; Mastroeni *et al.*, 2001) highlighting the importance of not extrapolating data from one bacterial-host combination to another.

#### *1.9.3.3 Resistance to the bactericidal action of serum*

The ability of bacteria to resist serum killing may be viewed as a virulence factor that facilitates survival within the extracellular environment of the host. The length of the bacterial LPS has been shown to influence the degree of serum resistance as certain 'O' side chains stimulate complement deposition by the alternative pathway better than others (Grossman *et al.*, 1986). Furthermore, smooth strains are typically more resistant to complement mediated lysis than rough mutants (Joiner, 1985; Grossman *et al.*, 1987; Nnalue *et al.*, 1990). This has been attributed to increased steric hinderance which potentially allows complement factors to be deposited on the ends of the LPS thereby reducing the amount of membrane attack complex (MAC) reaching the bacterial cytoplasmic membrane (Joiner, 1982)

Three plasmid-encoded genetic loci have been identified in some *Salmonella* serotypes that are believed to be important in the resistance to complement-mediated serum lysis namely *traT*, *rsk*, and *rck* (reviewed by Rotger and Casadesús, 1999). The *S. typhimurium traT* gene appears to influence plasmid-mediated serum resistance and virulence following intraperitoneal inoculation of mice, but not net growth within liver macrophages (Rhen and Sukuplovi *et al.*, 1988). The regulation of serum killing (*rsk)* gene is proposed to be involved in the regulation of serum resistance in *S. typhimurium* (Vandenbosch *et al.*, 1989a; Vandenbosch *et al.*, 1989b), while the *rck* gene is believed to confer resistance of *S. typhimurium* to human serum possibly by blocking the MAC on the bacterial surface (Heffernan *et al.*, 1992a; Heffernan *et al.*, 1992b Guiney *et al.*, 1994).

The contribution of serum resistance to the host specific phenotype is at present inconclusive. For example, it could be speculated that survival of different serotypes within the serum of their natural host species might offer a pathogenic advantage. As such, it has been reported that *S. gallinarum*, but not *S. enteritidis*, is resistant to chicken serum (Collins, 1967). Conversely, it is postulated that host-restricted or host-specific serotypes gain access to systemic sites via pathways that facilitate translocation in an intracellular niche (Pascopella *et al.*, 1995; Henderson *et al.*, 1999; Vazques-Torres *et al.*, 1999) suggesting that resistance to serum killing may be less important. Furthermore, the ability of different ubiquitous, host-specific or host-resistant serotypes to survive within serum from different animals shows no

correlation with the host specific phenotype (Barrow *et al.*, 1994; Wallis *et al.*, 1997; Uzzau *et al.*, 2000a).

### *1.9.4 Molecular contribution to Salmonella survival and persistence*

*Salmonella* pathogenicity island-2 (SPI-2), which encodes more than 40 genes including several putative effector proteins, two putative chaperones and a second type three secretion system (TTSS-2) (Hensel, 2000) (figure 1.9), contributes to the systemic phase of pathogenesis in mice and calves (Shea *et al.*, 1996; Bispham *et al.*, 2001). This locus has at least two distinct functions. SPI-2 has been shown to promote net intracellular growth in macrophages and epithelial cells both *in vitro* and *in vivo* and is discussed in section 1.9.5. In addition SPI-2 is believed to protect *Salmonella* from oxidative killing within infected macrophages and this is discussed in section 1.9.5.1.

Regulation of SPI-2 encoded genes *in vitro* (Deiwick *et al.*, 1999) and *in vivo* (Cirillo *et al.*, 1998) is dependent on the local two-component regulatory system *ssrA* and *ssrB* which in turn is modulated by global regulatory systems outside of SPI-2 including OmpR/EnvZ (Lee *et al.*, 2000) and PhoP/PhoQ (Deiwick *et al.*, 1999) (section 1.9.5.2).

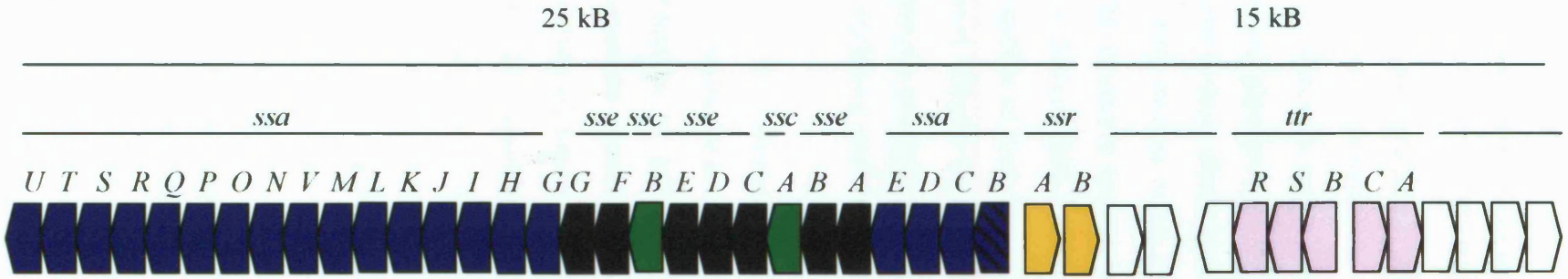



**Figure 1.9 Genetic organisation of SPI-2**

Genes encoding proteins involved in apparatus assembly or structure (blue); putative effectors (black); chaperones (green); regulators (yellow); structural components of the anaerobic tetrathionate reductase (pink); uncharacterised proteins (white).

Gene abbreviations: *ssa*, secretion system apparatus; *sse*, secretion system effector; *ssc*, secretion system chaperone; *ssr* secretion system regulator; *ttr*, tetrathionate reductase

Reference sources: Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Hensel, 2000

### *1.9.5 Bacterial survival within the Salmonella containing vacuole (SCV)*

Following entry into host cells, bacteria become localised within a membrane-bound 'spacious phagosome' or SCV. It is believed that the SCV undergoes transient interactions with early endosomes but does not fuse with late endosomes or mature lysosomes (Garcia-del Portillo and Finlay, 1995a; Garcia-del Portillo and Finlay, 1995b; Rathman *et al.*, 1997; Méresse *et al.*, 1999; Steele-Mortimer *et al.*, 1999). This process appears to be regulated by at least one putative SPI-2 effector protein, SsaB, which is translocated into the cytoplasm of infected macrophages where it appears to influence intracellular trafficking (Uchiya *et al.*, 1999). Although not associated with the normal endocytic pathway, the SCV acquires lysosomal glycoproteins (lgp), such as LAMP-1, by recruitment of pre-existing lgp-containing vesicles without direct interaction with lysosomes (Méresse *et al.*, 1999; Steele-Mortimer *et al.*, 1999). Several hours after invasion of epithelial cells, bacteria become localised within tubular structures known as *Salmonella*-induced filaments (Sifs) (Garcia-del Portillo *et al.*, 1993). Several SPI-2 and non-SPI-2 encoded proteins are believed to be important for aggregation of host endosomes (Guy *et al.*, 2000). These include SifA, a putative TTSS-2 secreted effector protein (Stein *et al.*, 1996; Brumell *et al.*, 2001), that is induced after *Salmonella* enters host cells and functions to maintain the integrity of the intracellular vacuole thus enclosing replicating bacteria (Beuzón *et al.*, 2000).

#### *1.9.5.1 Resistance to oxygen dependent killing mechanisms*

Professional phagocytes posses both oxygen-dependent and oxygen-independent mechanisms to kill intracellular bacteria. Oxygen-dependent mechanisms involve the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which catalyses the production of superoxide leading to the metabolism of a variety of toxic reactive oxygen species (De Groote *et al.*, 1997; Mastroeni *et al.*, 2000). Potentially lethal levels of reactive oxygen species are produced by the host during this phagocytic oxidative burst, which can damage bacterial DNA, proteins and cell membranes, (Babior, 1992). The oxidative burst is followed by a prolonged nitrosative bacteriostatic phase, which is dependent on inducible nitric oxide synthase (iNOS) (Shiloh *et al.*, 1999).

Many pathogens have developed strategies to resist the antimicrobial effects of reactive oxygen and nitrogen species by the production of molecular scavengers, antioxidant enzymes, repair systems and expression of specific antioxidant regulons (Farr and Kogma, 1991). For example, resistance to nitric oxide (NO) and similar nitrogen compounds is mediated in part by synthesis of homocysteine, a possible antagonist of NO (De Groote *et al.*, 1996). Furthermore, *Salmonella* produce at least one, and sometimes two superoxide dismutases (Sod). One of these dismutases, SodC, is required for full virulence in several

*S. enterica* serotypes following oral inoculation of mice (Farrant *et al.*, 1997). However, no difference was observed between *sodC* or the parental strain for survival in murine peritoneal or J774 macrophages.

It has recently been proposed that through the action of unknown SPI-2 effectors *Salmonella* inhibits trafficking of NADPH oxidase to the SCV (Vazquez-Torres *et al.*, 2000) thereby reducing the oxidant stress encountered by the bacteria and maintaining an intracellular environment conducive to bacterial replication. Consistent with this hypothesis, the ability of *S. typhimurium* SPI-2 mutant strains to survive in macrophages and cause lethal infection can be restored when administered intraperitoneally to mice containing macrophages unable to undergo the oxidative burst (deficient in the gp91 *phox* subunit of the phagocyte NADPH oxidase) (Vazquez-Torres *et al.*, 2000). As such, exclusion of the NADPH oxidase from the phagosome may contribute to the virulence of intracellular pathogens.

#### *1.9.5.2 Resistance to oxygen-independent killing mechanisms and regulation of intramacrophage gene expression*

Oxygen independent bactericidal mechanisms of phagocytes include acidification of the SCV and the secretion of bactericidal peptides into this compartment (Ganz and Weiss, 1997). Once inside the phagocyte, *Salmonella* possess an array of co-ordinately-regulated genes to facilitate survival within the hostile intracellular environment. These include genes necessary for repair of macrophage-induced damage and for adaptation to stresses induced by changes in pH, osmolarity and nutrient availability (Mekalanos, 1992; Bajaj *et al.*, 1996; Heithoff *et al.*, 1999; Lucas and Lee, 2000). A large number of these genes have been shown to be positively regulated by the PhoP/PhoQ two component system (Groisman *et al.*, 1989; Miller *et al.*, 1989). In low $Mg^{2+}$ and low $Ca^{2+}$ conditions, typical of those encountered in the intracellular niche, there is expression of PhoP-activated genes (*pag*) and repression of PhoP repressed genes (*prg*) (reviewed by Cotter, 2000). Other genes necessary for intramacrophage survival include those encoded by the two component regulatory system *pmrAB* (required for resistance to cationic antimicrobial peptides) (Gunn and Miller, 1996; Soncini and Groisman, 1996) and those required for regulation of the acid tolerance response (Bearson *et al.*, 1998). Additionally, while expression of the *Salmonella* plasmid virulence (*spv*) operon is controlled directly by *spvR* (section 1.9.6), a role for both PhoP/PhoQ and RpoS in regulation of SpvR has also been proposed (Wilson and Gulig, 1998; Heithoff *et al.*, 1999).

### *1.9.6 Role of the Salmonella virulence plasmid during systemic pathogenesis*

At least sixteen serotypes, including *S. typhimurium*, *S. choleraesuis*, *S. dublin*, *S. abortusovis* and *S. gallinarum* are known to posses a high molecular weight (50 to 90 kb) 'virulence plasmid' (Williamson *et al.*, 1988a; Gulig, 1990; Boyd and Hartl, 1998). To date, the exact role of this plasmid in *Salmonella* pathogenesis still remains unclear (reviewed by Rotger and Casadesús, 1999). For example, the host-specific serotype *S. typhi* does not contain a typical *Salmonella* virulence plasmid, but rather some strains posses a cryptic plasmid which shows recent common ancestry with a *Yersinia pestis* virulence plasmid (Parkhill *et al.*, 2001). Furthermore, introduction of the *S. dublin* virulence plasmid into *S. typhi* does not confer mouse virulence to the hybrid *S. typhi* strain (Roudier *et al.*, 1990).

A conserved 7.8-kb region, encoding the *Salmonella* plasmid virulence (*spv*) operon, is required for expression of full infectivity (Williamson *et al.*, 1988b; Lax *et al.*, 1990) and this region can restore murine virulence to plasmid-cured strains (Gulig *et al.*, 1993). The *spv* region encodes five genes, *spvR* (regulator) and the *spvABCD* operon (Gulig *et al.*, 1993), which are expressed during the stationary growth phase and the intracellular stages of infection (Norel *et al.*, 1992). Studies comparing isogenic plasmid-cured or naturally plasmid-free strains with the wild type parent have demonstrated that the virulence plasmid appears to contribute to the systemic phase of infection in different hosts including mice (Jones *et al.*, 1982; Nakamura *et al.*, 1985; Gulig and Curtiss III, 1987), pigs (Danbara *et al.*, 1992), calves (Wallis *et al.*, 1995; Libby *et al.*, 1997), sheep (Uzzau *et al.*, 2000b) and fowl (Barrow and Lovell, 1988).

In mice, plasmid genes have been implicated in increasing the growth rate of bacteria in the liver and spleen possibly within macrophages (Gulig and Doyle, 1993; Gulig *et al.*, 1993; Guilloteau *et al.*, 1996a; Gulig *et al.*, 1998; Matsui *et al.*, 2000b) but not non-phagocytic cells (Gulig *et al.*, 1998). In calves it has been suggested that the *S. dublin* virulence plasmid is required for systemic, but not enteric, virulence possibly by mediating persistence of this serotype within the liver and spleen (Wallis *et al.*, 1995). By contrast, it has also been proposed that in calves the *S. dublin spvR* gene is required for enhanced intracellular proliferation in both intestinal tissues and at extraintestinal sites (Libby *et al.*, 1997). These contrasting observations relating to the role to the virulence plasmid in the induction of bovine enteritis could possibly be attributed to the use of different inocula sizes, strains and mutations in addition to the experimental techniques used to assess enteropathogenesis or diarrhoea.

The virulence plasmids of several serotypes including *S. dublin*, *S. typhimurium*, *S. abortusovis* and *S. choleraesuis* are also involved in the non-apoptotic lysis of resident or activated murine peritoneal macrophages *in vitro* and recruitment of inflammatory cells to the

foci of infection *in vivo* (Guilloteau *et al.*, 1996a; Gautier *et al.*, 1997). However expression of the *spv* operon does not appear to be involved in this process (Guilloteau *et al.*, 1996b) suggesting the presence of as yet uncharacterised plasmid genes involved in macrophage lysis. In contrast, a recent study proposed that lysis of human macrophages requires expression of the *spv* operon and occurs as a result of apoptosis (Libby *et al.*, 2000). These results implicate a role for different virulence plasmid genes in lysing macrophages by distinct mechanisms, which may or may not be dependent on the bacterial growth phase within cells (Gautier *et al.*, 1997; Libby *et al.*, 2000; Van der Velden *et al.*, 2000).

Other loci found on the *Salmonella* virulence plasmid, such as the fimbrial operon *pef* (section 1.6.6.1) and genes required for serum resistance (section1.9.3.3) may play a role in other stages of pathogenesis.

### *1.9.7 Contribution of bacterial-induced cell death to Salmonella pathogenesis*

Apoptosis is a normal cellular process, characterised by the maintenance of membrane integrity during cell shrinkage, membrane blebbing, nuclear condensation and DNA fragmentation resulting in cell death in the absence of an immune response. In contrast, necrotic cell death results from acute cellular injury and may be characterised by rapid cell swelling and lysis resulting in a strong pro-inflammatory response (Majno and Joris, 1995; McConkey, 1998; Weinrauch and Zychlinsky, 1999). Induction of apoptosis may contribute significantly to pathogenesis as a bacterial strategy to evade the immune response to infection or to assist exploitation of a new niche. Alternatively, this process may be a host response facilitating removal of the *Salmonella*-containing phagocytes thereby limiting pathogen spread.

While the ability of many pathogens to reside within cells of the reticuloendothelial system is frequently cited as contributing to pathogenesis (Barrow *et al.*, 1994), several bacterial species, including *Salmonella*, *Shigella* and *Yersinia* have been observed to kill target host cells (Weinrauch and Zychlinsky, 1999). These observations appear to contradict the requirement for bacterial proliferation within an intracellular niche however, to date the precise mechanisms by which host cells die remains controversial. For example, numerous reports have proposed that *Salmonella*-induced cell death occurs by apoptosis (Chen *et al.*, 1996; Lindgren *et al.*, 1996; Monack *et al.*, 1996; Richter-Dahlfors *et al.*, 1997; Kim *et al.*, 1998) although evidence to support a role for this form of host cell death warrants careful consideration. For example, several studies have monitored cell death by the uptake of non-membrane permeative dyes such as trypan blue or ethidium bromide (Chen *et al.*, 1996; Monack *et al.*, 1996). However, the documented steady uptake over time of these dyes is inconsistent with cell death by apoptosis where membrane integrity is retained until the

induction of secondary necrosis. Furthermore, at least two frequently used methods for the detection of cell death, namely terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling (TUNEL) staining and other assays detecting DNA fragmentation, are not entirely specific for detection of apoptosis as DNA fragmentation may also occur during necrosis (Bortner *et al.*, 1995; Grasl-Kraupp *et al.*, 1995).

Cell lysis has been shown to be dependent on the SPI-1 encoded protein SipB (Hersh *et* al., 1999) and the logarithmic phase of bacterial growth (Lundberg *et al.*, 1999). SipB is believed to bind to, and activate, caspase-1, a pro-apoptotic, pro-inflammatory protease that converts the precursor form of interleukin 1β (IL-1β) to its mature form (Hilbi *et al.*, 1998; Hersh *et* al., 1999). Although caspase-1 is required for inflammation, the role for this protease in the induction of apoptosis is disputed. For example, caspase-1 deficient mice do not produce IL-1β and resist endotoxic shock but demonstrate no significant defects in apoptosis (Kuida *et al.*, 1995; Li *et al.*, 1995; Li *et al.*, 1997). In addition, the proposal that cell death may increase the local inflammatory response, via the eukaryotic release of IL-1β (Zychlinsky and Sansonetti, 1997) is inconsistent with the widely held view that apoptosis limits inflammation (Savill, 1997). It is conceivable that the precise mechanism of cell death depends on physiological conditions, bacterial location and the time cause of infection. For example, it has been proposed that in addition to SPI-1 dependent cell death, a delayed SPI-1 independent mechanism of host cell killing occurs in *S. typhimurium*-infected murine bone marrow-derived (Van der Velden *et al.*, 2000), human (Libby *et al.*, 2000) and bovine monocyte-derived macrophages (Santos *et al.*, 2001a). This delayed cell death might contribute to the systemic phase of infection as both SPI-2 (Van der Velden *et al.*, 2000) and *spv* genes (Libby *et al.*, 2000) have been implicated in this process.

Recently, it has been demonstrated that *S. dublin* and *S. typhimurium* are capable of lysing bovine and porcine alveolar macrophages *in vitro* by a SipB, caspase 1-dependent mechanism distinct from apoptosis (Watson *et al.*, 2000b; Watson *et al.*, 2000c). Furthermore, *S. typhimurium* has been demonstrated to lyse murine peritoneal macrophages by a similar mechanism (Brennan and Cookson, 2000). As such, it has been proposed that *Salmonella*-induced cell death may be the result of necrosis rather than apoptosis. While the fate of intracellular bacteria trapped within apoptotic cells remains unclear, necrotic cell death might represent an integral part of pathogenesis potentiating escape from host cells, followed by extracellular growth or the reinfection of other immune cells. This hypothesis is supported by the observations that many serotypes are able to withstand the bactericidal activities of complement *in vitro* (Wallis *et al.*, 1997; Uzzau *et al.*, 2000a). The ability to lyse bovine, porcine or cultured murine macrophages *in vitro* does not appear to correlate with host specificity (Chen *et al.*, 1996; Wallis *et al.*, 1997; Watson *et al.*, 2000b), but may depend on

serotype specific factors as *S. choleraesuis* is less able to lyse both porcine and bovine alveolar macrophages than *S. dublin* or *S. typhimurium* (Wallis *et al.*, 1997; Watson *et al.*, 2000c).

To date only a few studies have examined the role of cell death *in vivo*. CLSM examination of immunolabelled sections from infected murine liver has shown that apoptosis of phagocytic cells is induced following intravenous inoculation with *S. typhimurium* 3 days after infection (Richter-Dahlfors *et al.*, 1997). However, another study demonstrated that mice deficient for Caspase-1 (*casp-1*$^{-/-}$) were resistant to colonisation by wild type *S. typhimurium* and the oral, but not the intraperitoneal, 50% lethal dose was 1000 fold higher than that of normal mice (Monack *et al.*, 2000). Furthermore, 1 hour after infection of murine ligated ileal loops, Peyer's patches from wild type, but not *casp-1*$^{-/-}$ mice, contained an increase in the number of cells undergoing apoptosis. Together these results suggest that caspase-1 is required for colonisation of the murine intestine and subsequent induction of systemic disease. However, the induction of apoptosis following intravenous inoculation may be independent of caspase-1. In calves it has recently been shown that *S. typhimurium*-induced cell death is not required for induction of fluid secretion and inflammation in ligated ileal loops (Santos *et al.*, 2001b). This observation cannot however be directly compared with results obtained in the murine model due to the contrasting nature of the disease, typically systemic in mice and enteric disease in calves, resulting from infection with *S. typhimurium*.

### 1.10 Immunity and host resistance to *Salmonella*

Resistance to infectious diseases relies on the activation of the innate (aspecific) and acquired (specific) immune systems. The innate system of the gastrointestinal tract is activated in response to invading pathogens and is composed of physical and chemical barriers to infection. These include cellular components for example PMNs, macrophages, DC, natural killer (NK) cells and molecular components for example activation of complement, acute phase proteins and antibacterial cationic peptides. Specific immunity rests on recognition and binding of non-self antigens (pathogens) by cell receptors. This is followed by a cascade of mechanisms, which ultimately inactivate the pathogen. Simultaneously memory, for the specific pathogen, is established such that in the case of renewed contact a fast and efficient specific immune response will follow.

Much of our understanding of mechanisms contributing to immunity to *Salmonella* and determination of the immunogenicity and safety of *Salmonella* vaccines have been derived from studies using the murine typhoid fever model (reviewed by Hormaeche *et al.*, 1993; Hormaeche *et al.*, 1995; Mastroeni *et al.*, 2001). The progression of salmonellosis following sub-lethal intravenous inoculation of naïve mice can be divided into at least four

distinct phases, which will be discussed in brief in the following sections. These include the initial inactivation of a large part of the inoculum, the exponential growth in the reticuloendothelial system (RES), the plateau phase and clearance of bacteria from the systemic tissues (Collins, 1974; Hormaeche *et al.*, 1993).

### *1.10.1 Initial inactivation of the inoculum*

Following intravenous inoculation of *S. typhimurium*, initial inactivation of a large proportion of the challenge dose is primarily controlled by the activities of PMNs and mononuclear phagocytes that may engulf invading pathogens. Phagocytic cells are able to control the growth of virulent *Salmonella* by the production of both NADPH oxidase, leading to the metabolism of a variety of toxic reactive oxygen species and reactive nitrogen intermediates, generated via the inducible nitric oxide synthase (iNOS) (De Groote *et al.*, 1997; Shiloh *et al.*, 1999; Mastroeni *et al.*, 2000). Recently it has been demonstrated that reactive oxygen intermediates are required for host resistance during the early stages of infection whilst reactive nitrogen intermediates are important during the later stages of the disease (Mastroeni *et al.*, 2000; Vazquez-Torres and Fang, 2001). In addition, oxygen-independent mechanisms such as the actions of defensins, lysozyme, cathepsin G and azurocidin (Groisman *et al.*, 1992) are also believed to contribute to the initial elimination of *Salmonella*. The phagocytic secretion of pro-inflammatory cytokines, including IL-1, IL-6 and TNF-α, have important local and systemic effects and mediate recruitment of more inflammatory cells and effector molecules to the site of infection. Furthermore, attack by the alternative pathway of complement activation can opsonise or destroy bacteria providing a non-adaptive early line of defence against many pathogens.

### *1.10.2 The exponential growth phase*

Between 2 and 7 days after inoculation, bacteria grow exponentially within the host reticuloendothelial system. The rate of growth during this phase depends on several factors including the virulence of the strain, the bacterial load and the immune status and genetic susceptibility of the host (Hormaeche *et al.*, 1993). The host response is largely determined by the phagocytic activities of PMNs and macrophages.

In mice, the major locus influencing host resistance to immunity is *Nramp1* (formally referred to as *immunity to typhimurium* [*Ity*], *Lsh* or *Bcg*) (Plant and Glynn, 1974) which is found on chromosome 1 (Plant and Glynn, 1979). *Nramp1* is predominantly expressed on macrophages (Govoni *et al.*, 1995; Lang *et al.*, 1997), and possibly granulocytes (Van Dissel *et al.*, 1986), and encodes a protein called natural resistance-associated macrophage protein1 (Nramp1) (Vidal *et al.*, 1993). Nramp1 is believed to mediate a bactericidal role, potentially

by pumping $Fe^{2+}$ into the phagosome thereby catalysing the formation of oxygen radicals within the *Salmonella*-containing vacuole (Vidal *et al.*, 1993; Cellier *et al.*, 1994; Gunshin *et al.*, 1997). Susceptibility to infection in mice is the result of a single point mutation in *nramp1* (Vidal *et al.*, 1995). Mice expressing a resistant allele (*Nramp*$^r$) are more able to survive the early stages of salmonellosis than susceptible mice (*Nramp*$^s$) (Vidal *et al.*, 1995; Govoni *et al.*, 1999).

Homologous *Nramp1* genes have been characterised in many phylogenetically distinct organisms including cattle (Feng *et al.*, 1996), pigs (Tuggle *et al.*, 1997), birds (Hu *et al.*, 1995), insects and plants (Cellier *et al.*, 1996). It remains to be determined whether the role of *Nramp1* in different vertebrates is similar to its function in mice. In poultry, however this gene only has a small influence on resistance to salmonellosis (Hu *et al.*, 1997).

In addition to *Nramp1* several other genetic loci, which influence host resistance to infection, have been characterised. In mice, the chronic phase of the infection is affected by, among others, the *Lps* (lipopolysaccharide) gene that determines the responsiveness of macrophages to bacterial LPS (O'Brien and Rosenstreich, 1983: Kovárová *et al.*, 1997). In chickens the salmonellosis-resistance gene1 (*sal1*), which is located on chromosome 5, influences the rate of multiplication of *Salmonella* within macrophages and as such is responsible for some of the differences in susceptibility of chickens to salmonellosis. Furthermore, another gene has been identified in chickens that influences intestinal colonisation, but not systemic disease, of *S. typhimurium*, *S. enteritidis* and *S. infantis* (Dr Paul Barrow, personal communication).

### *1.10.3 The plateau phase*

In immunocompetent mice, bacterial growth in the reticuloendothelial system is controlled by the end of the first week of infection resulting in the establishment of the plateau phase (O'Brien and Metcalf, 1982; Maskell *et al.*, 1987; Hormaeche *et al.*, 1990; Nauciel, 1990). This host response requires bone marrow-derived cells, leading to the formation of granulomas that contain numerous mononuclear cells (Hormaeche *et al.*, 1990; Mastroeni *et al.*, 1995). Several cytokines including tumour necrosis factor α (TNF-α), interferon γ (IFN-γ), IL-12, IL-15 and IL-18 are important mediators of this phase and are involved in the suppression of bacterial growth within systemic sites (reviewed by Mastroeni *et al.*, 2001). TNF-α is required primarily for granuloma formation (Mastroeni *et al.*, 1992; Mastroeni *et al.*, 1995) whereas IFN-γ is important for macrophage activation and contributes to the formation of focal granulomas in the tissues (Kagaya *et al.*, 1989; Mastroeni *et al.*, 1992; Muotiala and Mäkelä, 1993). IL-12 and IL-18 are necessary for the induction of IFN-γ (Mastroeni *et al.*, 1996; Mastroeni *et al.*, 1999), while IL-15 is thought to be responsible for

the activation of natural killer cells at infected sites together with the production of IFN-γ (Hirose *et al.*, 1999).

### *1.10.4 Bacterial clearance*

Following the plateau phase, bacteria are cleared from the reticuloendothelial system by a process that is dependent on αβ $TCR^+$ $CD4^+$ T-lymphocytes (O'Brien and Metcalf, 1992; Nauciel, 1990; Hess *et al.*, 1996). In the absence of $CD4^+$ T-lymphocytes, a persistent infection can develop which may lead to the re-initiation of bacterial growth and death of the infected host. Failure of the bacterial clearance mechanisms, in any host, can lead to persistent, low level infection and intermittent shedding (Collins, 1974). In mice, host genes, both within and outside the major histocompatibility complex (Hormaeche *et al.*, 1985; Nauciel *et al.*, 1990) modulate the rate of bacterial clearance.

### *1.10.5 Acquired immunity*

Acquired immunity is the response of antigen-specific lymphocytes to antigen, including the development of immunological memory. Acquired immunity is generated by the clonal selection of lymphocytes.

Following the use of effective live *Salmonella* vaccines, bacterial clearance can provide long lasting immunity to rechallenge with virulent organisms. Both cell-mediated, involving antigen-specific T-lymphocytes, and humoral immune responses, antibody-mediated involving B-lymphocytes, appear to be necessary to modulate clearance of *S. typhimurium* infection in mice (reviewed by Mastroeni *et al.*, 2001). Many studies determining the effectiveness of live *Salmonella* vaccines have used innately susceptible mice in which both arms of the immune response are required for protection. In contrast killed vaccines, which induce humoral immune responses, yet elicit poor cell-mediated responses, can protect innately resistant mice better than innately susceptible animals (Eisenstein *et al.*, 1984; Hormaeche *et al.*, 1993; Hormaeche *et al.* 1995).

### *1.10.6 Cell mediated immune responses*

T-lymphocytes, in addition to their role in the clearance phase of infection, participate in the protective immunity that develops after live *Salmonella* vaccination of different hosts including mice (Villarreal *et al.*, 1992; McSorley *et al.*, 2000a), calves (Villarreal-Ramos *et al.*, 1998) and sheep (Gohin *et al.*, 1997).

The recall of immunity and protection to oral challenge in susceptible mice requires $CD4^+$ and $CD8^+$ T-lymphocytes, IFN-γ and TNF-α (Nauciel, 1990; Mastroeni *et al.*, 1992; Nauciel and Espinasse-Maes, 1992). $CD4^+$ T-lymphocytes are believed to have a particularly

important role as depletion of $CD4^+$, as opposed to $CD8^+$ T-lymphocytes, has been shown to have a more pronounced effect on the control of both primary *Salmonella* infection and on protection induced following vaccination (Nauciel, 1990; Mastroeni *et al.*, 1992; Pie *et al.*, 1997). However, there is also evidence of a role for $CD8^+$ T-lymphocytes in immunity to *Salmonella* as depletion of this cell phenotype reduces the ability to transfer protection against virulent *S. typhimurium* (Nauciel, 1990; Mastroeni *et al.*, 1992). Despite the importance of cell-mediated immunity during responses to *Salmonella*, the mechanisms underlying protection are not yet fully understood. It is widely accepted that macrophage activation is one of the pivotal elements for protective immunity against intracellular bacteria (Mackaness, 1971). As such production of the macrophage-activating cytokines, particularly IFN-γ is important in the host response against these pathogens (Kaufmann, 1993). Infection of mice with *Salmonella* induces a Th1 response, characterised by the production of large amounts of IFN-γ (Thatte *et al.*, 1993; Pie *et al.*, 1997) and mice deficient in the IFN-γ receptor have been shown to be highly susceptible to attenuated *Salmonella* (Hess *et al.*, 1996). However it has been suggested that IFN-γ is only important during the initial phase of *Salmonella* infection and that neutralisation of IFN-γ at later stages of infection is not important for host defence (Muotiala and Mäkelä, 1993; Pie *et al.*, 1997). It is possible that IFN-γ-independent T-lymphocyte mechanisms are also involved in the control of *S. typhimurium* which could include production of other macrophage-activating cytokines such as IL-4 and IL-10 (Pie *et al.*, 1996; Everest *et al.*, 1997; Pie *et al.*, 1997).

### *1.10.7 Humoral immune responses*

Infection of mice with *S. typhimurium* results in a potent antibody response against bacterial surface components, such as LPS, porins, outer membrane proteins, flagella and fimbriae (Brown and Hormaeche, 1989; Harrison *et al.*, 1997; McSorley *et al.*, 2000b). However the precise identification of the protective antigens in immunised animals and the role of antibodies in protection remains poorly defined (Eisenstein, 1999).

The role of antibodies in protection against *Salmonella* appears to depend, in part, on the genetic background of the host. Vaccination of resistant mice with either attenuated or killed *Salmonella* has been shown to induce protection against subsequent challenge that could be passively transferred to naïve mice by serum (Eisenstein *et al.*, 1984). These results suggest that antibodies alone are sufficient for the control of virulent bacteria. In contrast, the use of innately susceptible mice has shown that killed bacterial vaccines elicit only partial protection against subsequent challenge and protection could not be transferred by serum (Eisenstein *et al.*, 1984). Vaccination of susceptible mice with live vaccines induced protection against challenge with virulent bacteria (Killar and Eisenstein, 1985) suggesting

that both serum and T-lymphocytes were required for transferring full protection (Mastroeni *et al.*, 1993).

## 1.11 Conclusion to introduction

A summary of *Salmonella* pathogenesis and possible mechanisms contributing to serotype-host specificity have been discussed in this introduction. Although our current understanding of host-range determining factors by different serotypes is limited, it is clear that the use of biologically relevant models is imperative for such studies. It can be speculated that, among other host and bacterial factors, specific adhesins might mediate serotype or host-specific attachment to epithelial cells. In addition, the ability to invade the intestinal mucosa, by a specific route, and/or induce enteropathogenic responses, resulting in disruption of the epithelial barrier, may contribute to the outcome of infection. Furthermore, the bacterial ability to disseminate, survive and replicate within specific host cells or systemic tissues may influence host specificity. A schematic overview of possible mechanisms contributing to systemic disease and host specificity are shown in figure 1.10.

### *1.11.1 Thesis aims and objectives*

The objective of this thesis is to contribute to a better understanding of *Salmonella* serotype-host specificity in calves by characterising the association of five serotypes, of diverse natural host prevalence, for their ability to interact with enteric and systemic tissues *in vivo*.

The specific aims of this thesis are as follows:

1. Determine the virulence of *S. dublin*, *S. typhimurium*, *S. choleraesuis*, *S. gallinarum* and *S. abortusovis* following both oral and intravenous inoculation of calves, for up to seven days *post* infection.
2. Quantify the magnitude of invasion of different serotypes both *in vitro*, using cultured epithelial cells, and *in vivo*, using the bovine ligated ileal loop model, and assess the relative importance of Peyer's patch tissue, compared with absorptive epithelium, in the invasion process *in vivo*.
3. Quantify the magnitude of the *Salmonella*-induced enteropathogenic response following infection of bovine ligated ileal loops with different serotypes.
4. Develop a model to study *Salmonella* dissemination from the intestine to systemic sites.
5. Compare the route and kinetics of *S. dublin* and *S. gallinarum* translocation from the intestinal mucosa to systemic tissues.

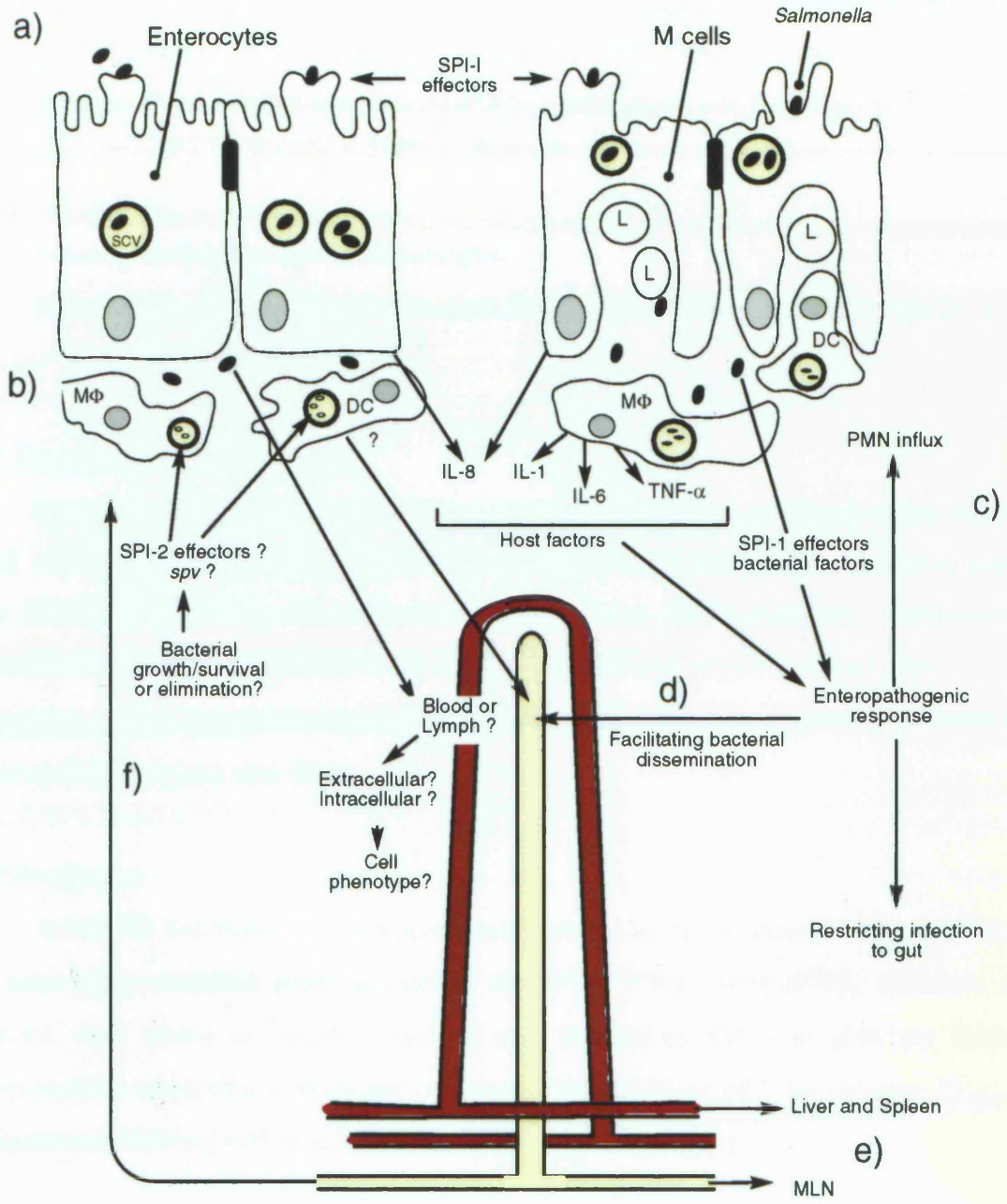



**Figure 1:10 Schemtic representation of some of the possible mechanisms contributing to *Salmonella* serotype-host specificity in calves that will be investigated during the generation of this thesis (section 1.11).** a) Intestinal invasion via M cells and/or enterocytes; b) Bacterial interaction with immune cells in the lamina propria. Bacterial net growth or target cell killing; c) Induction of enteric disease; d) Translocation to systemic sites; e) Increased growth or resistance to killing within systemic tissues; f) Re-seeding of the gastro-intestinal tract.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Introduction

The procedures outlined in this chapter are those that were used in the following results sections. The manufacturers and suppliers of equipment and reagents used in this study are listed in appendix 2.1 (at the end of this chapter).

## 2.2 Chemicals, antibiotics and radioisotopes

A list of the solutions and stains most frequently used in this study is given in table 2.1.

### *2.2.1 Chemicals*

Chemicals and antibiotics were either obtained from Sigma or Merck unless otherwise stated, and unless specified all solutions were prepared using sterile water (Super Q polished water [SQW] at 18.2 Mega Ohm conductivity adjusted to the required pH). Solutions were prepared under aseptic conditions using autoclaved glass and plasticware and were sterilised, if necessary, either by autoclaving (115°C, 20 min at 10 pounds *per* square inch [psi]) or by filtration (0.22 μm pore size filter).

### *2.2.2 Antibiotics*

Antibiotic solutions were prepared using either SQW or ethanol (chloramphenicol) and were filter sterilised prior to storage at -20°C. Where appropriate, antibiotic stock solutions were added to liquid or molten agar (cooled to 55°C) to give the following concentrations: ampicillin, 100 μg $ml^{-1}$; chloramphenicol, 30 μg $ml^{-1}$; kanamycin, 75 μg $ml^{-1}$; sulphadiazine, 120 mg $ml^{-1}$ or gentamicin, 100 μg $ml^{-1}$.

### *2.2.3 Radioisotopes*

$^{111}$Indium oxinate, with a specific activity of 37 MBq $ml^{-1}$, was obtained from Mallinckrodt.

## 2.3 Media

All liquid and solid media used in this study were prepared by Mrs C. Powers and Mr P. Green (Institute for Animal Health [I.A.H.], Compton, Berkshire, U.K.) and a list of the composition and preparation conditions is given in table 2.2. Dehydrated media were obtained from Oxoid Ltd, Becton Dickinson or Merck Ltd and made according to the manufacturers instructions. Plates and liquid media were either used immediately or stored at 4°C until

required, and plates supplemented with antibiotics were stored for a maximum of 2 weeks before use.

## 2.4 Tissue culture cell lines and reagents

The cultured intestinal epithelial cell line Int 407 (also known as Henle 407) and the cervical cell line HeLa used in this study were obtained from the European collection of animal cell cultures (ECACC) and were maintained by Mrs E. Bennett (I.A.H., Compton).

Media, buffers and antibiotics used in tissue culture analysis were obtained from Sigma, Life Technologies Ltd or JRH Biosciences Ltd and were filter sterilised and stored at 4°C prior to use.

### *2.4.1 Preparation of cultured cells*

Tissue culture flasks (75 $cm^2$) were seeded with HeLa or Int 407 cells, approximately 0.5 ml in 30 ml complete eagles minimal essential medium (CEMEM), at 6 $log_{10}$ or 5.9 $log_{10}$ respectively and incubated (5% $CO_2$, 37°C) for 5-7 days to form confluent monolayers. For subculture, it was first necessary to remove adherent cells from the base of the flask by enzyme treatment. The overlying medium was poured off and the monolayer rinsed with sterile phosphate buffered saline (PBS). The monolayer was washed once (150 X *g*, 5 min, 4°C), with a solution containing 1 part trypsin 0.25% (w/v) to 8 parts versene (NaCl 0.14 M; KCl 2.7 mM; $Na_2HPO_4$, 8.1 mM; $KH_2PO_4$, 1.5 mM; EDTA, 0.5 mM; pH 7.2) and incubated at 37°C in trypsin/versene solution for approximately 5 minutes. The cells were dislodged by agitation and the trypsin neutralised by the addition of CEMEM containing 10% foetal calf serum (FCS); 1% non-essential amino acids; L-glutamine, 2 mM; $NaHCO_3$, 23.8 mM; penicillin, 100 units $ml^{-1}$; streptomicin, 100 µg $ml^{-1}$ and mycostatin, 25 units $ml^{-1}$. The cells were pelleted by centrifugation (150 X *g*, 5 min, 4°C) and resuspended in fresh CEMEM.

The number of cells recovered was determined using an improved neubauer haemocytometer. The cell suspension was diluted to the required concentration, in CEMEM, and Int 407 or HeLa cells were added in 1 ml or 100 µl volumes to 24 well or 96 well flat-bottomed tissue culture plates respectively at a seeding rate of 5.3 (HeLa) or 5.7 (Int 407) $log_{10}$ cells *per* $ml^{-1}$. The cells were incubated in an atmosphere of 5% $CO_2$ at 37°C for approximately 24 h before use.

**Table 2.1 Solutions and stains most frequently used in this study**

| Solution | Chemical | Final concentration |
|---|---|---|
| **Lysis buffer** | Glucose | 50 mM |
| | Tris-HCL | 25 mM |
| | EDTA | 10 mM |
| | pH 8.0 (autoclave and store at 4°C) | |
| **TAE buffer** | Tris-acetate | 40 mM |
| | EDTA | 1 mM |
| | pH 8.0 (autoclave and store at 4°C) | |
| **Loading buffer** | Xylene cyanol | 4.6 mM |
| | Ficoll type 400 | 150 g $l^{-1}$ |
| | Bromophenol blue | 2.5 g $l^{-1}$ |
| | (store at 4°C) | |
| **SOB medium** | NaCl | 0.17 M |
| | KCl | 33.5 mM |
| | Bacto tryptone | 20 g $l^{-1}$ |
| | Yeast extract | 5 g $l^{-1}$ |
| | (autoclave and store at 4°C) | |
| **Phosphate buffer (0.0132M)** | $KH_2PO_4$ | 0.13 M |
| | $Na_2HPO_4$ | 0.13 M |
| | pH 6.8 (store at 4°C) | |
| **$Ca^{2+}$-free Tyrodes buffer** | NaCl | 0.14 M |
| | $NaHCO_3$ | 11.9 mM |
| | D-glucose | 5.6 mM |
| | KCl | 2.7 mM |
| | $MgCl_2.6H_2O$ | 2.0 mM |
| | $NaH_2PO_4$ | 0.4 mM |
| | pH 6.5 (autoclave and store at 4°C) | |
| **Alkaline buffered glycerol** | $NaHCO_3$ | 2.1 mM |
| | $Na_2CO_3$ | 1.5 mM |
| | Glycerol | 90% (v/v) |
| | (store at 4°C) | |

| Solution | Chemical | Final concentration |
|---|---|---|
| **Geys solution:** (mix 20 parts solution A; 5 parts solution B; 5 parts solution C and 70 parts SQW) | **Solution A** | |
| | $NH_4Cl$ | 0.7 M |
| | Glucose | 27.8 mM |
| | KCl | 24.8 mM |
| | $Na_2HPO_4.12H_2O$ | 4.2 mM |
| | $KH_2PO_4$ | 0.9 mM |
| | (filter sterilise and store at 4°C) | |
| | **Solution B** | |
| | $CaCl_2.2H_2O$ | 30.6 mM |
| | $MgCl_2.6H_2O$ | 20.7 mM |
| | $MgSO_2.7H_2O$ | 11.6 mM |
| | (autoclave and store at 4°C) | |
| | **Solution C** | |
| | $NaHCO_3$ | 0.3 M |
| | (autoclave and store at 4°C) | |
| **GCTcm10 medium:** (to 350 ml of solution A, add 50 ml of solution B and 44 ml of solution C) | **Solution A** | |
| | Choline chloride | 68.5 mM |
| | KCl | 22.9 mM |
| | (autoclave and store at 4°C) | |
| | **Solution B** | |
| | Glucose | 0.9 M |
| | Choline bicarbonate | 5% (v/v) |
| | (filter sterilise before use) | |
| | **Solution C** | |
| | $NaHCO_3$ | 26.8 mM |
| | L-glutamine | 2 mM |
| | EMEM x 10 | 10% (v/v) |
| | FCS | 10% (v/v) |
| | (make up in class II cabinet) | |

| Solution | Chemical | Final concentration |
|---|---|---|
| **Fluorescent antibody phosphate buffered saline (FA-PBS)** | NaCl | 0.14 M |
| | $Na_2HPO_4$ | 7.5 mM |
| | $NaH_2PO_4$ | 3.3 mM |
| | pH 7.1 | |
| **Alkaline buffered glycerol** | $NaHCO_3$ | 2.1 mM |
| | $Na_2CO_3$ | 1.5 mM |
| | Glycerol | 90% (v/v) |
| | (store at 4°C) | |

**Table 2.2 Composition of media used in this study**

| Media | Chemical | Concent-ration | Strains | Sterilisation conditions |
|---|---|---|---|---|
| **Bacto-tryptose (BT) broth (Jones, 1975)** | NaCl | 85.6 mM | All strains | 115°C, 20 min, 10 psi |
| | β-sodium glycerophosphate | 6.3 mM | | |
| | D-glucose | 5.6 mM | | |
| | Bacto-tryptose | 20 g $l^{-1}$ | | |
| | pH 7.4 | | | |
| **Brilliant green agar (BGA) Oxoid CM 263 (modified)** | NaCl | 85.6 mM | *S. abortusovis* (SAO15/5) | 121°C, 15 min, 15 psi |
| | Sucrose | 29.2 mM | | |
| | Lactose | 27.8 mM | | |
| | Agar | 12 g $l^{-1}$ | | |
| | Proteose peptone | 10 g $l^{-1}$ | | |
| | Yeast extract | 3 g $l^{-1}$ | | |
| | Brilliant green | 0.12 g $l^{-1}$ | | |
| | Phenol red | 80 mg $l^{-1}$ | | |
| | pH 6.9 | | | |
| **Brilliant green agar (BGA) Oxoid CM 329 (modified)** | Sucrose | 29.2 mM | All strains except *S. abortusovis* | 100°C, 3 min (in agarclave) |
| | Lactose | 27.8 mM | | |
| | $Na_2HPO_4$ | 7 mM | | |
| | $NaH_2PO_4$ | 5 mM | | |
| | Agar | 12 g $l^{-1}$ | | |
| | Peptone | 10 g $l^{-1}$ | | |
| | Lab-Lemco powder | 5 g $l^{-1}$ | | |
| | Yeast extract | 3 g $l^{-1}$ | | |
| | Sulphadiazine | 120 mg $l^{-1}$ | | |
| | Phenol red | 90 mg $l^{-1}$ | | |
| | Brilliant green | 4 mg $l^{-1}$ | | |
| | pH 6.9 | | | |
| **Luria Bertani (LB) broth** | NaCl | 0.2 M | All strains | 115°C, 20 min, 10 psi |
| | Bacto-tryptone | 10 g $l^{-1}$ | | |
| | Yeast extract | 5 g $l^{-1}$ | | |

| Media | Chemical | Concent-ration | Strains | Sterilisation conditions |
|---|---|---|---|---|
| **LB agar** | As for LB broth plus Bacto-agar | 15 g $l^{-1}$ | All strains | 121°C, 15 min, 15 psi |
| **MacConkey agar (Oxoid CM7)** | Lactose | 27.8 mM | All strains except *S. abortusovis* (SAO44) | 121°C, 15 min, 15 psi |
| | NaCl | 85.5 mM | | |
| | Peptone | 20 g $l^{-1}$ | | |
| | Agar | 12 g $l^{-1}$ | | |
| | Bile salts | 5 g $l^{-1}$ | | |
| | Neutral red | 75 mg $l^{-1}$ | | |
| | pH 7.4 | | | |
| **Phosphate buffered saline (PBS)** | NaCl | 0.2 M | All strains | 115°C, 20 min, 10 psi |
| | $Na_2HPO_4$ | 10.1 mM | | |
| | KCl | 3.4 mM | | |
| | $KH_2HPO_4$ | 1.8 mM | | |
| | pH 7.2 | | | |
| **Rambach agar (Merck Ltd)** | NaCl | 85.5 mM | *S. abortusovis* (SAO44) | 100°C, 3 min (in agarclave) |
| | Sodium deoxycholate | 2.4 mM | | |
| | Agar | 15 g $l^{-1}$ | | |
| | Propylene glycol | 10.5 g $l^{-1}$ | | |
| | Peptone | 8 g $l^{-1}$ | | |
| | Chromogenic mix | 1.5 g $l^{-1}$ | | |
| | pH 7.3 | | | |
| **Rappaport broth (Rappaport *et al.*, 1956)** | NaCl | 0.12 M | All strains | 115°C, 20 min, 10 psi |
| | $MgCl_2.6H_2O$ | 17.4 mM | | |
| | $KH_2PO_4$ | 10.4 mM | | |
| | Tryptone | 4.42 g $l^{-1}$ | | |
| | Malachite green | 11 mg $l^{-1}$ | | |
| **Selenite brilliant green broth Becton Dickinson (Difco 0661)** | D-mannitol | 27.4 mM | All serotypes except *S. choleraesuis* | Steam10 min |
| | $K_2HPO_4$ | 15.2 mM | | |
| | $KH_2PO_4$ | 7.5 mM | | |
| | Peptone | 5 g $l^{-1}$ | | |
| | Yeast extract | 5 g $l^{-1}$ | | |
| | Sodium taurocholate | 1 g $l^{-1}$ | | |
| | Brilliant green | 5 mg $l^{-1}$ | | |
| | pH 7.3 | | | |

## 2.5 Animals

All animal experiments were performed under the requirements of the Animals Scientific Procedures Act, 1986.

### *2.5.1 Calves*

Male Friesian calves, which were born and reared at I.A.H. farms, Compton Berkshire, U.K. or obtained from local farms in Berkshire, were used in this study.

From birth to 2 days of age calves received colostrum from their dam. From 2 days to 6 weeks they received either reconstituted dried milk (Gold top milk replacement), or milk from the bulk storage tank (2 L twice daily). Additionally from 2 days until the termination of experimental procedures calves also received 250 g of calf starter (C2) proprietary unmedicated weaner nuts twice daily with water available *ad libitum* from birth.

Where possible animals were moved from the farm to an isolated calf rearing unit at 1-2 weeks of age where they were housed in single cubicles on straw bedding. Calves were moved to a high containment unit (maintained at 18-20°C) at approximately 4 weeks of age where they were either used immediately, for surgical procedures, or randomly allocated into treatment groups and penned singly, on a bed of shavings, for use in challenge studies.

Rectal swabs were taken from calves at 2 weeks and 1 week prior to either infection or surgery, to ensure that the animals were not excreting salmonellas. Samples were enriched in Rappaport broth (37°C, 18 h) and selenite brilliant green broth (43°C, 18 h) followed by incubation of the enrichment cultures on Oxoid CM 329 modified brilliant green agar plates (table 2.2) at 37°C for 18 h.

## 2.6 Bacteria

### *2.6.1 Storage, recovery and routine growth of bacteria*

Stocks of bacterial strains for routine use were prepared by inoculating Luria Bertani (LB) broth (10 ml) (table 2.2) with several colonies of the required strain. Cultures were incubated at 37°C, with shaking at 150 rpm, for 5 h. Sterile glycerol, at a concentration of 10% (v/v), was added and aliquots of broth (100 µl) were stored, in 2 ml glass vials, at -70°C until required. For recovery the glycerol stock was thawed and streaked, to obtain single colonies, onto both LB and MacConkey agar (LB and Rambach agar for *S. abortusovis* SAO44) followed by incubation at 37°C for approximately 18 h (36 h for *S. abortusovis* strains). The serotype of each strain was confirmed using the appropriate *Salmonella* typing sera.

Broth cultures were routinely prepared by inoculating 10 ml of LB broth with several individual bacterial colonies (from LB plates) of no more that 48 h old and incubating at

either 25°C or 37°C for up to 18 h, depending on the experimental procedure used. Log phase cultures were prepared, if necessary, as described in individual sections.

### *2.6.2. Bacterial strains, mutants and plasmids*

A list of the bacterial strains used in this study, together with their respective source and any relevant references is given in table 2.3. A list of the mutants and plasmids used in this study, together with any antibiotic requirements, and relevant references is given in table 2.4.

### *2.6.3. Enumeration of bacteria*

#### *2.6.3.1. Nephelometry*

To estimate the number of bacteria within broth cultures, grown under similar conditions, the optical density (OD), at $600_{nm}$ was taken and the value read off a standard growth curve. The estimate of bacterial numbers was confirmed by viable count (section 2.6.3.2).

A standard growth curve was prepared for several serotypes used in this study as follows. LB broth (100 ml in 500 ml conical flasks) was inoculated with 200 µl of an overnight bacterial culture and incubated at 37°C with shaking at 150 rpm. The OD of the cultures was measured at thirty minute intervals for 8 h after subculture and the number of bacteria present at each time point was determined by viable count (section 2.6.3.2.[i]). A best-fit line was drawn through a graphical plot of the OD (x-axis) against the viable count $\log_{10}$ (y-axis).

#### *2.6.3.2. Viable counts*

Viable counts of bacterial suspensions were performed using two different methods as follows.

i) Using a modification of the method of Miles and Misra, (1938), dilutions (1:10) of the bacterial suspension were made in sterile 96 well U bottom microtitre plates using 180 µl of sterile PBS. The dilutions (20 µl) were dropped, in triplicate, onto quarter sections of agar plates and the number of colony forming units *per* millilitre (cfu $ml^{-1}$) was recorded from dilutions that produced between 20-80 colonies after overnight growth at 37°C.

ii) Serial dilutions of the bacterial suspension were made, in glass universals, using sterile PBS or isotonic saline. The aliquots (100 µl) were dispensed onto agar plates in triplicate, and distributed across the surface of the agar using a sterile glass spreader. The number of cfu $ml^{-1}$ was recorded from dilutions that produced 20-200 colonies after overnight growth at 37°C.

**Table 2.3 Bacterial strains used in this study**

| Serotype (strain) | Source | Reference |
|---|---|---|
| ***S. dublin* (SD2229)** | Bovine isolate | Baird *et al.*, 1985 |
| ***S. dublin* (SD3246)** | Bovine isolate | Hall and Jones, 1976 |
| ***S. choleraesuis* (var. *kunzendorf*) (SCSA50)** | Porcine isolate | Veterinary Investigation Centre, Cardiff, U.K. (1972) |
| ***S. choleraesuis* (var. *kunzendorf*) (SCS14/74)** | Porcine isolate | Veterinary Investigation Centre, Cardiff, U.K. (1972) |
| ***S. typhimurium* (ST4/74)** | Bovine isolate | Jones *et al.*, 1988 |
| ***S. typhimurium* (ST12/75)** | Bovine isolate | Baird *et al.*, 1985 |
| ***S. gallinarum* (SG9)** | Fowl isolate | Williams-Smith, 1955 |
| ***S. gallinarum* (SGJ91)** | Fowl isolate | Christensen *et al.*, 1992 |
| ***S. abortusovis* (SAO44)** | Ovine isolate | Colombo *et al.*, 1992 |
| ***S. abortusovis* (SAO15/5)** | Ovine isolate | Lantier *et al.*, 1983 |

**Table 2.4 Mutations and plasmids used in this study**

| Name of mutant/plasmid | Major phenotypic alteration | Antibiotic resistance | Transform-ation of strains | Reference |
|---|---|---|---|---|
| ***invH*201::Tn*phoA*** | Reduced invasion/enteropat-hogenesis in calves (encodes TTSS-1 apparatus protein) | Kanamycin (75 μg $ml^{-1}$) | ST4/74 and ST12/75 | (Watson *et al.*, 1995; Watson *et al.*, 1998) |
| ***sipB*::pB1** | Reduced invasion/enteropat-hogenesis in calves (encodes TTSS-1 translocator/effect-or protein) | Chloramph-enicol (30 μg $ml^{-1}$) | SD2229 and SG9 | (Wood *et al.*, 1996) |
| **pTECH2 expressing GFP** | Plasmid encoded expression of the green fluorescent protein | Ampicillin (100 μg $ml^{-1}$) | SD3246 and SG9 | Gift from Professor G. Dougan, Imperial College of Science, Technology and Medicine, London, U.K. (Khan *et al.*, 1994) |

## 2.7 Molecular techniques (adapted from Sambrook *et al.*, 1989)

### *2.7.1 Extraction of pTECH2GFP plasmid DNA from Salmonella using the 'mini-prep' method*

One millilitre of an overnight LB broth culture was pelleted, in an eppendorf tube, by centrifugation at 7,000 X *g*, room temperature for 1 min and the pellet resuspended in 100 µl of cold lysis buffer (table 2.1) containing 5 mg $ml^{-1}$ lysozyme. The suspension was vortexed, incubated at 37°C for 10 min and placed on ice for a further 2 min. Two hundred microlitres of freshly prepared NaOH/SDS (0.2 M/1% [w/v]) were added and the suspension mixed gently and incubated on ice for 5 min. Ice cold 3 M sodium acetate pH 4.8 (150 µl), was added and the mixture left on ice for 10 min followed by centrifugation at room temperature (7,000 X *g*, 10 min). The supernatant was transferred to a fresh eppendorf tube and an equal volume of 1:1 (v/v) Tris-saturated phenol:chloroform (pH 8) was added and the eppendorf vortexed to mix the contents. The suspension was centrifuged (7,000 X *g*, room temperature, 7 min) and the upper aqueous layer transferred to a clean eppendorf where plasmid DNA was precipitated by adding 2 volumes of ice-cold ethanol. After 2 min at room temperature the DNA was pelleted by centrifugation (7,000 X *g*, room temperature, 5 min) and washed once with 70% ethanol. The DNA was dried in a vacuum dessicator, dissolved in 10-20 µl SQW containing 0.1 mg $ml^{-1}$ ribonuclease A and stored at -20°C.

#### *2.7.1.1 Restriction enzyme digestion of plasmid DNA*

In order to linearise the plasmid DNA 5 µl, containing approximately 250 ng of DNA, was digested (37°C for 1 h) with 1 µl the restriction enzyme *Bam*HI and 2 µl of buffer, as indicated by the manufacturer, in a total volume of 20 µl.

#### *2.7.1.2 Agarose gel electrophoresis of DNA*

To confirm its size, isolated plasmid DNA was electrophoresed in a 0.7% agarose TAE (table 2.1) gel at a constant voltage (70 V) for approximately 1 h. The gel was stained with 0.5 µg $ml^{-1}$ ethidium bromide added to the positively charged end of the tank. Ethidium bromide in TAE electrophoresis buffer binds to nucleic acids and causes them to fluoresce when irradiated with ultra-violet (UV) light. Each sample of DNA was mixed approximately 3:1 (v/v) with loading buffer (table 2.1). A 1 kb DNA ladder diluted 1:1:1 (v/v) with SQW and loading buffer was included in one of the empty wells in order to determine the correct size of the sample DNA. The DNA was visualised by trans-illumination with UV light at a wavelength of 300-365 nm.

### *2.7.2 Transformation of Salmonella*

#### *2.7.2.1 Preparation of competent cells*

A 10 ml LB broth culture was grown for 18 h at 37°C. One millilitre was transferred into 100 ml LB broth in a sterile 250 ml flask, and incubated with shaking for 4 h at 37°C. The culture was centrifuged (6,000 X *g*, 15 min, 4°C) and the pellet washed once in 10% (v/v) sterile glycerol prior to final suspension in 600 µl of 10% (v/v) sterile glycerol.

#### *2.7.2.2 Transformation of Salmonella with plasmid DNA*

Ice cold competent bacterial cells (100 µl) and plasmid DNA 5 µl were added to a 2 mm gene pulser cuvette. Electroporation was performed using a gene pulser and controller set at 2.4 kV, 25 µFD with 200 Ω resistance. SOB medium (1 ml) (table 2.1) was immediately added to the cuvette, followed by incubation at 37°C for 1 hour. Aliquots (100 µl) were plated onto LB agar containing 100 µg $ml^{-1}$ ampicillin. Any antibiotic resistant transformants recovered after overnight incubation at 37°C were confirmed to contain the plasmid by performing a 'mini-prep', linearising the DNA, running the sample on an agarose gel and checking for the presence of the appropriate sized band. Competent cells alone plated onto LB agar, with and without antibiotics, were included to serve as positive and negative controls. Frozen stocks of the transformed *Salmonella* strains were prepared and stored at - 70°C as described in section 2.6.1.

## 2.8 Cultured cell invasion assay

Int 407 cultured epithelial cells were used to assess invasion of different *Salmonella* serotypes *in vitro*, using a modification of the method of Galán and Curtiss III (1989). Int 407 cell monolayers were seeded into flat bottomed 24 (1 ml) or 96 (100 µl) well plates at 5.7 $\log_{10}$ cells $ml^{-1}$ as described in section 2.4.1.

### *2.8.1 Preparation of inocula*

From an overnight broth culture, 100 µl were subcultured into 10 ml fresh LB broth and incubated for a further 4 h (37°C, 150 rpm) to obtain a mid-log phase culture. The number of cfu $ml^{-1}$ in each culture was estimated by nephelometry (section 2.6.3.1). Cultures were diluted to approximately 6.4 $\log_{10}$ cfu $ml^{-1}$ (to give a multiplicity of infection [moi] of 5:1) in pre-warmed Roswell Park Memorial Institute (RPMI) 1640 medium without phenol red, supplemented with 5% FCS; Hepes buffer, 17.6 mM and L-glutamine, 2 mM (supplemented RPMI). The exact number of salmonellas in the RPMI suspension was determined by viable count (section 2.6.3.2.[i]).

#### *2.8.2.1 Rate of in vitro extracellular growth*

To determine whether all strains used grew at the same rate, 1 ml of inocula were incubated with supplemented RPMI media alone for 2 h (37°C, 5% $CO_2$) and the change in bacterial numbers during incubation assessed by viable count (section 2.6.3.2[i]).

### *2.8.2 Infection of Int 407 cell monolayers and recovery of bacteria*

Monolayers of Int 407 cells, seeded into 24 well plates, were washed once with supplemented RPMI medium 30 min prior to the addition of 1 ml of inocula into triplicate wells. After incubation of infected monolayers for 1 hour at 37°C with 5% $CO_2$, cells were washed twice with RPMI and lysed with 1 ml of PBS containing 0.1% (w/v) sodium deoxycholate to quantify the total number of bacteria associated with the Int 407 cells. In addition, infected monolayers were incubated for a further hour in supplemented RPMI containing 100 µg $ml^{-1}$ gentamicin prior to washing and lysing with 0.1% sodium deoxycholate to determine the number of intracellular bacteria only. The bacteria were enumerated on MacConkey agar plates, or LB agar for the *S. abortusovis* strains, as described in section 2.6.3.2.(i). In order to increase the interaction between the non-motile serotype *S. gallinarum* and the Int 407 cells, a separate experiment was performed in which the infected monolayers were centrifuged (162 X *g*, 10 mins, room temperature) subsequent to the addition of inocula.

### *2.8.3 Cytotoxicity of salmonellas for Int 407 cells*

The amount of cytotoxicity manifested on Int 407 epithelial cells following infection with salmonellas was determined by measuring the release of the intracellular enzyme lactate dehydrogenase (LDH) using the CytoTox 96$^{TM}$ assay kit (Promega Ltd). The manufacturers instructions were followed with several modifications as follows. Monolayers of Int 407 cells were seeded into flat bottomed 96 well plates as described in section 2.4.1. Two hours and immediately prior to infection, the overlying medium was replaced with either 100 µl or 90 µl of supplemented RPMI (without phenol red) respectively. Ten microlitres of inocula, prepared as described in section 2.8.1, were added to each monolayer in triplicate, and the plates incubated at 37°C with 5% $CO_2$ for 2 h. Seventy five minutes after infection, lysis buffer, supplied in the kit, was added to three uninfected monolayers and 3 wells that contained medium alone in order to obtain 100% cytotoxicity. Two hours after bacterial infection the amount of lactate dehydrogenase released by damaged epithelial cells was determined by an enzymatic colourimetric reaction and measured by optical density. The percentage cytotoxicity of Int 407 cells was calculated as follows:

$$\frac{[OD_{492}\text{ (test strain)} - OD_{492}\text{ (medium)}] \times 100}{OD_{492}\text{ (Int 407 cells + lysis buffer)} - OD_{492}\text{ (medium + lysis buffer)}}$$

## 2.9 Oral and intravenous inoculation of calves

Male Friesian calves, aged 25-31 days, were reared and housed as described in section 2.5.1, and were not excreting salmonellas as determined by enrichment of faecal swabs in Rappaport and selenite brilliant green broths. Two days before inoculation the calves were moved into a high containment animal house where they were randomly allocated into treatment groups.

For calves inoculated by the oral route, the morning feed was withheld until after the animals had been infected.

### *2.9.1 Preparation and administration of inocula for oral challenge*

Bacterial cultures were prepared by inoculating Bacto-Tryptose (BT) broth (table 2.2) with several colonies and incubating statically for 18 h at 37°C. The $OD_{600nm}$ was taken and the culture volume adjusted to give a final concentration of 8.7 to 8.9 $\log_{10}$ cfu. The number of bacteria present in the inocula were confirmed by plating onto either brilliant green or Rambach agar as described in section 2.6.3.2.(ii).

The bacterial suspension was mixed with 20 ml of ant-acid (SQW containing 5% [wt/vol] $Mg(SiO_3)_3$, 5% [wt/vol] $MgCO_3$, and 5% [wt/vol] $NaHCO_3$) and administered orally to calves, with the aid of a syringe, before the morning feed.

### *2.9.2 Preparation and administration of inocula for intravenous challenge*

The inocula were grown as described in section 2.9.1. Cultures were diluted in 1 ml of sterile isotonic saline to give a final concentration of approximately 6.0 $\log_{10}$ cfu injected directly into the jugular vein.

### *2.9.3 Monitoring of calves*

Following inoculation of calves, virulence was assessed in terms of pyrexic responses, composition and consistency of diarrhoea produced, general demeanour and the recovery of bacteria from selected systemic and enteric sites (section 2.9.4).

Rectal temperatures were recorded and calves monitored for clinical signs of disease twice daily after infection. A cumulative daily scour-scoring scheme was used to record the severity of diarrhoea based on the consistency of the faeces and the presence of either sloughed mucosa or blood. Scores for consistency were as follows: 0, normal; +1, semi-solid; +2, liquid; or +3, watery. Scores for the faecal content were as follows: +1, fresh blood; +2, sloughed mucosa.

Calves were killed by intravenous injection, using an overdose of pentobarbitone sodium (Euthatal 66 mg $kg^{-1}$), at 24 h or 7 days *post* inoculation (dpi) or if any of the following occurred during infection: a cumulative scour score of greater than 20; prolonged, severe dehydration due to scouring and anorexia (no milk consumption for 2 days or less than half milk consumption for 4 days); the inability to stand unaided or a drop in rectal temperature from greater than 39°C to less than 38°C over a twelve hour period.

### *2.9.4 Enumeration of salmonellas from tissues*

Samples were taken in triplicate from selected systemic and enteric sites. The systemic organs were sampled first to avoid contamination from intestinal contents. All tissues were washed thoroughly in cold tap water and instruments were washed between samples. Biopsies were taken from the lung, spleen, liver, ileum, caecum, colon and their associated nodes and intestinal contents. Additionally, mucosa and contents were sampled from the abomasum, omasum, rumen and reticulum in the calves used for 24 h colonisation studies.

To determine the number of salmonellas present, 1 g of tissue was homogenised in 9 ml of isotonic saline (-1 diln). The homogenates were further diluted 1:100 in saline (-3 diln.) and 100 µl of both dilutions spread onto brilliant green or Rambach agar plates, as appropriate, in triplicate (section 2.6.3.2 [ii]). The number of colonies obtained after overnight incubation of the plates at 37°C was recorded and further dilutions were performed if necessary. The limit of accurate quantification using this technique is 2.0 $\log_{10}$ cfu $g^{-1}$ tissue. Any samples containing fewer bacteria were enriched in Rappaport and selenite brilliant

green broth as described in section 2.5.1. Samples that were positive on enrichment were given a value of 2.0 $\log_{10}$ cfu $g^{-1}$ and those that were negative were given a value of 0.

## 2.10 Bovine ligated ileal loop model

This model was used for quantification of intestinal invasion *in vivo* (section 2.11) and for determination of enteropathogenic responses to different *Salmonella* serotypes (section 2.12). Furthermore, together with the cannulation of the venous blood supply draining infected intestinal mucosa and efferent lymphatics draining regional MLN (section 2.13), this model was used to assess the route and magnitude of bacterial translocation in addition to host and bacterial responses associated with this process.

### *2.10.1 Anaesthesia and surgical technique*

Conventionally reared male Friesian calves (25-31 days old) (section 2.5.1) were used throughout. The morning feed was withheld and calves were anaesthetised by intravenous administration of Sagatal (pentobarbitone sodium, 20 mg $kg^{-1}$) for the duration of the experiment. The animals were intubated prior to surgery (endotracheal tube number 12 or 13) and the ventral abdominal area was shaved, and disinfected with hibiscrub. The abdominal and peritoneal walls were opened by a mid-line incision and the ileum exteriorised. Ligated loops were either constructed in the distal ileum (containing both Peyer's patch mucosa and absorptive epithelia) or in the mid-ileum (containing absorptive epithelia only), depending on the aim of the experiment.

For loop preparation, a length of intestine was isolated and a ligature placed at the proximal end. The intestine was flushed back with PBS so that the first loop could be constructed either adjacent to the caecum (distal ileum) or after the termination of continuous Peyer's patch mucosa (mid-ileum). A series of loops, with 1 cm spacers, were then constructed and ligated using 3 metric braided silk suture. The ileal loops were carefully injected, with 5 ml of either the bacterial strain in LB or LB alone as a negative control, and returned to the abdominal cavity, which was temporarily sutured.

Calves were positioned on their sides and turned every 2–3 hours to prevent both a build up of gas in the intestines and excessive pressure on the internal organs. The temperature, pulse and respiration rates were measured every hour. Animals were kept hydrated by the intravenous administration of a drip, containing 500 ml of 5% (w/v) glucose in saline, every 6 hours.

## 2.11 Quantification of intestinal invasion using the bovine ligated ileal loop model

The method of Watson *et al*, (1995) for determining the relative invasiveness of different *Salmonella* serotypes was adapted as follows.

### *2.11.1 Preparation of inocula*

Two different sizes of inocula were used. The method of inocula preparation optimised by Watson *et al*, (1995) was used initially (i). Studies were then extended to include a temperature shift (ii) which increases the expression of secreted effector proteins necessary for intestinal invasion and induction of enteropathogenesis (Wood *et al.*, 1996; Galyov *et al.*, 1997).

i) An 18 h LB broth culture grown at 37°C was subcultured (1:100 dilution) into fresh LB and incubated at 37°C with shaking (150 rpm) for a further 4 h. The $OD_{600nm}$ was taken and adjusted so that all cultures were similar. Bacteria were harvested by centrifugation (3,500 X *g*, 4°C, 10 min) and resuspended in half the original volume of LB broth to give a bacterial concentration of approximately 8.9 $\log_{10}$ cfu $ml^{-1}$ .

ii) The $OD_{600nm}$ of a 24 h broth culture, grown at 25°C with shaking, was adjusted to approximately 1.00 in a volume of 6 ml. Cultures were temperature shifted by incubating for a further 2 h at 37°C with shaking and finally diluted in LB to give a bacterial concentration of approximately 6.7 $\log_{10}$ cfu $ml^{-1}$.

For both i) and ii), the numbers of bacteria present in the inocula were determined by viable count (section 2.6.3.2.[i]).

### *2.11.2 Construction and inoculation of ligated ileal loops*

Up to 15 loops in the distal and/or 20 loops in the mid-ileum, of 9 cm in length, were constructed and inoculated as described in section 2.10.1.

One hour after inoculation the loops were exteriorised and 5 ml of GCTcm10 medium (Amin *et al.*, 1994) (table 2.1) containing 300 μg $ml^{-1}$ gentamicin was injected into loops to be processed for viable counts, but not those to be sampled for microscopic analysis. The amount of gentamicin used is equivalent to a working concentration of approximately 150 μg $ml^{-1}$ when the dilution with the loop contents is accounted for. The loops were returned to the abdominal cavity for a further hour and the wound re-sutured.

### *2.11.3 Processing the ileal mucosa*

Two hours after loop inoculation the ileum was exteriorised and 5 ml of 10% (v/v) neutral buffered formalin (NBF) or 4% (w/v) paraformaldehyde (PFA) were injected *in situ* into the loops to be sectioned for histological analysis. Cross section biopsies were taken from

these loops and placed into fixative for a minimum of 24 h prior to sectioning (section 2.15). The animals were then killed with an overdose of pentobarbitone sodium (section 2.9.3).

Individual unfixed loops for assessing viable counts were excised, opened longitudinally and placed in 50 ml of ice cold GCTcm10 medium to dilute the gentamicin and maintain tissue integrity prior to processing. The loops were washed gently in PBS and circular biopsies of 7 mm diameter were removed from the middle of each loop (6 biopsies in the distal ileum, 3 which contained Peyer's patch mucosa and 3 which contained absorptive epithelia, and 3 biopsies in the mid-ileum containing absorptive epithelia only). The biopsies were homogenised in 3 ml of PBS containing 1% (v/v) triton X-100 and the bacteria were enumerated (section 2.6.3.2.[i]) on MacConkey or Rambach agar (*S. abortusovis* SAO44).

## 2.12 Assessment of enteropathogenic responses using the bovine ligated ileal loop model

The method of Wallis *et al*, (1995) for assessing the enteropathogenic responses (PMN influx, induction of fluid secretion and damage to mucosal integrity) to different *Salmonella* serotypes was adapted as follows.

### *2.12.1 Preparation of inocula*

Inocula were prepared using temperature shifted bacteria (section 2.11.1.[ii]) to give approximately 8.3 $\log_{10}$ cfu $ml^{-1}$.

### *2.12.2 Construction and inoculation of ligated ileal loops*

Up to 35 loops, of 6 cm in length were constructed in the mid-ileum and inoculated as described in section 2.10.1.

### *2.12.3 $^{111}$ Indium labelling of leucocytes*

#### *2.12.3.1 Preparation of bovine polymorphonuclear leucocytes (PMNs)*

Labelled PMN leucocytes were prepared with the help of Dr P. Watson and Dr J. Bispham. Immediately after loop inoculation 50 ml of jugular blood was withdrawn and mixed with 9 ml acid citrate (tri-sodium citrate, 85 mM; citric acid, 46 mM) to prevent blood clotting. PMNs were isolated using a modification of the method of Carlson and Kaneko (1973) as follows. Blood was centrifuged (1,000 X *g*, 4°C, 15 min) and the plasma and buffy coat discarded. The cell pellet was weighed and 4 ml of sterile SQW *per* g of cells was added and mixed for 30 to 45 seconds to lyse the erythrocytes. The isotonicity was restored by adding 2 ml *per* g of 0.0132 M phosphate buffer (table 2.1) containing NaCl, 0.5 M *per* g of cells. The PMN leucocytes were collected by centrifugation (200 X *g*, 4°C, 10 min) and resuspended in 20 ml $Ca^{2+}$ -free Tyrodes buffer (table 2.1), containing 0.42 $\mu$g $ml^{-1}$

prostaglandin $E_1$. The PMN leucocytes were centrifuged (640 X *g*, room temperature, 5 min) and resuspended finally in 2 ml of Tyrodes buffer containing 0.42 $\mu$g $ml^{-1}$ prostaglandin $E_1$.

#### *2.12.3.2 $^{111}$Indium labelling*

The PMN leucocyte suspension was mixed with 3.7 MBq $^{111}$Indium oxinate, buffered with 0.2 M Tris, pH 7.0, and incubated for 2 min at room temperature, followed by the addition of 8 ml $Ca^{2+}$-free Tyrodes buffer. The leucocytes were collected by centrifugation (640 X *g*, room temperature, 5 min) and resuspended in 5.5 ml $Ca^{2+}$ -free Tyrodes buffer, followed by reinjection back into the donor calf.

### *2.12.4 Experimental termination and processing of intestinal mucosa*

Twelve hours after loop inoculation the ileum was exteriorised and 5 ml of 10% (v/v) NBF injected *in situ* into the loops to be sectioned for histological analysis. Cross section biopsies were taken from these loops and placed into NBF prior to sectioning (section 2.15). The animals were then killed using an overdose of pentobarbitone sodium (section 2.9.3).

Individual, unfixed loops were excised and the length of each loop (cm) together with the volume of fluid it contained (ml) were recorded. The ratio of the loop volume to loop length was termed the secretory response. The PMN influx was expressed as the ratio of $\gamma$-irradiation (counts *per* minute) emitted from the test loops compared with that emitted from the negative control loops, and was determined by passing both loop contents and mucosa through a gamma irradiation counter. In order to obtain an average PMN influx from all calves, the results *per* animal were expressed as the mean percentage minus 1 (the value for the LB control loops) compared to the mean value for *S. typhimurium* (ST4/74) (the strain that induced the greatest response).

## 2.13 Cannulation of venous blood supply draining infected intestinal mucosa and efferent lymphatics draining regional mesenteric lymph nodes (MLN)

To aid bacterial detection by flow cytometry and microscopy, salmonellas were transformed with the plasmid pTECH2 expressing GFP. Bacterial interactions with the intestinal mucosa and MLN (section 2.13.5.4 and 2.15.3.1) together with the kinetics of bacterial translocation via the venous drainage or efferent lymphatics (figure 2.1) were assessed. Host responses to infection were characterised including cell phenotypic alterations (section 2.17), morphological changes to the intestinal mucosa and MLN (section 2.15.1) and characterisation of the niche occupied by *Salmonella* during translocation (section 2.14 and 2.15.3.1). Additionally, the contribution of host cell death to pathogenesis was determined by

Annexin V staining and flow cytometric analysis of infected lymph and MLN (section 2.16.1) and/or TUNEL staining of infected tissues (section 2.16.2 and figure 2.2).

Efferent lymph, venous blood, MLN and intestinal mucosa obtained from these experiments were used for all of the experimental procedures described in sections 2.13 to 2.17.

### *2.13.1 Preparation of inocula*

Inocula were prepared using temperature shifted bacteria (section 2.11.1.[ii]) to give approximately 8.9 $\log_{10}$ cfu $ml^{-1}$ (10.5 to 11.0 $\log_{10}$ cfu in 50 ml).

### *2.13.2 Construction of heparin-coated cannulas*

The cannulas used were either commercially pre-coated CBAS, size 3 or 3.5 french, or portex tubing (0.4 mm to 0.9 mm internal diameter) which was cut into lengths of approximately 80 cm and coated using the following protocol. The inside of the cannula was rinsed with acetone and coated with 5% (v/v) 3-aminopropyltriethoxy silane for 10 min. The tubing was rinsed once with acetone followed by sterile saline, then filled with heparin sulphate (5000 units $ml^{-1}$) and stored at 4°C until required.

### *2.13.3 Cannulation of venous blood supply and efferent lymphatics*

These experiments were performed with help from Dr B. Charleston. Calves were anaesthetised and prepared for surgery as described in section 2.10.1. The abdominal wall was opened by a mid-line incision and the distal ileum exteriorised. A suitably sized efferent lymphatic vessel, draining several MLN, was isolated and exposed by dissection. Adrenalin (1 mg $ml^{-1}$) was added directly onto the operation site to reduce localised haemorrhaging, and a ligature was securely tied around the efferent lymphatic to prevent lymph flow and to enlarge the vessel for easier manipulation. A second ligature was loosely applied approximately 2 cm proximal to the first. The enlarged vessel was nicked using Castroviejo's ophthalmic scissors, and a heparin-coated cannula inserted into the draining efferent lymphatic. The cannula was secured in place by tightening both the loose ligature and the cut ends of silk suture from the first ligature around the tube and the vessel. The same process was repeated to cannulate the venous blood supply directly draining the intestinal mucosa.

The areas of intestine containing the cannulated vessels were carefully sutured onto a piece of corrugated plastic, approximately 6 cm x 6 cm, so that the intestines could be replaced inside the abdominal cavity and sample flow maintained.

A single distal ileal loop of 60-150 cm was flushed with PBS prior to construction and flanked at each end with a 1 cm loosely tied spacer. The loop encompassed the cannulated

venous vessel and several MLN draining to the cannulated lymphatics (figure 2.1). The ligated loop and attached cannulae were replaced back into the abdominal cavity. Blood and lymph were continuously collected in 60 ml Falcon tubes, containing 1000 units of heparin (final concentration 50 units $ml^{-1}$) at room temperature for 1-2 h *prior* to loop infection.

#### *2.13.3.1 Infection of ligated ileal loop*

Following collection of pre-infection samples the distal ileum was exteriorised and the inoculum (50-100 ml) injected into the ligated loop via both loosely tied spacers using a 3.25 IN (16 G) Angiocath intravenous catheter. The spacer ligatures were tightened and the inoculum moved throughout the length of the ligated mucosa by gentle manipulation prior to re-internalisation, wound repair and continuous monitoring of calves as described in section 2.10.1. It was assumed that by not injecting directly into the loop, the risk of accidental administration of salmonellas into a blood vessel was reduced.

#### *2.13.3.2 Collection of samples*

Efferent lymph and venous blood were collected continuously, in heparinised tubes, at room temperature for up to 12 hours *post* loop infection. In addition, a sample of jugular blood (20 ml) was collected every 2 hours into heparinised tubes. Samples were returned to the laboratory for harvesting every 2 hours.

### *2.13.4 Termination of the experiment*

Up to 12 hours after loop inoculation the ileum was exteriorised and cross section biopsies taken from both the intestine and MLN were placed in 10% (v/v) NBF, 4% (w/v) PFA or snap frozen for histological sectioning and analysis (section 2.15). The animals were killed using an overdose of pentobarbitone sodium (section 2.9.3).

Tissue samples were immediately removed for subsequent processing from 3 distal ileal MLN (from which draining lymph was collected), 1 mid-ileal control MLN, distal ileal mucosa, loop contents, liver and spleen.

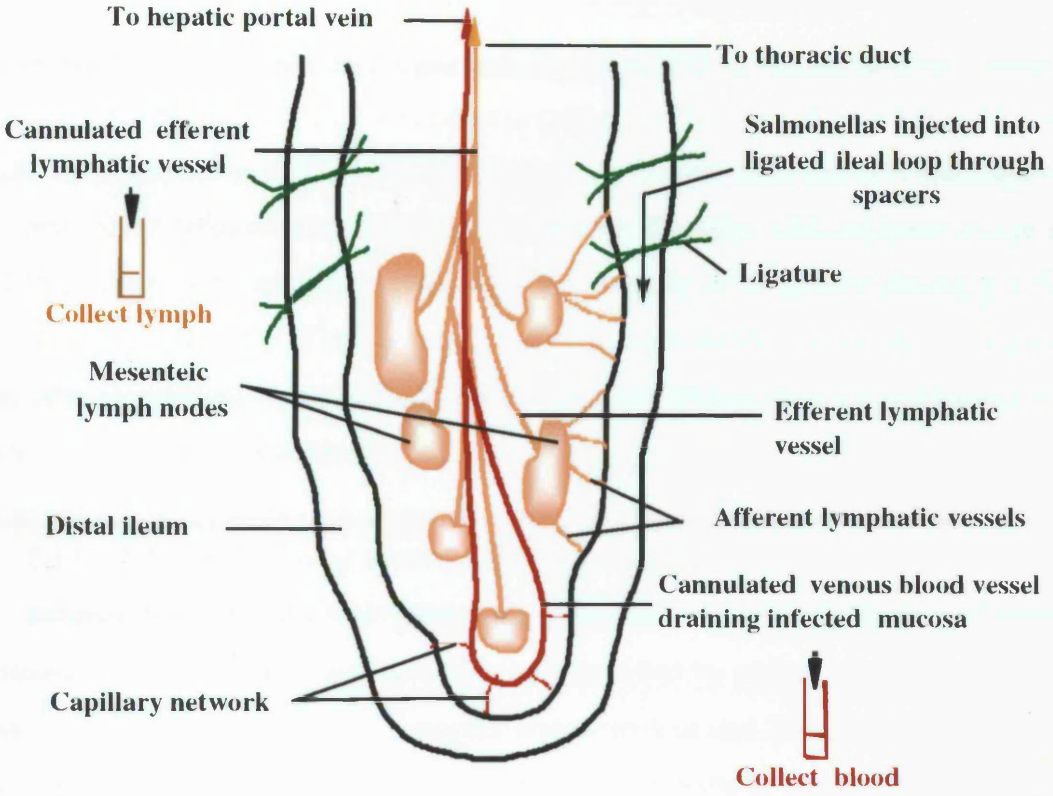



**Figure 2.1 Modification of the bovine ligated ileal loop model to enable collection of venous blood and efferent lymph, directly draining infected intestinal mucosa or MLN, respectively via cannulation of these vessels.**

### *2.13.5 Processing of blood, lymph and tissue samples from cannulation experiments*

#### *2.13.5.1 Optimisation of conditions for enumeration of gentamicin protected bacteria*

To assess the bactericidal activity of gentamicin for *Salmonella*, experiments were performed using efferent lymph from uninfected calves. The duration of sample incubation with gentamicin was optimised using different bacterial concentrations. Briefly, bacteria were grown as described in section 2.11.1 (ii) and diluted in PBS to obtain bacterial concentrations of between 2 and 6 $\log_{10}$ cfu $ml^{-1}$. Gentamicin (100 μg $ml^{-1}$) was added to 1 ml of bacteria and the cultures incubated at 37°C for 30 min, 1 h or 1 h 30 min followed by centrifugation (200 X *g*, 3 min, room temperature) and 2 washes in PBS. Bacteria (100 μl) were plated (section 2.6.3.2 [ii]) in triplicate onto MacConkey agar followed by overnight incubation at 37°C.

As more than 99% of bacteria at the highest concentration were killed by gentamicin 30 min after incubation, these conditions were used for enumeration of gentamicin-protected bacteria in subsequent experiments.

#### *2.13.5.2 Enumeration of bacteria in efferent lymph and venous blood*

Enumeration of total and gentamicin-protected bacteria in blood and lymph was determined at 2 h intervals. Total bacteria were quantified by adding whole blood or lymph to 10% (v/v) triton X-100 (9:1 ratio). Samples were vortexed and 200 μl plated (section 2.6.3.2 [ii]) in triplicate onto MacConkey agar plates, with and without ampicillin (100 μg $ml^{-1}$) (to determine plasmid stability), followed by overnight incubation at 37°C.

Gentamicin-protected bacteria, in lymph only, were enumerated by incubation of the sample at 37°C with gentamicin (100 μg $ml^{-1}$) for 30 min, followed by centrifugation (200 X g, 3 min, room temperature) and 2 washes with PBS. Samples were mixed with 10% triton X-100 and plated in triplicate as described above.

#### *2.13.5.3 Enrichment of blood and lymph samples*

Venous blood and efferent lymph (1 ml) were added to 9 ml of LB broth and incubated at 37°C for 18 h. Samples (100 μl) were spread plated onto MacConkey agar and examined for the presence of salmonellas after overnight incubation at 37°C.

#### *2.13.5.4 Enumeration of salmonellas from tissues*

Tissue samples (2 g) taken at *post mortem* (section 2.13.4) were finely chopped, teased apart into a sterile petri dish containing 18 ml PBS, and gently disrupted by passing through a fine meshed steel sieve using a syringe plunger. To determine the total number of salmonellas in each sample the cell suspensions were vortexed with 1/10 volume of 10% triton X-100 and

plated (100 µl) onto MacConkey (MLN, liver, spleen) or brilliant green (intestinal mucosa and contents) agar with and without ampicillin (100 µg $ml^{-1}$). To determine the number of gentamicin protected bacteria, tissue homogenates were centrifuged (470 X *g*, 5 min, 4°C) and the cell pellet washed in PBS. The sample was incubated for 30 min at 37°C, with gentamicin (100 µg $ml^{-1}$) followed by 2 washes in PBS. Samples were mixed with 10% triton X-100 and plated in triplicate as described above.

## 2.14 Enumeration of salmonellas associated with $CD14^+$ cells

### *2.14.1 Preparation of cells for $CD14^+$ purification*

Cell suspensions from infected MLN (4-8 g in total taken from 3 nodes) were prepared by maceration of MLN in 10 ml PBS (section 2.13.5.4). Cell suspensions (2 x 5 ml) were diluted in an equal volume of PBS, containing 200 µg $ml^{-1}$ gentamicin, and carefully underlayed with 5 ml Histopaque 1083 containing 100 µg $ml^{-1}$ gentamicin. The interface (mononuclear cells) was isolated after density gradient centrifugation (1,200 X *g*, 30 min, 20°C, no brake). Cells in this fraction were washed twice in PBS (470 X *g*, 10 min, 4°C) and finally resuspended in 5 ml RPMI medium containing 100 µg $ml^{-1}$ gentamicin. Following incubation at 37°C for 30 min, cells were washed twice in PBS, the two samples were pooled and resuspended in 5 ml PBS containing 10% FCS. Viable eukaryotic cells were enumerated (section 2.17.1.1) prior to isolation of $CD14^+$ cells and quantification of bacteria associated with these cells.

### *2.14.2 Isolation of $CD14^+$ cells*

With the help of Ms. V. Chance, I.A.H. Compton U.K., $CD14^+$ cells were isolated from infected MLN using MACS $CD14^+$ MicroBeads (Miltenyi Biotec). The manufacturers instructions were followed. In brief, mononuclear cells isolated by histopaque density gradient were mixed with $CD14^+$ MicroBeads (10 µl *per* 7 $\log_{10}$ cells) and incubated for 10 min at room temperature. Cells were washed twice with PBS, pH 6.8 (300 X *g*, 10 min, 4°C) and the cell pellet resuspended in 3 ml of PBS mixed with 2% FCS. A positive selection separation column was placed in the magnetic field of a MACS separator and the column washed once with buffer. The cell suspension was added to the column allowing the magnetically labelled $CD14^+$ beads to be retained in the column and the negative cells to pass through. The column was rinsed with RPMI containing 10% FCS, removed from the separator and the positive cells eluted and flushed out.

Cells with the $CD14^+$ phenotype were enumerated (section 2.17.1.1) and analysed for purity by flow cytometry (section 2.17.2.1).

### *2.14.3 Quantification of salmonellas associated with CD14$^+$ cells from infected MLN*

The number of gentamicin-protected bacteria associated with CD14$^+$ cells in the MLN were determined by lysing the MACS purified cell suspension with 10% triton X-100 and plating (section 2.6.3.2 [ii]) 200 µl onto MacConkey agar followed by bacterial enumeration after overnight incubation of the plates at 37°C.

## 2.15 Tissue sectioning, immunolabelling and microscopic examination

Intestinal mucosa or MLN were sectioned, stained and analysed by light microscopy (section 2.15.1). These samples, in addition to agar embedded infected lymph, were also cryosectioned (section 2.15.2) or sectioned using a vibrating microtome (section 2.15.3) and immunolabelled (sections 2.15.2 and 2.15.3.1) followed by examination using confocal laser scanning microscopy (CLSM). Additionally, whole lymph or CD14$^+$ cells, purified from infected MLN (section 2.14.2), were adhered to glass slides by cytocentrifugation (section 2.15.4), immunolabelled and examined by UV microscopy or CLSM.

### *2.15.1 Preparation and analysis of Haematoxylin and Eosin (H and E) stained sections*

Tissues for H and E staining were processed by Mrs. S. Hacker and Ms. H. Matthews, I.A.H., Compton.

Fixed intestinal mucosa or MLN, for examination by light microscopy, was embedded in paraffin wax prior to sectioning and drying on glass slides. Cut sections (2–3 µm thickness) were de-waxed using 'safe clear', rehydrated through graded alcohols to water and stained with haematoxylin gill 3 and 0.5% aqueous eosin yellowish. Prior to examination and scoring sections were dehydrated, through graded alcohols, cleared in 'safe clear' and mounted in safeclear mountant.

#### *2.15.1.1 Microscopic examination of haematoxylin and eosin stained sections*

H and E stained intestinal mucosa and MLN sections were examined using light microscopy (X 10, X 25 or X 40 lens) and scored 'blind', for structural tissue changes, by 2 individuals.

The amount of mucosal damage was scored on a scale of 0 to 3 and assessed in terms of disruption to the enterocyte monolayer, extrusion of enterocytes, stunting of villi and infiltration of inflammatory cells into the lamina propria, submucosa and lumen. The scoring system used represented no damage (0), mild (1), moderate (2) or severe damage (3) respectively.

A semi-quantitative scoring system for MLN sections was designed, based on the magnitude of the PMN influx into different areas of the node. The overall numbers of PMNs

were assessed as being discrete within a particular area or diffuse throughout the entire section. Additionally, the total number of layers of PMNs were assessed over 10 fields of view in the subcapsular sinus, cortex/paracortex, cortical trabeculae and medullary sinus and were scored on a scale of 0 to 3. The scoring system used represented an average of 1 or fewer layers of PMNs in the subcapsular sinus and around the cortical trabeculae (0), 1 to 3 layers (1), 3 to 5 layers (2) or more than 5 layers (3). The scoring system in the cortex/paracortex represented 0-20 PMNs *per* field (0) or more than 20 PMNs *per* field (1) and the scoring system used in the medullary sinuses represented 0-20 PMNs *per* field (0), 20-50 PMNs *per* field (1), 50-70 PMNs *per* field (2) or more than 70 PMNs *per* field (3).

For photographic records, images were taken using a digital camera (X 10 lens) and files were processed on a Macintosh personal computer using Adobe photoshop.

### *2.15.2 Preparation and visualisation of cryosectioned tissue*

Examination of tissues infected with salmonellas containing pTECH2 expressing GFP were sectioned, counterstained, mounted and examined under UV light to determine whether fluorescent bacteria could be visualised in tissues without the need for immunolabelling.

Infected bovine intestinal mucosa was fixed *in situ* in 4% PFA. Tissue biopsies were placed in foil boats filled with O.C.T. embedding medium and snap frozen by placing the sample in a metal holder containing iso-pentane and lowering this into liquid nitrogen for approximately 1 minute. Samples were placed on dry ice for transportation and were subsequently stored at -70°C.

Sections of 6-10 μm were cut using a Leica CM 1900 cryostat, picked up onto glass slides and stored in foil at -20°C. For visualisation of bacteria, slides were brought to room temperature, washed carefully in FA-PBS (table 2.1) and incubated for 30 min in FA-PBS containing wheatgerm/Texas red (1:50). Unbound conjugate was removed by washing for 30 minutes at room temperature in FA-PBS as described above. The sections were mounted in alkaline buffered glycerol (table 2.1) and viewed, either immediately or after overnight storage at 4°C, with light exclusion using a Leica confocal microscope (section 2.15.3.2).

### *2.15.3 Tissue sectioning using a vibrating microtome*

Whole lymph embedded in agar blocks (1 ml lymph centrifuged in 1 ml molten 1.5% agar in PBS), intestinal mucosa or MLN samples, fixed in 4% PFA overnight followed by long term storage in 1% PFA at 4°C with light exclusion, were sectioned using a vibrating microtome. Use of this equipment overcomes the need to embed in wax thus minimising tissue processing and associated denaturation of antigens. Additionally, the vibrating action of

the blade during sectioning protects the tissue integrity to a greater degree than conventional cryosectioning (Monaghan *et al.*, 2001).

Thick sections of agar embedded lymph (100 μm thickness) or tissue (50 μm thickness) were either used immediately for immunolabelling or were stored in 1% PFA at 4°C with light exclusion until required.

### *2.15.3.1 Immunofluorescent labelling of microtome sectioned agar embedded lymph, MLN or intestinal mucosa*

The immunolabelling protocol was initially optimised using HeLa cultured cells (5.3 $\log_{10}$ cells $ml^{-1}$), grown on round coverslips in 24 well plates, infected with *Salmonella* (6.7 $\log_{10}$ cfu $ml^{-1}$). The permeabilisation agents and antibodies used, together with the duration of exposure to these reagents, were then re-optimised using mucosa and MLN sections in order to achieve the best possible sample penetration and labelling.

Although salmonellas containing pTECH2 expressing GFP were used for these experiments, only weak fluorescence was observed following cryosectioning and tissue counterstaining (section 2.15.2). Therefore to increase the intensity of the signal, bacteria were additionally labelled with antibodies specific to *Salmonella* 'O' antigens.

Sectioned tissue or agar-embedded lymph were manipulated for immunolabelling using a fine paintbrush, with most of the bristles removed, into solutions contained on parafilm that had been taped onto a large tile. Coverslips, tissue or agar-embedded sections were washed 3 times in PBS (5 min at room temperature) prior to and *post* permeabilisation in 0.1% saponin in PBS (30-45 min, room temperature). Non-specific binding sites were blocked by the addition of 0.5% bovine serum albumin (BSA) in PBS (BSA/PBS) (60 min at room temperature). Tissue sections were incubated in the primary antibodies diluted 1:100 (rabbit anti-*Salmonella* LPS) or 1:2000 (mouse anti-α-tubulin to label the cell cytoskeleton) respectively in BSA/PBS for 1 h at room temperature in a humidified chamber. Samples were washed 3 times in PBS and incubated for 1 h at room temperature in a humidified chamber with either anti-rabbit IgG conjugated to Alexa Fluor 568 (red), anti-mouse IgG conjugated to Alexa Fluor 488 (green) or anti-mouse IgG conjugated to Cy5 (blue) diluted 1:200 in BSA/PBS. Samples were washed 3 times, mounted in aqueous mounting medium (Vectashield) and the coverslips sealed with nail varnish prior to visualisation of samples using confocal laser scanning microscopy (CLSM).

### *2.15.3.2 Visualisation of immunolabelled sections using CLSM*

Confocal images were generated with help from Dr P. Mongaghan and Dr P. Watson. Images were collected on a Leica microscope attached to a confocal laser-scanning

microscope. The laser lines on the krypton/argon laser were $488_{nm}$ (FITC), $568_{nm}$ (TxR) and $647_{nm}$ (Cy5). Typically, 10-15 images were taken as projections of a 10-20 μm z-stack through the section. Single or composite images were collected through the X 63 oil immersion objective. Confocal images were saved in TIFF format and imported into Adobe Photoshop to provide the final image of the immunolabelled section.

### *2.15.4. Cytocentrifugation of efferent lymph or CD14$^+$ cell suspensions for labelling and examination by immunofluorescent microscopy*

Leucocytes isolated from efferent lymph or CD14$^+$ purified cells from infected MLN (section 2.14.2) were adjusted to a concentration of 5 $\log_{10}$ cells ml$^{-1}$ in 10% FCS. Glass slides (Snowcoat X-tra) and a holed filter card were aligned with a cytospin block and inserted into the centrifuge. Cells (300 μl) were added to each block and spun onto slides 45 X *g*, 7 min, room temperature using a cytocentrifuge. The slides were dried, fixed (1% PFA, 1 h, room temperature) immunolabelled (section 2.15.3.1), mounted and viewed with light exclusion (X 50 water immersion) by UV microscopy.

## 2.16 Quantification of bacterial-induced cell death in efferent lymph, MLN or intestinal mucosa

The contribution of cell death, in infected efferent lymph, MLN or intestinal mucosa, to *Salmonella* pathogenesis was assessed by Annexin V staining and flow cytometric analysis and/or by the presence of *in situ* Terminal deoxynucleotidyl transferase d Uridine triphosphate Nick End Labelling (TUNEL) positive cells.

### *2.16.1 Annexin V staining*

Annexin V is a $Ca^2$-dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS). During the early stages of apoptosis PS is translocated to the outer layer of the plasma membrane and as such becomes exposed at the external cell surface. Therefore, Annexin-V can be used as a probe for PS exposure and detection of apoptotic cells. As both apoptotic and necrotic cells expose PS, necrotic cells can be differentiated by their uptake of propidium iodide (PI).

For labelling, the Annexin-V-FLUOS assay kit was used (Boehringer Mannheim U.K.) and the manufacturers instructions were followed. Briefly leucocytes isolated from efferent lymph and MLN suspensions (sections 2.17.1) were aliquoted into 96-well U bottom plates at a concentration of 6 $\log_{10}$ cells *per* well. Cells were pelleted by centrifugation (200 X *g*, 4°C, 2 min) and resuspended in 100 μl of labelling solution (Annexin-V-FLUOS labelling reagent, 20 μl; propidium iodide (PI), 1 μg ml$^{-1}$; incubation buffer 1 ml [hepes/NaOH, 10 mM

pH 7.4; NaCl, 140 mM; $CaCl_2$, 5 mM]) for 10 min at room temperature. The cells were washed 3 times (200 X *g*, 4°C, 2 min), resuspended finally in 600 µl of incubation buffer and analysed by flow cytometry (section 2.17.2.1).

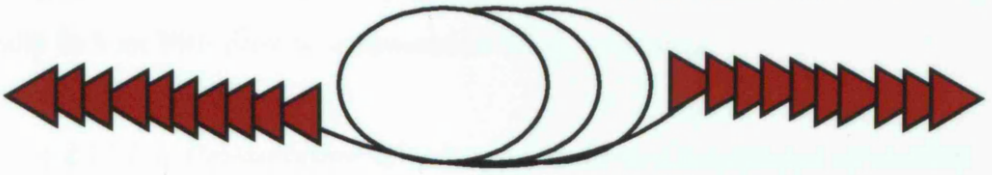
### *2.16.2 Detection of TUNEL positive cells in intestinal mucosa and MLN*

The presence of TUNEL positive cells in the intestinal mucosa (2 and 12 hours after infection) or MLN (12 hours after infection) were determined using the ApopTag$^{TM}$ plus *in situ* apoptosis detection kit (Intergen, Europe). In theory, 3′-OH ends generated by DNA fragmentation in apoptotic nuclei can be catalytically extended with residues of digoxigenin-nucleotides using terminal deoxynucleotidyl transferase (TdT). After incubation with FITC-conjugated anti-digoxigenin antibody, TUNEL positive cells can be visualised by fluorescent microscopy (figure 2.2).

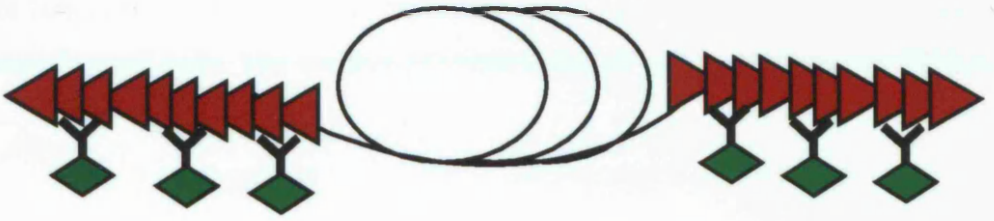
Briefly, fixed tissue sections were washed 3 times in PBS prior and subsequent to permeabilisation (section 2.15.3.1). Equilibration buffer (75 µl) was added to the sample and incubated at room temperature for 5 min, followed by the addition of 54 µl of working strength TdT enzyme (diluted 1:2 with reaction buffer) and incubation in a humidified chamber (37°C, room temperature, 1 h). Tissues were placed in working strength stop/wash buffer (1:35 with SQW) for 30 min at 37°C followed by 3 washes in PBS. Working strength anti-digoxigenin-fluorescein (24.5 µl antibody diluted in 28 µl blocking solution) was added and the samples were incubated in a humidified chamber for 30 min at room temperature. Sections were washed a further 3 times and immunolabelled with rabbit anti-*Salmonella* LPS or mouse anti-α-tubulin followed by anti-rabbit IgG conjugated to Alexa Fluor 568 (red) or anti-mouse IgG conjugated to Cy5 (blue) respectively (section 2.15.3.1). Sections were then mounted and visualised using CLSM (section 2.15.3.2). A negative control sample containing water instead of TdT and a positive control slide (supplied with the kit) of rat mammary gland cells, that naturally undergo programmed cell death during tissue regression following lactation, were included each time the protocol was followed

An unbiased, semi-quantitative scoring system was developed whereby 10 independent fields of view were examined from each section and the number of bacteria, TUNEL positive cells and TUNEL positive cells associated with salmonellas were counted.

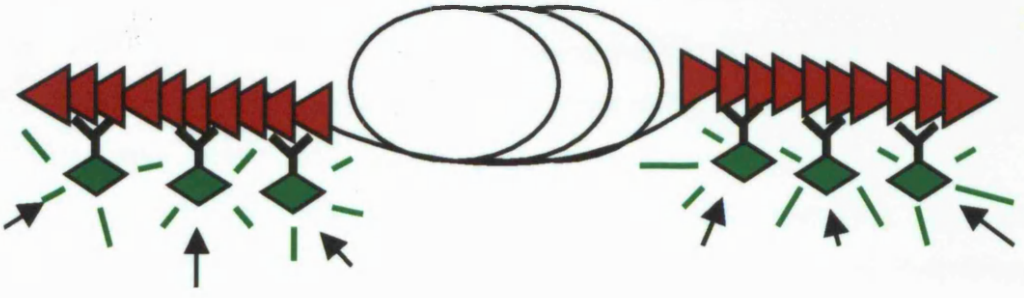
Apoptosis of cells results in DNA fragments of about 180 bp nucleosomal units



Residues of digoxigenin-nucleotide are catalytically added to the DNA fragments by the enzyme TdT



Anti-digoxigenin fluorescein conjugate is bound to the catalytically added digoxigenin-nucleotide



When excited by light at $494_{nm}$ fluorescin produces a signal at $523_{nm}$, which can be detected by fluorescent microscopy.

**Figure 2.2 Principle of the TUNEL reaction for *in situ* determination of apoptotic/necrotic nuclei (adapted from the Oncor ApopTag instruction manual)**

## 2.17 Eukaryotic cell isolation and phenotypic analysis

Eukaryotic cells were isolated from efferent lymph, blood (pre-anaesthesia jugular blood only) and MLN up to 12 hours after loop infection in order to assess changes in cell numbers and phenotype.

### *2.17.1 Isolation of leucocytes from bovine blood, lymph or MLN*

Heparinised blood or lymph or MLN suspension was mixed with Gey's solution (table 2.1), at a ratio of 1:5, and incubated on ice for 4 min to lyse the erythrocytes. The leucocytes were pelleted by centrifugation (470 X *g*, 5 min, 4°C), washed twice in PBS and resuspended finally in 5 ml PBS prior to enumeration for cell viability.

#### *2.17.1.1. Enumeration of eukaryotic cells and assessment of viability*

Viability of leucocytes was determined by a trypan blue dye-exclusion method.

Isolated eukaryotic cells were mixed with PBS and 0.1% (w/v) trypan blue in PBS (1:9:10). Microscopic examination using a neubauer haemacytometer enabled enumeration of viable (exclusion of trypan blue) and dead (stain blue due to entry of dye through damaged cell membranes) cells. The number of viable cells $ml^{-1}$ were calculated as follows:

$$\frac{\text{Viable cells in both sides of the chamber}}{2} \times 20 \times 10\,000$$

### *2.17.2. Phenotypic analysis of bovine leucocytes by immunofluorescent staining*

A list of the monoclonal antibodies (mAbs) used in the phenotypic analysis of leucocytes is given in table 2.5. Antibodies, derived from culture supernatant, at 10-100 $\mu g\,ml^{-1}$ were used either neat (CD14) or 1:10 (CD21, WC1, CD4, CD8, Myd-1, TRT1, CD11a, CD11b, CD11c, and MHC II) while those derived from ascitic fluid (CD32, FCγIIR and CD3) were diluted 1:1000 prior to use.

Monoclonal antibodies were diluted in PBS containing 10% (v/v) normal rabbit serum, 0.5% (w/v) BSA and 0.05% (w/v) sodium azide (PBS/BSA/Az). Propidium iodide (PI) or the secondary antibody phycoerythrin (PE) were diluted to 25 $\mu g\,ml^{-1}$ or 2.5 $\mu g\,ml^{-1}$ respectively in PBS/BSA/Az prior to use.

#### *2.17.2.1 Two colour flow cytometry using fluorescent activated cell sorting (FACS)*

Viable leucocytes isolated from efferent lymph, pre-anaesthetic blood or MLN were aliquoted into 96-well U bottom plates at a concentration of 5.7 $\log_{10}$ cells *per* well. Cells were pelleted by centrifugation (200 X *g*, 4°C, 2 min) and resuspended in 25 µl of PI (for determining cell viability) PE (negative control) or the primary antibody, followed by

incubation at room temperature for 10 min. Leucocytes were washed 3 times in PBS/BSA/Az and resuspended in 25 µl of the isotype-specific secondary antibody conjugated to PE. The cells were incubated for a further 10 minutes at room temperature followed by 3 washes and final resuspension in 100 µl of PBS/BSA/Az. Cells were diluted in 0.5 ml FACSFlow optimised sheath fluid. Data was acquired using a FACSCalibur cell sorter together with the Cellquest programme and analysed on the basis of size, granularity or intensity of fluorescence using FCS Express (Denovo software [http://www.denovosoftware.com]).

**Table 2.5 Characteristics of monoclonal antibodies used in this study**

| Monoclonal antibody | Specificity | Isotype | Cell distribution | Reference or supplier |
|---|---|---|---|---|
| **MM1A** | CD3 | IgG1 | Pan T cells | Davis *et al.*, 1993 |
| **CC30** | CD4 | IgG1 | Class II restricted T cells (helper/inducer) | Bensaid and Hadam, 1991 |
| **CC58** | CD8 β chain | IgG1 | Class I restricted T cells (cytotoxic/suppressor) | Howard *et al.*, 1991 |
| **IL-A99** | CD11a | $IgG2_a$ | Leucocytes | Naessens and Howard, 1993 |
| **IL-A15** | CD11b | IgG1 | Monocytes, macrophages, granulocytes, B subset | Splitter and Morrison, 1991 |
| **IL-A16** | CD11c | IgG1 | Monocytes, macrophages, granulocytes (CR4) | Splitter and Morrison, 1991 |
| **CCG33** | CD14 | IgG1 | Monocytes/macrophages | Sopp *et al.*, 1996 |
| **CC21** | CD21 | IgG1 | B cells | Naessens *et al.*, 1990 |
| **CCG36** | FCγRII (CD32) | IgG1 | Monocytes, granulocytes, B cells, eosinophils | Zhang *et al.*, 1994 |
| **CCG24** | FCγIIR | IgG2 | Monocytes, granulocytes, B cells | Zhang *et al.*, 1995 |
| **IL-A24** | MyD-1 | IgG1 | Monocytes, macrophages, dendritic cells | Ellis *et al.*, 1988; Naessens and Howard, 1993 |
| **IL-A21** | MHC class II | $IgG2_a$ | Monoctyes, macrophages, dendritic cells | Baldwin *et al.*, 1988 |
| **CC15** | WC1 | $IgG2_a$ | Peripheral γδ T cells | Morrison and Davis, 1991 |
| **TRT1** | G surface glycoprotein of turkey rhinotracheitis virus | IgG1 | _ | Cavanagh, 1992 |

## 2.18 Statistical analysis

All data, unless otherwise stated, were statistically analysed for the effect of serotype, and when appropriate strain, using a two-way analysis of variance (ANOVA) and the general linear model (GLM) (Minitab Inc. statistical package). The effects of serotype on rectal temperature were tested by means of an F test with the data taken as repeated measurements (proc mixed, statistical analysis systems [SAS] 1995). Analyses of data from the cannulation studies (section 2.13) were performed by means of paired t-tests (Minitab Inc.).

For all statistical analyses, the viable count data was normalised by logarithmic transformation. Subsequently pair-wise comparisons between serotypes/strains were performed. In the event that any differences were found, the Students t-test was applied. The variance used in the t-test was obtained from the adjusted mean square calculated in the analysis of variance. Bars on the figures represent the standard error of the mean based on an estimate of the variance calculated from the observations for each group and were not calculated using the pooled variance from the analysis of variance. *P* values of greater than 0.1 were considered statistically insignificant. *P* values between 0.05 and 0.1 were considered as showing a tendency towards significance while *P* values of less than 0.05 were considered significant and those less than 0.01 were considered highly significant.

**Appendix 2.1 Manufacturers and suppliers of chemicals and equipment used in this study**

| Manufacturer/supplier | Product(s) | Addresses |
|---|---|---|
| **Anachem Ltd** | Eppendorf tubes | 20 Charles Street, Luton, Bedfordshire LU2 OEB |
| **Arnolds Veterinary products** | Castroviejo's ophthalmic scissors,<br>Portex tubing | Cartmel Drive, Harlescott Industrial Estate, Shrewsbury, Shropshire S71 37B |
| **Bayer PLC** | O.C.T. embedding medium | Bayer House, Strawberry Hill, Newbury, Berkshire RG14 1JA |
| **Becton Dickinson** | Dehydrated media,<br>Trypsin 0.25%,<br>FACSFlow optimised sheath fluid,<br>FACSCalibur cell sorter and Cellquest programme | Between Towns Road, Cowley, Oxford, Oxfordshire OX4 3LY |
| **Bibby Sterilin Ltd** | Petri dishes,<br>96 well U bottom microtitre plates | Tilling Drive, Stone, Staffordshire ST15 O5A |
| **Bio-Rad Laboratories Ltd** | Gel tray,<br>Electrophoresis tank,<br>Ethidium bromide,<br>2 mm gene pulser cuvettes,<br>Gene pulser and controller | Bio-Rad House, Maylands Avenue, Hemel Hempstead Hertfordshire HP2 7TD |
| **BOCM Pauls Ltd** | Gold top milk replacement,<br>Cotswold Earliwean calf nuts | PO Box 39, 47 Key Street, Ipswich, Suffolk IP4 1BX |
| **Boehringer Mannheim U.K.** | Annexin-V-FLUOS assay kit | Bell Lane, Lewes, East Sussex BN7 1LG |
| **Cambridge Bioscience** | Propidium iodide (PI),<br>Phycoerythrin (PE) | 24-25 Signet Court, Newmarket Road, Cambridge CB5 8LA |
| **Chance propper Ltd** | Round cover slips | Smethwick, Warley, U.K. |
| **Chemix U.K. Ltd** | Safeclear | Unit 58, Bradley Trading Estate, Bradley Lane, Standish, Nr Wigan, Greater Manchester WN6 OXQ |
| **Corning-Costar U.K.** | 96 well flat bottom plates | 1, The Valle Centre, Gordon Road, High Wycombe, Buckinghamshire HP13 6EQ |
| **Cyanamid** | 3 metric braided silk suture | 154 Fashan Road, Gosport, Hampshire O13 OAS |

| Manufacturer/supplier | Product(s) | Addresses |
|---|---|---|
| **European collection of animal cell cultures (ECACC)** | Int 407 intestinal epithelial cell line<br>HeLa cervical cell line | Centre for applied microbiology and research, Salisbury, Wiltshire SP4 0JG |
| **Fahrenheit Laboratory Services** | Falcon tubes<br>Falcon tissue culture flasks | 21 Alston Drive, Bradwell Abbey, Milton Keynes, Buckinghamshire MK13 9HA |
| **Imperial College of Science, Technology and Medicine** | pTECH2 expressing GFP | University of London |
| **Institute for Animal Health (I.A.H.)** | Friesian male calves | Compton Laboratory, Nr Newbury, Berkshire RG20 7NN |
| **Integra Biosciences Ltd** | Automated technomat agar dispenser | Unit 9, Ascot Industrial Estate, Icknield Way, Letchworth, Hertfordshire SG6 1TD |
| **Intergen Europe** | ApopTag™ | Oxford, U. K. |
| **Jencons PLC Ltd** | UV transilluminator | Cherrycourt Way Industrial Estate, Stanbridge Road, Leighton Buzzard, Bedfordshire LU7 8UA |
| **JRH Biosciences Ltd** | Chemicals, media and antibiotics | Smeaton Road, West Portland Industrial Estate, Andover, Hampshire SP10 3LF |
| **Laboratory Sales Ltd** | 2 ml glass vials | 20/21 Transpennine Trading Estate, Rochdale, Lancashire OL11 2PX |
| **Leica U.K. Ltd** | Cryostat CM 1900,<br>Confocal microscope,<br>Vibrating microtome | Davy Avenue, Knowhill, Milton Keynes, Buckinghamshire MK5 8LB |
| **Leo Laboratories** | Heparin | Longwick Road, Princes Risborough, Aylesbury, Buckinghamshire HP17 9RR |
| **Life Technologies Ltd** | Chemicals, media and antibiotics,<br>Restriction enzyme BamH1,<br>1 Kb DNA ladder,<br>Gentamicin | 3 Fountain Drive, Inchinnan Business Park, Inchinnan, Renfrewshire, Scotland PA4 9RF |
| **Mallinckrodt** | $^{111}$Indium oxinate | 10 Talisman Business Centre, London Road, Bicester, Oxfordshire OX6 OJX |
| **Martindale Pharmaceuticals** | Adrenalin | Bampton Road, Harold Hill, Romford, Essex RM3 8UG |

| Manufacturer/supplier | Product(s) | Addresses |
|---|---|---|
| **Merck Ltd** | Chemicals,<br>Laboratory sundries,<br>Parafilm<br>Dehydrated media,<br>Ultra-turrax homogeniser,<br>Improved neubauer haemocytometer,<br>0.5% aqueous eosin yellowish,<br>Square coverslips,<br>Glass microscope slides,<br>24 well flat bottom plates,<br>Safeclear clearing agent<br>Safeclear mountant<br>Trypan blue | Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN |
| **Millipore** | Super Q polished water | The Boulevard, Blackmoor Lane, Watford, Hertfordshire WD1 8YW |
| **Miltenyi Biotec** | $CD14^+$ beads,<br>MACS purification column | MACS CD14 MicroBeads |
| **Minitab Inc.** | Minitab statistical software | State College, PA, U.S.A. |
| **Molecular probes** | Wheatgerm/Texas red,<br>α-rabbit IgG conjugated to Alexa Fluor 568,<br>α-mouse IgG conjugated to Alexa Fluor 488,<br>α-mouse IgG conjugated to Cy5 | PoortGebouw,<br>Rijnsburgerweg 10,<br>2333, AA Leider,<br>The Netherlands |
| **Murex Biotech (U.K.) Ltd** | Salmonella antisera | Central Road, Temple Hill, Dartford, Kent DA1 5LR |
| **Nikon Europe BV** | Eclipse 400 microscope<br>Coolpix 950 digital camera | Schipholweg 321,<br>1171 PI Badhoevedorp,<br>The Netherlands |
| **Oxoid Ltd** | Dehydrated media | Wade Road, Basingstoke, Hampshire RG24 8PW |
| **Promega Ltd** | Cytotox $96^{TM}$ LDH assay kit | Delta House, Chilworth Research Centre, Southampton, Hampshire SO1 7NS |
| **Rhône Mérieux Ltd** | Euthatal,<br>Sagatal | Spire Green Centre,<br>Pinnacles West, Harlow,<br>Essex CM19 5TS |
| **Sartorius Instruments Ltd** | 0.22 μm pore size filters | Longmead Business Centre,<br>Blenheim Road, Epsom,<br>Surrey KT19 9QN |
| **SAS Institute Inc.** | SAS statistical package | Cary, N.C, U.S.A. |
| **Schering-Plough Animal Health** | Hibiscrub | Welwyn Garden City,<br>Hertfordshire, AL7 1TW |

| Manufacturer/supplier | Product(s) | Addresses |
|---|---|---|
| **Shandon Scientific Ltd** | Holed filter cards,<br>Cytocentrifuge | 93-95 Chadwick Road,<br>Astmoor, Runcorn, Cheshire<br>WA7 1PR |
| **Sigma Chemical Company Ltd** | Chemicals and antibiotics,<br>Lysozyme,<br>Tris-saturated phenol:chloroform,<br>Ribonuclease A,<br>Agarose,<br>Sodium deoxycholate,<br>Triton X-100,<br>Prostaglandin $E_1$,<br>3-aminopropyltriethoxy silane,<br>Paraformaldehyde,<br>Isopentane,<br>Normal rabbit serum (NRS),<br>Bovine serum albumin (BSA),<br>Sodium axide (Az),<br>Saponin,<br>Histopaque 1083 | The Old Brickyard, New Road, Gillingham, Dorset<br>SP8 4XT |
| **Surgipath Europe Ltd** | Neutral buffered formalin (NBF),<br>Snowcoat X-tra microscope slides,<br>Haematoxylin gill 3 | Venture Park, Stirling Way,<br>Bretton, Peterborough,<br>Cambridgeshire PE3 8YD |
| **UNO Roestvaststaal BV** | CBAS pre-coated cannulas | Postbus 15, 6900 AA,<br>Zevenaar, Holland |
| **Vector Laboratories** | Vectashield mounting medium | 16 Wolfic Square,<br>Peterborough,<br>Cambridgeshire PE3 8RF |
| **Veterinary Drug Company Ltd** | 5% (w/v) glucose in saline (drips),<br>Endotracheal tubes,<br>16 G Angiocath intravenous catheters,<br>Selection of needles and syringes | Unit 8, Lakeside Industrial Estate, Colnbrook by-pass,<br>Colnbrook, Slough,<br>Buckinghamshire SL3 OED |
| **Wallac (EGG)** | Gamma irradiation counter | 20 Vincent Avenue, Crown Business Centre, Crownhill,<br>Milton Keynes,<br>Buckinghamshire MK8 OAB |
| **Western Laboratory Services Ltd** | Glass universals | Unit 8, Redan Hill Estate,<br>Redan Road, Aldershot,<br>Hampshire GU12 4SJ |

# CHAPTER 3

# CHARACTERISATION OF THE VIRULENCE OF DIFFERENT *SALMONELLA* SEROTYPES FOLLOWING ORAL INOCULATION OF CALVES.

## 3.1 Introduction

Salmonellosis is a zoonosis resulting in symptoms which, depending on the particular host-pathogen combination, range from mild enteritis to severe systemic disease. Serotypes within *S. enterica* sub-species I can be loosely divided into three groups, according to host prevalence. First, non-host specific serotypes, for example, *S. typhimurium* and *S. enteritidis* typically cause enteric infections in a wide range of hosts (Lax *et al.*, 1995). Second, host-restricted serotypes infect a limited number of phylogenetically related host species, typically resulting in systemic and enteric disease. For example, *S. dublin* and *S. choleraesuis* are predominantly associated with disease in cattle (Gibson, 1961) and pigs (Sojka *et al.*, 1977) respectively, however these serotypes are occasionally associated with infections of other mammalian hosts including man (Buxton, 1957; Allison *et al.*, 1969). Third, host-specific serotypes for example, *S. typhi*, *S. gallinarum* and *S. abortusovis* are almost exclusively associated with severe systemic disease and/or abortion in primates (Hornick *et al.*, 1970a; Hornick *et al.*, 1970b), fowl (Barrow *et al.*, 1994; Shivaprased, 2000) and sheep (Jack, 1968; Jack, 1971) respectively.

The factors influencing *Salmonella* serotype-host specificity remain unclear. Environmental elements, such as selective mixing of livestock, poor husbandry techniques or the use of contaminated feed could result in the increased exposure of mammalian or avian species to a particular serotype, leading to an association of certain serotypes with specific hosts. Alternatively, genetic differences between serotypes could influence the infectivity of particular serotypes for different host species.

## 3.2 Aim

In order to understand the phenomenon of *Salmonella* serotype-host specificity it is important to determine whether environmental or innate genetic factors influence the outcome of infection with particular combinations of serotype and host. Therefore to address this question, the work described in this chapter aimed to characterise the virulence of several *Salmonella* serotypes, naturally associated with different host species, following oral inoculation of calves under controlled experimental conditions.

### 3.3 Approaches and experimental design

The experimental procedures used are described in section 2.9. To assess virulence, as described below, two groups of six calves and two groups of three calves were inoculated orally with *S. dublin* (SD3246), *S. choleraesuis* (SCSA50), *S. gallinarum* (SG9) or *S. abortusovis* (SAO44) respectively and the infection monitored for up to seven days *post* infection (dpi). In order to simplify the data, *post mortem* bacterial counts were analysed separately from calves that survived for seven days compared with calves that reached end point criteria.

To assess initial intestinal colonisation, two groups of five calves and two groups of three calves were orally inoculated with *S. dublin* (SD3246), *S. gallinarum* (SG9), *S. abortusovis* (SAO44) or *S. abortusovis* (SAO15/5) respectively and the infection monitored for twenty four hours.

Virulence was assessed as described in section 2.9.3. Tissue counts at *post mortem* examination were derived from triplicate tissue samples in each calf and the mean recovery *per* animal was presented graphically together with the SEM. Any animals meeting end point criteria, before the planned termination date were humanely killed as described in section 2.9.3. Details of the inocula used for these experiments are given in tables 3.1 and 3.2.

### 3.4 Kinetics of infection following oral inoculation of calves with *S. dublin*, *S. choleraesuis*, *S. gallinarum* or *S. abortusovis* for up to seven days.

#### *3.4.1 Temperature responses*

Calves infected with all serotypes developed a pyrexic response 36-48 hours after oral inoculation. This response was mild and transient in calves inoculated with either *S. gallinarum* or *S. abortusovis*, but severe and prolonged in calves inoculated with either *S. dublin* or *S. choleraesuis* (figure 3.1). From two days after inoculation, pyrexic responses from *S. dublin* and *S. choleraesuis* infected calves were significantly greater, with only a few exceptions, than in those calves infected with either *S. gallinarum* or *S. abortusovis* ($P < 0.08$ to $P < 0.0001$). There were no significant differences in the magnitude of the pyrexic response, with only a few exceptions, in the calves infected with either *S. dublin* or *S. choleraesuis* regardless of whether the end point criteria were reached before the termination of the experiment or not ($P > 0.1$). Furthermore, there were no differences in the magnitude of the pyrexic response, with the exception of day 4.5 ($P < 0.07$), in the calves inoculated with either *S. gallinarum* or *S. abortusovis* ($P > 0.1$).

In conclusion, regardless of the serotype, all calves responded to the *Salmonella* infection. However, differences were observed in the severity and duration of the pyrexic response that correlated with the clinical signs following inoculation.

### *3.4.2 Faecal consistency*

The faeces of calves infected with either *S. dublin* or *S. choleraesuis* became semi-solid or watery, and contained fresh blood and sloughed mucosa, 36-48 hours after oral inoculation. In these animals diarrhoea persisted for the remainder of the experiment.

The faeces of one calf infected with either *S. gallinarum* or *S. abortusovis* became semi-solid, but contained no blood or sloughed mucosa, 36-48 hours after oral inoculation. The change in consistency was transient, and faeces returned to normal after 1-2 days (table 3.1).

### *3.4.3 General demeanour*

Only calves inoculated with either *S. dublin* or *S. choleraesuis* showed symptoms typical of severe salmonellosis, resulting in a 50% mortality rate within the seven day period (table 3.1). Calves inoculated with these two serotypes began to refuse milk, or showed a partial loss of appetite, from day 2-3 onwards. In general calves inoculated with *S. dublin* became dehydrated, dull and anorexic and were humanely killed, if necessary, due to severe diarrhoea resulting in scour score endpoints being met. Calves inoculated with *S. choleraesuis* exhibited similar symptoms but were humanely killed, if necessary, because they were unable to stand unaided or they showed a large reduction in rectal temperature. The latter observations are more typically associated with systemic disease, which can result in terminal collapse due to lack of homeostasis.

In contrast, calves inoculated with either *S. gallinarum* or *S. abortusovis* displayed no symptoms typical of salmonellosis throughout the duration of the experiment.

### *3.4.4 Bacterial recovery from systemic and enteric tissues*

Bacterial recovery data from calves that survived for seven days and data from calves that succumbed to salmonellosis before this time point were analysed separately and results are presented in figures 3.2a, b, c and d.

In calves that survived for seven days, *S. dublin* and *S. choleraesuis* were recovered from systemic and enteric tissues in similar numbers, with the exception of the spleen where recovery of *S. dublin* was significantly greater ($P < 0.01$). Bacteria could only be detected by enrichment from the intestinal walls and nodes of calves inoculated with either *S. gallinarum* or *S. abortusovis*.

In *S. dublin* and *S. choleraesuis* infected calves that were humanely killed at less than seven days after challenge, *S. choleraesuis* was recovered in greater numbers than *S. dublin* from the hepatic lymph node, intestinal contents, nodes and ileal mucosa ($P < 0.1$ to $P < 0.02$). A comparison of *S. dublin* infected calves that survived 7 dpi with those that succumbed to infection showed that there were no differences in bacterial recovery from any samples ($P > 0.1$) except for the colon contents ($P < 0.05$) where counts were significantly higher in calves that were killed earlier than 7 dpi. In contrast, when comparing bacterial counts from *S. choleraesuis* infected calves, this serotype was recovered in greater numbers from all tissues in animals that were killed before 7 dpi ($P < 0.1$ to $P < 0.01$) with the exception of the lung and mediastinal lymph node ($P > 0.1$).

At *post mortem* examination, animals infected with either *S. dublin* or *S. choleraesuis* had intestinal pathology typical of acute salmonellosis including necrotic, reddened ileal and colonic mucosa and enlarged necrotic mesenteric lymph nodes. These findings correlated with both clinical symptoms and recovery of bacteria in large numbers from intestinal contents, mucosa and lymph nodes. In contrast, calves inoculated with either *S. gallinarum* or *S. abortusovis* showed no gross pathological changes.

In summary, these results demonstrate that, under experimental conditions of infection, *S. dublin* and *S. choleraesuis*, but not *S. gallinarum* or *S. abortusovis* are highly virulent for calves. However, the symptoms leading to end point criteria being met in either *S. dublin* or *S. choleraesuis* infected animals appear to differ.

| Serotype (strain) | Calf number | Inocula ($log_{10}$ cfu) | Day of death *post* inoculation | Scour score | Reason for euthanasia |
|---|---|---|---|---|---|
| ***S. dublin*** | 3.1 | 8.74 | 7 | ND | End of experiment |
| **(SD3246)** | 3.2 | 8.74 | 7 | ND | End of experiment |
| | 3.3 | 9 | 5 | 20 | Scour score =20 |
| | 3.4 | 9 | 3 | 6 | Dehydrated, dull, unable to stand unaided |
| | 3.5 | 8.56 | 6 | 20 | Scour score =20 |
| | 3.6 | 8.56 | 7 | 5 | End of experiment |
| **Mean scour score SD3246** | | | | **12.75** | |
| ***S. choleraesuis*** | 3.7 | 8.92 | 7 | ND | End of experiment |
| **(SCSA50)** | 3.8 | 8.92 | 7 | ND | End of experiment |
| | 3.9 | 8.9 | 2 | 6 | Dull, unable to stand unaided |
| | 3.10 | 8.9 | 5 | 16 | Dull, rapid temperature drop |
| | 3.11 | 8.68 | 7 | 15 | End of experiment |
| | 3.12 | 8.68 | 4 | 10 | Dull, unable to stand unaided |
| **Mean scour score SCSA50** | | | | **11.75** | |
| ***S. gallinarum*** | 3.13 | 9.14 | 7 | 2 | End of experiment |
| **(SG9)** | 3.14 | 9.14 | 7 | 0 | End of experiment |
| | 3.15 | 8.81 | 7 | 0 | End of experiment |
| **Mean scour score SG9** | | | | **0.66** | |
| ***S. abortusovis*** | 3.16 | 9 | 7 | 0 | End of experiment |
| **(SAO44)** | 3.17 | 9 | 7 | 1 | End of experiment |
| | 3.18 | 9 | 7 | 0 | End of experiment |
| **Mean scour score SAO44** | | | | **0.33** | |

**Table 3.1 Inocula used and clinical findings of calves infected orally with *S. dublin* (SD3246), *S. choleraesuis* (SCSA50), *S. gallinarum* (SG9) or *S. abortusovis* (SAO44) for up to 7 dpi. (ND – not determined)**

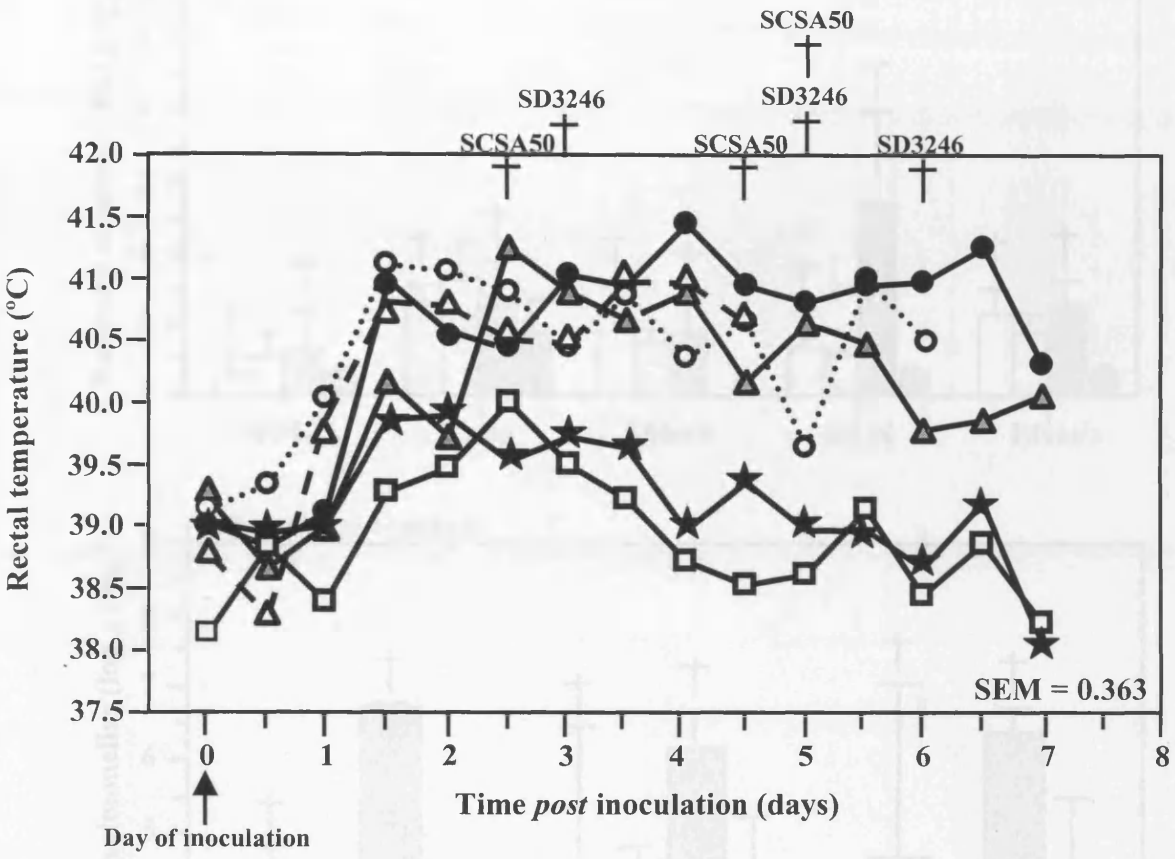



**Figure 3.1 Least square mean (SEM) daily rectal temperature of calves following oral inoculation with approximately 8.9 $\log_{10}$ cfu of *S. dublin* (survivors) (-●-); *S. dublin* (moribund) (·○·); *S. choleraesuis* (survivors) (-△- ); *S. choleraesuis* (moribund) (-△-); *S. gallinarum* (survivors) (-★-) or *S. abortusovis* (survivors) (-□-).** Each datum point is derived from 3 calves *per* group. The times to death of *S. dublin* or *S. choleraesuis* infected calves that reached end point criteria are depicted in table 3.1 and as such, temperature responses are derived from either 1, 2 or 3 calves as appropriate (time of death is indicated with†).

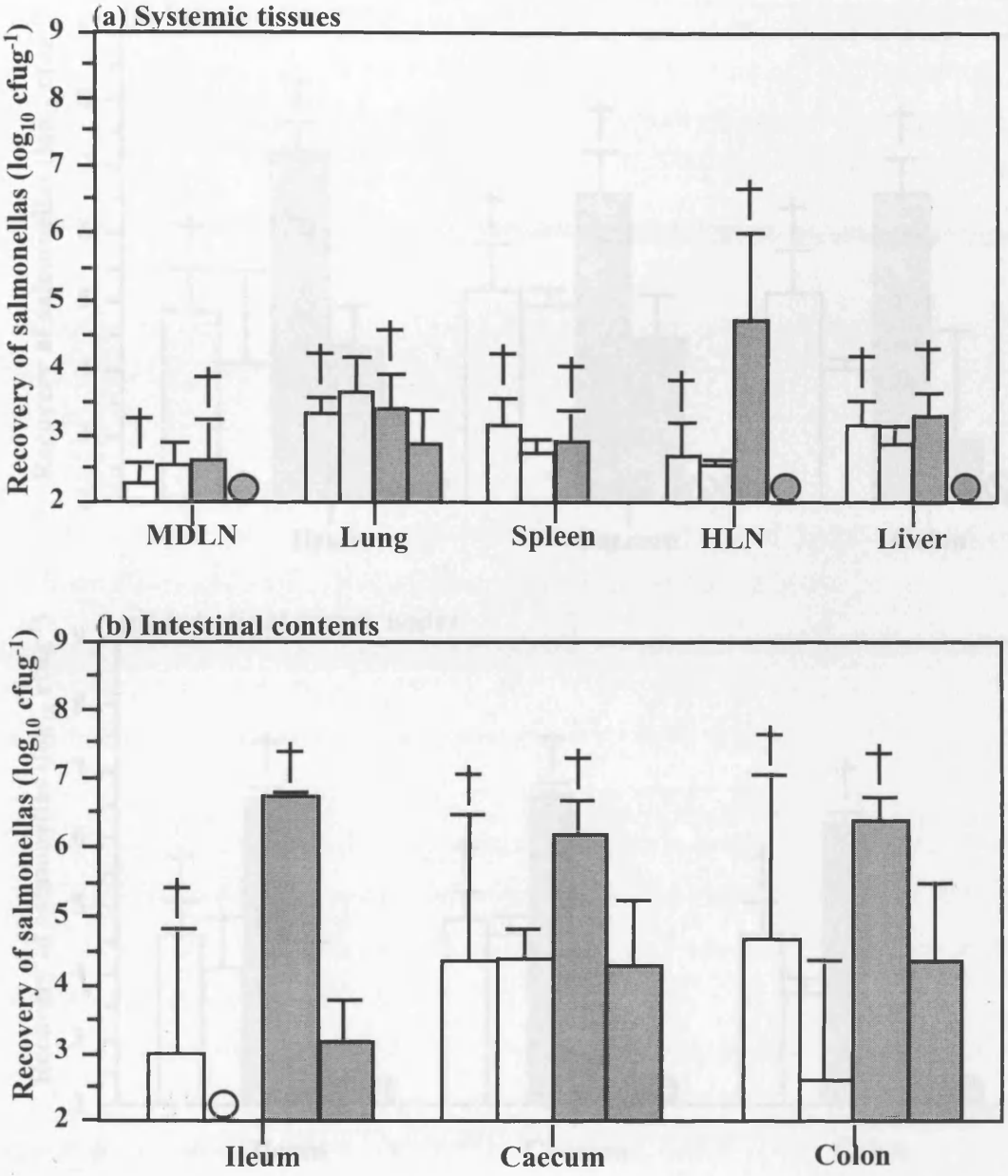



**Figure 3.2 Recovery of *Salmonella* from systemic tissues (a) and intestinal contents (b) of calves at up to 7 days *post* oral inoculation.** Triplicate samples were taken from each tissue and means were calculated to give a value *per* animal. Each bar represents the mean of 3 calves infected with *S. dublin* (moribund and survivors) [□ ] or *S. choleraesuis* (moribund and survivors) [▩ ] and is presented with the standard error of the mean. There was no bacterial recovery from *S. gallinarum* or *S. abortusovis* infected calves. Data from moribund calves were analysed separately and tissues are represented with †. Details of calves that succumbed to salmonellosis before 7 dpi are given in table 3.1.○ Samples that were positive on enrichment only. MDLN and HLN represent mediastinal and hepatic lymph nodes respectively.

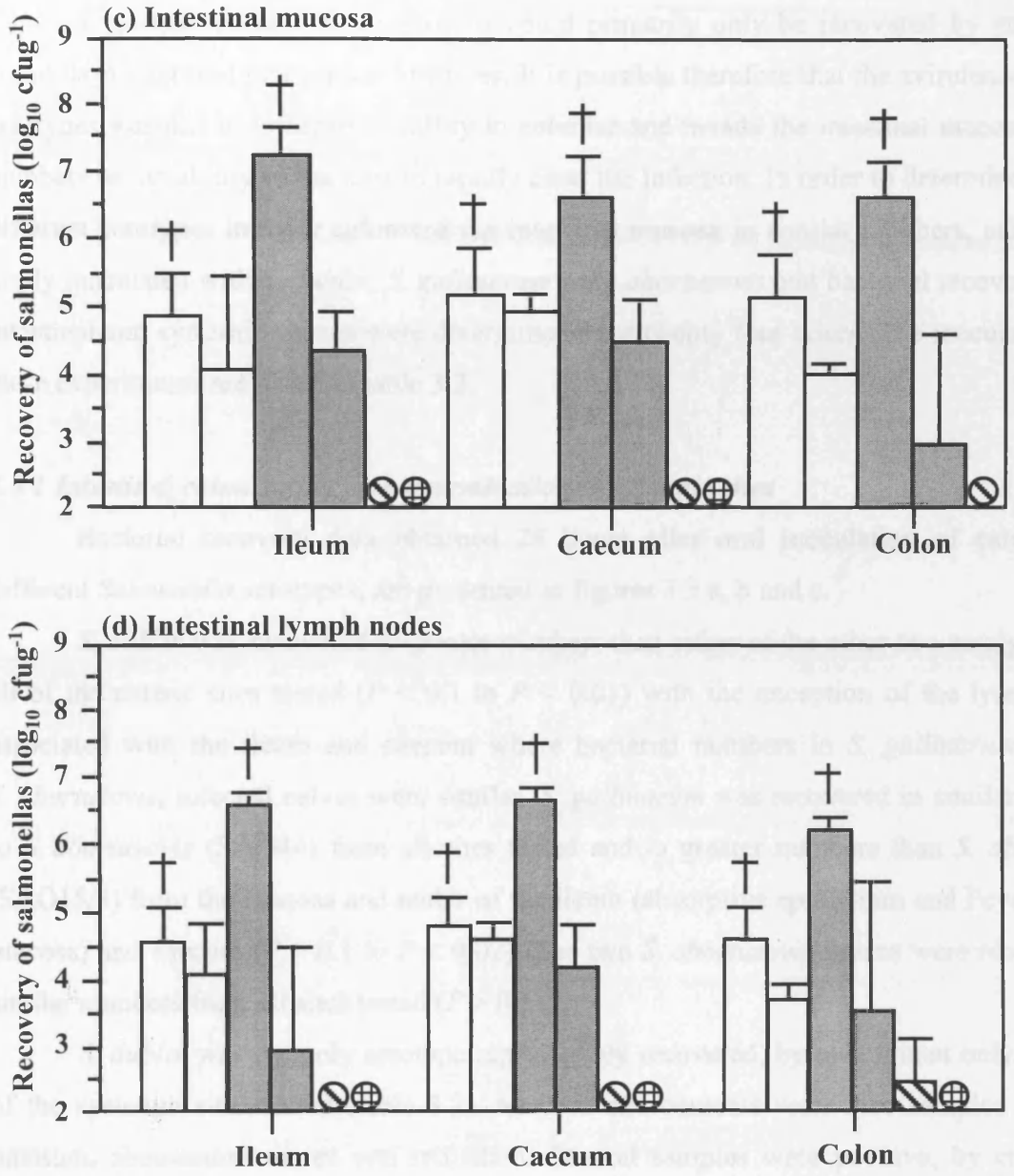



**Figure 3.2 cont. Recovery of *Salmonella* from intestinal mucosa (c) and intestinal nodes (d) of calves at up to 7 days *post* oral inoculation.** Triplicate samples were taken from each tissue and means were calculated to give a value *per* animal. Each bar represents the mean of 3 calves infected with *S. dublin* (moribund and survivors) [ □ ], *S. choleraesuis* (moribund and survivors) [ ▩ ], *S. gallinarum* (survivors) [ ▨ ] or *S. abortusovis* (survivors) [ ⊞ ] and is presented with the standard error of the mean. Data from moribund calves were analysed separately and tissues are represented with †. Details of calves that succumbed to salmonellosis before 7 dpi are given in table 3.1. ○ Samples that were positive on enrichment only.

## 3.5 Quantification of intestinal colonisation twenty four hours after oral inoculation of calves with *S. dublin*, *S. gallinarum* or *S. abortusovis*

*S. gallinarum* and *S. abortusovis* could primarily only be recovered by enrichment seven days after oral inoculation of calves. It is possible therefore that the avirulence of these serotypes was due to either an inability to colonise and invade the intestinal mucosa in high numbers or the ability of the host to rapidly clear the infection. In order to determine whether different serotypes initially colonised the intestinal mucosa in similar numbers, calves were orally inoculated with *S. dublin*, *S. gallinarum* or *S. abortusovis* and bacterial recoveries from intestinal and systemic tissues were determined after twenty four hours. The inocula used for these experiments are given in table 3.2.

### *3.5.1 Intestinal colonisation and dissemination to systemic sites*

Bacterial recovery data obtained 24 hours after oral inoculation of calves, with different *Salmonella* serotypes, are presented in figures 3.3 a, b and c.

*S. dublin* was recovered in greater numbers than either of the other two serotypes from all of the enteric sites tested ($P < 0.1$ to $P < 0.01$) with the exception of the lymph nodes associated with the ileum and caecum where bacterial numbers in *S. gallinarum*, but not *S. abortusovis*, infected calves were similar. *S. gallinarum* was recovered in similar numbers to *S. abortusovis* (SAO44) from all sites tested and in greater numbers than *S. abortusovis* (SAO15/5) from the mucosa and nodes of the ileum (absorptive epithelium and Peyer's patch mucosa) and caecum ($P < 0.1$ to $P < 0.02$). The two *S. abortusovis* strains were recovered in similar numbers from all sites tested ($P > 0.1$).

*S. dublin* was the only serotype reproducibly recovered, by enrichment only, from all of the systemic sites tested (table 3.2). Mucosa and contents were also sampled from the omasum, abomasum, rumen and reticulum. Several samples were positive, by enrichment only, from calves inoculated with *S. dublin*, *S. gallinarum* or *S. abortusovis* (SAO15/5), but not *S. abortusovis* (SAO44) (data not shown).

While it was not practical to quantify intestinal colonisation of two strains for each serotype, it was important to establish that strain specific differences were not evident. For this reason, colonisation of the bovine intestine was assessed using a second strain of *S. abortusovis*.

| Serotype (strain) | Calf number | Inocula ($log_{10}$ cfu) | Systemic tissue positive (+) or negative (-) by enrichment | | | | |
|---|---|---|---|---|---|---|---|
| | | | Mediastinal lymph node | Lung | Spleen | Hepatic lymph node | Liver |
| *S. dublin* (SD3246) | 3.19 | 8.78 | + | + | + | + | + |
| | 3.20 | 8.78 | – | + | + | + | + |
| | 3.21 | 8.78 | – | + | + | + | + |
| | 3.22 | 8.89 | + | + | + | + | + |
| | 3.23 | 8.84 | – | – | + | + | + |
| *S. gallinarum* (SG9) | 3.24 | 8.97 | – | + | + | – | + |
| | 3.25 | 8.97 | – | – | – | – | – |
| | 3.26 | 8.86 | – | – | – | – | – |
| | 3.27 | 8.89 | – | + | + | – | + |
| | 3.28 | 8.81 | – | – | – | – | – |
| *S. abortusovis* (SAO44) | 3.29 | 8.69 | – | – | – | – | – |
| | 3.30 | 8.69 | – | – | – | – | – |
| | 3.31 | 8.72 | – | – | – | – | – |
| *S. abortusovis* (SAO15/5) | 3.32 | 8.81 | – | – | + | – | – |
| | 3.33 | 8.65 | – | – | – | – | + |
| | 3.34 | 8.97 | – | – | – | + | – |

**Table 3.2 Inocula used and bacterial recovery, by enrichment only, from systemic tissues 24 hours after oral infection of calves with *S. dublin* (SD3246), *S. gallinarum* (SG9), *S. abortusovis* (SAO44) or *S. abortusovis* (SAO15/5).**

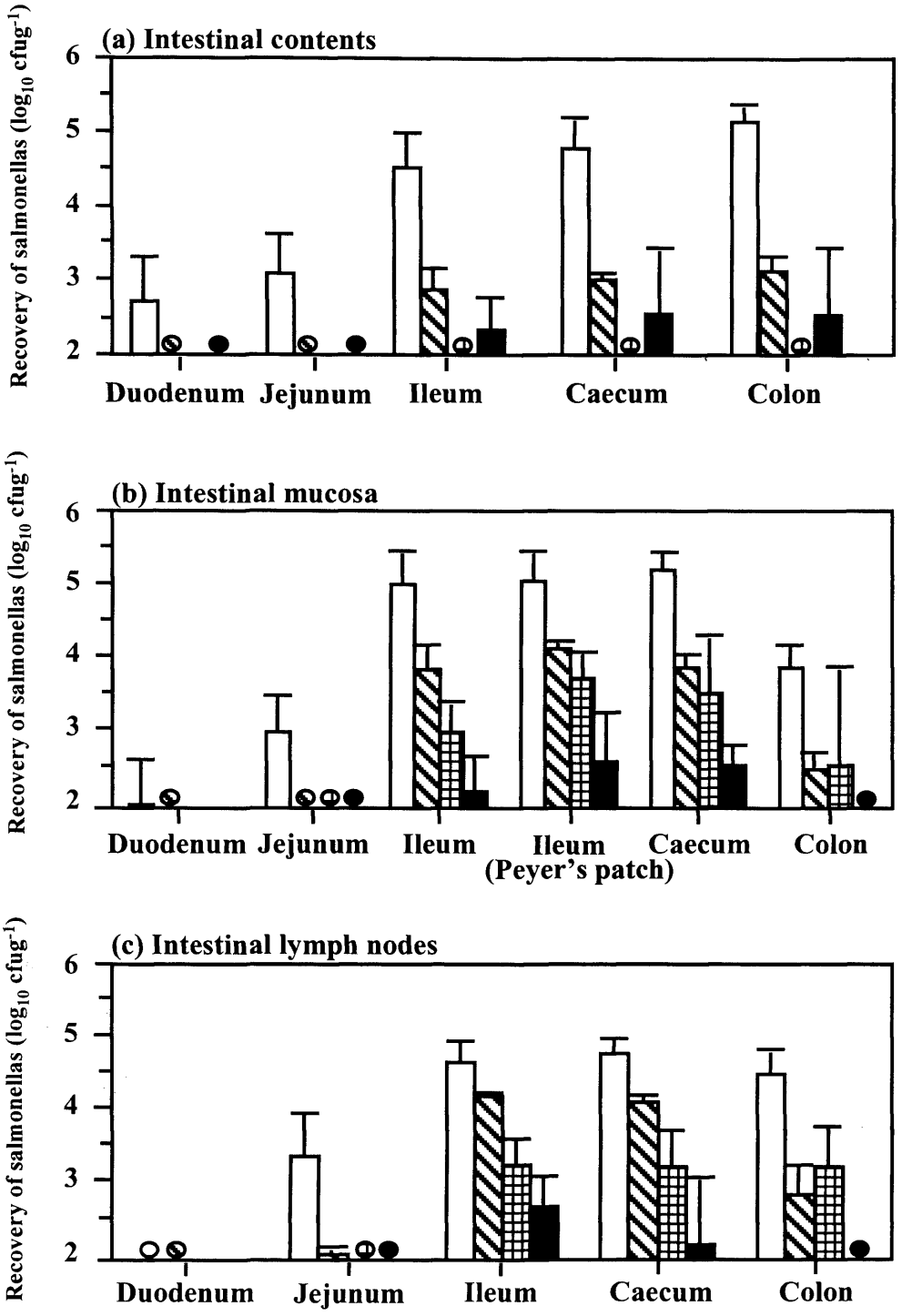



**Figure 3.3 Recovery of *Salmonella* from intestinal contents (a), mucosa (b) and nodes (c) of calves at 24 hours after oral inoculation.** Triplicate samples were taken from each tissue and means were calculated to give a value *per* animal. Each bar represents the mean of 5 calves infected with *S. dublin* SD3246 [□] or *S. gallinarum* SG9 [▨] and 3 calves each for *S. abortusovis* SAO44 [⊞] or SAO15/5 [■] and is presented with the standard error of the mean. ○ Samples that were positive on enrichment only.

### 3.6 Summary of results

1. *S. dublin* was highly virulent, while *S. gallinarum* and *S. abortusovis* were largely avirulent following oral inoculation of calves. These results correlated with the natural association of these serotypes in calves and suggested that there were genetic differences between the serotypes that accounted for *Salmonella* host specificity. *S. choleraesuis* was also highly virulent for calves suggesting that environmental factors potentially influence the low prevalence of this serotype in cattle.
2. There appeared to be differences in the pathogenesis of calves inoculated with either *S. dublin* or *S. choleraesuis*. *S. dublin* infected calves met end point criteria predominantly as a result of severe diarrhoea leading to dehydration. In contrast, while *S. choleraesuis* infected calves had the highest intestinal bacterial counts, these animals met end point criteria as a result of systemic-like symptoms including a dull demeanour and an inability to stand unaided.
3. *S. gallinarum* and *S. abortusovis* colonised the intestinal mucosa and were isolated from systemic sites in lower numbers than *S. dublin* twenty four hours after oral inoculation. This would suggest that initial *Salmonella*-intestinal interactions or dissemination and survival of bacteria within both enteric and systemic tissues might influence serotype-host specificity.

### 3.7 Discussion

Epidemiological evidence clearly suggests that there is an association of particular *Salmonella* serotypes with a limited number of host species (Sojka and Field, 1970; Sojka, *et al.*, 1977; figure 1.1). In order to determine whether this apparent serotype-host specificity could be explained by genetic differences between serotypes, virulence was assessed following oral inoculation of calves with one strain each of *S. dublin*, *S. choleraesuis*, *S. gallinarum* or *S. abortusovis*.

*S. dublin* was highly virulent, which correlated with a natural association of this serotype in cattle (Wray and Davies, 2000). In contrast, *S. gallinarum* and *S. abortusovis* were largely avirulent correlating with the natural specificities of these serotypes for fowl and sheep respectively (Jack, 1968; Barrow, *et al.*, 1994). These results clearly demonstrate that with some serotypes genetic differences contribute to pathogenesis in calves.

*S. choleraesuis* was also highly virulent in calves, an observation that was consistent with a previous experimental study (Smith & Halls, 1968), yet surprising as this serotype rarely causes disease in cattle naturally (Sojka and Field, 1970; Sojka, *et al.*, 1977). This clearly indicates that pathogenesis following experimental infection may not always correlate with epidemiological data and implies that environmental factors may, in part, determine the host restriction of this serotype. The results from the report of Smith and Halls, (1968) were difficult to interpret because the experimental infection model used was very severe and therefore differences in the virulence of the serotypes compared may have been missed. For example, a high inoculum and the use of very young Jersey calves, a breed known to be highly susceptible to *Salmonella* infection (Wray and Sojka, 1978), potentially contributed to the severity of the disease. In the current study, despite using the more resistant Friesian cattle breed (Jones, 1992), a lower inoculum and older animals the observations confirm that calves are susceptible to experimental *S. choleraesuis* infection. In addition to calves, this serotype can cause severe disease, under experimental conditions, in other mammalian species including pigs (Watson *et al.*, 2000c), sheep (Uzzau, personal communication), rats (Nnalue *et al.*, 1992), rabbits and guinea pigs (Barrow *et al.*, 1994). It is therefore possible that with *S. choleraesuis* the infectious dose and the virulence of the infecting strain may influence both the prevalence of incidents and the increased pathogenicity following experimental infection.

Due to pre-disposing environmental factors, it is also possible that natural infection may occur with an atypical combination of serotype and host. For example, during the late 1960s there was a sharp escalation in the number of *S. dublin* incidents in calves, which may in part be explained by the increased movement of calves and cattle from endemic areas due to an expansion in intensive rearing. This rise in *S. dublin* incidents correlated with an

increase in isolations of this serotype from both pigs and sheep (Sojka and Field, 1970; Sojka, *et al.*, 1977; VLA, 1978-1999; figure 1.1). While this was almost certainly due to a greater exposure of both hosts to *S. dublin*, this serotype is a common isolate from sheep, but not pigs, in the U.K. (VLA reports, 1978-1999) where it is associated with abortion, systemic and enteric salmonellosis (Gitter and Sojka, 1970; Walton, 1972). Interestingly, the bovine virulent *S. dublin* strain (SD3246) used in this study is virulent for sheep (Uzzau *et al.*, 2001) but avirulent for pigs following oral inoculation (Watson *et al.*, 2000c). These results highlight the complexity of *Salmonella*-host interactions and demonstrate that experimental data cannot be extrapolated from one species to another.

With some combinations of serotype and host there is a very strong correlation between experimental and natural infection. For example, neither *S. gallinarum* nor *S. abortusovis* were isolated, prior to their elimination from U.K. livestock, in any significant numbers from hosts other than fowl and sheep respectively (Sojka and Field, 1970; Sojka, et al., 1977). Likewise, under controlled experimental conditions, these serotypes fail to cause systemic disease in all but their naturally associated host species (S. Paulin, unpublished data; Chadfield *et al.*, 2001; Uzzau *et al.*, 2001) a fact which reflects the highly host-specific nature of these serotypes.

A comparison of the clinical and pathological signs observed in *S. dublin* and *S. choleraesuis* infected calves, which succumbed to salmonellosis before seven days *post* inoculation, suggested that the diseases caused by these serotypes were distinct. In these animals, *S. choleraesuis* was recovered in higher numbers than *S. dublin* from the intestinal sites, despite the fact that calves infected with *S. dublin* met end point criteria due to severe scouring leading to dehydration. In contrast, calves inoculated with *S. choleraesuis* had less severe scouring and showed symptoms more typical of systemic disease including an eventual inability to stand unaided and an inability to control homeostasis. Similar symptoms have been reported in pigs following both natural and experimental infection with *S. choleraesuis*, when the first signs of disease are often lethargy, pyrexia, depression and the finding of dead, or dying, animals with purple extremities and abdomens (Wilcock and Schwartz, 1992; Watson *et al.*, 2000c). Taken together, the clinical symptoms in both calves and pigs following infection with *S. choleraesuis* suggest that the resulting disease is often systemic in nature but is also dependent on the host species. For example, in pigs despite infecting both the intestinal mucosa and systemic tissues in high numbers diarrhoea is often absent. In calves however, diarrhoea is typically observed together with high bacterial recoveries from intestinal sites and low recoveries from systemic tissues suggesting that, in cattle at least, persistence and growth within enteric sites is pivotal in determining the outcome of infection.

Despite the avirulence of *S. gallinarum* and *S. abortusovis* in calves, both of these serotypes were able to elicit a transient pyrexic response following inoculation. This indicated that the bacteria had survived initial host defence mechanisms including low stomach pH, low oxygen levels, presence of intestinal bile salts, high osmolarity and competition with resident gut microflora and had penetrated the intestinal mucosa in sufficient numbers to activate the host immune system. *S. gallinarum* and *S. abortusovis* were clearly able to colonise the intestinal mucosa and reach the regional lymph nodes. However, these serotypes were recovered in lower numbers than *S. dublin* from all intestinal sites after twenty four hours and only reached systemic tissues intermittently. Neither *S. gallinarum* nor *S. abortusovis* were recovered from the intestinal contents after seven days and both of these serotypes were present within the contents in lower numbers than *S. dublin* after twenty four hours. In addition to the magnitude of intestinal invasion, many factors influence the ability of salmonellas to persist within a given host. These include the *in vivo* growth and death rates, the resulting ability to disseminate and persist within enteric and systemic tissues and the ability to re-seed and grow within the gut lumen. It could be speculated therefore that initial interactions with the intestinal mucosa might result in the differences in colonisation observed with *S. gallinarum*, and *S. abortusovis* compared to *S. dublin*.

In conclusion, *Salmonella* serotypes, with different natural host preferences, have been characterised for their virulence up to seven days after oral inoculation of calves. *S. dublin* and *S. choleraesuis* were highly virulent, while *S. gallinarum* and *S. abortusovis* were avirulent. In addition, *S. gallinarum* and *S. abortusovis* were unable to colonise the intestinal mucosa in similar numbers to *S. dublin* after twenty four hours. These results suggest that genetic differences between serotypes exist, which may determine their host specificity, and implicate initial intestinal interactions between serotype and host in influencing the outcome of disease. Therefore these strains, of defined virulence, were assessed for their ability to invade intestinal epithelial cells *in vitro* and bovine intestinal mucosa *in vivo*.

# CHAPTER 4

# CHARACTERISATION OF THE INVASIVE PHENOTYPES OF *SALMONELLA* SEROTYPES WITH DISTINCT HOST SPECIFICITIES.

## 4.1 Introduction

Attachment to, and invasion of, intestinal epithelial cells is an essential SPI-1-dependent feature of *Salmonella* pathogenesis enabling bacteria to gain access to the host and facilitating translocation to deeper tissues.

Several studies have suggested that initial interactions with the intestinal mucosa may influence the outcome of infection in different species (Pospischil *et al.*, 1990; Jones *et al.*, 1994; Pascopella *et al.*, 1995; Henderson *et al.*, 1999). For example, following oral inoculation of mice *S. typhimurium*, which causes a disease in this host with similarities to typhoid fever in humans, preferentially enters (Carter and Collins, 1974; Jones *et al.*, 1994) and subsequently destroys (Jones *et al.*, 1994; Pascopella *et al.*, 1995) 'M' cells overlying the follicle associated epithelium of Peyer's patch mucosa. The murine avirulent serotypes *S. typhi*, and *S. gallinarum*, are either able to invade, but not destroy, or are simply unable to invade 'M' cells respectively (Pascopella *et al.*, 1995), which suggests that both the route and magnitude of intestinal invasion correlate with the host specific phenotype. In contrast to the situation reported in mice, within fifteen minutes of injecting *S. typhimurium* into bovine ligated ileal loops salmonellas can be seen associating with both 'M' cells and enterocytes overlying domed and absorptive villi (Frost *et al.*, 1997). Therefore, as a result of initial interactions with the intestinal mucosa, the foci of infection appear to develop in murine, but not bovine, Peyer's patch mucosa.

While the implications of these observations are unknown, it is possible that the nature of the bacterial-host interaction with the intestinal mucosa may influence the subsequent infection. Consequently, it is likely that serotype and host factors influence the invasive phenotype of different *Salmonella* serotypes.

## 4.2 Aim

In the previous chapter it was established that following oral inoculation, the outcome of disease in calves was dependent on the infecting *Salmonella* serotype. Intestinal invasion is believed to be essential for *Salmonella* pathogenesis, therefore the relative abilities of different serotypes to invade both cultured epithelial cells and bovine intestinal mucosa were determined. As the importance of enterocytes, compared with M cells, in the context of host specificity remains poorly defined the ability of different *Salmonella* serotypes to preferentially invade Peyer's patch mucosa or absorptive epithelia was also determined.

## 4.3 Approaches and experimental design

The invasive phenotype of two strains each of *S. dublin* (SD3246 and SD2229), *S. typhimurium* (ST4/74 and ST12/75), *S. choleraesuis* (SCSA50 and SCS14/74), *S. gallinarum* (SG9 and SGJ91) or *S. abortusovis* (SAO44 and SAO15/5) was assessed using both *in vitro* and *in vivo* techniques.

*In vitro* invasion was quantified using the human intestinal epithelial cell line Int 407. The experimental procedure used has been optimised previously (Watson *et al.,* 1995) and was modified as described in section 2.8.

The route and magnitude of intestinal invasion and the amount of disruption to mucosal integrity induced by different *Salmonella* serotypes *in vivo* were determined using the bovine ligated ileal loop model. The experimental procedure used has been optimised previously (Watson *et al.*, 1995) and was modified as described in sections 2.10 and 2.11.

Data from the *in vitro* assays was derived from the mean of triplicate wells in either two or four separate experiments. Data from the *in vivo* experiments was derived from the mean strain values *per* animal in 2 to 6 separate calves (2 or 3 loops *per* strain *per* animal). For simplicity of interpretation, the mean values for 2 strains within a given serotype were determined and statistical analyses based on this data were used in the discussion of results. Where appropriate, any strain specific differences were reported.

Inocula for the *in vitro* assays and *in vivo* ligated ileal loops at the high dose were prepared as described in section 2.11.1 (i). Inocula for the low dose ligated ileal loop assays were prepared as described in section 2.11.1 (ii). Details of inocula used are given in tables 4.1a, 4.2 and 4.3.

## 4.4 Quantification of *in vitro* invasion into cultured epithelial cells

The use of the epithelial cell line Int 407 provides a rapid initial assessment of the invasive phenotypes of different *Salmonella* serotypes. In addition to quantifying the magnitude of invasion, the release of the intracellular enzyme lactate dehydrogenase (LDH) was measured two hours after infection to ensure that the monolayer integrity remained intact. Damage to epithelial cells could result in the subsequent killing, by gentamicin, of intracellular bacteria that had been released into the culture media, thereby artificially lowering the estimation of the number of intracellular salmonellas. Details of inocula used are given in table 4.1a

### *4.4.1 Determination of the growth rate, in RPMI media, of different Salmonella serotypes after two hours*

In order to check that all serotypes grew at a comparable rate *in vitro*, RPMI medium, supplemented with 5% FCS, was seeded with one strain *per* serotype and the total number of bacteria recovered after incubation for two hours was determined (table 4.1b). All strains appeared to grow at a comparable rate suggesting that growth in tissue culture media should not influence the intracellular bacterial recovery from Int 407 cells.

### *4.4.2 Magnitude of Int 407 epithelial cell invasion by different Salmonella serotypes*

All serotypes invaded Int 407 cells, although the magnitude of invasion varied (figure 4.1). *S. dublin* and *S. typhimurium* were significantly more invasive than *S. choleraesuis* or *S. abortusovis* ($P < 0.002$ to $P < 0.001$), while *S. gallinarum* was significantly less invasive than all of the other serotypes ($P < 0.001$). The reduced invasion mutant ST4/74 *invH*201::Tn*phoA* (hereafter referred as *invH* mutant) was recovered at 1.3 logs lower than the corresponding wild type strain. This is consistent with previous observations (Watson *et al.*, 1995; Watson *et al.*, 1998) demonstrating that the assay was discriminatory for different invasive phenotypes. Additionally, there were no significant strain specific differences within a given serotype.

### *4.4.3 Lactate dehydrogenase (LDH) release following infection of Int 407 cells with different Salmonella serotypes*

All strains tested resulted in the release of similar amounts of LDH to the negative control (figure 4.2). The results from this series of experiments suggest that the serotypes typically associated with infection of mammalian species are significantly more invasive for a mammalian cell line than the avian associated serotype *S. gallinarum*. This could be due to a host-specific phenomenon, alternatively it could be due to the non-motility of *S. gallinarum in vitro*. To investigate this, the invasion assay was repeated including a centrifugation step in an attempt to increase bacterial-cell interactions and therefore overcome potential differences in invasion of the different serotypes due to the lack of motility of *S. gallinarum*.

### *4.4.4 Effect of centrifugation of Int 407 cells on total and intracellular recovery of S. typhimurium and S. gallinarum*

The total (intracellular and adherent) and intracellular recoveries of one strain of *S. typhimurium* (ST4/74) and two strains of *S. gallinarum* (SG9 and SGJ91) were compared following centrifugation of the Int 407 cell monolayers immediately after infection (section 2.8.2). The total and intracellular recoveries of *S. typhimurium* were similar either with or

without centrifugation whereas the recoveries of *S. gallinarum* were reduced by approximately 1–1.5 logs in the absence of the centrifugation step (figure 4.3a, b). Total recovery of the reduced invasion mutant SG9 *sipB*::pB1 (hereafter referred to as SG9*sipB*) was similar whether a centrifugation step was included or not (figure 4.3a), but intracellular recovery could only be detected if the monolayer was centrifuged (figure 4.3b). As the results were derived from a single experiment statistical analyses were not performed. None of the strains resulted in significant LDH release from the cell monolayer following centrifugation (figure 4.3c).

It can be concluded that centrifugation does increase the uptake of *S. gallinarum*, but not to the level seen with *S. typhimurium* suggesting that serotype specific factors influence *in vitro* invasion. To determine whether these differences were evident *in vivo*, the ability of different *Salmonella* serotypes to invade bovine intestinal mucosa was assessed.

(a)

| Strain | Inocula ($log_{10}$ cfu $ml^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | Total number of wells | Int 407 assay 4.1 | Int 407 assay 4.2 | Int 407 assay 4.3 | Int 407 assay 4.4 | Average inocula ± SEM |
| **SD2229** | 12 | 6.52 | 6.42 | 6.41 | 6.36 | 6.43±0.03 |
| **SD3246** | 12 | 6.52 | 6.46 | 6.35 | 6.38 | 6.43±0.04 |
| **SCSA50** | 12 | 6.58 | 6.45 | 6.48 | 6.33 | 6.46±0.05 |
| **SCS 14/74** | 12 | 6.59 | 6.49 | 6.52 | 6.31 | 6.48±0.06 |
| **ST4/74** | 12 | 6.44 | 6.32 | 6.26 | 6.2 | 6.31±0.05 |
| ***invH* mutant** | 12 | 6.39 | 6.29 | 6.36 | 6.15 | 6.30±0.05 |
| **ST12/75** | 12 | 6.50 | 6.48 | 6.37 | 6.34 | 6.42±0.04 |
| **SAO44** | 6 | _ | _ | 6.05 | 6 | 6.03±0.02 |
| **SAO15/5** | 6 | _ | _ | 5.97 | 5.9 | 5.94±0.03 |
| **SG9** | 6 | _ | _ | 6.46 | 6.21 | 6.34±0.13 |
| **SGJ91** | 6 | _ | _ | 6.29 | 6.03 | 6.16±0.13 |

(b)

| Strain | Average inocula ($log_{10}$ cfu $ml^{-1}$ ± SEM) | Bacterial counts 2 hours *post* inoculation ($log_{10}$ cfu $ml^{-1}$ ± SEM) | Growth ($log_{10}$ cfu $ml^{-1}$) |
|---|---|---|---|
| **SD2229** | 5.85 ± 0.56 | 6.76 ± 0.41 | 0.91 |
| **SCSA50** | 6.05 ± 0.43 | 6.90 ± 0.41 | 0.85 |
| **ST4/74** | 5.75 ± 0.50 | 6.87 ± 0.38 | 1.12 |
| ***invH* mutant** | 5.81 ± 0.55 | 6.68 ± 0.57 | 0.87 |
| **SAO44** | 5.68 ± 0.37 | 6.52 ± 0.28 | 0.84 |
| **SG9** | 5.94 ± 0.52 | 6.65 ± 0.78 | 0.71 |

**Table 4.1 Inocula used to determine Int 407 epithelial cell invasion by *Salmonella* serotypes (a) and growth of *Salmonella* in RPMI media (b)** (data derived from triplicate wells in two separate experiments).

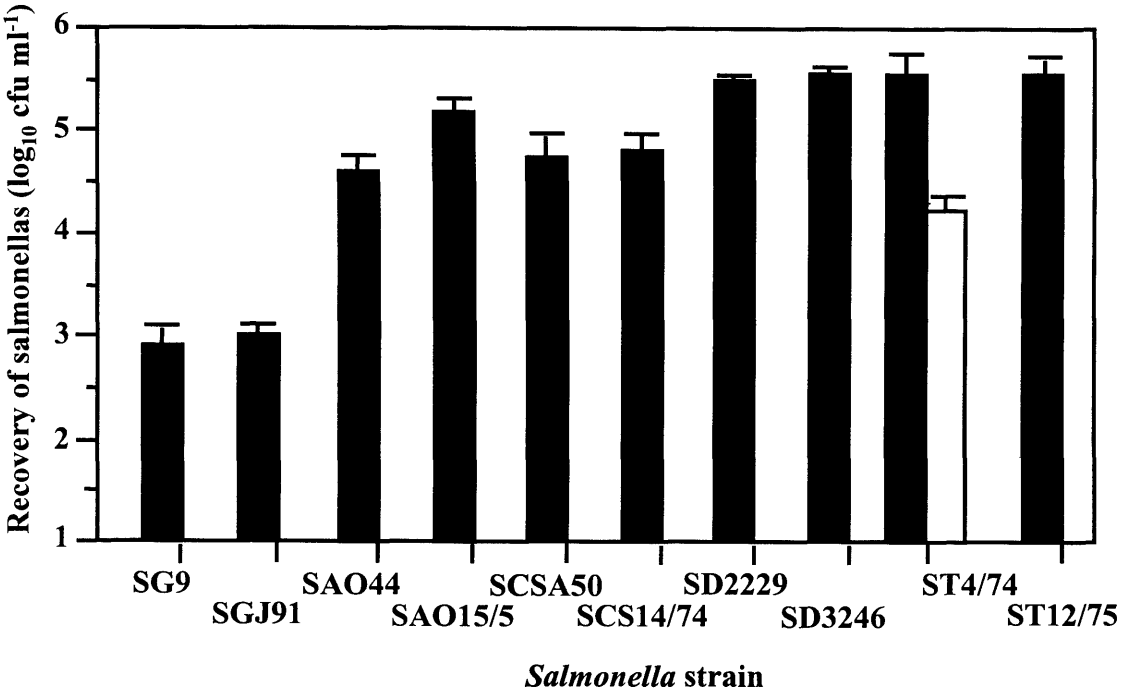



**Figure 4.1 Intracellular recovery of *Salmonella* serotypes 2 hours after infection of Int 407 cells with approximately 6.5 $log_{10}$ cfu $ml^{-1}$.** Each bar represents the mean value taken from triplicate wells in either 2 or 4 separate experiments and is presented with the standard error of the mean.

(□) represents mean values for the ST4/74*invH* mutant.

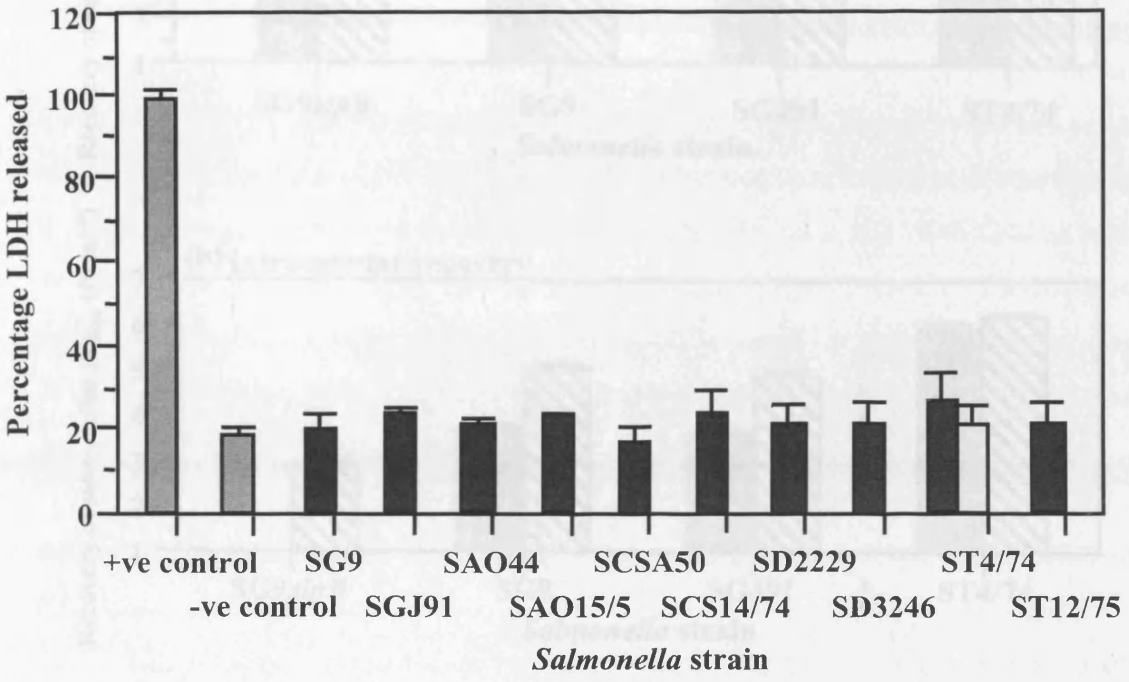



**Figure 4.2 Magnitude of lactate dehydrogenase (LDH) released by Int 407 cells 2 hours after infection with *Salmonella* serotypes.** Each bar represents the mean value taken from triplicate wells in either 2 or 4 separate experiments and is presented with the standard error of the mean. The LDH release is expressed as a percentage compared to the positive control. (□) represents mean values for the ST4/74*invH* mutant.

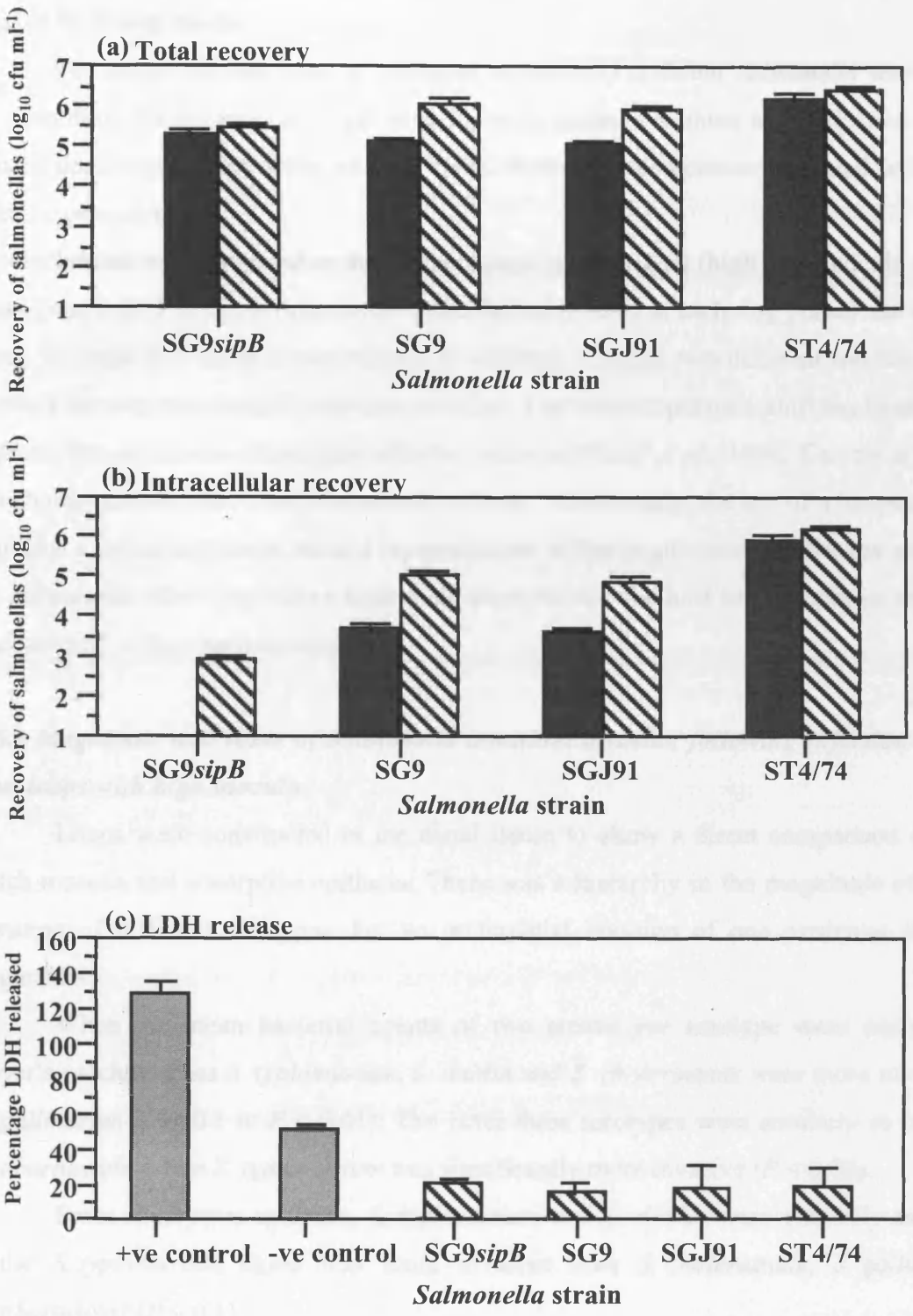



**Figure 4.3 Effect of centrifugation on total (a) and intracellular (b) recovery of *Salmonella* from Int 407 cells and resulting LDH release (c).** Each bar represents the mean value from triplicate wells in 1 experiment only and is presented with the standard error of the mean (based on 3 wells in a single experiment). The average inocula (in $log_{10}$ cfu $ml^{-1}$) used were ST4/74, 6.43; SG9, 6.55; SG9*sipB*, 6.54; SGJ91, 6.59. (■) without centrifugation; (▨) with centrifugation.

## 4.5 Characterisation of the magnitude and route of intestinal invasion using the bovine ligated ileal loop model

The magnitude and route of intestinal invasion by different *Salmonella* serotypes, and any resulting disruption to mucosal integrity were assessed 2 hours after infection of bovine ligated ileal loops to determine whether initial intestinal interactions correlated with the host specific phenotype.

Inocula were prepared as described in sections 2.11.1 (i) (high dose inocula at mid-log phase) and 2.11.1 (ii) (low dose temperature shifted inocula at early-log phase) and details are given in tables 4.2 and 4.3 respectively. In addition to using two different inocula sizes, the method for preparing inocula was also modified. Use of a temperature shift has been shown to increase the expression of secreted effector proteins (Wood *et al.*, 1996; Galyov *et al.*, 1997) which play an essential role in intestinal invasion. Additionally, the use of a temperature shift provides a potentially more natural representation of the environmental changes encountered by *Salmonella* when they move from a situation outside the host to the ambient temperature encountered within the intestines.

### *4.5.1 Magnitude and route of Salmonella intestinal invasion following infection of ligated ileal loops with high inocula*

Loops were constructed in the distal ileum to allow a direct comparison of Peyer's patch mucosa and absorptive epithelia. There was a hierarchy in the magnitude of intestinal invasion of different serotypes, but no preferential invasion of one particular tissue type (figure 4.4).

When the mean bacterial counts of two strains *per* serotype were analysed from Peyer's patch mucosa *S. typhimurium*, *S. dublin* and *S. choleraesuis* were more invasive than *S. gallinarum* ($P < 0.1$ to $P < 0.01$). The latter three serotypes were similarly as invasive as *S. abortusovis*, while *S. typhimurium* was significantly more invasive ($P < 0.05$).

From absorptive epithelia, *S. typhimurium* and *S. dublin* were similarly as invasive, while *S. typhimurium* alone was more invasive than *S. choleraesuis*, *S. gallinarum* or *S. abortusovis* ($P < 0.1$).

The *invH* mutant was recovered at approximately one log lower than the comparable wild type strain ($P < 0.05$). The reduced invasion mutant SD2229*sipB* was recovered at approximately three logs lower than the comparable wild type strain ($P < 0.001$), demonstrating the sensitivity of the assay.

In conclusion, with the use of high inocula there was no clear correlation between *Salmonella* intestinal invasion and host specificity. To confirm this result the invasion assay was repeated using lower inocula that had been temperature shifted for maximal expression of

SPI-1 dependent secreted effector proteins. Prior to these experiments the inocula was titrated to determine the optimum low dose.

### *4.5.2 Effect of inocula size on the intracellular recovery of S. dublin*

The ability of *S. dublin* (SD2229) to invade the mid-ileal absorptive epithelia, at a range of inocula sizes, was determined. The relationship between inocula size and intracellular recovery was linear with the exception of recoveries at 8 and 8.8 $\log_{10}$ cfu loop which were similar, suggesting saturation of the assay (figure 4.5). At a dose of 7 $\log_{10}$ cfu loop, mucosal counts fell mid range of recovery in the assay and numbers of intracellular bacteria were more than 2 logs above the limit of detection and approximately 1.5 logs lower than the recovery obtained when using the high inocula (figure 4.4). Therefore, 7 $\log_{10}$ cfu loop (prepared using a temperature shift) was used in subsequent assays.

### *4.5.3 Magnitude and route of Salmonella intestinal invasion following infection of ligated ileal loops infection with low inocula*

The use of lower inocula clearly demonstrated a hierarchy in the magnitude of intestinal invasion of different serotypes within the distal ileum, in the absence of preferential invasion of tissue either with or without Peyer's patches (figure 4.6a).

When the mean bacterial recoveries of two strains *per* serotype were analysed, *S. typhimurium* was significantly more invasive than *S. dublin* from absorptive epithelia ($P < 0.01$) and similarly as invasive from Peyer's patch mucosa. In both tissue types these serotypes were significantly more invasive than *S. gallinarum* ($P < 0.05$ to $P < 0.01$), which was significantly more invasive than *S. choleraesuis* ($P < 0.05$ to $P < 0.01$) which in turn was on average significantly more invasive than *S. abortusovis* ($P < 0.05$).

All strains were recovered in significantly higher numbers from test loops compared with the LB control with the exception of *S. abortusovis* (SAO44) however, there was a strain specific difference between SAO44 and SAO15/5 ($P < 0.01$).

In conclusion, although there were differences in the magnitude of intracellular bacterial recovery, there was no clear correlation between *Salmonella* intestinal invasion and host specificity.

### *4.5.4 Magnitude of intestinal invasion of different Salmonella serotypes in the mid ileum*

In order to determine whether the magnitude of invasion was similar, irrespective of the region of intestinal mucosa used, loops were constructed in the mid ileum from absorptive epithelia. Loops were infected with approximately 7 $\log_{10}$ cfu loop prepared using a temperature shift (table 4.3).

There was a hierarchy in the magnitude of intestinal invasion in the mid ileum which was similar to that observed in the distal ileum (figure 4.6b). The ability of *S. dublin* SD2229*sipB* to invade the intestinal mucosa was reduced by approximately 3 logs compared to the corresponding wild type strain which suggests that this area of intestinal mucosa is equally able to discriminate between strains of different invasive phenotypes as the distal ileum.

These results suggest that invasion of *Salmonella* serotypes is not dependent on the region of intestinal mucosa used, at least within the distal part of the gastro-intestinal tract.

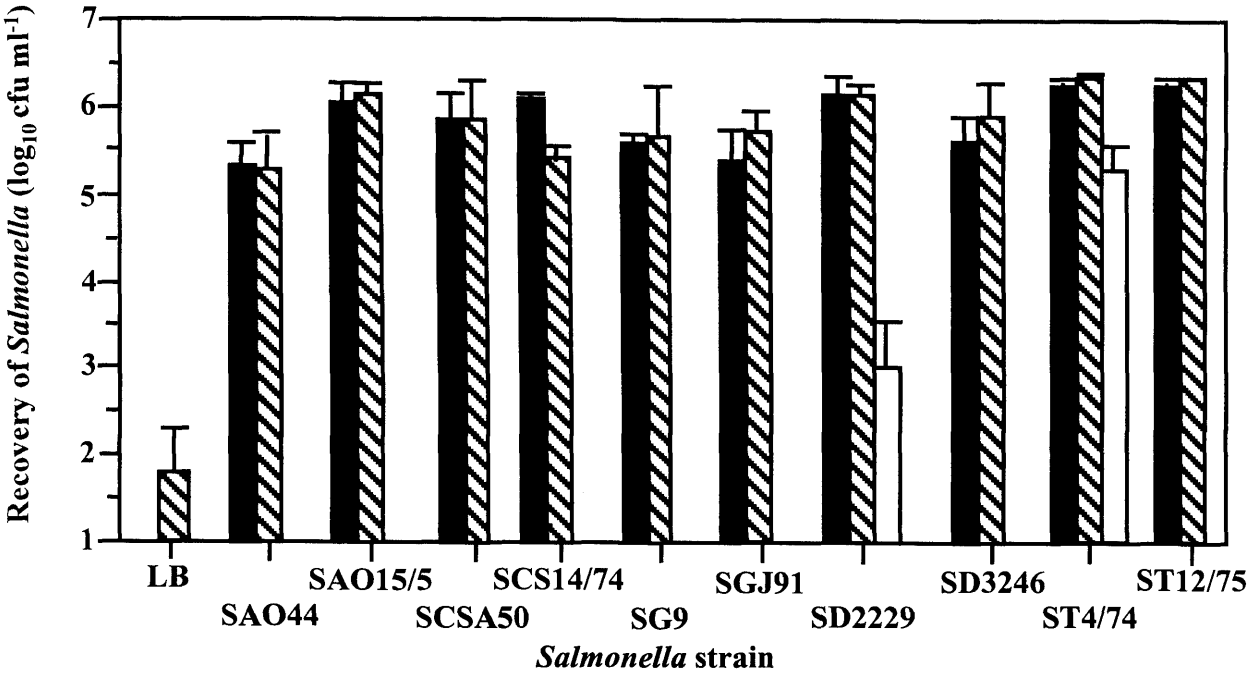
### *4.5.5 Amount of mucosal damage induced by Salmonella serotypes following infection of bovine ligated ileal loops with high and low inocula*

The amount of damage to the intestinal mucosa following infection of the different *Salmonella* serotypes was semi-quantified by scoring H and E stained sections as described in 2.15.1.1.

When inocula of approximately 7 $\log_{10}$ cfu loop were used very little mucosal damage was observed following infection with any serotype (table 4.4 and figure 4.7). However, when inocula of approximately 9.5 $\log_{10}$ cfu loop were used *S. typhimurium* and *S. dublin* induced the most severe mucosal damage (table 4.5 and figure 4.8). This suggests that the number of gentamicin protected bacteria enumerated following infection with these two serotypes might be a slight underestimation as a result of subsequent antibiotic killing of released intracellular bacteria.

| Strain | Inocula ($log_{10}$ cfu loop) derived from 5 ml of bacterial culture | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Total number of loops** | **Calf 4.1** (3 loops *per* strain) | **calf 4.2** (2 loops *per* strain) | **calf 4.3** (2 loops *per* strain) | **calf 4.4** (2 loops *per* strain) | **calf 4.5** (2 loops *per* strain) | **Average inocula *per* loop ± SEM** |
| **SD2229** | 4 | _ | _ | _ | 9.6 | 9.65 | 9.63 ± 0.02 |
| **SD2229 *sipB*** | 4 | _ | _ | _ | 9.62 | 9.87 | 9.75 ± 0.13 |
| **SD3246** | 4 | _ | 9.52 | 9.3 | _ | _ | 9.41 ± 0.11 |
| **SCSA50** | 4 | _ | 9.55 | 9.58 | _ | _ | 9.57 ± 0.01 |
| **SCS14/74** | 4 | _ | _ | _ | 9.78 | 9.79 | 9.79 ± 0 |
| **ST4/74** | 7 | 9.73 | 9.5 | 9.36 | _ | _ | 9.53 ± 0.11 |
| ***invH* mutant** | 7 | 9.78 | 9.58 | 9.27 | _ | _ | 9.54 ± 0.15 |
| **ST12/75** | 4 | _ | _ | _ | 9.69 | 9.9 | 9.80 ± 0,10 |
| **SAO44** | 4 | _ | 9.38 | 9.25 | _ | _ | 9.32 ± 0.06 |
| **SAO15/5** | 4 | _ | _ | _ | 9.06 | 8.96 | 9.01 ± 0.05 |
| **SG9** | 4 | _ | 9.58 | 9.27 | _ | _ | 9.43 ± 0.15 |
| **SGJ91** | 4 | _ | _ | _ | 9.43 | 10.01 | 9.72 ± 0.29 |

**Table 4.2 High dose inocula used to quantify the magnitude of *in vivo* intestinal invasion by different *Salmonella* serotypes.**




| Serotype | Mean bacterial recovery of two strains *per* serotype ($log_{10}$ cfu $ml^{-1}$) | |
|---|---|---|
| | Peyer's patch mucosa | Absorptive epithelium |
| ***S. typhimurium*** | 6.22 | 6.33 |
| ***S. dublin*** | 5.88 | 6.02 |
| ***S. gallinarum*** | 5.45 | 5.67 |
| ***S. choleraesuis*** | 5.96 | 5.65 |
| ***S. abortusovis*** | 5.66 | 5.70 |

**Figure 4.4 Magnitude of *Salmonella* intestinal invasion 2 hours after infection of bovine ligated ileal loops with approximately 9.5 $log_{10}$ cfu loop.**

Loops were constructed in the distal ileum from either Peyer's patch mucosa ( ■ ) or absorptive epithelia ( ▨ ). Triplicate samples were taken from 2 loops in each animal and means were calculated to give a value *per* animal. Each bar on the graph represents the mean of either 2 or 3 calves and is presented with the standard error of the mean. The mean values *per* serotype are given in the above table. ( □ ) represents mean values for the SD2229*sipB* and ST4/74*invH* mutants.

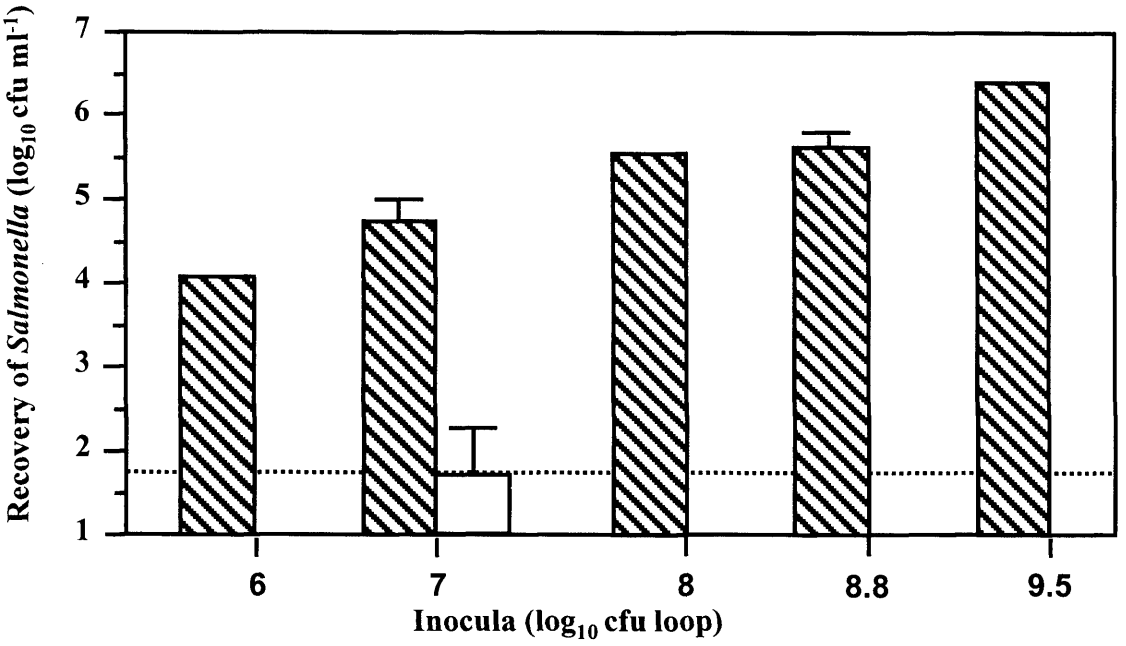
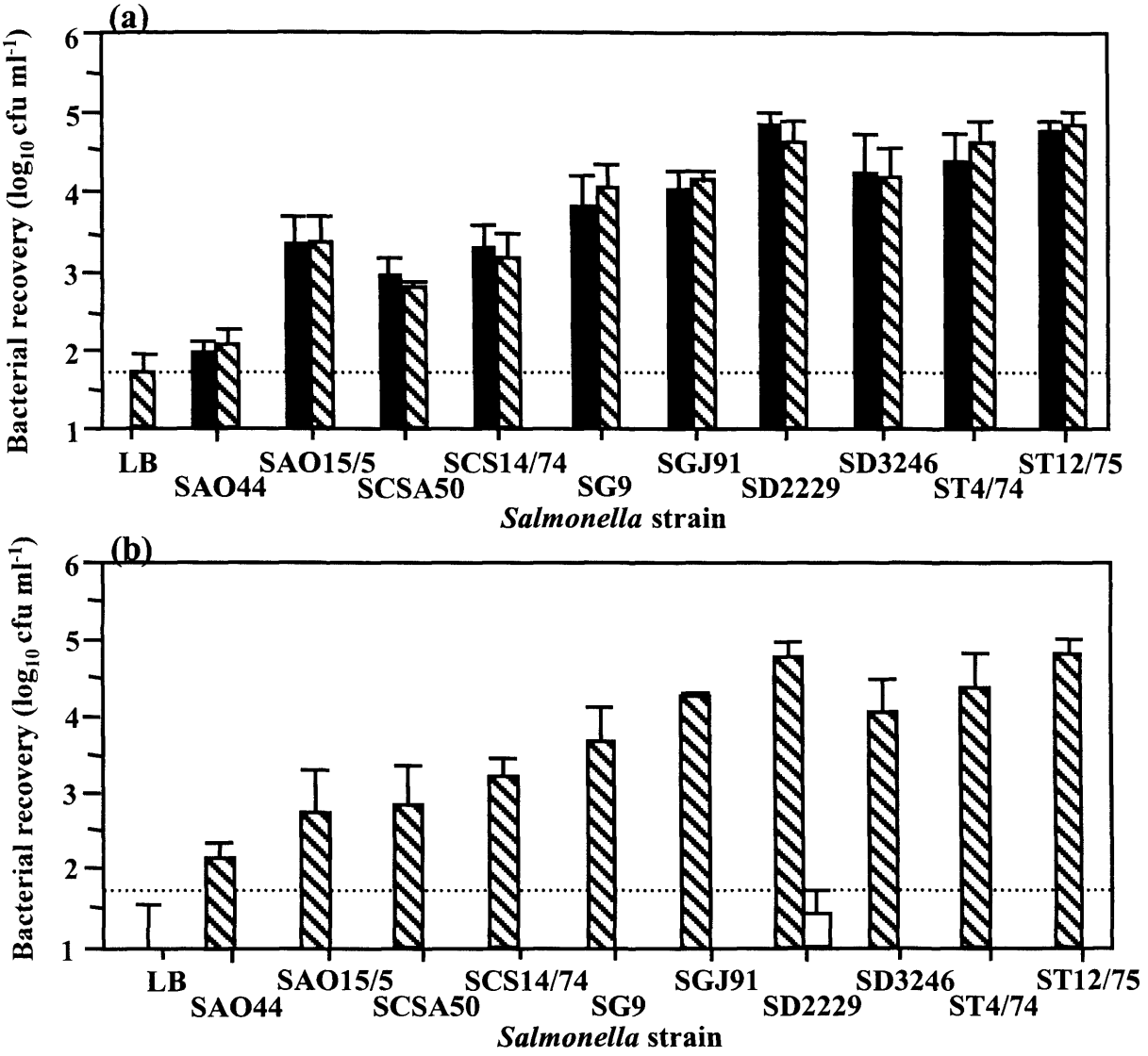



**Figure 4.5 Effect of inocula size on intracellular recovery of *S. dublin* (SD2229).** Loops were constructed in the mid ileum from absorptive epithelial tissue. Triplicate samples were taken from 3 loops in each animal and means were calculated to give a value *per* animal. Each bar represents the mean of either 1 or 2 calves and, where appropriate, is presented with the standard error of the mean.

(□) represents mean values for the reduced invasion mutant SD2229*sipB*. The dotted line represents the limit of accurate detection.

| Strain | Inocula ($log_{10}$ cfu loop) derived from 5 ml of bacterial culture | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Total number of loops in distal ileum** | **Total number of loops in mid ileum** | **Calf 4.6** | **Calf 4.7** | **Calf 4.8** | **Calf 4.9** | **Calf 4.10** | **Calf 4.11** | **Average inocula *per* loop ± SEM** |
| **SD2229** | 8 | 6 | - | - | 7.45 | 7.31 | 7.29 | 7.35 | 7.35 ± 0.04 |
| **SD2229 *sipB*** | - | 12 | 7.04 | 6.98 | 6.52 | 7.38 | 7.15 | 7.07 | 7.02 ± 0.13 |
| **SD3246** | 8 | 6 | 7.1 | 6.77 | - | - | 7.27 | 7.55 | 7.17 ± 0.16 |
| **SCSA50** | 4 | 4 | 7.36 | 7.1 | - | - | - | - | 7.23 ± 0.13 |
| **SCS14/74** | 4 | 4 | - | - | 6.95 | 7.61 | - | - | 7.28 ± 0.33 |
| **ST4/74** | 8 | 6 | 7.08 | 7/04 | - | - | 7.1 | 7.6 | 7.21 ± 0.13 |
| **ST12/75** | 8 | 6 | - | - | 6.55 | 7.22 | 7.35 | 7.63 | 7.19 ± 0.23 |
| **SAO44** | 4 | 4 | - | - | 6.75 | 7.39 | - | - | 7.07 ± 0.32 |
| **SA015/5** | 6 | 4 | 6.53 | 6.24 | - | - | 6.68 | - | 6.48 ± 0.13 |
| **SG9** | 6 | 4 | 7.38 | 7.22 | - | - | 7.48 | 7.75 | 7.46 ± 0.11 |
| **SGJ91** | 6 | 4 | - | - | 6.62 | 7.61 | - | 7.75 | 7.33 ± 0.36 |

**Table 4.3 Low dose temperature shifted inocula used to quantify the magnitude of *in vivo* intestinal invasion by different *Salmonella* serotypes.** Figures underlined represent inocula used for distal ileum loops only.




| Serotype | Mean bacterial recovery of two strains *per* serotype ($log_{10}$ cfu $ml^{-1}$) | | |
|---|---|---|---|
| | Peyer's patch mucosa | Absorptive epithelium (distal ileum) | Absorptive epithelium (mid ileum) |
| *S. typhimurium* | 4.42 | 4.54 | 4.60 |
| *S. dublin* | 4.39 | 4.25 | 4.43 |
| *S. gallinarum* | 3.80 | 4.03 | 3.99 |
| *S. choleraesuis* | 3.35 | 3.22 | 3.00 |
| *S. abortusovis* | 2.69 | 2.76 | 2.47 |

**Figure 4.6 Magnitude of *Salmonella* intestinal invasion 2 hours after infection of bovine ligated ileal loops with approximately 7 $log_{10}$ cfu loop.** Loops were constructed in the distal (a) or mid ileum (b) from Peyer's patch mucosa (■) or absorptive epithelia (▨). Triplicate samples were taken from 2 loops in each animal and means were calculated to give a value *per* animal. Each bar represents the mean of 2, 3, 4 or 6 calves and is presented with the standard error of the mean. The mean values *per* serotype are given in the above table. (□) represents mean values for SD2229*sipB*. The dotted line represents the limit of accurate detection.

| Serotype | Disruption of enterocyte monolayer | Extrusion of enterocytes | Stunting of villi | Influx of PMNs into submucosa and lamina propria | Mean score |
|---|---|---|---|---|---|
| **LB** | 0 | 0 | 0 | 1 | 0.25 |
| **SD3246** | 0 | 0 | 0 | 1 | 0.25 |
| **SCSA50** | 0 (0) | 0 (0) | 1 (1) | 1 (1) | 0.5 (0.5) |
| **ST4/74** | 0 | 0 | 1 | 2 | 0.75 |
| **SAO15/5** | 0 | 0 | 0 | 1 | 0.25 |
| **SG9** | 0 (0) | 0 (0) | 1 (1) | 1 (1) | 0.5 (0.5) |

**Table 4.4 Amount of *Salmonella*-induced mucosal damage 2 hours after inoculation of bovine ligated ileal loops with approximately 7 $\log_{10}$ cfu loop, grown by the temperature shift method.** The scoring system used represents no damage (0), mild (1), moderate (2) or severe (3) damage respectively and is described in section 2.15.1.1. Figures within brackets represent Peyer's patch mucosa and figures without brackets represent absorptive epithelia. Scores are derived from calf number 4.7 only (table 4.3).

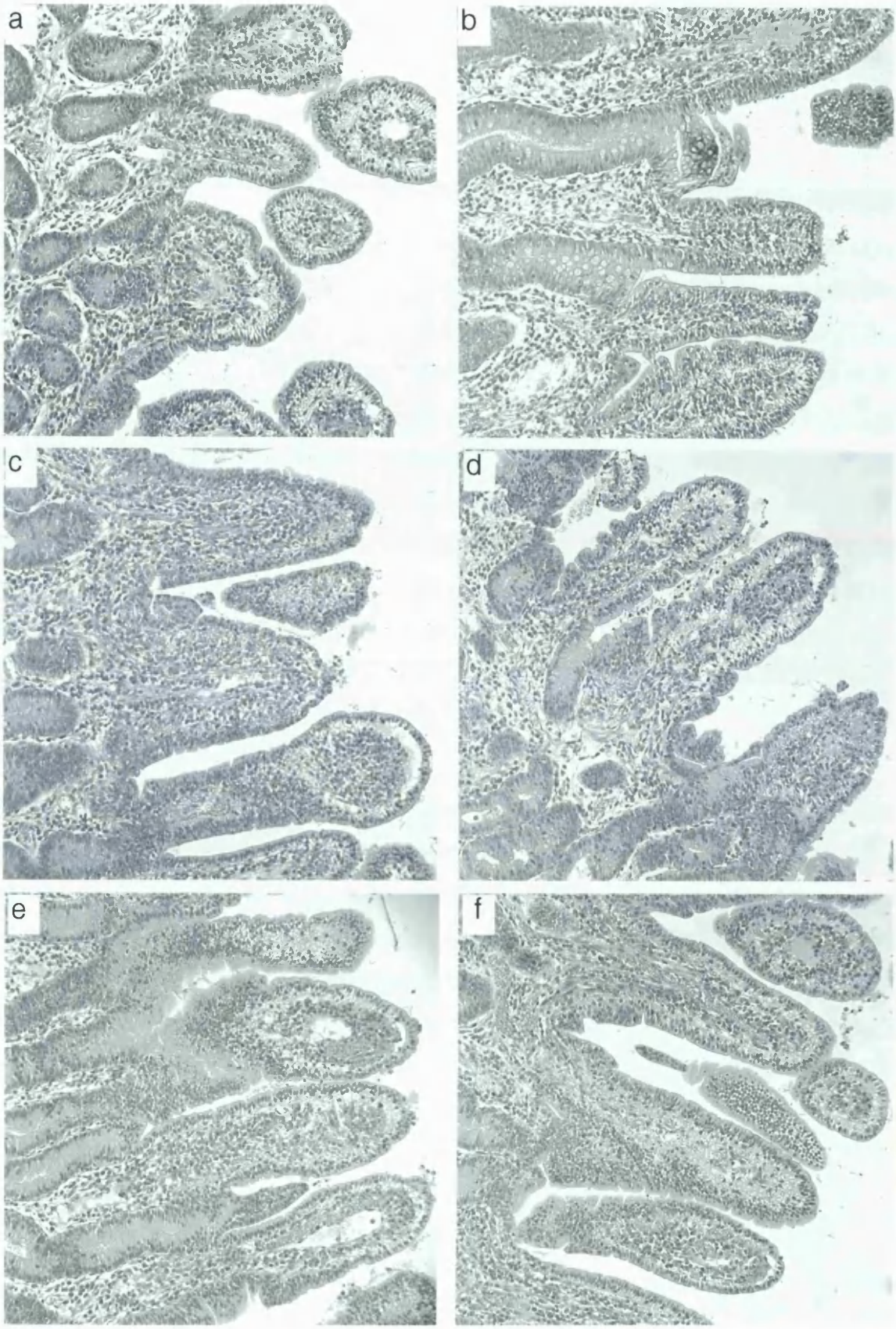



**Figure 4.7 Histological changes induced to intestinal absorptive epithelia 2 hours after injection of bovine ligated ileal loops with approx. 7 $log_{10}$ cfu loop**. a) LB broth; b) SD3246; c) ST4/74; d) SCSA50; e) SG9; f) SAO15/5. Details of the scoring system are given in section 2.15.1.1. Magnification x94

| Serotype | Disruption of enterocyte monolayer (mean) | Extrusion of enterocytes (mean) | Stunting of villi (mean) | Influx of PMNs into submucosa and lamina propria (mean) | Number of loops | Mean total score |
|---|---|---|---|---|---|---|
| **LB** | 0.1 (0) | 0.1 (0) | 0 (0) | 0 (0) | 6 (2) | 0.1 (0) **(0)** |
| **SD2229** | 0.8 (0.5) | 0.8 (0.6) | 0.8 (0.5) | 1.4 (1.5) | 5 (3) | 0.95 (1) **(1.5)** |
| **SD3246** | 1 (1) | 2 (1) | 1 (1) | 1.5 (2) | 2 (1) | 1.4 (1.25) **(1.5)** |
| **SCSA50** | 1 (1) | 0 (1) | 0 (1) | 1 (3) | 1 (1) | 0.5 (1.5) **(1.5)** |
| **SCS14/74** | 0 (0) | 0 (0) | 0 (0) | 1 (1) | 3 (2) | 0.25 (0.25) **(1)** |
| **ST4/74** | 1.5 (2) | 2 (2) | 1.5 (2) | 2 (2) | 2 (1) | 1.75 (2) **(3.5)** |
| **ST12/75** | 1.5 (1) | 1.75 (1) | 1.25 (1) | 1.75 (1) | 4 (1) | 1.6 (1) **(1.5)** |
| **SAO44** | 0 (0) | 0 (0) | 0 (0) | 1 (1) | 3 (2) | 0.25 (0.25) **(0.5)** |
| **SAO15/5** | 0 (0) | 0 (0) | 0 (0) | 1 (1) | 2 (1) | 0.25 (0.25) **(1)** |
| **SG9** | 0.3 (1) | 0.3 (1) | 0.3 (1) | 0.6 (1) | 3 (1) | 0.4 (1) **(1.5)** |
| **SGJ91** | 0.25 (0) | 0 (0.5) | 0.25 (0) | 1 (0.5) | 4 (2) | 0.4 (0.25) **(0.5)** |

**Table 4.5 Amount of *Salmonella*-induced mucosal damage 2 hours after inoculation of bovine ligated ileal loops with approximately 9.5 $\log_{10}$ cfu loop.** The scoring system represents no damage (0), mild (1), moderate (2) or severe (3) damage respectively and is described in section 2.15.1.1. Figures within brackets represent Peyer's patch mucosa and figures without brackets represent absorptive epithelia. Scores are derived from the mean of one loop in 1 to 6 calves. Results in normal type were derived from early-log phase inocula grown by the temperature shift method (section 2.11.1[ii]) and results underlined in bold (mean total score only) were derived from mid-log phase inocula (section 2.11.1 [i]).

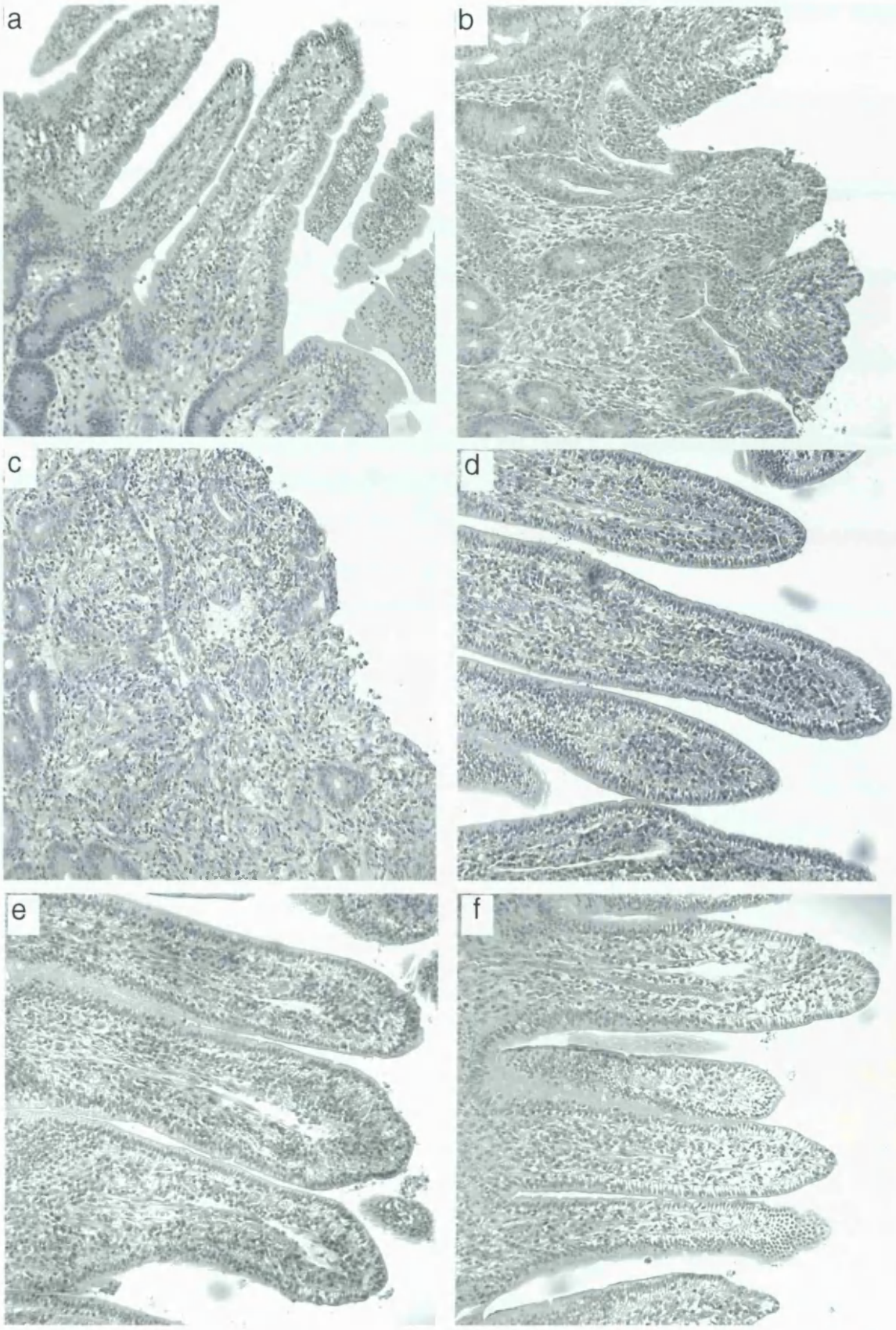



**Figure 4.8 Histological changes induced to intestinal absorptive epithelia 2 hours after injection of bovine ligated ileal loops with approx. 9.5 $\log_{10}$ cfu loop.** a) LB broth; b) SD3246; c) ST4/74; d) SCSA50; e) SG9; f) SAO15/5. Details of the scoring system used are given in section 2.15.1.1. Magnification x94

### 4.6 Summary of results

1. *Salmonella* serotypes associated with infection of mammalian species were significantly more invasive for the mammalian cell line Int 407 than the avian associated serotype *S. gallinarum*.
2. There was no direct correlation between invasion of epithelial cells *in vitro* and intestinal invasion *in vivo*.
3. The use of lower inocula, to compare the relative invasiveness of different *Salmonella* serotypes, increased the sensitivity of the ligated ileal loop assay.
4. The magnitude of intestinal invasion was similar regardless of whether the loops were constructed in the mid or distal ileum.
5. There was no direct correlation between the route or magnitude of *Salmonella* intestinal invasion *in vivo* and serotype-host specificity in calves.
6. There was no direct correlation between the magnitude of *Salmonella* intestinal invasion and *Salmonella* virulence following oral challenge.
7. There was no direct correlation between the relative invasiveness of different serotypes and their ability to induce mucosal damage.

## 4.7 Discussion

The ability to invade the intestinal mucosa by a particular route represents an important early stage in *Salmonella* pathogenesis that potentially facilitates bacterial translocation to systemic sites. As such, initial intestinal interactions have been correlated with the host specific phenotype in both mammalian (Pospischil *et al.*, 1990; Pascopella *et al.*, 1995) and avian (Henderson *et al.*, 1999) species. Therefore, in order to ascertain whether there was a correlation between intestinal invasion and serotype-host specificity in calves, the magnitude of invasion of different serotypes was assessed both *in vitro* and *in vivo*. In addition, the bacterial ability to preferentially associate with either Peyer's patch mucosa or absorptive epithelia was determined.

*S. typhimurium* and *S. dublin* were the most invasive serotypes *in vitro*, which correlated with their propensity to induce severe enteritis following oral inoculation of calves (Chapter 3; Jones *et al.*, 1991). *S. choleraesuis*, which results in severe disease following oral inoculation of calves, and *S. abortusovis*, which is largely asymptomatic in this host, were moderately invasive suggesting that the ability to enter cultured epithelial cells *in vitro* does not correlate with the host specific phenotype. *S. gallinarum*, which is non-motile (Li *et al.*, 1993), was poorly invasive *in vitro* and even following a centrifugation step to increase the bacterial-epithelial cell interaction, this serotype still remained less invasive than *S. typhimurium*. This suggests that expression of flagella may be important for invasion of epithelial cells *in vitro*, possibly by increasing the frequency of contact between bacteria and host cells. Alternatively, *S. gallinarum* may be relatively poor at invading mammalian cells, which could explain the naturally low virulence of this serotype for mammalian species. However *S. gallinarum*, and the closely related biotype *S. pullorum*, have been reported previously to invade both mammalian (Barrow and Lovell, 1989; Henderson *et al.*, 1999; Wilson *et al.*, 2000; Uzzau *et al.*, 2001) and avian (Barrow and Lovell, 1989; Henderson *et al.*, 1999) cell lines at a lower rate than *S. typhimurium*. This suggests that additional factors, apart from the origin of the cell line, account for the poor invasive phenotype of this serotype *in vitro*.

Cultured epithelial cell lines have been widely exploited to characterise the molecular basis of *Salmonella* invasion (reviewed by Finlay and Falkow, 1989; Finlay and Falkow, 1990). Recently MDCK and Hep-2 cultured cell lines have been used to assess the invasive properties of different *Salmonella* serotypes. As with Int 407 cells, the ability, of the same serotypes used in this study, to invade these cell lines does not appear to correlate with host specificity in sheep (Uzzau *et al.*, 2001) or chickens (Chadfield *et al.*, 2001) respectively. *In*

*vitro* techniques cannot fully model the complex interactions that occur *in vivo* between bacteria and infected mucosa and are therefore only of limited value for studying invasion, particularly in the context of host specificity. For this reason, the invasiveness of different *Salmonella* serotypes was also determined *in vivo*, using a bovine ligated ileal loop model specifically adapted to quantify the magnitude of intestinal invasion by different routes (Watson *et al.*, 1995). In calves the distal ileum, immediately proximal to the ileocaecal valve, contains a continuous strip of Peyer's patch mucosa (Parsons, 1991). 'M' cells, overlying this specialised tissue, potentially represent an important cell type apparently facilitating *Salmonella* serotype-specific intestinal invasion in mice (Jones *et al.*, 1994; Pascopella *et al.*, 1995). Use of the bovine distal ileum enabled the relative affinity of the different serotypes for both Peyer's patch mucosa and the immediately adjacent absorptive epithelium to be determined. In addition, the use of the mid-ileum enabled an assessment of *Salmonella* intestinal invasion within different areas of the bovine gastro-intestinal tract.

Initially all strains were tested *in vivo*, using an inocula size of approximately 9.5 $\log_{10}$ cfu loop, with growth conditions that had been established previously (Watson *et al.*, 1995). At this dose, the relative magnitude of intestinal invasion of the different serotypes was similar to that seen *in vitro*, with the exception of *S. gallinarum* which was highly invasive *in vivo*. As described previously *S. gallinarum* and non-flagellated or non-motile mutants of *S. typhimurium* (Lockman and Curtiss III, 1990; Henderson *et al.*, 1999; Uzzau *et al.*, 2001) exhibit reduced invasion of cultured epithelial cells, despite being virulent or invasive *in vivo*. It is known that *S. gallinarum* possesses the flagellin C (*fliC*) gene which encodes the phase 1 structural flagella protein (Li *et al.*, 1993; Kwon *et al.*, 2000). However, it is unknown whether *S. gallinarum* flagella are expressed *in vivo* either in avian or mammalian hosts. It is possible that differential regulation of flagella gene expression may account for the poor *in vitro* invasion seen with this serotype. These results further highlight the limitations of using cell monolayers for studying *Salmonella* invasion and, as such these assays are clearly not predictive of *Salmonella* interactions with intestinal mucosa *in vivo*.

At the high inocula there was an increase in the amount of mucosal damage induced by *S. typhimurium* and *S. dublin* compared with other serotypes. This fact has been reported previously in sheep (Uzzau *et al.*, 2001), rabbits (Bolton *et al* 1999a), calves (Watson *et al.*, 1995) and pigs (Bolton *et al.*, 1999b) and suggests the possibility of an under-estimation of bacterial recoveries associated with these serotypes due to killing of gentamicin protected bacteria released through the damaged mucosa. It is possible that the degree of damage associated with *S. typhimurium* in particular correlates with the ubiquitous nature of this

serotype resulting in enteric salmonellosis in a wide range of hosts. Additionally, it has been suggested that this tissue damage may be due to the presence of a serotype-specific histotoxin (Lodge *et al.*, 1999; Bolton *et al.*, 1999a; Bolton *et al.*, 1999b), which may open up tight junctions between enterocytes facilitating exposure of sub-epithelial cells to invading bacteria. In contrast to *S. typhimurium, S. gallinarum,* which was largely avirulent in calves but invasive *in vivo*, and *S. choleraesuis*, which despite being slightly less invasive than *S. typhimurium in vivo* was virulent in calves, both failed to induce significant mucosal damage. This would suggest that the magnitude of intestinal invasion and the ultimate severity of disease in calves do not correlate with the ability to damage intestinal mucosa.

Experiments using the same strains of defined virulence, as those in this study, in other host species including pigs (Bolton *et al.*, 1999b), rabbits (Bolton *et al.*, 1999a), sheep (Uzzau *et al.*, 2001) and fowl (Chadfield *et al.*, 2001) have found there to be no correlation between intestinal invasion and host specificity. However, the use of high inocula in all of these experiments has not highlighted much variation between the invasive capabilities of different host-restricted or host-specific serotypes. It is possible therefore that such results may reflect a relative insensitivity of the invasion assay thereby limiting the detection of small differences in the invasive phenotypes of different *Salmonella* serotypes. Additionally, it is possible that saturation of the mucosal binding sites may have occurred again reducing the potential to accurately detect serotype differences. These studies were extended by decreasing the inocula, and as such increasing the sensitivity of the assay, to assess the differential ability of serotypes to invade bovine intestinal mucosa.

At approximately 7 $\log_{10}$ cfu loop, a hierarchy in the magnitude of intestinal invasion of the serotypes was observed and the differences between the serotypes were more marked, although invasion of the bovine intestinal mucosa *per se* did not correlate with the host specific phenotype. There was however a direct correlation between invasion of the distal and mid ileum suggesting that invasion is similar regardless of the area of intestine used. The importance of this observation will be described in Chapter 5. As observed *in vitro* and at the high inocula *in vivo*, *S. typhimurium* and *S. dublin* were the most invasive serotypes. This suggests that invasion of the intestinal mucosa in high numbers is an important pathogenic feature contributing to the induction of severe enteric disease following oral inoculation of calves.

The high invasiveness of *S. gallinarum* for bovine intestinal mucosa was surprising given the avirulence of this serotype following oral inoculation of calves and the low invasiveness both *in vitro* and *in vivo* in mice (Pascopella *et al.*, 1995). However, it has recently been reported that *S. gallinarum* is able to invade ovine intestinal mucosa, at a high

inocula, in comparable numbers to *S. dublin* and *S. typhimurium* three hours after ligated ileal loop infection (Uzzau *et al.*, 2001). Additionally, 24 hours after oral inoculation *S. gallinarum* is able to invade the bovine intestinal mucosa in sufficient numbers to reach the ileal and caecal lymph nodes in comparable numbers to *S. dublin* (Chapter 3). Therefore given that this serotype is clearly able to invade mammalian tissues, it would appear that the avirulence for these hosts might be related to the inability of *S. gallinarum* to survive within eukaryotic cells.

Despite being highly invasive *in vitro*, *S. abortusovis* was poorly invasive for bovine intestinal mucosa at low inocula, which correlated with an inability to cause enteric or systemic disease in calves. *S. abortusovis* which has a restricted natural host range and is largely asymptomatic even in adult sheep, is also unable to invade ovine intestinal mucosa in high numbers (Uzzau *et al.*, 2001). This suggests that the poor invasive phenotype is serotype rather than host specific. It could be speculated that the specificity of *S. abortusovis* to sheep, due to its mode of infection, is associated with evolution towards reduced virulence in adult animals but with retention of virulence for foetal and newborn lambs.

One of the most surprising results from the *in vivo* studies was the relatively low invasiveness of *S. choleraesuis*, considering this serotype is highly virulent for calves and is recovered in high numbers from the intestinal mucosa seven days after oral inoculation. A similarly low invasive, yet highly virulent, phenotype has been observed following experimental infection of pigs (Bolton *et al.*, 1999b; Watson *et al.*, 2000c). Taken together, it is possible that pathogenesis following infection with *S. choleraesuis*, results from a strategy of 'stealth' whereby relatively few bacteria are required for a highly virulent phenotype. This interesting hypothesis is discussed further in Chapter 8.

To date there is conflicting evidence that the route of intestinal invasion correlates with host specificity in some species. Experimental infection of mice with *S. typhimurium* or *S. enteritidis* has shown an almost exclusive ability to invade and subsequently destroy 'M' cells overlying Peyer's patch mucosa (Carter and Collins, 1974; Jones *et al.*, 1994) apparently exploiting this route to access systemic sites. To further complicate this issue, it has been suggested that serotype specific preferential invasion of Peyer's patch mucosa influences the invasive phenotype in several hosts including pigs (Pospischil *et al.*, 1990), mice (Pascopella *et al.*, 1995) and chickens (Henderson *et al.*, 1999). These observations suggest that host and serotype factors are influencing bacterial uptake, persistence and potentially host specificity. However, most of these studies have used the rather subjective approach of microscopic analysis, as opposed to viable counts, to interpret the interaction of *Salmonella* serotypes with the intestinal mucosa. Additionally, with the exception of *S. gallinarum* and *S. typhi* in the

murine model (Pascopella *et al.*, 1995), these studies were performed with only one strain of each serotype. As the virulence of strains within a serotype may differ, it is preferable to compare observations using more than one strain. Furthermore, these studies used inocula conditions that are now known to be sub-optimal for expression of SPI-1 dependent secreted effector proteins required for *Salmonella* intestinal invasion (Wood *et al.*, 1996; Galyov *et al.*, 1997).

In other species including pigs (Bolton *et al.*, 1999b), rabbits (Bolton *et al.*, 1999a), sheep (Uzzau *et al.*, 2001), chickens (Chadfield *et al.*, 2001) and cattle (Watson *et* al., 1995; Frost *et al.*, 1997; Bolton *et al.*, 1999b) it has been demonstrated that the foci of infection does not develop exclusively in one tissue type in a particular host although initial preferential interactions cannot be ruled out. The studies cited above, using the well-characterised strains of defined virulence and identical experimental protocols to those used in the present investigations, have to date provided the best-co-ordinated examination into *Salmonella* intestinal invasion with respect to host specificity. Furthermore, the non-preferential route of invasion was also confirmed in calves using low inocula.

In conclusion, intestinal invasion of some serotypes in high numbers appears to be an important mechanism leading to the induction of salmonellosis in calves. However, initial interactions between host and pathogen and subsequent damage to the mucosal integrity *per se* do not correlate with serotype-host specificity or the production of diarrhoea. In addition the focus of infection does not develop in either Peyer's patch mucosa or absorptive epithelia suggesting that in calves the route of bacterial invasion does not influence the outcome of disease.

Having invaded the intestinal mucosa it has been shown that pathogenic salmonellas actively recruit PMNs to the foci of infection, elicit intestinal fluid secretion and induce mucosal damage (reviewed by Wallis and Galyov, 2000). Therefore, to determine whether disruption to the normal integrity of the intestinal mucosa correlated with the host specific phenotype in calves, enteropathogenic responses to different serotypes were assessed.

# CHAPTER 5

# QUANTIFICATION OF THE BOVINE ENTEROPATHOGENIC RESPONSE INDUCED BY DIFFERENT *SALMONELLA* SEROTYPES OF DEFINED VIRULENCE.

## 5.1 Introduction

The production of watery diarrhoea is a characteristic symptom induced by non-typhoidal *Salmonella* serotypes. There are three key pathological features resulting in this 'enteropathogenic' response (reviewed by Wallis and Galyov, 2000; section 1.8). These include first, the influx of polymorphonuclear leucocytes (PMNs) into the intestinal lumen and mucosa (Takeuchi *et al.*, 1967; Wallis *et al.*, 1989). Second, net electrolyte and associated fluid secretion across the epithelium and into the gut lumen (Rout *et al.*, 1974). Third, disruption to mucosal architecture, including extrusion of enterocytes and 'M' cells, resulting in villous atrophy (Rout *et al.*, 1974; Wallis *et al.*, 1986b; Clarke and Gyles *et al.*, 1987).

Salmonellosis in mammals and birds may result in systemic or enteric disease and/or abortion, depending on the infecting serotype and the infected host. Consequently, diarrhoea and disruption of mucosal architecture are not always observed. Ubiquitous serotypes, for example, *S. typhimurium* and *S. enteritidis* are often associated with the enteric form of the disease. In contrast the host-specific or host-restricted serotypes typically result in septicaemia and/or enteritis (reviewed by Uzzau *et al.*, 2000a) (section 1.2.2).

Interactions between *Salmonella* and intestinal epithelial cells appear to be important in mediating the enteropathogenic response. For example, the recruitment of inflammatory cells to the foci of infection is dependent on the release of several chemoattractant mediators by *Salmonella*-infected epithelial cells (Eckmann *et al.*, 1993; McCormick *et al.*, 1993; Jung *et al.*, 1995; Eckmann *et al.*, 1997b; McCormick *et al.*, 1998). In addition, it has been shown that this response is dependent on the SPI-1 encoded type three secretion system (reviewed by Wallis and Galyov, 2000), which is responsible for the translocation of effector proteins into target eukaryotic cells (Gálan, 1996; Hueck, 1998). Recently, three such effector proteins SopA (Wood *et al.*, 2000), SopB (Galyov *et al.*, 1997), and SopD (Jones *et al.*, 1998) have been identified in *S. dublin*. These proteins, which are conserved in several ubiquitous and host-specific/restricted serotypes, act together in cattle to influence *Salmonella*-induced secretory and inflammatory responses (Jones *et al.*, 1998; Wood *et al.*, 2000).

## 5.2 Aim

In the previous chapter it was established that different *Salmonella* serotypes did not preferentially invade either absorptive epithelia or Peyer's patch mucosa. As such, neither the

route nor the magnitude of intestinal invasion correlated with host specificity in calves. Having invaded the intestinal mucosa, pathogenic salmonellas actively recruit inflammatory cells to the foci of infection, induce fluid secretion into the gut lumen and cause mucosal damage. Therefore in order to determine whether *Salmonella*-induced disruption of the mucosal barrier influences host specificity in calves, enteropathogenic responses, resulting from infection with different *Salmonella* serotypes, were quantified using an *in vivo* model.

### 5.3 Approaches and experimental design

The enteropathogenic responses induced by two strains each of *S. dublin* (SD3246 and SD2229), *S. typhimurium* (ST4/74 and ST12/75), *S. choleraesuis* (SCSA50 and SCS14/74), *S. gallinarum* (SG9 and SGJ91) or *S. abortusovis* (SAO44 and SAO15/5) were determined using the bovine ligated ileal loop model as described in sections 2.10 and 2.12. The use of this model has been validated previously by the observations that isogenic mutants with reduced enteropathogenicicty in bovine ligated ileal loops were attenuated following oral inoculation of calves (Watson *et al.*, 1998). Furthermore, mutants that do not affect enteropathogenicity in ligated loops do not affect the severity of enteritis in orally inoculated calves (Wallis *et al.*, 1995). Ligated ileal loops were constructed in the mid ileum as the greater length of this part of the gastrointestinal tract, compared to the distal ileum, enabled the fluid accumulation (referred to throughout as the secretory) and inflammatory responses of all strains to be tested in duplicate or triplicate within a single animal. In addition, it has been shown that regardless of whether the loops were constructed in the mid or distal ileum the invasive phenotypes of different serotypes were similar (Chapter 4).

Data were derived from the mean strain responses *per* animal in three separate calves (8 loops per strain in total). For simplicity of interpretation, the mean values of 2 strains within a given serotype were determined and statistical analyses based on this data were used in the discussion of results. Where appropriate any strain specific differences were reported. In addition, disruption to mucosal integrity was assessed, from one loop *per* serotype, in a single animal using tissue derived from loops constructed in the distal ileum (table 5.2). These results enabled the magnitude of damage to be compared in both absorptive epithelium and Peyer's patch mucosa as described in section 2.15.1.1.

Details of inocula preparation are given in section 2.11.1 (ii), and inocula used for each experiment are given in table 5.1.

## 5.4 Magnitude of enteropathogenic responses induced by different *Salmonella* serotypes

All serotypes, with the exception of *S. abortusovis*, induced an enteropathogenic response however the magnitude of this response varied. Sterile LB broth did not induce an enteropathogenic response in any negative control loop tested.

### *5.4.1 Secretory and inflammatory responses*

On average, *S. typhimurium* and *S. gallinarum* induced similar secretory responses ($P > 0.1$), while the inflammatory response to *S. typhimurium* was significantly greater than that to *S. gallinarum* ($P < 0.05$). These two serotypes induced significantly greater responses than *S. dublin* ($P < 0.001$). The secretory and inflammatory responses resulting from infection with *S. choleraesuis* were significantly lower than those to *S. dublin* ($P < 0.001$), but significantly greater than the magnitude of fluid secretion ($P < 0.02$) or influx of inflammatory cells ($P < 0.05$) induced by *S. abortusovis* (figures 5.1a and b).

There was a positive correlation between the magnitude of the secretory and inflammatory responses for all the strains tested, a fact that has been reported previously in calves (Galyov *et al.*, 1997; Jones *et al.*, 1998; Watson *et al.*, 1998; Wood *et al.*, 1998; Watson *et al.*, 1999) suggesting that these parameters are in some way related.

No differences were observed in the responses obtained for two strains of a given serotype with the exception of the secretory ($P < 0.02$) and inflammatory responses ($P < 0.1$) to *S. gallinarum* and the inflammatory responses only to *S. typhimurium* ($P < 0.05$) and *S. dublin* ($P < 0.1$). It is unlikely that these relatively minor strain specific differences were of great significance because the overall trend of the serotype was not altered.

The *S. abortusovis* inocula used in these experiments was on average slightly lower than the inocula used for the other serotypes. However, in calf number 5.3, similar inocula were used for all strains (table 5.1) suggesting that factors independent of initial inocula influence the magnitude of secretory and inflammatory responses to this serotype.

### *5.4.2 Mucosal damage*

The magnitude of *Salmonella*-induced damage varied depending on the particular serotype (table 5.2 and figure 5.2). *S. typhimurium* and *S. dublin* induced the most severe damage, *S. gallinarum* and *S. choleraesuis* induced moderate damage, while the *S. abortusovis* infected mucosa appeared relatively unchanged and comparable with the LB negative control. In addition, the amount of *Salmonella*-induced damage to both absorptive epithelia and Peyer's patch mucosa was similar. Due to the limited number of samples examined for mucosal damage, this result awaits further confirmation in an additional experiment.

| Strain | Inocula ($log_{10}$ cfu loop) derived from 5 ml of bacterial culture | | | | | |
|---|---|---|---|---|---|---|
| | **Total number of loops** | **Calf 5.1** (3 loops *per* strain) | **Calf 5.2** (3 loops *per* strain) | **Calf 5.3** (2 loops *per* strain) | **Calf 5.4** (1 loop *per* strain) (Microscopy) | **Average inocula *per* strain + SEM** |
| **SD2229** | 8 | 9.63 | 8.9 | 9.12 | _ | 9.22 ± 0.22 |
| **SD3246** | 9 | 9.77 | 9.05 | 9.05 | 9.07 | 9.24 ± 0.18 |
| **SCSA50** | 9 | 9.91 | 9.23 | 9.11 | 9.26 | 9.42 ± 0.25 |
| **SCS14/74** | 8 | 9.73 | 9.23 | 9.16 | _ | 9.35 ± 0.13 |
| **ST4/74** | 9 | 9.67 | 9.06 | 9.13 | 9.15 | 9.25 ± 0.14 |
| **ST12/75** | 8 | 9.74 | 9.15 | 9.23 | _ | 9.37 ± 0.18 |
| **SAO44** | 8 | * | 8.56 | 9.01 | _ | 8.81 ± 0.11 |
| **SAO15/5** | 9 | 8.82 | 7.75 | 8.94 | 8.86 | 8.50 ± 0.36 |
| **SG9** | 9 | 9.85 | 9.49 | 9.22 | 9.06 | 9.41 ± 0.17 |
| **SGJ91** | 8 | 9.88 | 9.49 | 9.18 | _ | 9.52 ± 0.20 |

**Table 5.1 Inocula used to determine enteropathogenic responses of different *Salmonella* serotypes.** * The inoculum for SAO 44 calf 5.1 was not determined however, the $OD_{600}$ after 90-120 minutes growth at 37°C was 0.61 which correlates to an approximate bacterial count of 9.0 $log_{10}$ cfu loop.

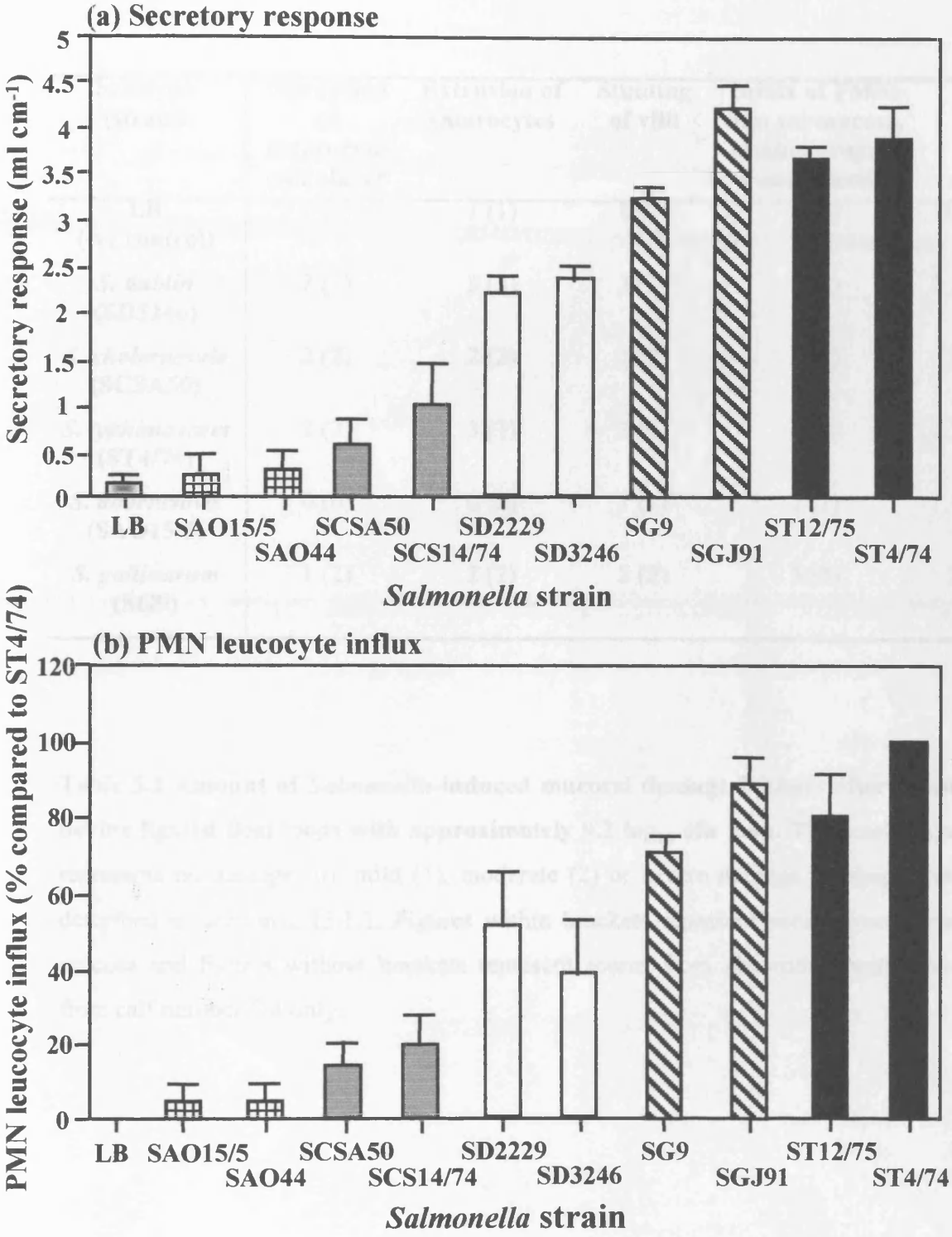



**Figure 5.1 Secretory response and PMN leucoctye influx induced by different *Salmonella* serotypes 12 hours after infection of bovine ligated ileal loops.** Each bar represents the mean response derived from two or three loops *per* strain in 3 separate calves (8 loops in total) and is presented with the standard error of the mean. The secretory response (a) represents the mean V/L ratio and the PMN influx (b) is presented as a percentage compared to *S. typhimurium*, strain 4/74.

| Serotype (strain) | Disruption of enterocyte monolayer | Extrusion of enterocytes | Stunting of villi | Influx of PMNs into submucosa, lamina propria and lumen | Mean score |
|---|---|---|---|---|---|
| **LB (-ve control)** | 1 (1) | 1 (1) | 0 (1) | 0 (0) | 0.5 (0.75) |
| ***S. dublin* (SD3246)** | 2 (2) | 3 (3) | 3 (3) | 2 (3) | 2.25 (2.75) |
| ***S. choleraesuis* (SCSA50)** | 2 (2) | 2 (2) | 2 (2) | 1 (2) | 1.75 (2) |
| ***S. typhimurium* (ST4/74)** | 2 (2) | 3 (3) | 3 (3) | 3 (3) | 2.75 (2.75) |
| ***S. abortusovis* (SAO15/5)** | 0 (0) | 0 (2) | 1 (1) | 1 (1) | 0.5 (1) |
| ***S. gallinarum* (SG9)** | 1 (2) | 2 (2) | 2 (2) | 3 (3) | 2 (2.25) |

**Table 5.2 Amount of *Salmonella*-induced mucosal damage 2 hours after inoculation of bovine ligated ileal loops with approximately 9.2 $\log_{10}$ cfu loop.** The scoring system used represents no damage (0), mild (1), moderate (2) or severe damage (3) respectively and is described in section 2.15.1.1. Figures within brackets represent scores from Peyer's patch mucosa and figures without brackets represent scores from absorptive epithelium, derived from calf number 5.4 only.

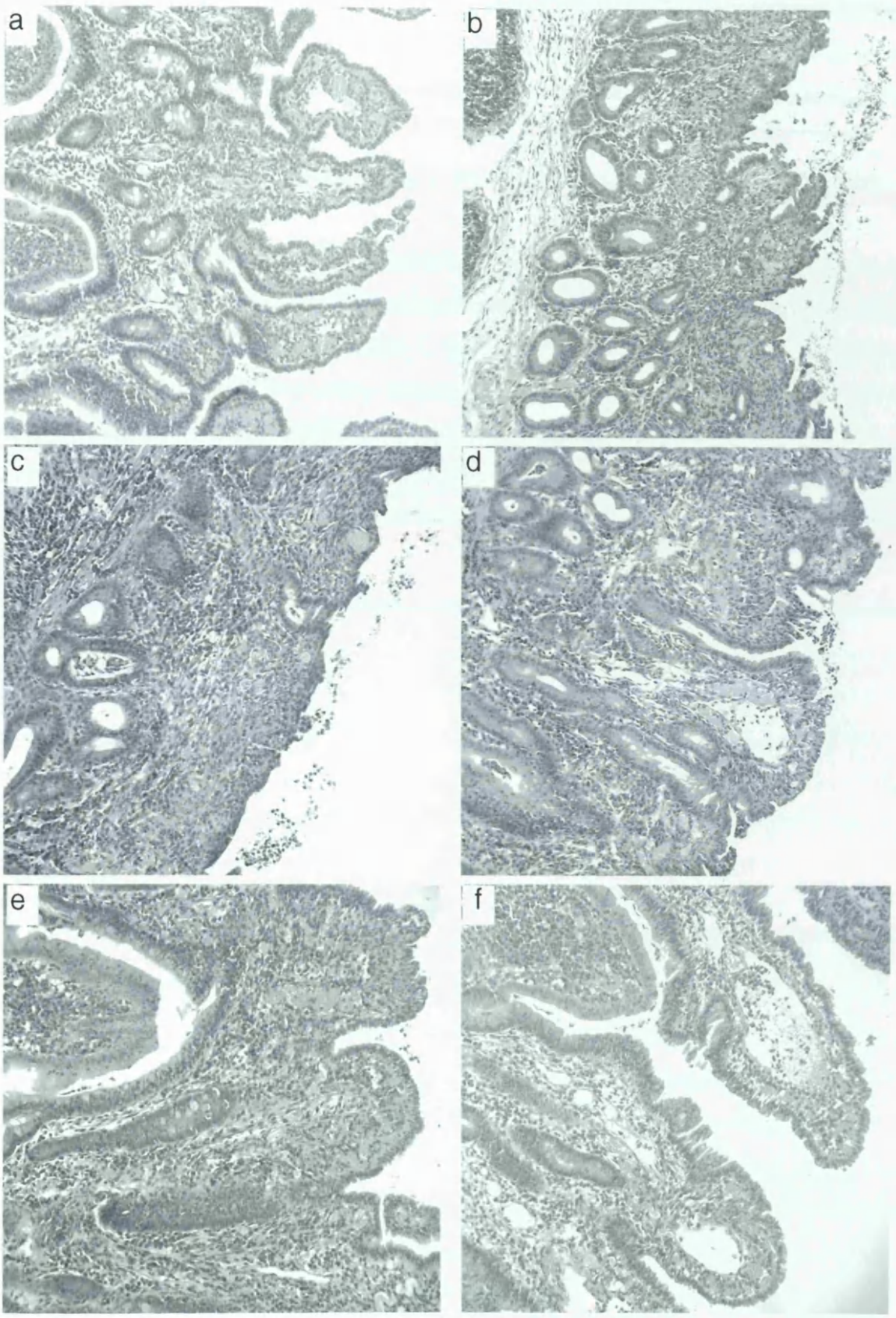



**Figure 5.2 Histological changes induced to Peyer's patch mucosa 12 hours after injection of bovine ligated ileal loops with approx. 9.2 $log_{10}$ cfu loop.** a) LB broth; b) SD3246; c) ST4/74; d) SCSA50; e) SG9; f) SAO15/5. Details of the scoring system used are given in section 2.15.1.1. Magnification x 94

## 5.5 Summary of results

1. There was a hierarchy in the magnitude of intestinal secretory and inflammatory responses induced by the different serotypes. *S. typhimurium* and *S. gallinarum* induced similarly potent responses which were greater than those induced by *S. dublin.* Infection with *S. choleraesuis* resulted in poor responses, while *S. abortusovis* failed to induce fluid secretion or recruitment of PMNs to the intestinal mucosa.
2. There was a positive correlation between the PMN influx and the secretory response for all strains.
3. The amount of mucosal damage induced by *S. typhimurium*, *S. choleraesuis* and *S. abortusovis* but not *S. dublin* or *S. gallinarum* correlated with the magnitude of the secretory and inflammatory responses.
4. The PMN influx and the secretory responses induced by the different serotypes did not directly correlate with the magnitude of intestinal invasion (Chapter 4).
5. The enteropathogenic responses induced by the different serotypes did not directly correlate with virulence following oral inoculation of calves or *Salmonella* host specificity (Chapter 3).

## 5.6 Discussion

Several studies have suggested that the intensity and outcome of disease induced by different serotypes depends on how these organisms interact with the intestinal mucosa (Pospischil *et al.*, 1991; Pascopella *et al.*, 1995; McCormick *et al.*, 1995b; Henderson *et al.*, 1999). Recently *in vitro* studies have shown that transepithelial signalling to neutrophils is crucial for those *Salmonella* serotypes known to elicit gastro-enteritis in humans (McCormick *et al.*, 1993; McCormick *et al.*, 1995b). Therefore in this study, the enteropathogenic responses to different serotypes were quantified in order to ascertain whether *Salmonella*-induced disruption of normal intestinal function correlated with, and therefore potentially influenced, the outcome of infection in calves. The use of the well-established bovine ligated ileal loop model to quantify *Salmonella* enteropathogenesis (Wallis *et al.*, 1995) provides the first documented use of an *in vivo* model to study these responses with respect to host specificity.

There were clear and unexpected differences in the magnitude of enteropathogenic responses observed for the different serotypes, which did not directly correlate with intestinal invasion or host specificity. *S. typhimurium* and remarkably *S. gallinarum*, a relatively avirulent serotype in calves, induced more potent secretory and inflammatory responses than *S. dublin*. Interestingly, *S. choleraesuis*, which is associated with systemic symptoms and diarrhoea following oral inoculation of calves, was poorly enteropathogenic, while the calf avirulent serotype *S. abortusovis* did not induce an enteropathogenic response. Taken together, these results indicate that factors additional to initial interactions between *Salmonella* and the bovine intestinal mucosa contribute to the outcome of disease.

While both *S. typhimurium* and *S. dublin* result in the induction of severe diarrhoea in calves (Chapter 3; Jones *et al.*, 1991; Wray and Davies, 2000), it has been previously established that *S. typhimurium* is more enteropathogenic than *S. dublin* (Watson *et al.*, 1998), which suggests that serotype specific factors influence enteropathogenicicty. In *S. dublin* several effector proteins secreted by the SPI-1 encoded TTSS-1 contribute to the induction of enteropathogenesis in calves (Galyov *et al.*, 1997; Jones *et al.*, 1998; Wood *et al.*, 1998; Wood *et al.*, 2000). However, the precise role of these proteins in the induction of secretory and inflammatory responses in different serotype-host combinations remains unknown and as such warrants further investigation.

The role of other pathogenicity islands in *Salmonella* enteropathogenesis is less well defined. However, it was recently shown that a mutation, *sseD*, in a SPI-2 gene encoding a putative secreted effector protein, significantly reduced *S. dublin*-induced enteritis in calves

and net growth in the ileal mucosa (Bispham *et al.*, 2001). SPI-2 is known to influence intracellular growth within epithelial and phagocytic cells (Crillo *et al.*, 1998; Hensel *et al.*, 1998; Shea *et al.*, 1998; Pfeifer *et al.*, 1999) and resistance to the bactericidal activities of macrophages (Vazquez-Torres *et al.*, 2000). While the precise role of SPI-2 in *Salmonella*-induced enteritis is unclear, it is possible that effector proteins secreted by the SPI-2 encoded TTSS-2 may influence the enteropathogenic response directly by modulating host cell function. Alternatively, *sseD* could influence enteropathogenesis through an effect on *Salmonella* intracellular growth within the intestinal mucosa. Reduced growth would indirectly affect the delivery of virulence factors including Sop proteins into target cells, simply by the presence of lower bacterial numbers. Therefore, the magnitude of enteropathogenic responses to *Salmonella* serotypes could be directly attributable to differences in their net growth rates *in vivo*.

The finding that *S. gallinarum* was invasive and enteropathogenic in bovine ligated ileal loops, yet largely avirulent following oral inoculation of calves was surprising. These observations require careful consideration, particularly as many studies have shown a good correlation between the ligated loop model and oral challenge studies using isogenic mutants (Wallis *et al.*, 1995; Watson *et al.*, 1998; Watson *et al.*, 1999).

Clinical diarrhoea results from an inability of the infected colon to reabsorb the increasing volume of fluid entering from the small intestine, which is in a net secretory state, following infection with salmonellas (Formal *et al.*, 1976; Clarke and Gyles, 1987). Twenty four hours after oral inoculation of calves, *S. gallinarum* is recovered in lower numbers than *S. dublin* from the colon contents and wall (Chapter 3). This suggests that although *S. gallinarum* can disrupt the absorptive capacity of the ileal mucosa, resulting in net fluid secretion, the ability of the colon to reabsorb the secreted fluid is likely to be unaltered. This could be due to the lower numbers of invading bacteria or the inability of this serotype to survive within the intestinal mucosa. Consequently, in calves, overt signs of diarrhoea do not result following infection with this serotype.

Before the successful elimination of *S. gallinarum* from U.K. poultry flocks in the 1960s, cases of food poisoning associated with this serotype were occasionally reported in several mammalian species including humans (Müller, 1933; Kauffmann, 1934; Buxton, 1957; Sojka and Field, 1970; Gupta and Verma, 1993). This indicates that *S. gallinarum* apparently has the potential to elicit enteropathogenic responses, as demonstrated in this study. This interaction with mammalian hosts appears to be limited to the gastro-intestinal tract as *S. gallinarum* is typically only associated with severe systemic disease in fowl (Shivaprased, 2000). In contrast to this evidence, an *in vitro* study has recently shown that the

ability to induce transepithelial signalling to PMNs is predictive of serotypes capable of causing gastro-enteritis in humans (McCormick *et al.*, 1995b). In this model system, *S. pullorum*, a biotype of *S. gallinarum*, failed to induce PMN transepithelial migration suggesting that this serotype would likely be avirulent in humans. One possible explanation for this discrepancy lies with the fact that clearly *in vitro* assays cannot mimic the complex interactions that occur *in vivo* between pathogen and host. Additionally, the work of McCormick *et al*, (1995b) used culture conditions, which are now known to be sub-optimal for induction of potent enteropathogenic responses (Galyov *et al.*, 1997). Furthermore, as the non-motility of *S. gallinarum* and *S. pullorum* appears to influence host cell interactions *in vitro* (Chapter 4; Henderson *et al.*, 1999), the ability of these serotypes to induce PMN transepithelial migration might also be affected.

To date, the role of flagella or bacterial motility in the induction of *Salmonella*-induced enteropathogenic responses has been little studied. *S. gallinarum* and *S. pullorum* are the only non-flagellated and, at least *in vitro*, non-motile serotypes within *S. enterica* (Li *et al.*, 1993). A recent report using phase locked *S. typhimurium* mutants, that were unable to switch flagella type, demonstrated that while blocking the phase variation affected the virulence for mice it did not attenuate the enteropathogenic responses seen following infection of bovine ligated ileal loops (Ikeda *et al.*, 2001). Therefore it appears that motility is not required for either the invasive (Chapter 4) or enteropathogenic phenotypes in calves. Interestingly, *Shigella* Spp., which were widely considered to be non-flagellated and therefore non-motile (Hale, 1991; Sansonetti *et al.*, 1994), have recently been shown to express flagella *in vitro* (Girón, 1995).

The poor enteropathogenic response to infection with *S. choleraesuis* was surprising, as this serotype was poorly invasive at the low dose (Chapter 4), yet induced moderate diarrhoea in calves following oral inoculation (Chapter 3). Although the reasons for this observation are unknown, similar invasive and enteropathogenic phenotypes have been observed following infection of porcine ligated ileal loops (Bolton *et al.*, 1999b; S. Paulin, unpublished observations). Together these results suggest that there are fundamental differences in the mechanisms of pathogenesis of *S. dublin* and *S. typhimurium* compared with *S. choleraesuis* in calves. The reasons for this are unknown but imply that serotype, rather than host, factors appear to influence the initial interactions of *Salmonella* with the intestinal mucosa.

The inability of *S. abortusovis* to induce an enteropathogenic response correlated well with the low invasiveness of this serotype, and the poor virulence in calves. *S. abortusovis*

infections in adult sheep are not typically associated with diarrhoea (Jack, 1968), which suggests that only apathogenic interactions occur with adult intestinal mucosa before bacteria disseminate systemically. Alternatively systemic disease may occur following natural parenteral infection, for example, by venereal transmission.

*S. abortusovis* is rarely associated with disease in hosts other than sheep (Jack, 1971) however, this serotype has been isolated from children with enteric disease, following the consumption of contaminated kangaroo meat (Murray *et al.*, 1994). The enteropathogenic responses of these particular strains (SA0515, 516 and 517, which were a kind gift from Professor S. Rubino, Sassari, Sardinia) were assessed following infection of bovine ligated ileal loops and interestingly they elicited significantly greater secretory and inflammatory responses than the negative control (P. Watson, unpublished observations). It is therefore likely that these particular *S. abortusovis* isolates were atypical of the sheep adapted strains, particularly as they were known to lack the *Salmonella* virulence plasmid.

Extensive mucosal damage, which may occur as a result of intestinal invasion, the movement of PMNs and/or the release of inflammatory cytokines (Giannella *et al.*, 1973a; Rout *et* al., 1974; Wallis *et al.*, 1986b; Everest *et al.*, 1999), is a pathological feature of some *Salmonella* infections. It has been proposed that *S. typhimurium*-induced damage to murine 'M' cells is serotype specific mediating bacterial dissemination to systemic sites (Pascopella *et al.*, 1995). Additionally, production of pro-inflammatory cytokines and infiltration of PMNs may limit some serotypes to the gut by induction of a strong immune response (Kaiser *et al.*, 2000), but as a result cause tissue pathology and often gastroenteritis.

In this study, differences in the amount of mucosal damage were observed in calves that did not directly correlate with the magnitude of the inflammatory and secretory responses. Due to their ability to cause severe diarrhoea in calves and induce enteropathogenic responses, it was not surprising that *S. typhimurium* and *S. dublin* resulted in extensive mucosal damage following infection of bovine ligated ileal loops. However, both of these serotypes also induce disruption to mucosal architecture in several species including rabbits (Bolton *et al.*, 1999a; Everest *et al.*, 1999), pigs (Bolton *et al.*, 1999b); chickens (Henderson *et al.*, 1999) and sheep (Uzzau *et al.*, 2001). This observation has been correlated to the presence of a serotype-specific tissue damaging histotoxin (Lodge *et al.*, 1999) and suggests that serotype-specific factors contribute to the magnitude of mucosal damage.

As a result of a potent secretory and inflammatory response, *S. gallinarum* resulted in moderately severe mucosal damage, following inoculation of ligated ileal loops. However this calf avirulent serotype resulted in greater enteropathogenic responses yet caused less mucosal disruption than *S. dublin*. Following oral inoculation of chickens, *S. pullorum* does not induce

severe intestinal pathology or a massive influx of heterophils one day after oral challenge (Henderson *et al.*, 1999). Additionally, *S. gallinarum* infection of chicken kidney cells *in vitro* causes no increase in the release of IL-6, in contrast to *S. typhimurium* (Kaiser *et al.*, 2000). These results suggest that in chickens, infection with *S. gallinarum* and *S. pullorum* fail to trigger a strong intestinal immune response thus facilitating systemic spread in the absence of diarrhoea.

Infection of bovine ligated ileal loops with *S. abortusovis* induced no intestinal pathology, consistent with the poor secretory and inflammatory responses observed and the absence of diarrhoea following oral inoculation of calves. Furthermore, *S. abortusovis* does not result in mucosal damage following infection of ovine ligated ileal loops (Uzzau *et al.*, 2001). This is in accordance with the pathogenesis of this serotype in sheep, resulting in low virulence in adult sheep, targeting of foetal lambs and bacterial dissemination into the environment via abortion of infected lambs (Uzzau *et al.*, 2001).

*S. choleraesuis* induced very little intestinal pathology in bovine ligated ileal loops which is consistent with the poor secretory and inflammatory responses observed. A similar lack of mucosal damage has been observed following initial interactions with porcine mucosa (Bolton *et al.*, 1999b). These results suggest that factors, subsequent to initial interactions with the intestinal mucosa, are essential in mediating the different pathogenic phenotypes following *S. choleraesuis* infection in both calves and pigs. As with *S. gallinarum* in chickens and *S. abortusovis* in sheep, *S. choleraesuis* virulence may be related to a strategy whereby the relative inability to recruit inflammatory cells to the foci of infection facilitates bacterial dissemination and subsequent disease in the absence of a potent intestinal immune response.

In conclusion, induction of enteropathogenesis by different *Salmonella* serotypes did not correlate with serotype-host specificity in calves. The results highlight the importance of performing relevant challenge studies in combination with ligated loop assays as clearly factors additional to enteropathogenesis influence the host-specific phenotype. It can be suggested that initial interactions with the intestinal mucosa do not determine the outcome of infection. As such, it is likely that host-pathogen interactions beyond the gut are consequential in mediating the virulent phenotype. Therefore, in order to determine the importance of bacterial interactions with systemic tissues, the virulence of different serotypes, with defined intestinal characteristics, was assessed in calves following intravenous inoculation.

# CHAPTER 6

# CHARACTERISATION OF THE VIRULENCE OF DIFFERENT *SALMONELLA* SEROTYPES FOLLOWING INTRAVENOUS INOCULATION OF CALVES.

## 6.1 Introduction

The precise mechanisms by which some serotypes within *S. enterica* are able to disseminate throughout the body, survive and proliferate within systemic tissues, while other serotypes are restricted to infecting the gastrointestinal tract remain poorly defined. With some combinations of serotype and host, interactions with the intestinal mucosa appear to influence the outcome of infection (Pospischil *et al.*, 1990; Pascopella *et al.*, 1995; Henderson *et al.*, 1999). However, it has been established that initial *Salmonella*-intestinal interactions do not directly correlate with serotype-host specificity in calves (Chapters 4 and 5), which implicates that factors involved in later stages of infection might influence the outcome of disease.

There is evidence to suggest that bacterial survival within cells of the reticuloendothelial system is an important mechanism contributing to the development of systemic salmonellosis in both avian and mammalian species (Barrow *et al.*, 1994). Although the precise location where *Salmonella* reside during different stages of pathogenesis is still in dispute, it is widely accepted that macrophages play a key role in this process. These cells potentially provide a niche for both bacterial replication and avoidance of host immune responses (Fields *et al.*, 1986; Buchmeier & Heffron, 1989; Dunlap *et al.*, 1994; Hensel *et al.*, 1998). Furthermore, once activated, macrophages can orchestrate host defences against the invading bacteria (reviewed by Jones and Falkow, 1996).

## 6.2 Aim

In order to address the question of whether survival within systemic tissues contributes to the host specific phenotype, virulence was assessed following intravenous inoculation of calves using *Salmonella* serotypes previously characterised for their abilities to interact with the intestinal mucosa.

## 6.3 Approaches and experimental design

The experimental procedures used are described in section 2.9. To determine the virulence of different serotypes, five groups of three calves were inoculated intravenously with *S. dublin* (SD3246), *S. typhimurium* (ST4/74). *S. choleraesuis* (SCSA50), *S. gallinarum* (SG9) or *S. abortusovis* (SAO44) and the infection monitored for up to seven days *post* inoculation (dpi).

Virulence was assessed by daily monitoring of the pyrexic response, production and consistency of diarrhoea, alteration in general demeanour and recovery of *Salmonella* from selected systemic and enteric tissues. Tissue counts at *post mortem* examination were derived from triplicate samples in each calf (section 2.9.4). Any animals meeting end-point criteria before the experimental termination dates were humanely killed (section 2.9.3). Details of inocula used are given in table 6.1.

## 6.4 Kinetics of infection following intravenous inoculation of calves with *S. dublin*, *S. typhimurium*, *S. choleraesuis*, *S. gallinarum* or *S. abortusovis*

### *6.4.1 Temperature responses*

Calves infected with all serotypes developed a pyrexic response 24-36 hours *post* inoculation. This response was mild and transient in calves inoculated with either *S. gallinarum* or *S. abortusovis*, severe and prolonged following infection with either *S. dublin* or *S. typhimurium* or bi-phasic following infection with *S. choleraesuis* (figure 6.1). From two days after inoculation, pyrexic responses in *S. dublin* or *S. typhimurium* infected calves were generally significantly greater than those of calves infected with *S. gallinarum* or *S. abortusovis* ($P < 0.05$ to $P < 0.001$). Typically, pyrexic responses induced by *S. dublin* or *S. typhimurium* were similar ($P > 0.1$). Equally, those induced by *S. gallinarum* or *S. abortusovis* were similar ($P > 0.1$). In contrast, the febrile response to infection with *S. choleraesuis* showed a bi-phasic pattern. This serotype induced a similar response to *S. dublin* and *S. typhimurium* for up to 3.5 days after inoculation ($P > 0.1$) followed by a significant drop in temperature for approximately 24 hours ($P < 0.02$ to $P < 0.0001$) and an increase towards the end of the experiment.

### *6.4.2 Faecal consistency*

Calves inoculated with *S. dublin*, *S. typhimurium* or *S. choleraesuis* developed diarrhoea 36-48 hours *post* infection. Diarrhoea was only mild or transient in calves infected with *S. choleraesuis*. In contrast calves inoculated with either *S. dublin* or *S. typhimurium* developed diarrhoea that persisted for the duration of the experiment, the consistency of faeces being at worst watery, with the presence of blood and sloughed mucosa in one *S. typhimurium*-infected calf. The faeces of calves inoculated with either *S. gallinarum* or *S. abortusovis* were normal throughout (table 6.1).

### *6.4.3 General demeanour*

Calves inoculated with *S. dublin*, *S. choleraesuis* or *S. typhimurium* became dull, lethargic and anorexic approximately 36 hours *post* inoculation. These symptoms persisted, with different magnitudes of severity, in calves inoculated with either *S. dublin* or *S. typhimurium*. One calf inoculated with *S. typhimurium* was humanely killed on day four *post* inoculation due to general debilitation and a rapid drop in body temperature. Calves inoculated with *S. choleraesuis* appeared to recover around days 4-5 *post* inoculation and presented no other symptoms of salmonellosis for the duration of the experiment with the exception of an increasing pyrexic response on days 5-6. Calves inoculated with either *S. gallinarum* or *S. abortusovis* developed no symptoms typical of salmonellosis throughout the duration of the experiment (table 6.1).

### *6.4.4 Bacterial recovery from systemic and enteric tissues*

The bacterial recovery of different *Salmonella* serotypes following intravenous inoculation of calves is presented in figures 6.2a, b, c and d.

There were no significant differences between recovery of either *S. dublin* or *S. typhimurium* from any intestinal site sampled ($P > 0.1$) with the exception of the ileal contents where *S. typhimurium* was recovered in greater numbers ($P < 0.1$). Both *S. dublin* and *S. typhimurium* were recovered in greater numbers than *S. choleraesuis* from contents and mucosa of the ileum, caecum and colon ($P < 0.1$ to $P < 0.05$), but not the corresponding lymph nodes ($P > 0.1$). Bacteria were recovered in similar numbers from all of the systemic tissues of calves inoculated with *S. dublin*, *S. typhimurium* or *S. choleraesuis* ($P > 0.1$) with the exception of the spleen and liver where *S. choleraesuis* was recovered by enrichment only. In addition bacteraemia was observed in one of the *S. dublin* and two of the *S. typhimurium* infected calves.

Bacterial recovery was detected, by enrichment only, following inoculation with *S. gallinarum*, but not *S. abortusovis*, from the mediastinal and hepatic lymph nodes, ileal contents and ileal lymph node.

In summary following intravenous inoculation, the recovery of salmonellas from systemic and enteric tissues, correlated positively with the clinical symptoms and pyrexic responses induced by the different serotypes.

| Serotype (strain) | Calf number | Inocula ($log_{10}$ cfu) | Day of death *post* inoculation | Scour score | Reason for euthanasia |
|---|---|---|---|---|---|
| ***S. dublin*** | 6.1 | 6 | 7 | 6 | End of experiment |
| **(SD3246)** | 6.2 | 6 | 7 | 8 | End of experiment |
| | 6.3 | 5.96 | 7 | 7 | End of experiment |
| **Mean scour score SD3246** | | | | **7** | |
| ***S. choleraesuis*** | 6.4 | 6.3 | 7 | 3 | End of experiment |
| **(SCSA50)** | 6.5 | 6.38 | 7 | 2 | End of experiment |
| | 6.6 | 6.36 | 7 | 0 | End of experiment |
| **Mean scour score SCSA50** | | | | **1.6** | |
| ***S. typhimurium*** | 6.7 | 6.04 | 5 | 9 | Temperature drop-unable to stand unaided |
| **(ST4/74)** | 6.8 | 6.04 | 7 | 14 | End of experiment |
| | 6.9 | 6.00 | 7 | 6 | End of experiment |
| **Mean scour score ST4/74** | | | | **9.6** | |
| ***S. gallinarum*** | 6.10 | 6.18 | 7 | 0 | End of experiment |
| **(SG9)** | 6.11 | 6.04 | 7 | 0 | End of experiment |
| | 6.12 | 6.28 | 7 | 0 | End of experiment |
| **Mean scour score SG9** | | | | **0** | |
| ***S. abortusovis*** | 6.13 | 6.04 | 7 | 0 | End of experiment |
| **(SAO44)** | 6.14 | 5.95 | 7 | 0 | End of experiment |
| | 6.15 | 5.90 | 7 | 0 | End of experiment |
| **Mean scour score SAO44** | | | | **0** | |

**Table 6.1 Inocula used and clinical findings of calves infected intravenously with *S. dublin* (SD3246), *S. choleraesuis* (SCSA50), *S. typhimurium* (ST4/74), *S. gallinarum* (SG9) or *S. abortusovis* (SAO44) for up to 7 dpi.**

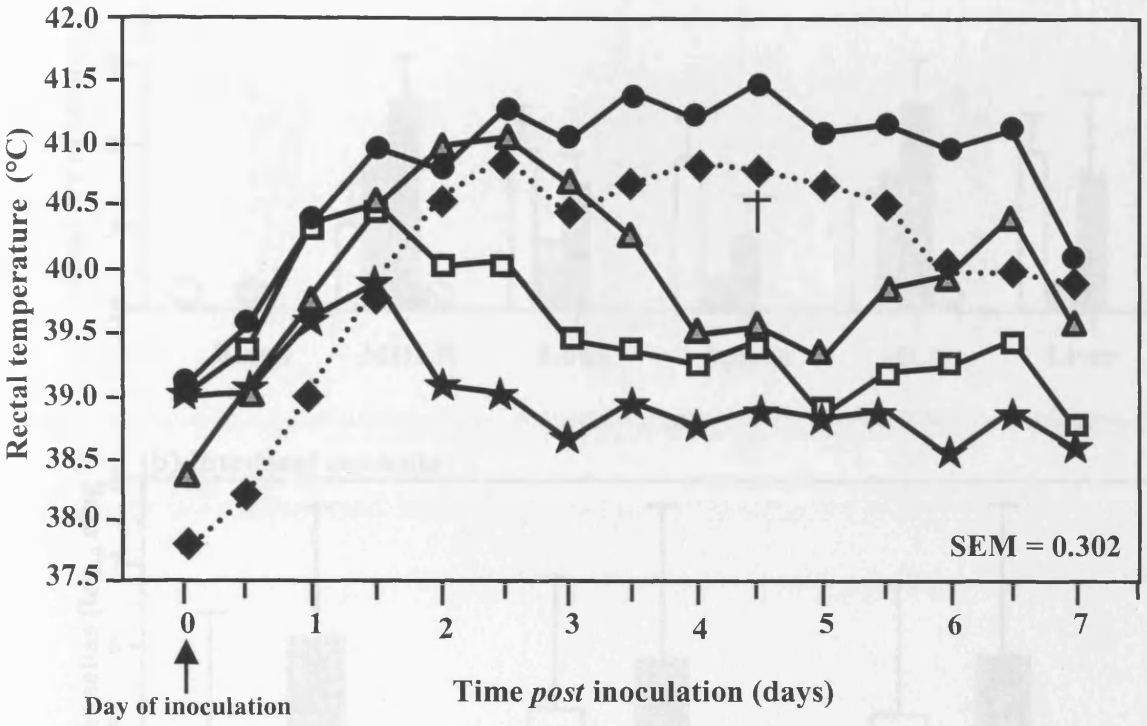



**Figure 6.1 Least square mean (SEM) daily rectal temperature of calves following intravenous inoculation with approximately 6.0 $\log_{10}$ cfu of either *S. dublin* SD3246 (-●-); *S. choleraesuis* SCSA50 (-△-); *S. typhimurium* ST4/74 (-◆-); *S. gallinarum* Sg9 (-★-) or *S. abortusovis* SAO44 (-□-).** Each datum point is derived from 3 calves *per* group with the exception of ST4/74 from day 5 onwards which is derived from 2 calves only (time of death is indicated with †)

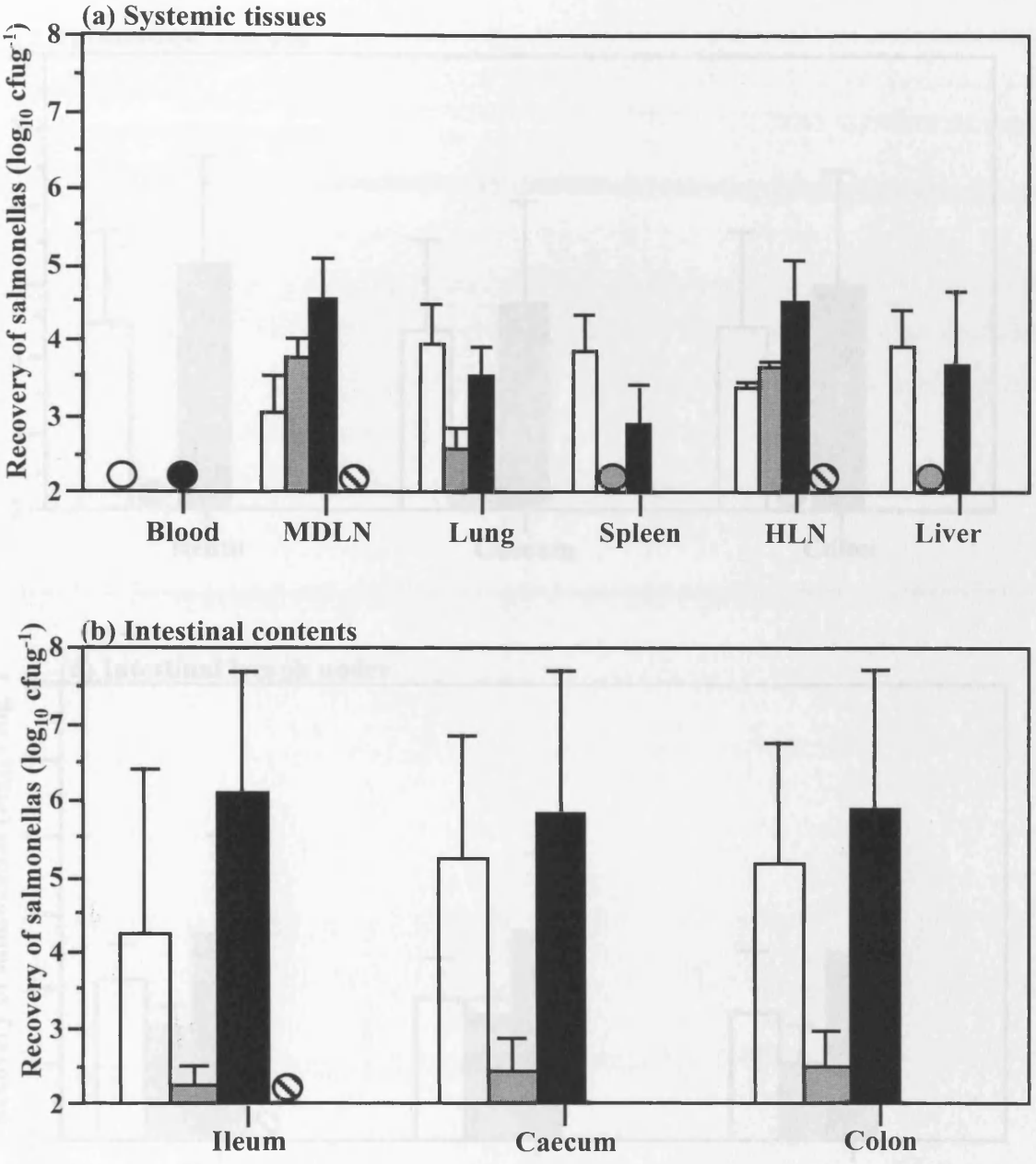



**Figure 6.2 Recovery of *Salmonella* from systemic tissues (a) and intestinal contents (b) of calves at up to 7 days *post* intravenous inoculation.** Triplicate samples were taken from each tissue and means were calculated to give a value *per* animal. Each bar represents the mean of 3 calves for *S. dublin* (SD3246 □ ]), *S. choleraesuis* (SCSA50 [▩]), *S. typhimurium* (ST4/74 [■]), or *S. gallinarum* (SG9 ▨ ]) and is presented with the standard error of the mean. There was no bacterial recovery from *S. abortusovis* inoculated calves.

○ Samples that were positive on enrichment only. MDLN and HLN represent mediastinal and hepatic lymph nodes respectively.

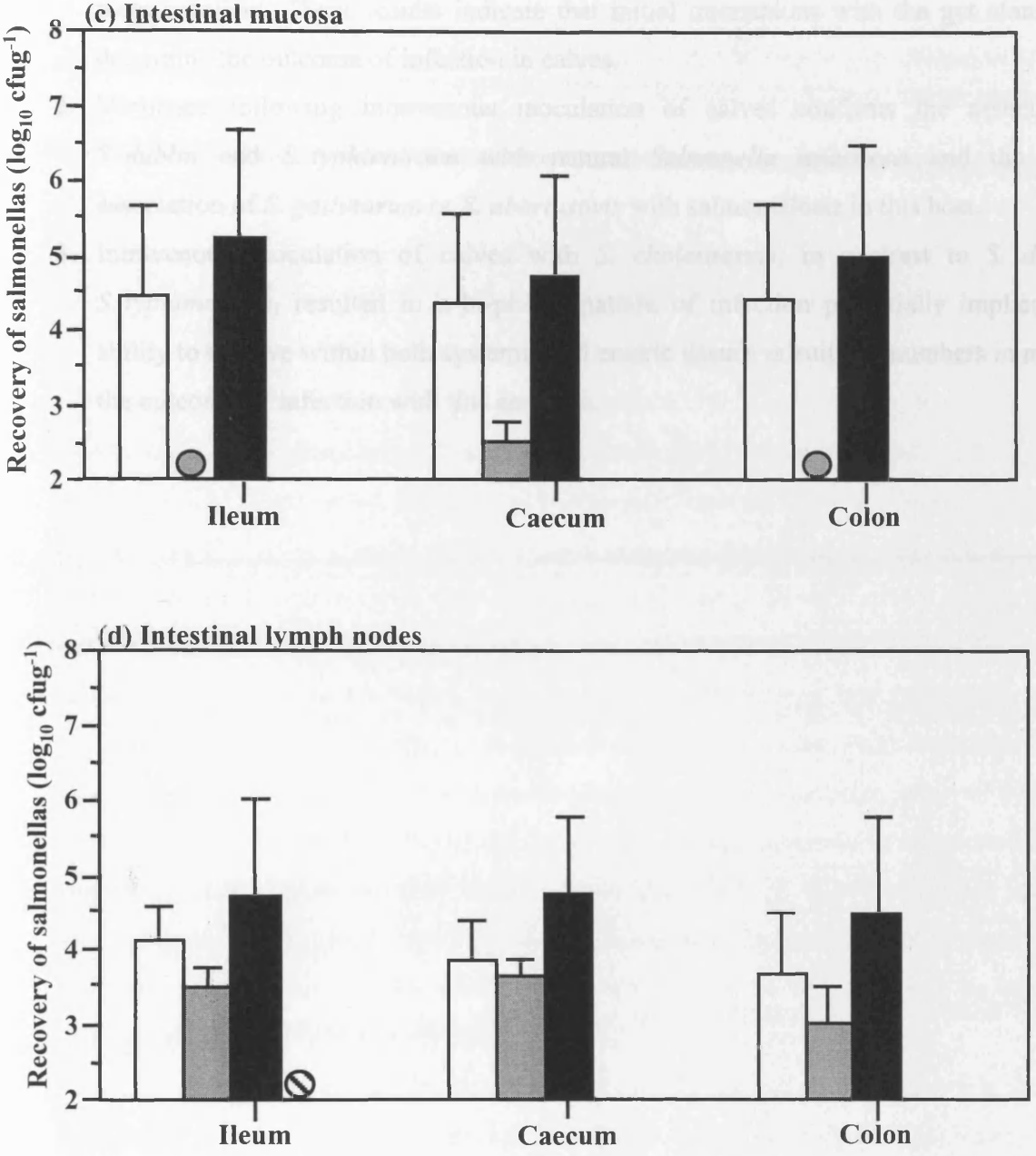



**Figure 6.2 (cont.) Recovery of salmonellas from intestinal mucosa (c) and lymph nodes (d) of calves at up to 7 days *post* intravenous infection.** Triplicate samples were taken from each tissue and means were calculated to give a value *per* animal. Each bar represents the mean of 3 calves for *S. dublin* (SD3246 [□]), *S. choleraesuis* (SCSA50 [▣]), *S. typhimurium* (ST4/74 [■]) or *S. gallinarum* (SG9 [▨]) and is presented with the standard error of the mean. There was no bacterial recovery from *S. abortusovis* inoculated calves.

○ Samples that were positive on enrichment only.

### 6.5 Summary of results

1. *S. dublin* and *S. typhimurium* were highly virulent following intravenous inoculation of calves, *S. choleraesuis* was moderately virulent while *S. gallinarum* and *S. abortusovis* were avirulent. These results indicate that initial interactions with the gut alone do not determine the outcome of infection in calves.
2. Virulence following intravenous inoculation of calves confirms the association of *S. dublin* and *S. typhimurium* with natural *Salmonella* infections and the lack of association of *S. gallinarum* or *S. abortusovis* with salmonellosis in this host.
3. Intravenous inoculation of calves with *S. choleraesuis*, in contrast to *S. dublin* or *S. typhimurium*, resulted in a bi-phasic pattern of infection potentially implicating the ability to survive within both systemic and enteric tissues in suitable numbers in mediating the outcome of infection with this serotype.

## 6.6 Discussion

It has been demonstrated that although intestinal invasion is necessary for the induction of systemic disease in calves, initial interactions with the intestinal mucosa do not directly correlate with *Salmonella* serotype-host specificity. This potentially implicates the ability to disseminate and interact with components of the innate immune system in determining the nature of disease following infection. To address the question of whether survival within systemic tissues influences the outcome of disease, the virulence of *S. dublin*, *S. choleraesuis*, *S. typhimurium*, *S. gallinarum* or *S. abortusovis* was determined following intravenous inoculation of calves.

*S. typhimurium* and *S. dublin* were the most virulent serotypes, eliciting symptoms that were similar to those observed during both natural *Salmonella* outbreaks and following experimental oral inoculation (Chapter 3) (Sojka & Field, 1970; Sojka *et al.*, 1977; Jones *et al.*, 1991). The observation that *S. typhimurium* was isolated from the blood and in similar numbers to *S. dublin* from systemic tissues was not surprising as this serotype, despite typically being associated with enteritis in calves (Wray & Davies, 2000), is able to breach intestinal barriers in young and immunocompromised hosts (Parker, 1990). Consequently, *S. typhimurium* is often recovered from systemic sites following oral inoculation of calves (Jones, *et al.*, 1991). Interestingly, regardless of whether calves were inoculated orally or intravenously with *S. dublin* or *S. typhimurium*, the onset of diarrhoea occurred 36-48 hours *post* infection. Consistent with natural infections, the pathogenesis of these two serotypes appears to be similar in that they both preferentially infect gut tissue where the bacteria are able to rapidly proliferate. Following intravenous inoculation, the gut would be seeded via the circulatory system or bacteria would enter directly via the bile into the duodenum thus avoiding the low pH of the stomachs encountered following oral inoculation.

*S. gallinarum* and *S. abortusovis* were largely asymptomatic following intravenous inoculation of calves, which correlated with the naturally specific host range of these serotypes (Jack, 1968; Shivaprased, 2000). *S. abortusovis* invades the bovine intestinal mucosa poorly and does not elicit an enteropathogenic response while *S. gallinarum* invades in high numbers *in vivo* and induces a potent enteropathogenic response. Despite the different interactions of these two serotypes with the intestinal mucosa, neither *S. gallinarum* nor *S. abortusovis* are able to survive within enteric (Chapter 3) or systemic (Chapters 3 and 6) sites. These results suggest that both systemic and intestinal factors are able to restrict the infection with certain serotype-host combinations.

*S. choleraesuis* was only moderately virulent, and interestingly exhibited a bi-phasic pattern of infection, following intravenous inoculation. This observation warrants explanation

as despite being both poorly invasive and enteropathogenic, this serotype was as virulent as *S. dublin* in calves infected orally. It could be speculated that due to the removal of *S. choleraesuis* from the blood and liver, this serotype was only able to re-seed the intestine in low numbers. This may have provided a 'lag' in the infection process prior to the establishment of *S. choleraesuis* in the intestinal mucosa and the onset of secondary pyrexia. While these are interesting observations relating to the pathogenesis of *S. choleraesuis*, further investigation is required as only a limited number of calves were used in this study. For example, it would be fascinating to determine whether severe clinical symptoms and diarrhoea resulted if calves infected intravenously with this serotype were monitored for a time period beyond seven days.

Collectively, following intravenous inoculation with *S. typhimurium*, *S. dublin*, *S. gallinarum* or *S. abortusovis* it can be concluded that the host specific phenotype is similar, regardless of whether the gut is bypassed or not. *S. choleraesuis* resulted in a bi-phasic response, which was distinct from the virulence of this serotype following oral inoculation. These results implicate a role for interactions with systemic tissues in determining the outcome of infection. However in cattle the enteric, rather than the systemic, sites appear to provide the primary niche for bacterial growth independent of the route of inoculation. This observation has also been demonstrated following oral or subcutaneous inoculation of calves with *S. typhimurium*, when the highest bacterial counts in 4/5 subcutaneously and 2/2 orally inoculated calves that succumbed to infection were associated with the gastro-intestinal tract (Villarreal-Ramos *et al.*, 2000).

It is interesting to make a comparison of the serotypes that were virulent in calves with the pathogenesis following both intravenous and oral inoculation of pigs. In the porcine model *S. choleraesuis* was the most virulent serotype by both routes of inoculation, and was the only serotype recovered in quantifiably high numbers from systemic sites. In contrast, *S. typhimurium* was moderately virulent by the oral route only and *S. dublin* was largely avirulent by both routes (Watson *et al.*, 2000c). These results suggest that neither *S. dublin* nor *S. typhimurium* are able to survive within porcine systemic or enteric tissues and implies that in pigs, as in calves, the mechanisms of host specificity do not depend on interactions with the gut alone. Additionally, these results highlight the differences in the pathogenesis of serotypes within particular hosts. For example, *S. choleraesuis* is typically associated with severe systemic disease in pigs (Wilcock and Schwartz, 1992). Accordingly, persistence at systemic sites is likely to be of pivotal importance in determining the outcome of infection. In contrast primarily enteric, but also systemic, persistence appears to influence the pathogenesis

of *S. typhimurium*, *S. dublin* and *S. choleraesuis* in calves suggesting that both host and serotype factors influence host specificity in different species.

Studies using *Salmonella* serotypes of defined virulence have shown, that as in calves, the ability to interact with the intestinal mucosa does not correlate with host specificity in pigs (Bolton *et al.*, 1999b), chickens (Chadfield *et al.*, 2001) or sheep (Uzzau *et al.*, 2001). However, all serotypes that are associated with systemic disease in a particular host can be isolated from systemic tissues following both oral and intravenous inoculation (Barrow, *et al.*, 1994; Uzzau, et al., 2001; Watson, et al., 2000c). In contrast, serotypes not typically associated with systemic disease are eliminated from these tissues within seven days (S. Paulin, unpublished observations; Barrow, *et al.*, 1994; Watson, *et al.*, 2000c; Uzzau, *et al.*, 2001). Accordingly, it has been speculated that host specificity results from the differential ability to survive and proliferate within tissues of the reticuloendothelial system (Barrow, *et al.*, 1994). As macrophages are thought to play a crucial role in *Salmonella* pathogenesis (reviewed by Jones and Falkow, 1996), several studies have addressed the question of whether persistence within these host cells correlates with host specificity (section 1.9.2.1). Results using primary macrophages of either human or murine origin have observed a correlation between the intracellular persistence of *S. typhi* or *S. typhimurium* respectively and the host specific phenotype (Lissner *et al.*, 1985; Vladoianu *et al.*, 1990; Alpuche-Aranda *et al.*, 1995; Ishibashi & Arai, 1996; Schwan & Kopecko 1997; Schwan *et al.*, 2000). Recently however, numerous studies have shown that *Salmonella* can lyse macrophages or Kupffer cells *in vitro* (Harrington and Hormaeche, 1986; Chen *et al.*, 1996; Guilloteau *et al.*, 1996b; Lindgren *et al.*, 1996; Monack *et al.*, 1996; Schwan, *et al.*, 2000; Watson *et al.*, 2000b; Watson *et al.*, 2000c). Therefore, failure to determine the amount of *Salmonella*-induced membrane integrity damage, for example by quantifying the release of intracellular enzymes (Guilloteau *et al.*, 1996b), makes such results difficult to interpret (Alpuche-Aranda *et al.*, 1995; Ishibashi & Arai, 1996). In addition, caution needs to be applied when comparing studies using macrophages derived from genetically susceptible hosts as responses to infection are likely to differ considerably to those obtained from naturally resistant animals (Harrington and Hormaeche 1986).

In contrast to the work with murine and human macrophages, the survival and persistence of well characterised *Salmonella* serotypes of defined virulence within primary macrophages of several host species including calves (Wallis, *et al.*, 1997), pigs (Watson, *et al.*, 2000c), sheep (Uzzau, personal communication) and chickens (Olsen, personal communication) does not appear to correlate with host specificity. This might suggest that

experimental infection of isolated macrophages *in vitro* does not mimic the complex interactions that take place *in vivo* between bacteria and host.

While it would be interesting to determine whether serotypes such as *S. gallinarum* and *S. abortusovis*, which do not cause disease in calves, are unable to survive within bovine macrophages there are complications underlying such *in vitro* approaches. First, *S. dublin* and *S. typhimurium* are known to induce rapid plasma membrane damage in bovine alveolar macrophages making comparisons with other serotypes difficult to interpret, due to loss of intracellular bacteria through gentamicin killing (Wallis, *et al.*, 1997; Watson *et al.*, 2000b). Second, several studies have compared the ability of both motile and non-motile serotypes to persist within macrophages. The ability to be taken up by avian macrophage-like cells is potentially influenced by motility (Henderson, *et al.*, 1999), making the intracellular persistence of motile and non-motile serotypes, such as *S. dublin* and *S. gallinarum* respectively, difficult to compare.

Although the studies mentioned above provide evidence that *Salmonella* can survive within macrophages *in vitro*, the *in vivo* evidence supporting a role for this cell type in *Salmonella* pathogenesis is less clear. Several studies have addressed the question of which host cells *S. typhimurium* reside in during systemic infections in mice. These results have been contradictory with several eukaryotic cell types being implicated including, neutrophils (Dunlap *et al.*, 1992), macrophages or Kupffer cells (Buchmeier and Heffron, 1989; Nnalue *et al.*, 1992) hepatocytes ( Lin *et al.*, 1987; Conlan & North, 1992), and even the extracellular space (Hsu, 1989). Many of these studies have used high inocula and relied on electron microscopic analysis to interpret results. However, a recent approach using Confocal Laser Scanning Microscopy (CLSM) in conjunction with immunostaining techniques and a low starting inocula was able to demonstrate an association of salmonellas with neutrophils during the early stages of infection and macrophages during the later stages (Richter-Dahlfors *et al.*, 1997). Exploitation of such techniques in the context of host specificity would provide valuable information regarding *in vivo* association of serotypes with particular eukaryotic cells in different tissues.

In conclusion, it would appear that survival and proliferation within systemic tissues is important in determining the host specific phenotype. However, relatively little is known about the interaction of salmonellas with cells of the innate immune system *in vivo*. In order to address the question of what happens after initial intestinal interactions that might influence the outcome of disease in calves, several factors, potentially influencing the virulence of *S. dublin* and *S. gallinarum*, were further characterised *in vivo* using a novel bovine cannulation model.

# CHAPTER 7

# CHARACTERISATION OF THE ROUTE OF BACTERIAL TRANSLOCATION TO SYSTEMIC TISSUES AND ASSESSMENT OF ACUTE HOST RESPONSES TO *S. DUBLIN* OR *S. GALLINARUM* INFECTION *IN VIVO*.

## 7.1 Introduction

It is generally believed that, given the right combination of *Salmonella* serotype and host, systemic infection ensues after translocation of intracellular salmonellas via the lymphatics (Carter and Collins, 1974) to organs such as the liver and spleen (Takeuchi and Sprinz, 1967; Barrow *et al.*, 1994). However, the precise route by which dissemination occurs and any interaction with particular eukaryotic cells during this process has not been unequivocally established, and may differ depending on the particular host and the infecting serotype (section 1.9.1).

Evidence to support a role for particular host cells in providing a bacterial niche during the systemic phase of infection is still unclear (section 1.9.2). For example, it has been shown that *Salmonella* survive within PMNs during the early stages of systemic disease (Dunlap *et al.*, 1994). These cells may then die potentially facilitating bacterial engulfment by other phagocytes, which are possibly macrophages (Richter-Dahlfors *et al.*, 1997). Examination of immunolabelled sections of murine liver by CLSM has shown that *S. typhimurium* is cytotoxic for both PMNs and macrophages *in vivo* (Richter-Dahlfors *et al.*, 1997). This fact has also been reported following *in vitro* infection of immortalised and primary macrophages (Chen *et al.*, 1996; Guilloteau *et al.*, 1996b; Monack *et al.*, 1996; Libby *et al.*, 2000), Kupffer cells (Harrington and Hormaeche, 1986) and epithelial cells (Kim *et al.*, 1998).

Following infection by intracellular pathogens, host cell death could represent a defence mechanism facilitating the elimination of infected cells, or a bacterial-induced virulence strategy enabling pathogen evasion of host defences and potentiating bacterial dissemination (reviewed by Weinrauch and Zychlinsky, 1999) (section 1.9.7). Several studies have suggested that *Salmonella*-induced cell death of macrophages *in vitro* and *in vivo* occurs by SPI-1 and caspase 1-dependent apoptosis (Chen *et al.*, 1996; Monack *et al.*, 1996; Richter-Dahlfors *et al.*, 1997; Hersh *et al.*, 1999; Monack *et al.*, 2000). In contrast, recent investigations have suggested that cell death, following *Salmonella* infection of bovine and porcine alveolar (Watson *et al.*, 2000b; Watson *et al.*, 2000c) or murine peritoneal exudate macrophages (Brennan and Cookson, 2000), may be the result of a SPI-1 and caspase 1-dependent necrosis. Furthermore, the cytotoxicity of *S. dublin* and *S. typhimurium* for murine peritoneal (Guilloteau *et al.*, 1996b; Gautier *et al.*, 1997) or human monocyte-derived (Libby *et al.*, 2000) macrophages *in vitro* has been shown to be mediated by genes encoded on the

virulence plasmid. However, evidence for a role of the *Salmonella* plasmid virulence (*spv*) operon in macrophage lysis is contradictory. For example, it has been suggested that lysis of murine macrophages is the result of a non-apoptotic mechanism that does not require expression of the *spv* operon (Guilloteau *et al.*, 1996b; Gautier *et al.*, 1997), while others found that lysis of human macrophages requires expression of the *spv* operon and occurs as a result of apoptosis (Libby *et al.*, 2000). Together these results potentially implicate a role for different virulence plasmid genes in lysing macrophages by distinct mechanisms.

Host immune responses, following infection with specific pathogens including *Salmonella*, have been determined following cannulation of pseudo-afferent or efferent lymphatics in large mammals such as sheep (Gohin *et al.*, 1997; Rothel *et al.*, 1998). Typically lymph vessels draining nodes that are relatively superficial and easily accessible are cannulated, for example lymph draining popliteal (Hall and Morris, 1962), pre-femoral (Entrican *et al.*, 1992), pre-scapular nodes (Bernard *et al.*, 1996; Doucet *et al.*, 1997) or the thoracic duct (Gershwin *et al.*, 1995). However to date, cannulation of efferent lymphatics draining intestinal mesenteric lymph nodes has not been used to determine the route of bacterial translocation and the immune responses to an enteric pathogen.

## 7.2 Aim

In order to determine whether host specificity could be attributed to a differential ability to translocate from the intestinal mucosa or MLN to systemic tissues, via venous blood or efferent lymph respectively, both the route and kinetics of bacterial dissemination were assessed. Furthermore, host inflammatory responses, association of salmonellas with specific cell types and the role of eukaryotic cell death in *Salmonella* pathogenesis were also evaluated *in vivo*.

## 7.3 Approaches and experimental design

A cannulation model, based on the bovine ligated ileal loop technique, was developed and exploited for the experiments described in this chapter (section 2.13 and figure 2.1). In total, ten calves comprising two controls, four infected with *S. dublin* (SD3246) and four infected with *S. gallinarum* (SG9) were used in these experiments. In addition, the effect of carriage of GFP *in vivo* was assessed using calves from which invasion (Chapter 4) and enteropathogenesis (Chapter 5) data had been obtained. Furthermore, sections of distal ileal mucosa used for immunolabelling following infection for two hours were also derived from calves that were used to assess intestinal invasion (Chapter 4).

Bacterial interactions with the intestinal mucosa and MLN (sections 2.13.5.3 and 2.15.3.1) and the kinetics of *S. dublin* and *S. gallinarum* dissemination to systemic sites

(section 2.13) were determined up to twelve hours after ligated ileal loop infection and cannulation of both efferent lymphatics and venous drainage. Host responses to infection were characterised including cell phenotypic alterations (section 2.17), morphological changes to the intestinal mucosa and MLN (section 2.15) and characterisation of the niche occupied by *Salmonella* during translocation (section 2.14). Additionally, the ability of *Salmonella* to induce cell death was determined by Annexin V staining and flow cytometric analysis of infected lymph and tissues (section 2.16.1) and/or TUNEL staining of infected tissues (section 2.16.2).

The cannulation model was initially optimised using calves infected with *S. dublin*. These studies were subsequently modified and extended to maximise the use of samples and gain further insight into specific host-pathogen interactions.

Details of inocula preparation are given in section 2.11.1[ii] and the inocula used for each of the cannulation experiments are given in table 7.1.

To ease detection of salmonellas, bacteria were tagged with a plasmid expressing the green fluorescent protein (GFP) as described in section 2.7. The effect of GFP expression was assessed for *in vitro* invasion of Int 407 cells (section 2.8.2 and 2.8.3), *in vivo* intestinal invasion (section 2.11) and induction of fluid secretion and inflammatory responses (section 2.12). All viable counts were plated onto agar with and without ampicillin (100 $\mu g\ ml^{-1}$) to determine plasmid stability *in vivo*.

## 7.4 Characterisation of salmonellas expressing GFP

The effect of GFP expression on *S. dublin* and *S. gallinarum*-host interactions was assessed to ensure that carriage of the plasmid did not alter the behaviour of *Salmonella in vivo*. The plasmid pTECH2GFP (section 2.7) was used with the idea that GFP would facilitate the detection of bacteria by both flow cytometry and CLSM analysis of immunostained sections.

### *7.4.1 Effect of GFP expression by salmonellas on invasion and lysis of Int 407 intestinal epithelial cells*

There were no differences in the magnitude of Int 407 invasion from wild type *Salmonella* compared with the corresponding GFP-tagged strains ($P > 0.1$) (figure 7.1a). Furthermore, two hours after infection of Int 407 cells all *Salmonella* serotypes tested induced LDH release in similar amounts to the negative control ($P > 0.1$) (figure 7.1b).

### *7.4.2 Effect of GFP expression by salmonellas on intestinal invasion and induction of fluid secretion and inflammatory responses in vivo*

*Salmonella* interactions with the intestinal mucosa were assessed using the bovine ligated ileal loop model (sections 2.11 and 2.12). There were no differences in the magnitude of intestinal invasion, from absorptive epithelia or Peyer's patch mucosa, of wild type *S. typhimurium* (ST4/74) compared to the corresponding GFP-tagged strain ($P > 0.1$) (figure 7.2). Additionally, there were no obvious differences in the magnitude of fluid secretion or the influx of inflammatory cells induced by wild type, compared to GFP-tagged salmonellas (figure 7.3). Similar responses were also obtained in a second calf (data not shown).

In summary, carriage of the plasmid expressing GFP was stable, as determined by enumerating salmonellas plated onto agar with and without antibiotic selection, and did not affect the behaviour of *Salmonella in vitro* or *in vivo*. Therefore the use of GFP-tagged bacteria appeared to be a valid and convenient tool for studying *Salmonella*-host interactions *in vivo*.

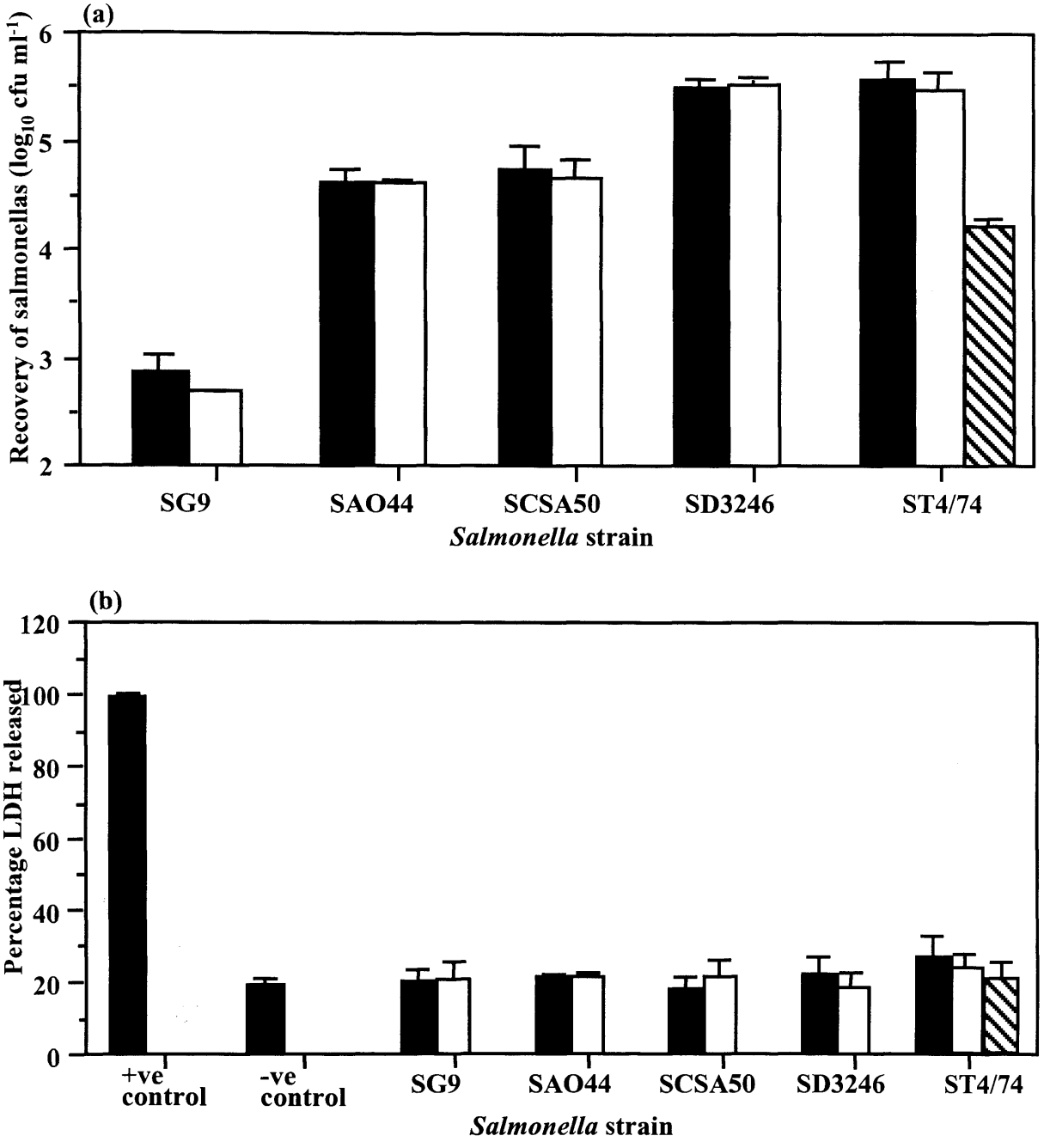



**Figure 7.1 Effect of GFP expression by *Salmonella* on invasion of epithelial cells *in vitro*.** Intracellular recovery of *Salmonella* 2 hours after infection of Int 407 cells (a) and corresponding LDH release (b). Each bar represents the mean value taken from triplicate wells in either 2 or 4 separate experiments and is presented with the standard error of the mean. Wild type (■); GFP-tagged (□) or the reduced invasion mutant *invH* (▨). Inocula used for the wild type and *invH* mutant are given in table 4.1. Average inocula ($log_{10}$ cfu $ml^{-1}$) for GFP tagged strains: SD3246, 6.41± 0.04; SCSA50, 6.51 ± 0.05; ST4/74, 6.32 ± 0.03; SAO44, 6.08 ± 0.05; SG9, 6.39 ± 0.11.

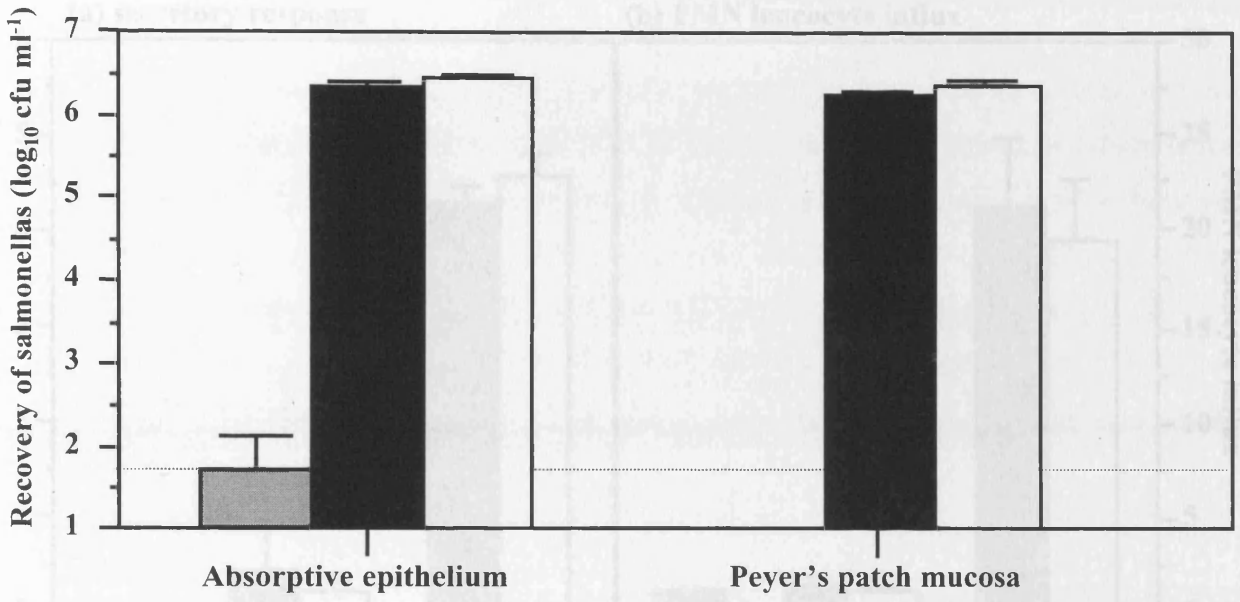



**Figure 7.2 Effect of GFP expression by *S. typhimurium* (ST4/74) on intestinal invasion *in vivo*.** Triplicate samples were taken from 2 or 3 loops *per* strain in each animal 2 hours after infection and means were calculated to give a value *per* calf. Each bar represents the mean of 3 separate calves and is presented with the standard error of the mean. Wild type (■); GFP-tagged (□) or LB negative control (▩). The dotted line represents the limit of accurate detection. Average inocula ($log_{10}$ cfu loop) used were: ST4/74, 9.5 ± 0.10; ST4/74-GFP, 9.7 ± 0.16

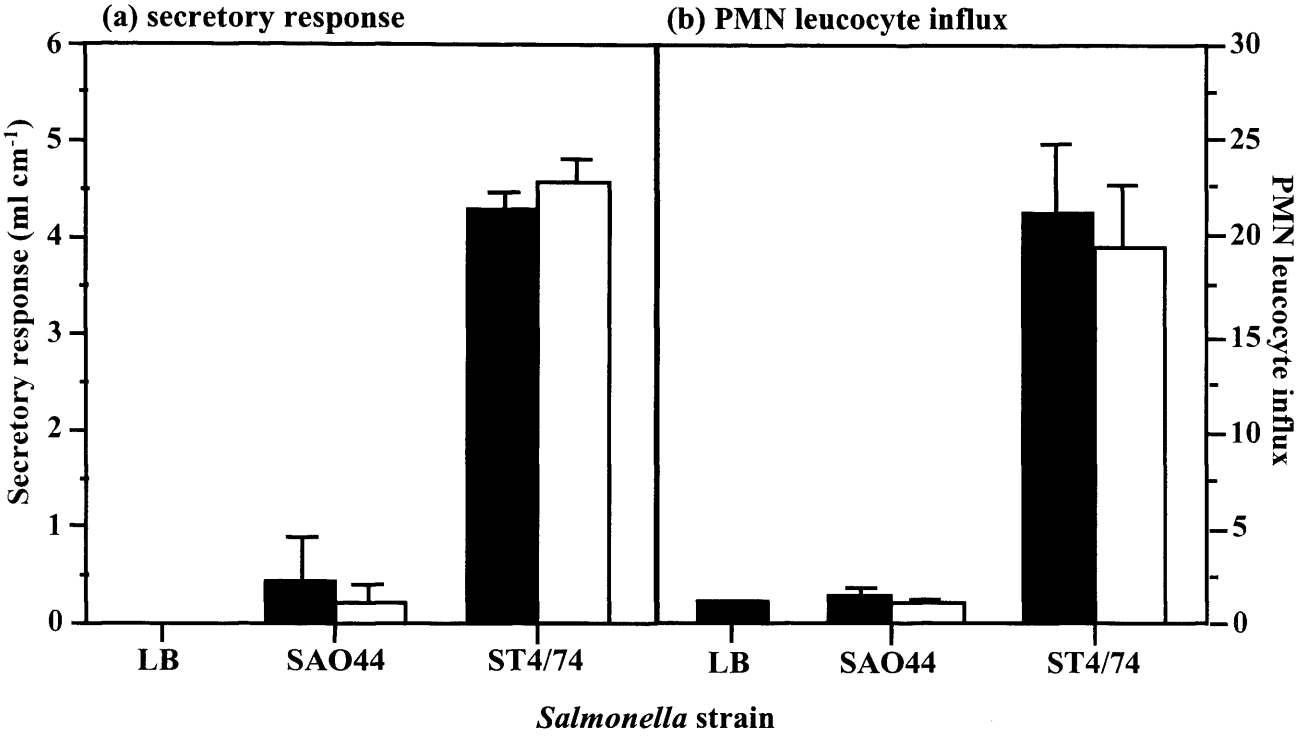



**Figure 7.3 Effect of GFP expression by *Salmonella* on bacterial-induced secretory (a) and inflammatory (b) responses 12 hours after infection of bovine ligated ileal loops.** Each bar represents the mean response derived from 3 loops in a single calf and is presented with the SEM. The secretory response (a) represents the mean V/L ratio and the PMN influx (b) is presented as a percentage compared to *S. typhimurium* strain ST4/74. Inocula ($log_{10}$ cfu loop) used were ST4/74, 9.04; ST4/74-GFP, 9.00; SAO44, 8.97; SAO44-GFP, 8.96.

### *7.4.3 Optimisation of fixation and sectioning techniques for visualisation of salmonellas expressing GFP*

To determine the best conditions for visualisation of *Salmonella* within tissue sections, bacteria expressing GFP were inactivated for 1 hour with a range of different fixatives, including neutral buffered formalin (NBF) and paraformaldehyde (PFA), followed by examination of slides under fluorescent microscopy. Paraformaldehyde, at concentrations up to 4%, was found to be the best fixative both in terms of brightness and duration of fluorescence (at least 5 days at room temperature).

Visualisation of GFP-tagged bacteria within intestinal mucosa was initially investigated using frozen sections embedded in O.C.T. and counterstained to reduce tissue autofluorescence (section 2.15.2). However, using this technique relatively weak bacterial fluorescence and poor tissue architecture were observed. Therefore in subsequent experiments, tissue sections were fixed and cut using a vibrating microtome (section 2.15.3), to preserve tissue architecture, and bacteria were immunolabelled (as described in section 2.15.3.1), to increase the sensitivity of the signal, followed by CLSM examination (section 2.15.3.2).

### *7.4.4 Detection of salmonellas expressing GFP by FACS analysis*

To confirm that bacteria expressing GFP could be detected by FACS analysis, *S. dublin* pTECH2GFP were fixed in 1% PFA, diluted in lymph and the percentage of fluorescing bacteria analysed by flow cytometry (section 2.17.2.1). At a concentration of 7 $\log_{10}$ cfu $ml^{-1}$, 72% of bacteria were FL1 positive (figure 7.4) confirming the ability to detect GFP-tagged *Salmonella* using this technique *in vitro*. However, the presence of a non-fluorescing bacterial population suggests that there is likely to be an under-estimation of *Salmonella in vivo*, particularly if the numbers of bacteria are relatively low and assay sensitivity is lost. As such, the usefulness of this construct for microscopy and FACS analysis was limited.

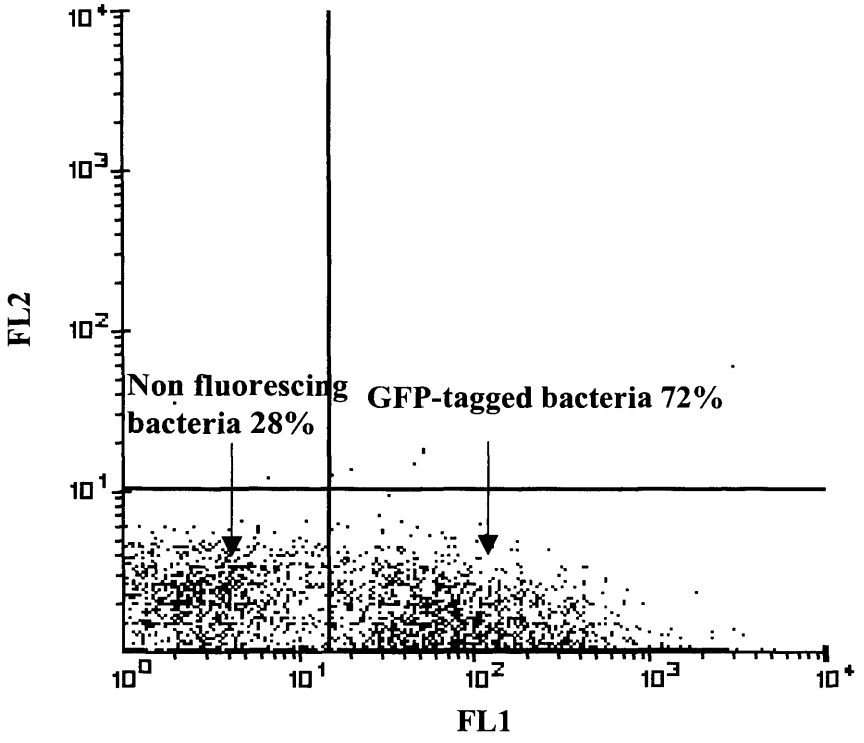



**Figure 7.4 Flow cytometric analysis to determine the percentage of FL1 positive SD3246 containing pTECH2GFP in control efferent lymph infected with 7 $\log_{10}$ cfu $ml^{-1}$ *in vitro*.**

## 7.5 Interaction of *S. dublin* or *S. gallinarum* with the intestinal mucosa and MLN up to twelve hours after infection of ligated ileal loops

To assess serotype-specific differences, the interactions of *S. dublin* and *S. gallinarum* with the intestinal mucosa and MLN were determined by CLSM analysis of immunolabelled sections at two and/or twelve hours after infection (section 2.15.3.1). *Salmonella* constructs were immunolabelled with rabbit anti-*Salmonella* LPS followed by anti-rabbit IgG conjugated to Alexa Fluor 568 (red). The leucocyte cytoskeleton was immunolabelled with mouse anti-α-tubulin followed by anti-mouse IgG conjugated to Alexa Fluor 488 (green). In addition, the numbers of bacteria present in the intestinal mucosa and MLN were quantified by viable count twelve hours after infection of ligated ileal loops and cannulation of efferent lymphatics and venous drainage (section 2.13.5.4). Prior to these cannulation experiments, *in vitro* optimisation of the bactericidal activity of gentamicin for *Salmonella* was determined (section 2.13.5.1). Inocula used and experimental details of calves are given in table 7.1

### *7.5.1 Optimisation of immunolabelling conditions*

Prior to sectioning of mucosa or MLN, the immunolabelling technique was optimised using HeLa cells as described in section 2.15.3.1. Coverslips were examined by CLSM and projected images through a Z-stack section of the HeLa cell showed intracellular bacteria and salmonellas associated with the cell surface (figure 7.5). This confirmed that the immunolabelling protocol was optimal for visualisation of bacteria associated with epithelial cells. The conditions were then re-optimised using 50 μm thick vibrating microtome sectioned mucosa and MLN (section 2.15.3). Due to the thickness of the sections and the cell density, the duration of time for permeabilisation and antibody penetration was increased to maximise the immunolabelling.

### *7.5.2 Visualisation of Salmonellas within the intestinal mucosa and MLN*

Two hours after infection of the intestinal mucosa similarly high numbers of *S. dublin* and *S. gallinarum* were observed primarily within epithelial cells and the lamina propria (figures 7.6 and 7.16c and d).

Twelve hours after infection, numerous *S. dublin*, both in clumps and singly, could be visualised predominantly within the lamina propria and submucosa. In contrast, only very low numbers of *S. gallinarum* could be seen within the same areas, usually as single bacteria, but occasionally as large clumps associated with a single cell (figures 7.7 and 7.17b, c and d).

Salmonellas could only be seen in the MLN of infected calves when the viable counts at *post mortem* were more than 5 $\log_{10}$ cfu g. Bacteria in the MLN of two *S. dublin*-infected calves and two nodes from one *S. gallinarum* infected animal were observed predominantly in

the sub-capsular sinuses and along the traberculae in the cortex, either singly or in clumps. Some of these salmonellas appeared to reside in an intracellular location, although the host cell type could not be determined (figures 7.8a, b and c and 7.15b, c and d). As observed in the mucosa, many host cells contained clumps of up to 10 bacteria and, particularly in the *S. gallinarum*-infected calves, some of these bacteria appeared to be degenerating within cells. Furthermore, particles of cell debris could occasionally be visualised on the eukaryotic cell surface or in close proximity to the infected host cell (figure 7.8c).

### *7.5.3 Bacterial recovery from systemic and enteric sites twelve hours after infection of bovine ileal mucosa*

In general *S. dublin* and *S. gallinarum* were recovered in similar numbers from the homogenised mucosa and MLN (section 2.13.5.4). However the total numbers of *S. gallinarum*, compared with *S. dublin*, were significantly greater in the intestinal contents ($P < 0.05$) while *S. dublin* was more prevalent than *S. gallinarum* in both the liver ($P < 0.1$) and spleen (figures 7.9a and b).

Gentamicin treatment of the ileal mucosa resulted in a reduction in bacterial recoveries, compared to untreated mucosa, of approximately 90% in *S. dublin*-infected calves ($P < 0.06$) and approximately 97% in *S. gallinarum*-infected calves ($P < 0.03$). As such, within the mucosa, the numbers of gentamicin resistant *S. dublin* were greater than the numbers of gentamicin resistant *S. gallinarum* (although the differences were not significant). Similar treatment of the MLN resulted in a reduction in bacterial recoveries by approximately 50% in *S. dublin* infected calves ($P > 0.1$) and approximately 75% in *S. gallinarum* infected animals ($P < 0.1$). As such, within the MLN, the numbers of gentamicin resistant *S. dublin* were greater than the numbers of gentamicin resistant *S. gallinarum* ($P = 0.1$).

Twelve hours after infection of calves with either *S. dublin* or *S. gallinarum*, the intestinal mucosa was reddened and the lumen contained a purulent secretion. The MLN from these animals were also enlarged and reddened. In contrast, tissues taken from control calves, or from control samples derived from the mid-ileum or MLN draining a non-infected area of the intestine within infected animals, appeared normal. Furthermore, no salmonellas were present in any tissue derived from enrichment cultures from negative control calves or control samples.

In summary, the differential ability of *S. dublin* or *S. gallinarum* to interact with host tissues appears pivotal in subsequent bacterial dissemination to systemic sites and therefore in potentially determining the outcome of infection.

| | Calf number | Inocula per loop ($log_{10}$ cfu) | Loop volume (ml)/ loop length (cm) | Time to death post loop inoculation |
|---|---|---|---|---|
| ***S. dublin*** | 7.1 | 10.47 | 50ml/65cm | 10 hours |
| **(SD3246-GFP)** | 7.2 | 10.6 | 50ml/65cm | 12 hours |
| | 7.3 | 11 | 100ml/150cm | 12 hours |
| | 7.4 | 10.59 | 50ml/60cm | 12 hours |
| ***S. gallinarum*** | 7.5 | 10.6 | 50ml/65cm | 12 hours |
| **(SG9-GFP)** | 7.6 | 10.64 | 50ml/80cm | 12 hours |
| | 7.7 | 10.74 | 50ml/85cm | 10 hours |
| | 7.8 | 10.6 | 50ml/75cm | 12 hours |
| **LB (negative** | 7.9 | Not applicable | 50ml/80cm | 8 hours |
| **control)** | 7.10 | Not applicable | 50ml/90cm | 8 hours |

**Table 7.1 Inocula and experimental details following infection of bovine ligated ileal loops subsequent to cannulation of both venous blood supply draining intestinal mucosa and efferent lymphatics draining regional MLN.**

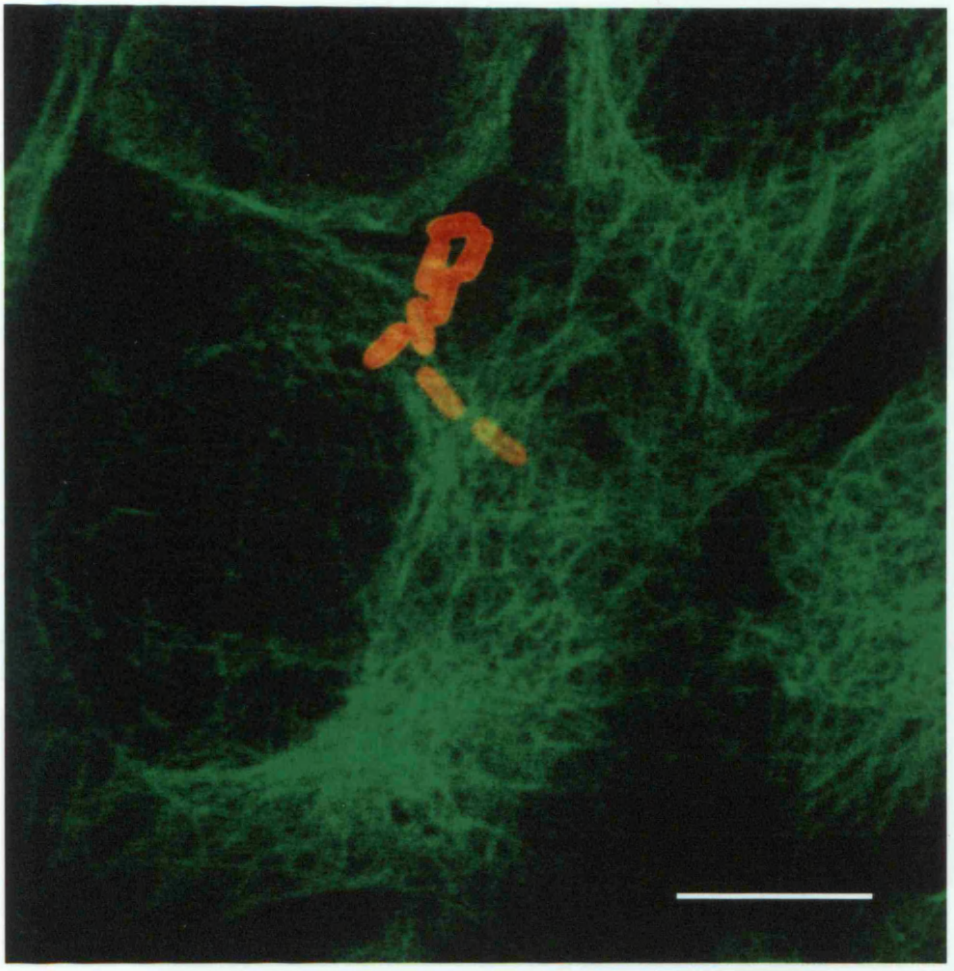


**Figure 7.5 Visualisation of intracellular and cell associated *S. dublin* following *in vitro* infection of HeLa cells.** Bacteria were immunolabelled with rabbit anti-*Salmonella* LPS and anti-rabbit IgG conjugated to Alexa Fluor 568 (red). Host cells were immunolabelled with mouse anti-α-tubulin and anti-mouse IgG conjugated to Alexa Fluor 488 (green). Sections were examined by CLSM. Scale bar 10 μm.

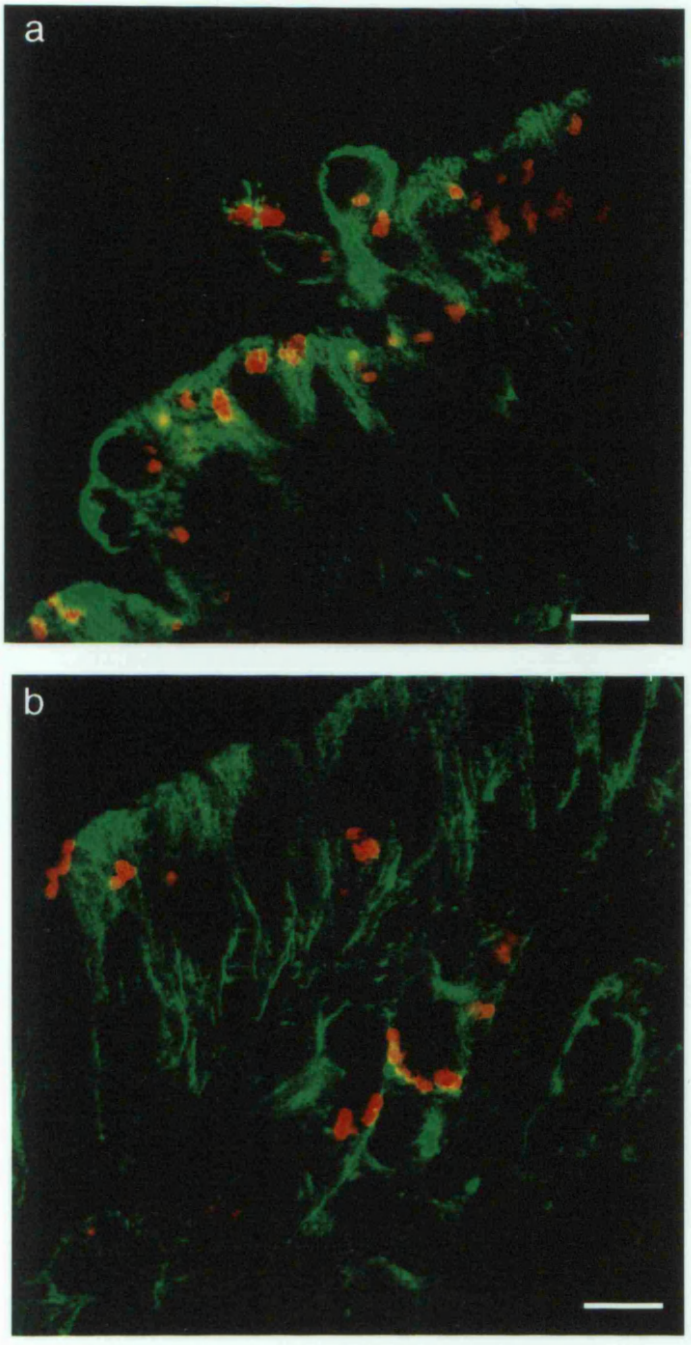



**Figure 7.6 Visualisation of cell-associated *S. dublin* (a) and (b) from intestinal mucosa two hours after infection of bovine ligated ileal loops.** Bacteria were immunolabelled with rabbit anti-*Salmonella* LPS and anti-rabbit IgG conjugated to Alexa Fluor 568 (red). Host cells were immunolabelled with mouse anti-α-tubulin and anti-mouse IgG conjugated to Alexa Fluor 488 (green). Sections were examined by CLSM. Scale bar 10 μm.

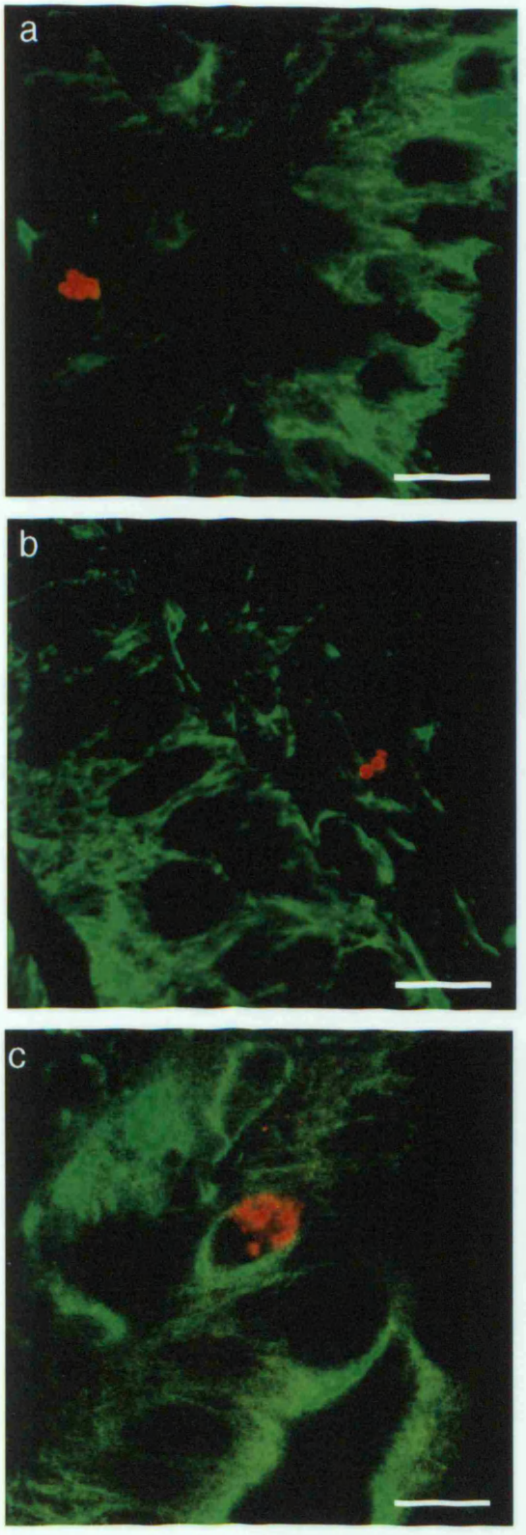



**Figure 7.7 Visualisation of cell-associated *S. dublin* (a) or *S. gallinarum* (b) and (c) from intestinal mucosa twelve hours after infection of bovine ligated ileal loops.** Bacteria were immunolabelled with rabbit anti-*Salmonella* LPS and anti-rabbit IgG conjugated to Alexa Fluor 568 (red). Host cells were immunolabelled with mouse anti-α-tubulin and anti-mouse IgG conjugated to Alexa Fluor 488 (green). Sections were examined by CLSM. Scale bar 10 μm.

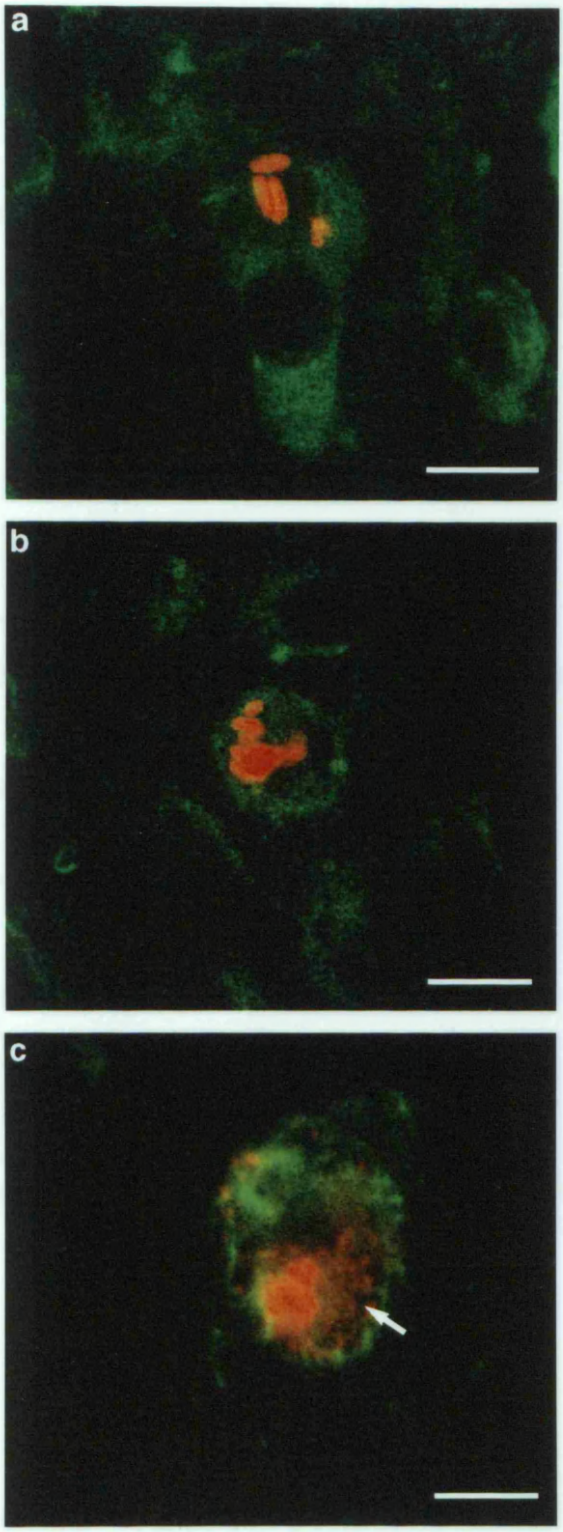



**Figure 7.8 Visualisation of cell-associated *S. dublin* (a) or *S. gallinarum* (b and c) from mesenteric lymph nodes twelve hours after infection of bovine ligated ileal loops.** Note the large clump of cell-associated *S. gallinarum* in (c) (arrow) Bacteria were immunolabelled with rabbit anti-*Salmonella* LPS and anti-rabbit IgG conjugated to Alexa Fluor 568 (red). Host cells were immunolabelled with mouse anti-α-tubulin and anti-mouse IgG conjugated to Alexa Fluor 488 (green). Sections were examined by CLSM. Scale bar 10 μm.

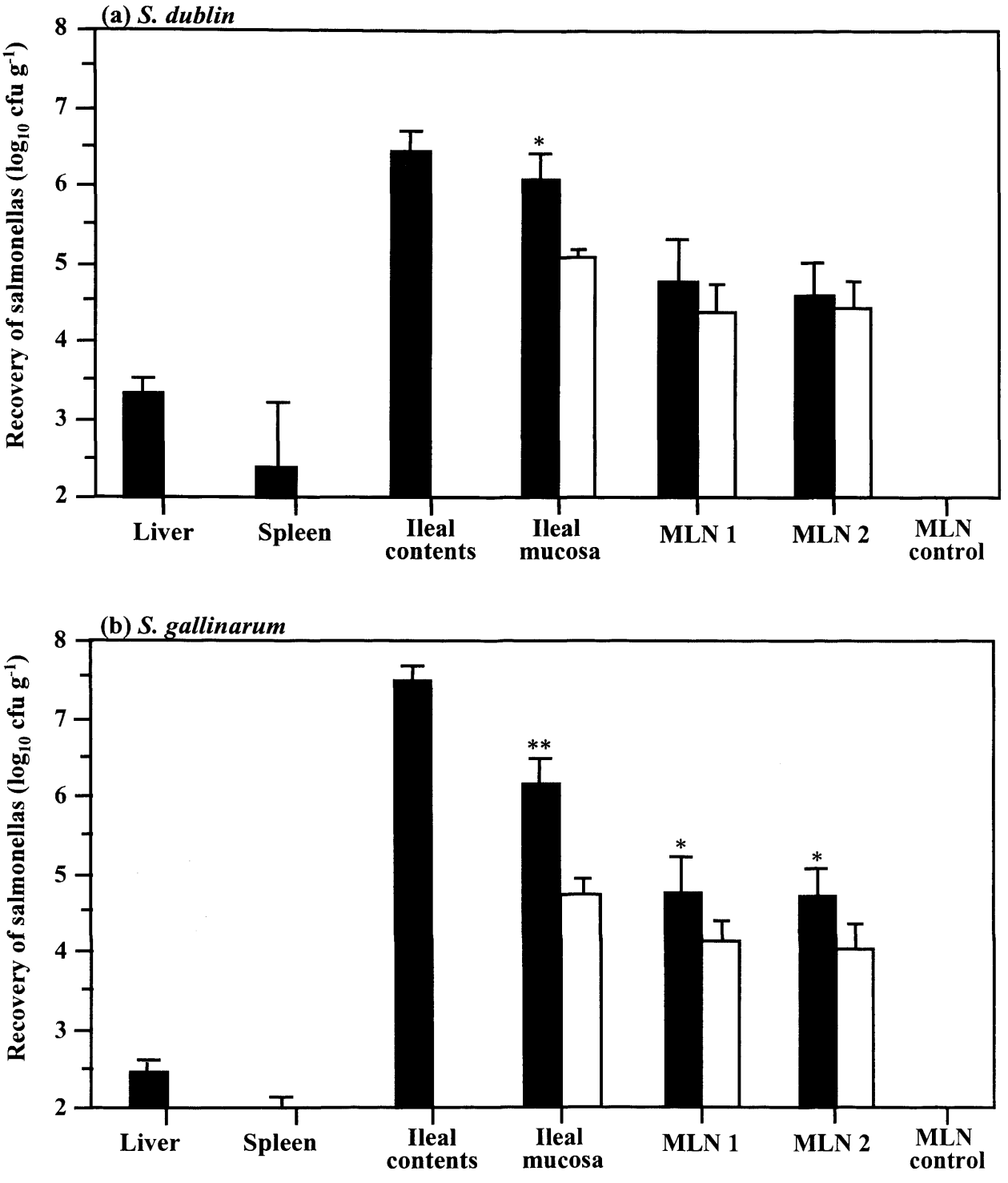



**Figure 7.9 Recovery of *S. dublin* (a) or *S. gallinarum* (b) from systemic and enteric tissues, with (□ ) or without (■ ) gentamicin treatment, 12 h after infection of ileal mucosa and cannulation of efferent lymphatics.** Each bar represents the mean of single tissue samples, taken from 4 calves *per* serotype, and is presented with the SEM. Statistical differences between treatments are represented by * ($P < 0.1$); ** ($P < 0.05$).

## 7.6 Morphological changes to infected or control intestinal mucosa and MLN assessed by haematoxylin and eosin staining

A semi-quantitative scoring system (section 2.15.1.1) was used to assess tissue damage and/or infiltration of inflammatory cells in the intestinal mucosa and MLN, of control calves or those infected with either *S. dublin* or *S. gallinarum*, twelve hours after loop inoculation. These results represent an extension of the studies from Chapter 5 when mucosal damage was determined in only one loop *per* serotype.

In the intestinal mucosa, *S. dublin* induced severe damage, *S. gallinarum* induced moderate to severe damage while the LB control mucosa appeared relatively undamaged. Additionally the amount of *Salmonella*-induced damage to absorptive epithelia and Peyer's patch mucosa was similar (table 7.2a and figures 7.10a-f) and mean scores were similar to those described in Chapter 5 thereby confirming previous observations.

Both *S. dublin* and *S. gallinarum* infected MLN contained high numbers of PMNs, particularly in the sub-capsular and medullary sinuses and along the cortical traberculae. In contrast control nodes, taken from the mid-ileum of infected calves and those taken from control animals, contained only low numbers of PMNs (table 7.2b and figures 7.11a-c).

| (a) | Mean score | | | | | |
|---|---|---|---|---|---|---|
| **Serotype** | **Disruption to enterocyte monolayer** | **Extrusion of enterocytes** | **Stunting of villi** | **Influx of PMNs** | **Number of calves** | **Mean total score** |
| ***S. dublin*** | 2.5 (2) | 3 (3) | 2 (2) | 2.5 (3) | 3 (2) | **2.5 (2.5)** |
| ***S. gallinarum*** | 1.6 (1) | 2 (2) | 1.6 (2) | 2.3 (2) | 3 (2) | **2 (2)** |
| **Negative control** | 0.5 (0.5) | 0 (0.5) | 0.5 (0.5) | 1 (1) | 2 (2) | **0.5 (0.6)** |

| (b) | Mean PMN influx into MLN | | | | | | |
|---|---|---|---|---|---|---|---|
| **Serotype** | **PMNs diffuse or discrete** | **PMNs in subcapsu -lar sinus** | **PMNs in cortex/pa-racortex** | **PMNs in cortical traberculae** | **PMNs in medull-ary sinus** | **Number of nodes** | **Mean total score** |
| ***S. dublin*** | Diffuse | 1.6 | 0.4 | 1.6 | 2 | 5 | **1.4** |
| ***S. dublin*** **(control)** | Discrete | 0 | 0.1 | 0 | 0.3 | 3 | **0.1** |
| ***S. gallinarum*** | Diffuse | 1.5 | 0.1 | 1.25 | 2.1 | 8 | **1.2** |
| ***S. gallinarum*** **(control)** | Discrete | 0.25 | 0 | 0.25 | 0.25 | 4 | **0.1** |
| **Negative control** | Discrete | 0.25 | 0 | 0.25 | 0.25 | 4 | **0.1** |

**Table 7.2 Magnitude of damage and/or influx of inflammatory cells into the intestinal mucosa (a) or MLN (b) 12 hours after inoculation of ligated ileal loops with *S. dublin*, *S. gallinarum* or LB broth.** Details of the scoring systems used are described in section 2.15.1.1. Figures without brackets represent scores from absorptive epithelium and figures within brackets represent scores from Peyer's patch mucosa. *S. dublin* or *S. gallinarum* (control) indicates MLN that were taken from the mid ileum of infected animals.

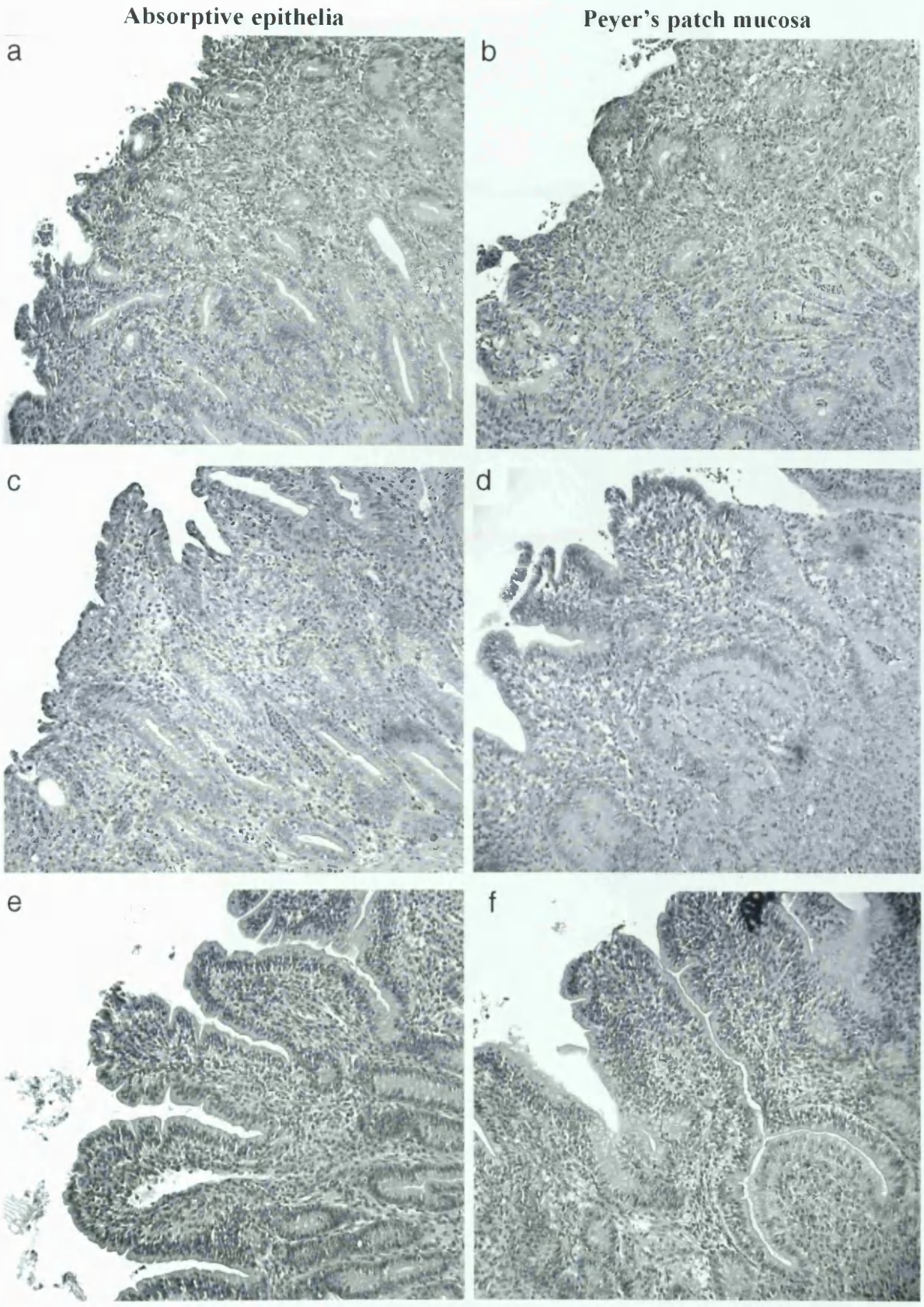



**Figure 7.10 Histological changes induced to intestinal absorptive epithelia (a, c and e) or Peyer's patch mucosa (b, d and f) 12 hours after injection of bovine ligated ileal loops with *S. dublin* (a and b), *S. gallinarum* (c and d) or LB control (e and f).** Details of the scoring system used are given in section 2.15.1.1. Magnification x94.

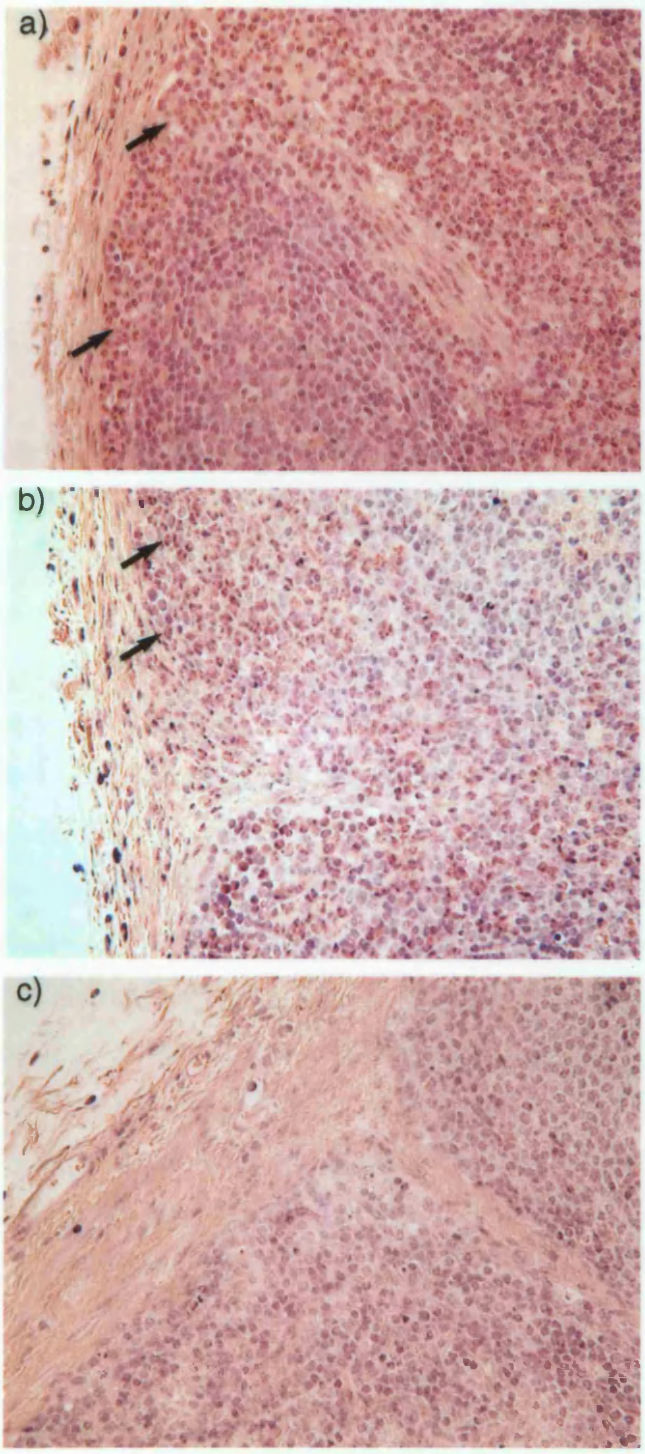



**Figure 7.11 Visualisation of the influx of inflammatory (salmon pink coloured) cells into mesenteric lymph nodes (arrows) 12 hours after injection of ligated ileal loops with *S. dublin* (a ), *S. gallinarum* (b) or LB control (c).** Details of the scoring system used are given in section 2.15.1.1. Magnification x 188

## 7.7 Quantification of bacteria associated with leucocytes of the monocyte/macrophage lineage within the MLN

While the majority of both *S. dublin* and *S. gallinarum* in the mucosa and *S. gallinarum* in the MLN appeared to reside in a gentamicin susceptible location, twelve hours after ligated ileal loop infection, clearly a proportion of these bacteria were within a gentamicin-protected niche (section 7.5.3 and figure 7.9).

Previous studies in mice have demonstrated that macrophages represent an important host cell occupied by salmonellas during systemic pathogenesis (Richter-Dahlfors *et al.*, 1997; Matsui *et al.*, 2000a). In calves leucocytes of the monocyte/macrophage lineage were increased within the MLN subsequent to infection of ligated ileal loops with *S. gallinarum* (section 7.10.3 and figure 7.21). Therefore to quantify the number of bacteria associated with cells of this phenotype, $CD14^+$ cells were purified from infected MLN (section 2.14) and gentamicin protected bacteria enumerated.

After MACS Microbead separation, the proportion of $CD14^+$ cells typically increased from 12-15% to 47-81% (figures 7.12a and b). However, less than 1% of these cells contained bacteria and typically only 10 to 26% of the gentamicin-protected *S. gallinarum* and 5% of the gentamicin protected *S. dublin* were associated with $CD14^+$ cells (<1% to 5% of the total number of salmonellas) (table 7.3).

Samples of $CD14^+$ enriched cells were centrifuged onto glass slides (section 2.15.4) and immunolabelled (section 2.15.3.1) prior to microscopic examination. The numbers of bacteria visualised were calculated to be similar to the numbers obtained following viable counts, indicating that the majority of cell-associated bacteria were alive (data not shown).

While viable *S. dublin* and *S. gallinarum* could be found within $CD14^+$ cells in the MLN, it is likely that both of these serotypes are able to reside within another, yet to be determined, intracellular niche as only a small percentage of the total *Salmonella* were found within cells of this phenotype.

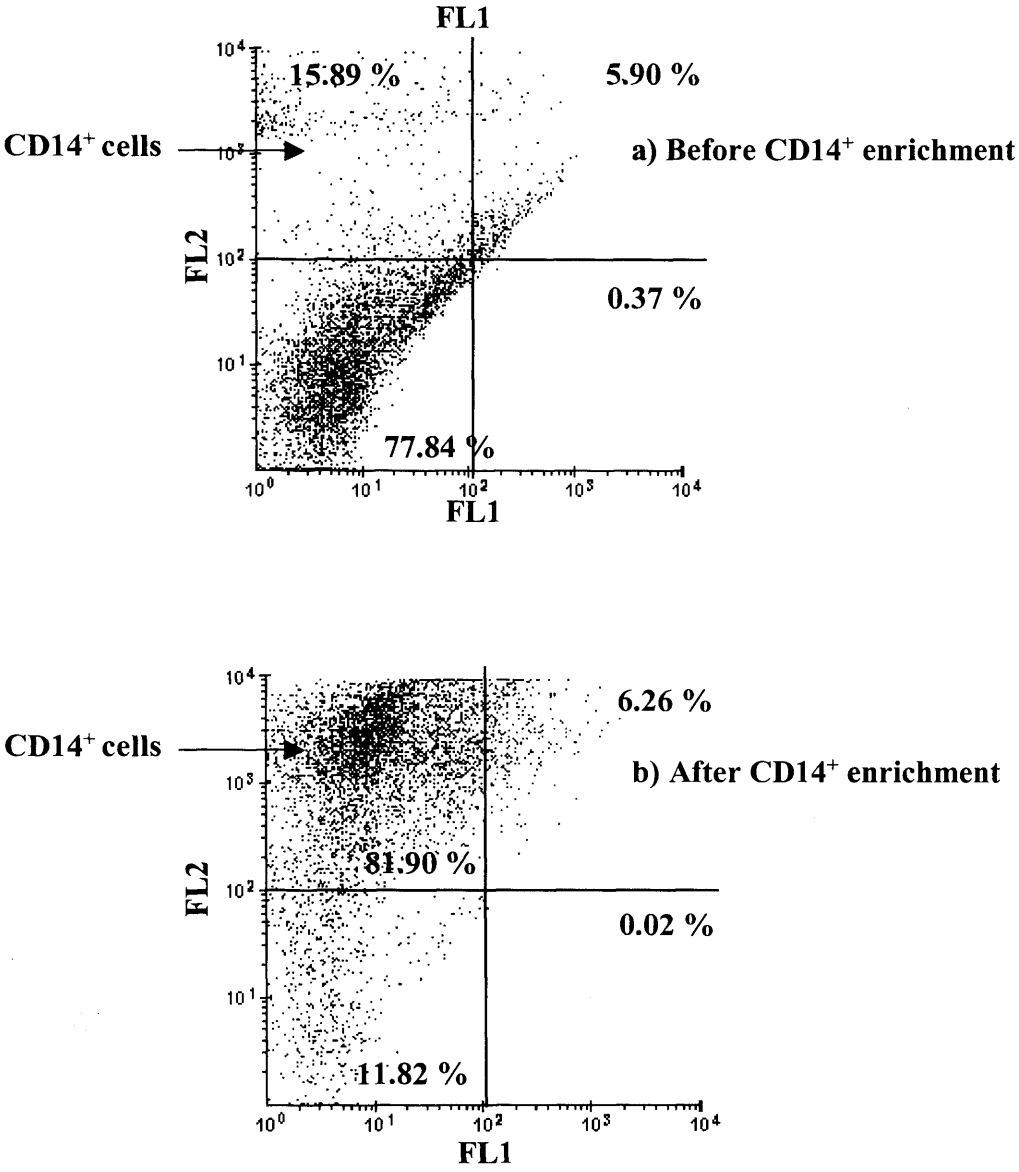



**Figure 7.12 Flow cytometric analysis of MLN from a representative *S. gallinarum* infected calf.** Before $CD14^+$ enrichment (a) or after $CD14^+$ enrichment (b).

| | | $Log_{10}$ cfu $g^{-1}$ | | | | Percentage | |
|---|---|---|---|---|---|---|---|
| **Strain expressing GFP** | **Calf number** | **Number of enriched $CD14^+$ cells** | ***Salmonella* in enriched $CD14^+$ cells** | **Gentamicin protected *Salmonella* in MLN** | **Total *Salmonella* in MLN** | **Enriched $CD14^+$ cells containing *Salmonella*** | **Gentamicin protected *Salmonella* in $CD14^+$ cells** |
| **SD3246** | 7.4 | 6.8 | 2.5 | 3.83 | 4.51 | <1 | 5 |
| **SG9** | 7.6 | 6.8 | 3.1 | 3.68 | 4.45 | <1 | 26 |
| **SG9** | 7.7 | 6.7 | 2.9 | 3.89 | 4.71 | <1 | 10 |
| **SG9** | 7.8 | 6.8 | 3.3 | 4.02 | 4.49 | <1 | 20 |

**Table 7.3 Association of salmonellas with $CD14^+$ cells in the MLN of 1 *S. dublin* or 3 *S. gallinarum* calves 12 hours after infection of intestinal mucosa.**

## 7.8 Evaluation of the route and kinetics of translocation of GFP-tagged *S. dublin* or *S. gallinarum* to systemic tissues

The route and kinetics of *Salmonella* dissemination to systemic tissues, following cannulation of venous blood supply and efferent lymphatics draining infected intestinal mucosa and MLN respectively, were determined. Furthermore, immunolabelling of agar-embedded lymph and examination of sections by CLSM was performed to confirm the specific niche occupied by *Salmonella* during the translocation process.

### *7.8.1 Route and magnitude of bacterial translocation*

By 2 hours *post* loop inoculation both *S. dublin* and *S. gallinarum* were recovered in comparable numbers from efferent lymph ($P > 0.1$) (figure 7.13a). The addition of gentamicin to lymph resulted in a 90 to 99% reduction in viable counts of both *S. dublin* and *S. gallinarum* ($P < 0.1$ to $P < 0.01$) (figure 7.13b) suggesting that these serotypes were present free in the lymph rather than in an intracellular niche. By 6 to 12 hours *post* loop inoculation, *S. dublin* was recovered from the lymph in higher numbers than *S. gallinarum* (figure 7.13a) suggesting that the greater ability of *S. dublin* to pass through the MLN potentially facilitates subsequent bacterial dissemination to systemic tissues.

While both serotypes translocated primarily via the efferent lymphatics, there remained the possibility that some bacteria could disseminate via the blood. However, recovery of salmonellas from cannulated venous drainage was typically lower than the accurate limit of detection (data not shown). As the blood was not the primary translocation route, only the efferent lymphatics were cannulated in the last four experiments. In these calves jugular blood was taken and enriched for salmonellas at each time point (section 2.13.5.3). Such samples from both *S. dublin* and *S. gallinarum*-infected calves were intermittently positive as infected lymph drains into the thoracic duct and subsequently into the left subclavian vein thereby providing access to the systemic circulation.

In summary, *S. dublin* and *S. gallinarum* translocate within the efferent lymph in a predominantly free, rather than gentamicin-protected, niche and the differential ability to pass through the MLN appears crucial in facilitating subsequent systemic dissemination of infection.

### *7.8.2 Immunolabelling of agar-embedded lymph*

The predominantly cell free location of *S. dublin* and *S. gallinarum* in efferent lymph was confirmed by immunolabelling agar embedded lymph and cytospins of infected lymph (figures 7.14a, b and c). No intracellular bacteria were observed, however cytocentrifugation had the effect of artificially increasing the contact between *Salmonella* and the host cells

(7.14c) thereby giving the appearance that the bacteria were possibly adhering to the leucocyte surface.

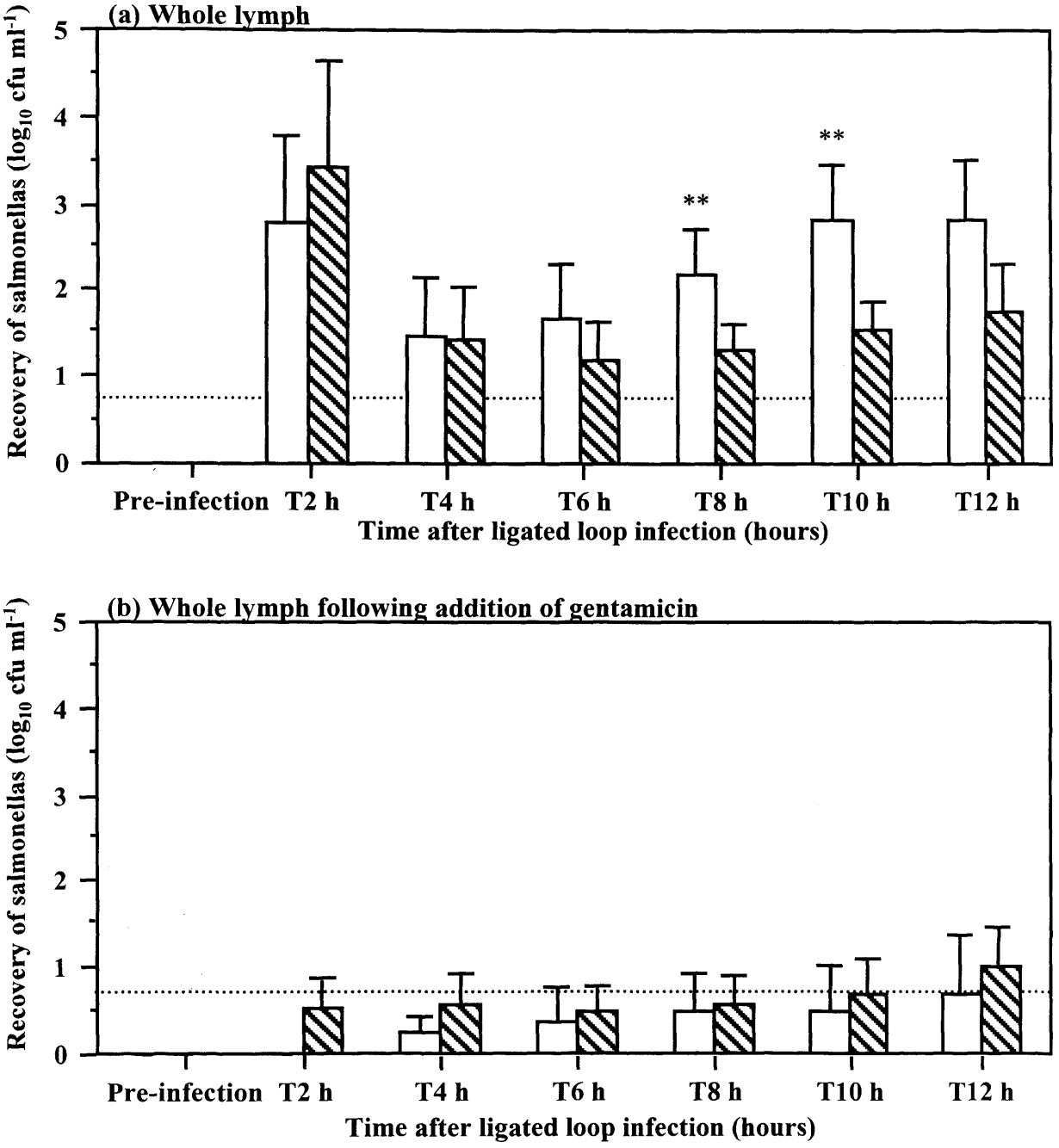



**Figure 7.13 Recovery of bacteria from efferent lymph, without (a) or with (b) the addition of gentamicin, up to 12 hours after infection of ileal mucosa.** Each bar represents the mean of individual lymph samples in 4 separate *S. dublin* (□ ) or *S. gallinarum* (▨ ) infected calves and is presented with the SEM. Statistically significant differences between serotypes are indicated by ** ($P < 0.05$). The dotted line represents the limit of accurate detection.

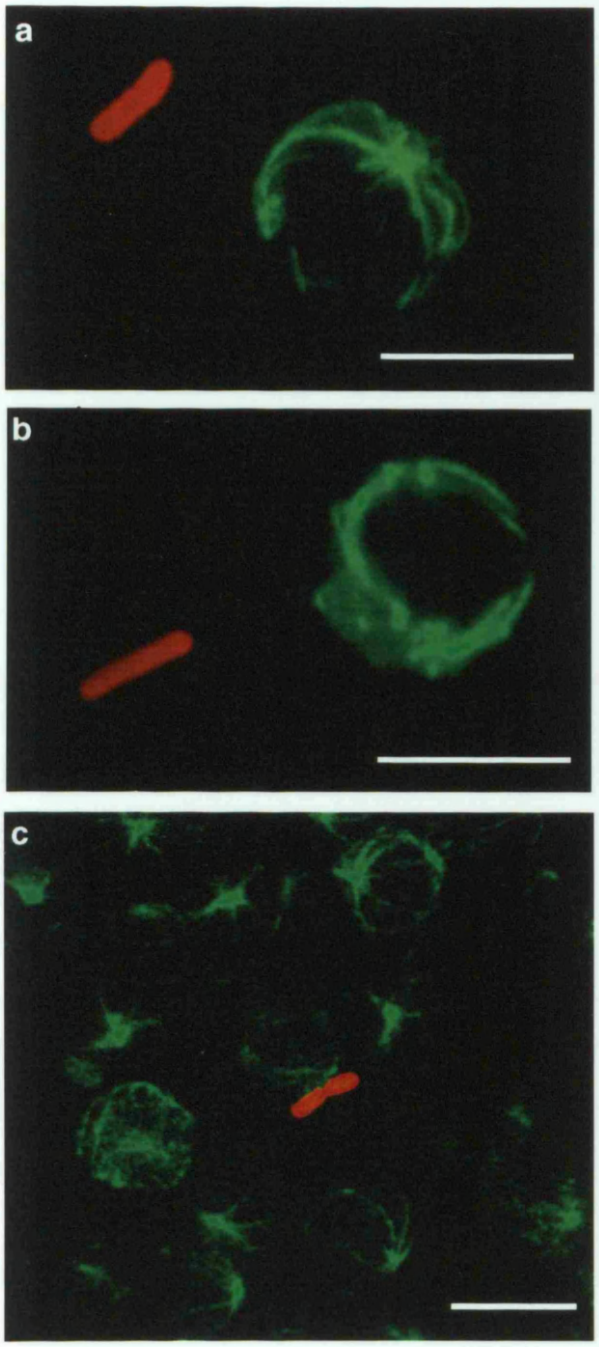



**Figure 7.14 Visualisation of cell-free *S. gallinarum* (a) or *S. dublin* (b and c) from efferent lymph embedded in agar (a and b) or following cytocentrifugation (c).** Bacteria were immunolabelled with rabbit anti-*Salmonella* LPS and anti-rabbit IgG conjugated to Alexa Fluor 568 (red). Host cells were immunolabelled with mouse anti-α-tubulin and anti-mouse IgG conjugated to Alexa Fluor 488 (green). Sections were examined by CLSM. Scale bar 10 μm.

## 7.9 Characterisation of host cell responses to infection with *Salmonella.*

The fate of host cells infected with *S. dublin* or *S. gallinarum* was assessed using the terminal deoxynucleotidyltransferase-mediated d UTP-biotin nick end labelling (TUNEL) assay (ApopTag in situ apoptosis kit [section 2.16.2 and figure 2.2]). TUNEL positive cells associated with these serotypes were visualised in the intestinal mucosa (2 and 12 h after loop inoculation) and MLN (12 h after loop inoculation). Use of the ApopTag kit does not permit absolute identification of apoptotic nuclei as such, fluorescein-labelled cells will be referred to simply as TUNEL positive. Typical examples of TUNEL positive cells showing marginalisation of condensed nuclear chromatin, a feature characteristic of apoptosis, are shown in figures 7.15a and 7.16a. In addition, quantitative assessment of apoptosis and necrosis was performed using flow cytometric analysis of Annexin V labelled eukaryotic lymph and MLN cells (section 2.16.1).

### *7.9.1 Bacterial association with TUNEL positive cells*

There was no association of bacteria with TUNEL positive cells in the MLN and no increase in TUNEL positive cells in the infected, compared with the control, nodes (figures 7.15b-d and table 7.4).

Two hours after infection of the intestinal mucosa there was little colocalisation of bacteria and TUNEL positive cells (figures 7.16c and d and table 7.4) and no apparent increase in these cells in response to infection. By twelve hours after infection there was a slight increase in the number of TUNEL positive cells associated with the intestinal mucosa from control and infected animals and as such there was a minor association of *S. dublin* with these labelled host cells (figures 7.17b, c and d and table 7.4). Positive and negative control samples were included for each experiment as described in section 2.16.2.

### *7.9.2 Detection of apoptotic or necrotic cells in efferent lymph and MLN by Annexin V labelling*

The majority of leucocytes isolated from efferent lymph were viable (negative for Annexin V and PI) at all time points tested. The numbers of apoptotic or necrotic cells in the lymph of control calves were slightly increased compared with numbers in infected animals, although there were no serotype-specific differences (figure 7.18a and table 7.5a). Within the MLN, there were slightly more apoptotic and necrotic cells, compared to those detected in lymph, but no major differences between infected and corresponding control nodes within the same calf (figure 7.18b and table 7.5b).

To summarise, there does not appear to be an increase in host cell death within efferent lymph or MLN of calves associated with infection of either *S. dublin* or *S. gallinarum*.

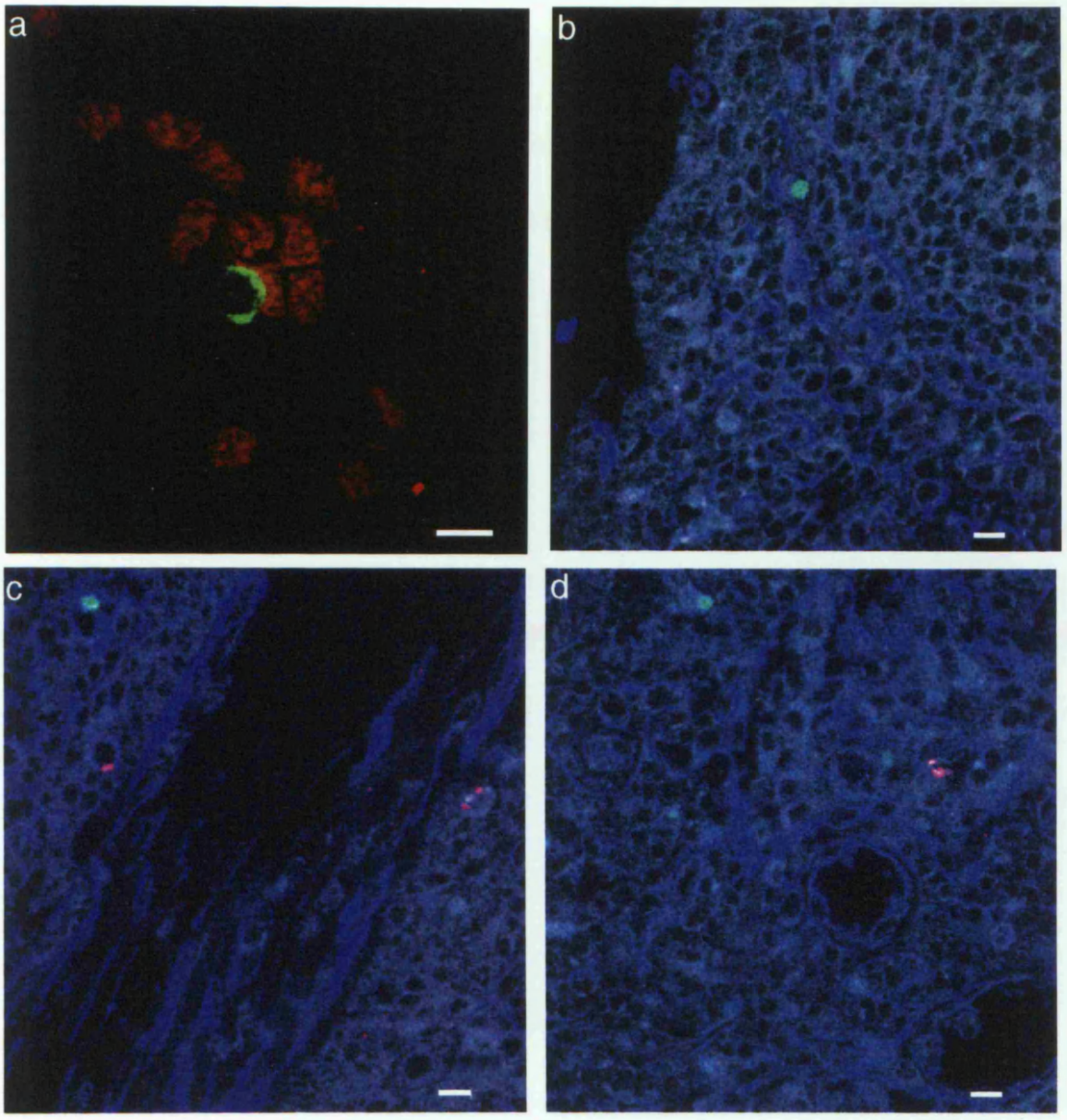



**Figure 7.15 Visualisation of the association of bacteria with TUNEL positive cells within the MLN. TUNEL positive control (a), section from control calf (b), *S. dublin* (c) or *S. gallinarum* (d) infected calves.** TUNEL positive cells were labelled with digoxigenin nucleotide conjugated to anti-digoxigenin fluorescein (green). Bacteria were immunolabelled with rabbit anti-*Salmonella* LPS and anti-rabbit IgG conjugated to Alexa Fluor 568 (red) (c and d only). Host cells were immunolabelled with mouse anti-α-tubulin and anti-mouse IgG conjugated to Alexa Fluor 568 (red) (a) or Cy5 (blue) (b,c and d). Sections were examined by CLSM. Scale bar 10 μm.

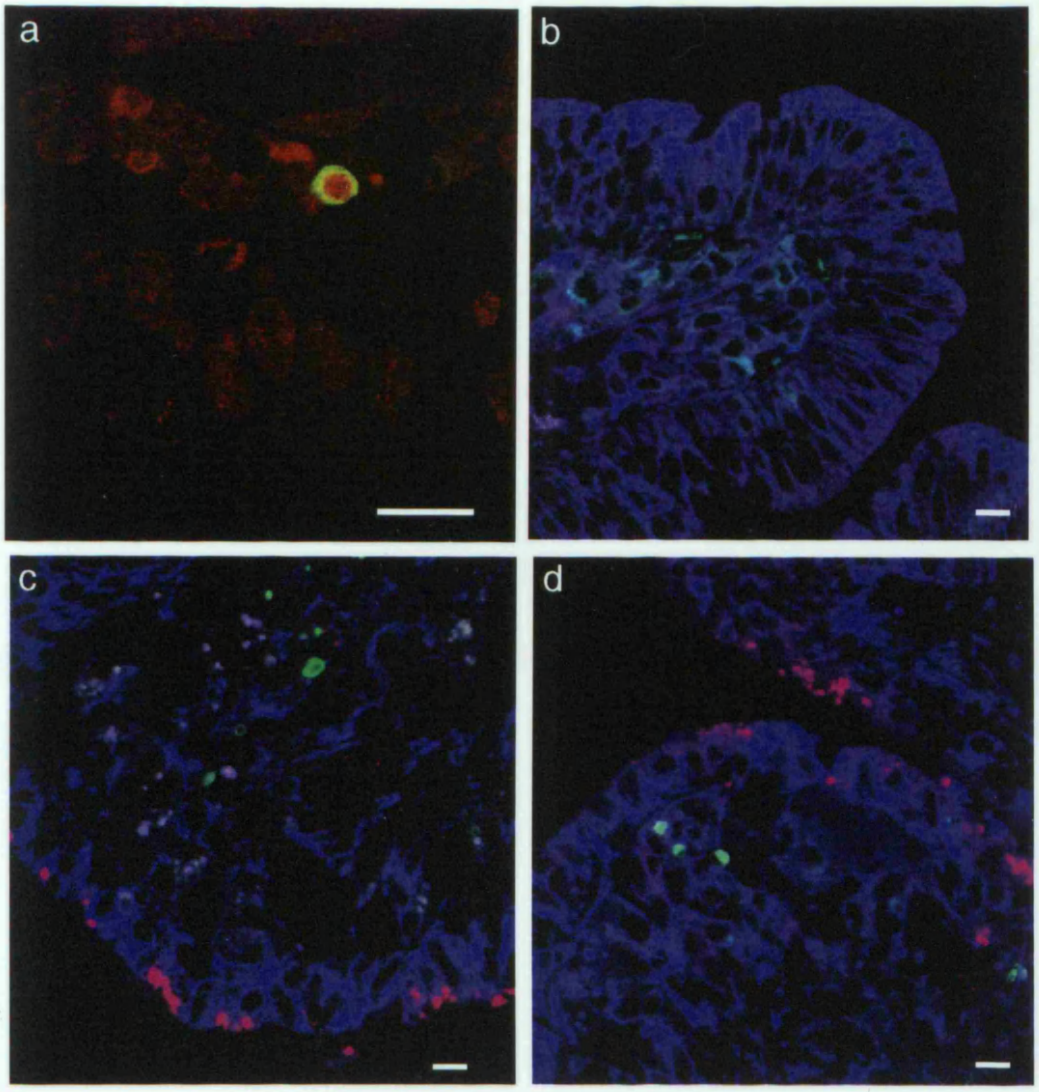



**Figure 7.16 Visualisation of the association of bacteria with TUNEL positive cells within the intestinal mucosa 2 h after loop inoculation. TUNEL positive control (a), section from control calf (b), *S. dublin* (c) or *S. gallinarum* (d) infected calves.** TUNEL positive cells were labelled with digoxigenin nucleotide conjugated to anti-digoxigenin fluorescein (green). Bacteria were immunolabelled with rabbit anti-*Salmonella* LPS and anti-rabbit IgG conjugated to Alexa Fluor 568 (red) (c and d). Host cells were immunolabelled with mouse anti-α-tubulin and anti-mouse IgG conjugated to to Alexa Fluor 568 (red) (a) or Cy5 (blue) (b, c and d). Sections were examined by CLSM. Scale bar 10 μm.

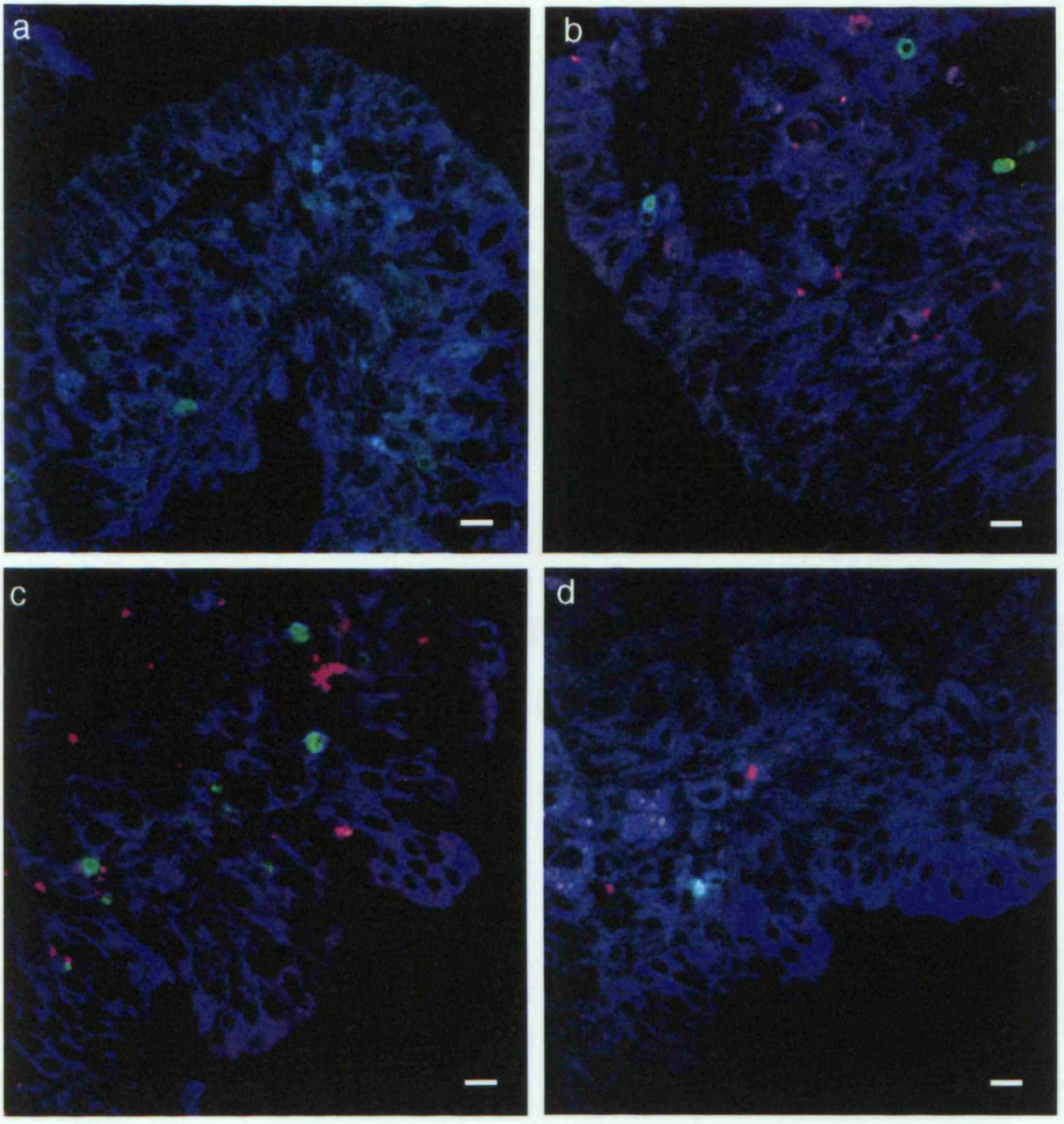



**Figure 7.17 Visualisation of the association of bacteria with TUNEL positive cells within the intestinal mucosa 12 h after loop inoculation of control calves (a) or those infected with *S. dublin* (b and c) or *S. gallinarum* (d).** TUNEL positive cells were labelled with digoxigenin nucleotide conjugated to anti-digoxigenin fluorescein (green). Bacteria were immunolabelled with rabbit anti-*Salmonella* LPS and anti-rabbit IgG conjugated to Alexa Fluor 568 (red). Host cells were immunolabelled with mouse anti-α-tubulin and anti-mouse IgG conjugated to Cy5 (blue). Sections were examined by CLSM Scale bar 10 μm.

| | Mean number of salmonellas or TUNEL positive cells in 10 fields of view | | | |
|---|---|---|---|---|
| **Strain** | **Number of bacteria** | **Position** | **Number of TUNEL +ve cells** | **Number of TUNEL +ve cells associated with number of bacteria (brackets)** |
| **2 hour mucosa** | | | | |
| **SD3246** | 51 | | 1 | <1 (<1) |
| **SD2229** | 30 | | 2 | <1 (<1) |
| **SG9** | 26 | Epithelium and | 1 | <1 (<1) |
| **SGJ91** | 19 | lamina propria | 1 | 0 (0) |
| **Control** | 0 | | 2 | 0 |
| **12 hour mucosa** | | | | |
| **SD3246-GFP (calf 7.3)** | 24 | | 5 | 2 (3) |
| **SD3246-GFP (calf 7.4)** | 15 | | 4 | <1 (<1) |
| **SG9-GFP (calf 7.5)** | 3 | Epithelium, lamina propria | 2 | <1 (<1) |
| **SG9-GFP (calf 7.8)** | 3 | and sub-mucosa | 2 | 0 (0) |
| **Control (calf 7.10)** | 0 | | 4 | 0 |
| **MLN** | | | | |
| **SD3246-GFP (calf 7.3-node 1)** | 5 | | 5 | <1 (<1) |
| **SD3246-GFP (calf 7.3-node 2)** | 4 | | 2 | <1 (<1) |
| **SG9-GFP (calf 7.5-node 1)** | 3 | Mainly sub-capsular sinus | 1 | 0 (0) |
| **SG9-GFP (calf 7.8-node 2)** | 4 | and traberculae | 2 | <1 (<1) |
| **Control (calf 7.10)** | 0 | | 7 | 0 |

**Table 7.4 Association of *Salmonella* with TUNEL positive cells in the intestinal mucosa, 2 or 12 hours after infection, and in the MLN 12 hours after infection.** Each result represents the mean number of bacteria and TUNEL positive cells from 10 independent fields of view.

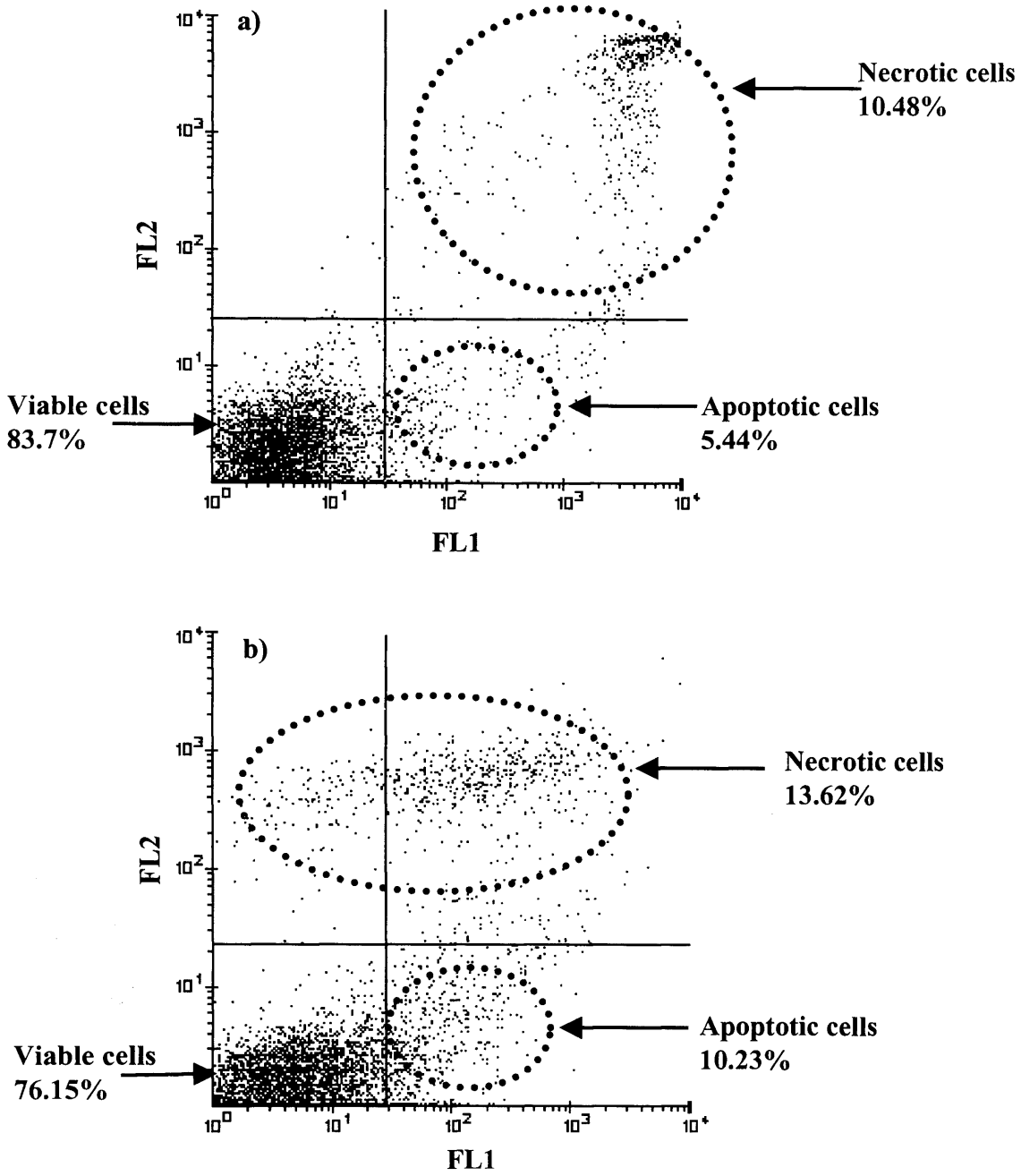



**Figure 7.18 Flow cytometric analysis of viable (Annexin V/PI negative), apoptotic (Annexin V positive) or necrotic (Annexin V positive/PI positive) cells in efferent lymph (a) or MLN (b) from a representative *S. dublin*-infected calf.**

| (a) | Efferent lymph | |
|---|---|---|
| | **Mean % necrotic cells ± SEM** | **Mean % apoptotic cells ± SEM** |
| ***S. dublin* (SD3246 GFP)** | 10.43 ± 2.55 | 8.57 ± 2.64 |
| ***S. gallinarum* (SG9 GFP)** | 10.63 ± 1.37 | 8.29 ± 1.56 |
| **LB (negative control)** | 14.3 ± 1.37 | 14.5 ± 2.27 |

| (b) | MLN | |
|---|---|---|
| | **Mean % necrotic cells ± SEM** | **Mean % apoptotic cells ± SEM** |
| ***S. dublin*** | 18.6 ± 4.24 | 10.1 ± 2.83 |
| ***S. dublin* (control MLN)** | 13.5 ± 3.78 | 15.2 ± 5.66 |
| ***S. gallinarum*** | 21 ± 1.24 | 16.5 ± 0.56 |
| ***S. gallinarum* (control MLN)** | 24 ± 0.71 | 11.7 ± 0.72 |

**Table 7.5 Percentage apoptotic and necrotic cells in efferent lymph (a) or MLN (b) as determined by annexin V immunolabelling and flow cytometric analysis.** Results are expressed as the mean ± SEM derived from two *S. dublin*-infected, four *S. gallinarum*-infected or 2 control calves. MLN control samples were derived from nodes draining a non-infected area of the intestine within infected calves.

## 7.10 Host innate immune responses to *Salmonella* infection

Host innate immune responses to infection were measured by determining eukaryotic cell phenotypic changes, in control calves or those infected with either *S. dublin* or *S. gallinarum*, up to 12 hours after infection of intestinal mucosa.

### *7.10.1 Influx of polymorphonuclear leucocytes (PMNs) into efferent lymph and MLN.*

The influx of PMNs into efferent lymph (both serotypes) and MLNs (*S. gallinarum* and control nodes draining a non-infected area of mid ileum within these animals) was determined by FACS analysis (section 2.17.2.1). There was an increase over time in the number of PMNs present in efferent lymph from both control and infected calves (table 7.6a), which suggests that surgery-induced trauma and/or anaesthesia influence this inflammatory influx and as such make changes due to *Salmonella* more difficult to interpret. However, there appeared to be a *Salmonella*-specific increase in the number of PMNs associated with infected, but not control nodes (table 7.6b) which correlates with the influx of inflammatory cells observed in the H and E stained MLN sections (section 7.6, table 7.2b and figures 7.11a-c).

### *7.10.2 Phenotypic changes associated with host cells in efferent lymph or jugular blood*

There were no major changes in the mean fluorescent intensity (MFI) of leucocytes incubated with a range of mAbs against cell surface markers when comparing pre or post anaesthesia jugular blood or venous blood derived from cannulation of the draining ligated ileal loop (data not shown). Therefore for convenience, a jugular blood and efferent lymph sample were analysed immediately post anaesthesia to check that the cell profile was within normal limits (figure 7.19a and b). Additionally, cell viability, as assessed by propidium iodide staining of eukaryotic cells (section 2.17.2) was determined at each time point and was typically greater than 95% (range 86-99% data not shown).

The MFI of host cells in efferent lymph was compared prior to and 8 hours after ligated ileal loop injection with either LB broth or *Salmonella*. By 8 hours there appeared to be a down regulation of several cell surface markers including CD3, CD4, CD8, CD11a and CD11b (figure 7.20a, b, c). No clear differences in the cell marker expression could be detected in lymph derived from control or infected calves, suggesting that *Salmonella*-specific factors were not influencing this host response.

### *7.10.3 Phenotypic changes associated with host cells in the MLN*

Changes in host cell surface markers were determined in the MLN draining infected mucosa and in the MLN draining uninfected mucosa within the mid-ileum of infected calves.

Comparisons were made from *S. gallinarum*-infected calves only, the reasons for which are explained in section 7.3.

The notable changes in the infected, compared to the control, nodes were an increase in those markers present particularly on monocytes, macrophages and dendritic cells (MHCII, CD32, Myd-1 and CD14) and a decrease in markers present on T cells (CD3, CD4 and CD8) (figure 7.21). The phenotypic changes in *Salmonella*-infected calves are consistent with an increase in phagocytic cells in response to infection and the subsequent up regulation of MHCII expression as a result of increased antigen presentation. Although it could be argued that these inflammatory changes were due to surgery or anaesthesia, this is unlikely because *Salmonella*-induced gross necrotic changes were associated with the infected, but not control, nodes and there was relatively little surgical trauma associated with the nodes during the cannulation procedure. Cell viability was typically greater than 85% in all nodes as determined by PI staining (figure 7.21).

| (a) | Mean PMN influx into efferent lymph (% ± SEM) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Serotype** | **Pre-infection** | **T2 h** | **T4 h** | **T6 h** | **T8 h** | **T10 h** | **T12 h** |
| ***S. dublin*** | 1 ± 0.3 | 1.6 ± 0.6 | 4.1 ± 1.4 | 8.5 ± 3.1 | 7.6 ± 3.3 | 7.3 ± 3.7 | 12.4 ± 6.7 |
| ***S. gallinarum*** | 0.1 ± 0 | 0.6 ± 0.5 | 2.4 ± 1.6 | 2.8 ± 1.5 | 3.9 ± 0.8 | 6.8 ± 1.7 | 9.1 ± 0.8 |
| **LB control** | 0.1 ± 0.1 | 0.4 ± 0.5 | 1.8 ± 1.2 | 2.9 ± 2.1 | 11 ± 10.6 | Not tested | Not tested |

| (b) | Mean PMN influx into MLN 12 hours after loop inoculation (% ± SEM) | | |
|---|---|---|---|
| **Serotype** | **MLN 1** | **MLN 2** | **Control** |
| ***S. gallinarum*** | 14 ± 3 | 17 | 4 ± 1.5 |

**Table 7.6 Flow cytometric analysis of the PMN influx into (a) efferent lymph from 2 control or 4 infected calves of each serotype and (b) MLN from 4 *S. gallinarum* infected calves or uninfected nodes taken from the mid-ileum of these animals (control).**

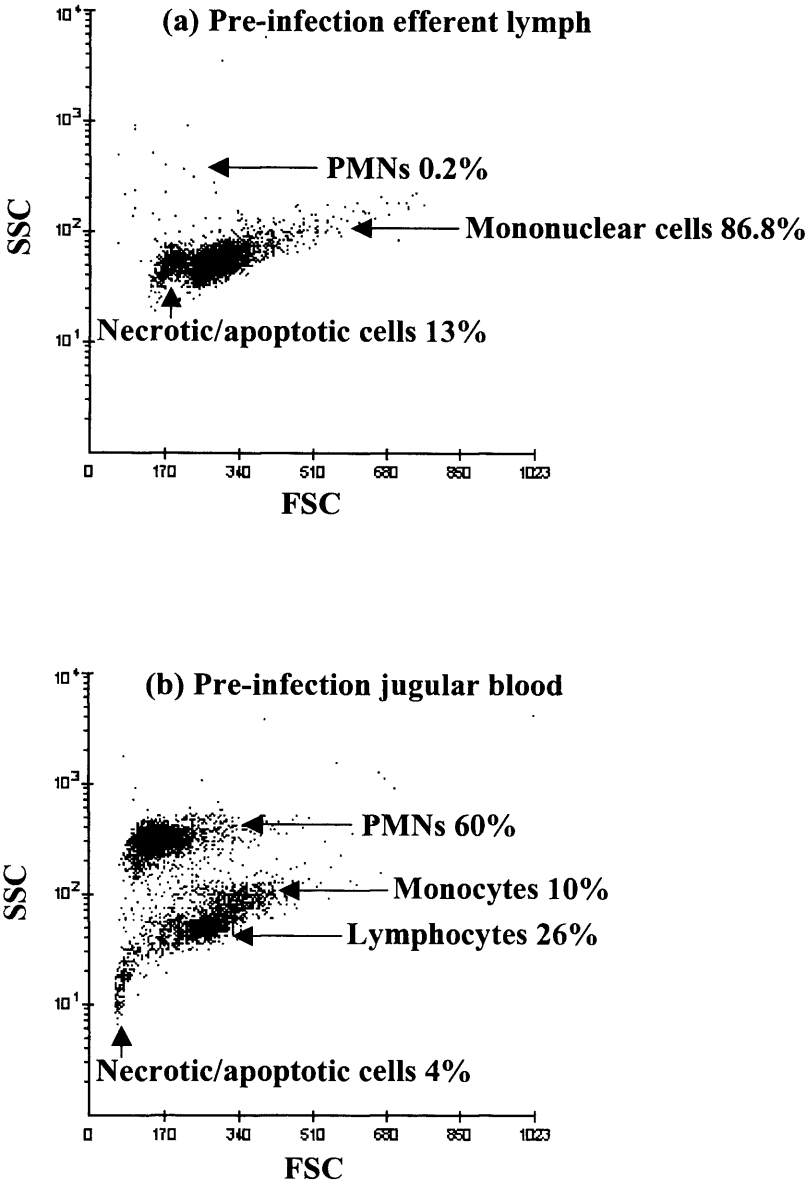



**Figure 7.19 Representative dot plots of pre-infection efferent lymph (a) or jugular blood (b).**

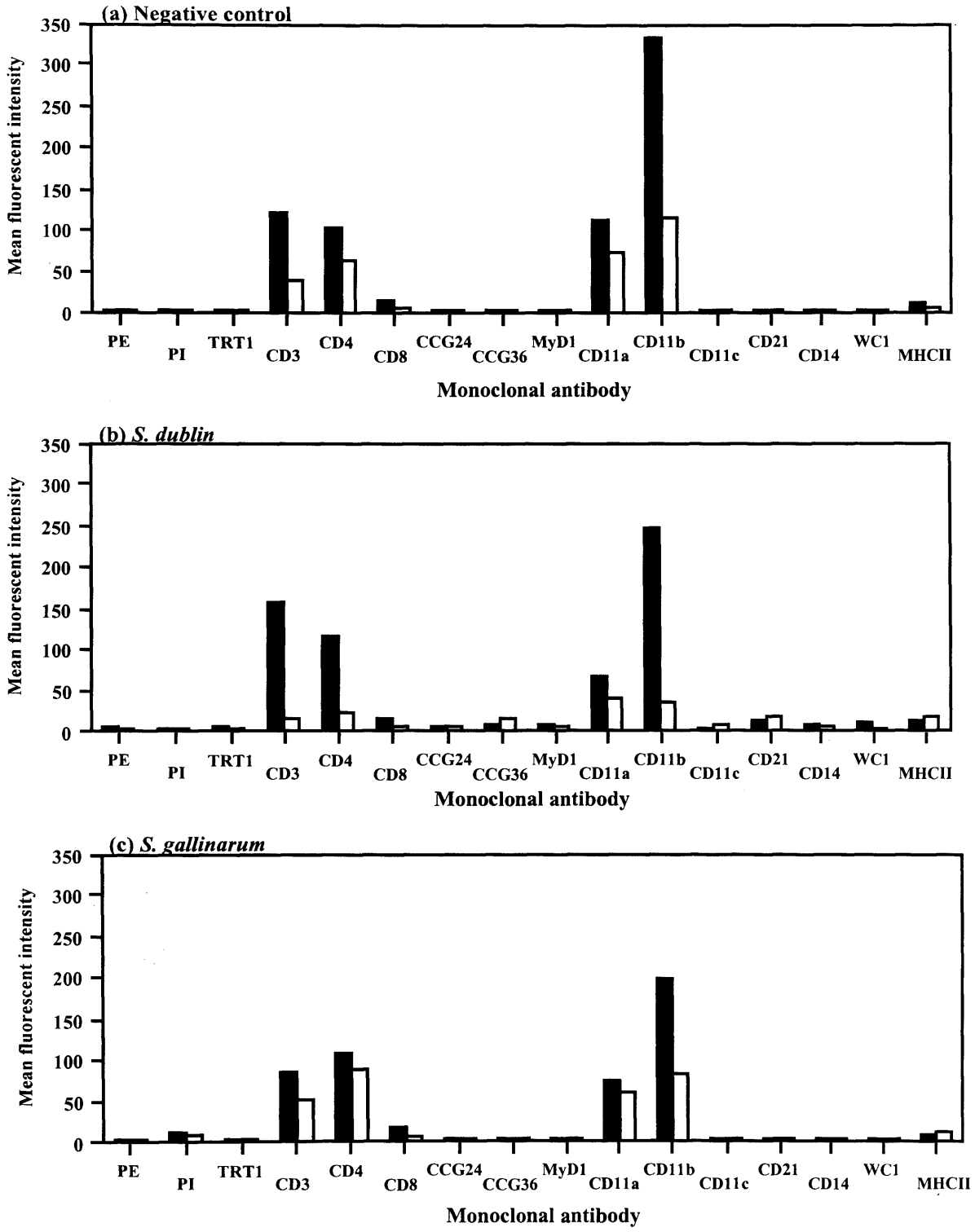



**Figure 7.20 Mean fluorescent intensity of efferent lymph cells incubated with a panel of mAbs to cell surface markers.** Pre (■) or 8 h *post* (□) loop inoculation of negative control (a), *S. dublin* infected (b) or *S. gallinarum* infected (c) calf. Data are presented from 1 representative calf *per* group. Details of mAbs used are given in table 2.5.

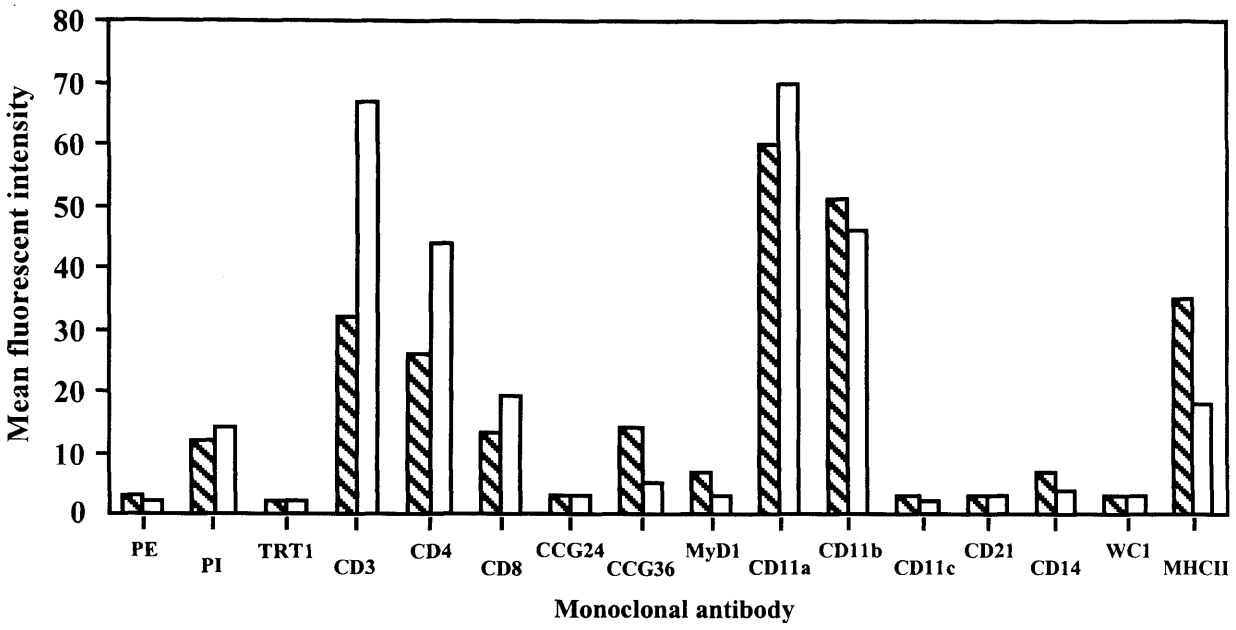



**Figure 7.21 Mean fluorescent intensity of MLN cells incubated with a panel of mAbs to cell surface markers.** MLN draining *S. gallinarum* infected mucosa (▨) or MLN draining uninfected mucosa (□). Data are presented from 1 representative calf *per* group. Details of mAbs used are given in table 2.5.

## 7.11 Summary of results

1. A novel model system has been developed for assessing *Salmonella* translocation kinetics and the subsequent acute host response to infection *in vivo*.
2. *S. dublin* and *S. gallinarum* were recovered in similar numbers from the intestinal mucosa and MLN twelve hours after infection of ligated ileal loops.
3. The majority of *S. dublin*, in the intestinal mucosa, and the majority of *S. gallinarum*, in both the mucosa and MLN, were within a gentamicin susceptible, rather than a gentamicin-protected niche. The proportion of bacteria residing in a gentamicin-protected, rather than a gentamicin-susceptible, environment appeared to be higher in the MLN compared with the ileal mucosa. Furthermore, the numbers of gentamicin-resistant *S. dublin* were greater than the numbers of gentamicin-resistant *S. gallinarum* from both tissue types.
4. There was an increase in cell surface expression of leucocytes of the monocyte/macrophage lineage in response to *Salmonella* infection, and some association of gentamicin protected *S. dublin* and *S. gallinarum* with $CD14^+$ cells in the MLN. These results indicate that, at least during certain stages of pathogenesis, a small proportion of bacteria is likely to reside within host cells of this phenotype
5. *S. dublin* and *S. gallinarum* translocate to systemic sites via the efferent lymphatics and are recovered from the lymph in a predominantly cell-free environment.
6. The ability to pass through the MLN is consistent with the host specific phenotype, as despite the similar recovery of both serotypes from this tissue, *S. gallinarum* translocated in the efferent lymph and was subsequently recovered from systemic sites in lower numbers than *S. dublin*.
7. There was little evidence of an increase in TUNEL or Annexin V positive cells in the efferent lymph, mucosa or MLN as a result of *Salmonella* infection or an association of either serotype with TUNEL positive cells within the mucosa and MLN.
8. There was an increase in the number of PMNs, associated with the MLN, in response to *Salmonella* infection.

## 7.12 Discussion

A novel venous blood and efferent lymphatic cannulation model was developed to assess the kinetics and route of bacterial translocation from bovine infected intestinal mucosa, through draining MLN to systemic sites. Immunolabelling of infected tissues and examination by CLSM aimed to determine specific serotype interactions with the intestinal mucosa and MLN and to evaluate the niche occupied by *Salmonella* during dissemination. In addition, acute host responses to infection were assessed including recruitment of inflammatory cells, phenotypic host cell changes, bacterial-induced tissue damage and the contribution of cell death to pathogenesis.

This is the first time that such an *in vivo* model has been used in the context of *Salmonella* host specificity and as such provides a unique 'real time' insight into interactions with the host subsequent to initial intestinal invasion. Furthermore, as *Salmonella* is an enteropathogen, the importance of studying both host and bacterial responses to infection within the gut are fundamental to increasing our understanding of the disease process. There are however obvious limitations to this approach, which need to be considered when interpreting results, for example the effect of surgical trauma on the innate immune system and the relatively short period of time with which responses to infection can be studied. In addition, it has been reported that, following cannulation of ovine lymph nodes, the use of barbiturate anaesthetics (such as Euthatal) can result in a transient period of decreased cell output in efferent lymph (termed 'shutdown') (Hall and Morris, 1965). This fall in cell output has also been correlated with a sustained increase in prostaglandin $E_2$ levels in efferent lymph (Hopkins *et al.*, 1981).

*S. dublin* and *S. gallinarum* were used for these studies because of their contrasting pathogenesis in calves. *S. dublin* (SD3246), a virulent serotype able to invade and induce enteropathogenic responses, is typically associated with severe disease in this host while *S. gallinarum* (SG9), is avirulent following oral challenge, but is as invasive as SD3246 *in vivo* and surprisingly more enteropathogenic in ligated ileal loops. Furthermore, *S. gallinarum* is able to reach the ileal and caecal lymph nodes in similar numbers to *S. dublin* yet in contrast, is only intermittently detected within systemic tissues 24 h after oral infection. These results suggest that while *S. gallinarum* has the potential to cause disease in mammalian species, the relative inability to reach systemic tissues may be a crucial factor influencing pathogenesis in calves.

Twelve hours after infection of bovine ligated ileal loops, *S. gallinarum* was recovered in higher numbers than *S. dublin* from the lumen contents despite similar recoveries of both

serotypes from the intestinal mucosa. While it is not possible to state specific reasons for this observation, it could be speculated that a high percentage of *S. gallinarum* were unable to interact with, or invade, the bovine intestine. Following oral infection, these bacteria would potentially be eliminated from the host. This observation correlates with a much-reduced recovery of *S. gallinarum*, compared with *S. dublin*, from the intestinal contents 24 hours after oral inoculation of calves (Chapter 3).

Despite the similar recoveries of *S. dublin* and *S. gallinarum* from the intestinal mucosa twelve hours after infection, the differential ability of these serotypes to survive subsequent to invasion might contribute to the outcome of disease. For example, immunolabelled *S. gallinarum* could be visualised within the epithelium and lamina propria in high numbers two hours *post* infection yet, in sharp contrast to *S. dublin*, were barely visible after twelve hours. These observations may be correlated to the presence of large numbers of apparently degenerating *S. gallinarum* that could occasionally be seen associated with cells in the intestinal mucosa at the later time point. Furthermore, by twelve hours after infection, while the majority of bacteria were in a cell-free environment, there were fewer gentamicin-protected *S. gallinarum*, compared with *S. dublin*, suggesting that the ability to survive within an intracellular niche may be crucial for the infection process.

It would appear unusual that despite high numbers of viable *S. gallinarum* in the mucosa 12 hours after infection, in contrast to *S. dublin*, these bacteria could not easily be visualised by CLSM. It is possible that the LPS of this serotype may have been down regulated thereby affecting the staining but not the viability of *S. gallinarum*. Alternatively, the fact that the tissue samples from the cannulation studies, but not the 2 hour invasion samples, were stored in PFA for several weeks prior to processing might have altered the tissue structure resulting in the reduced ability of *Salmonella* antibodies to bind to the *S. gallinarum* cell surface.

*S. dublin* and *S. gallinarum* were recovered from the MLN in similar numbers twelve hours after infection of bovine ligated ileal loops. As in the mucosa, the numbers of gentamicin resistant *S. dublin* were greater than the numbers of gentamicin resistant *S. gallinarum*. However given the smaller difference between gentamicin susceptible and gentamicin resistant bacteria of both serotypes within the MLN, it is possible that different niches are occupied depending on the stage of pathogenesis. Therefore assessment of the fate of both serotypes subsequent to leaving the mucosa might provide an insight as to whether the bacteria were cell-free or cell-associated when they arrived at the MLN. At present the possible scenarios concerning the mode of dissemination can only be speculated and clearly determination of the niche occupied by bacteria within afferent lymph, which most likely

represents the primary route of translocation, warrants further study. While the afferent lymphatics were not directly cannulated in these experiments, due to their minute size, this procedure is potentially possible in calves either by direct cannulation or by removal of the MLN and collection of pseudo-afferent lymph.

It is possible to make some conclusions from these results, regarding differences between *S. dublin* and *S. gallinarum* that potentially correlate with host specificity. For example, as in the intestinal mucosa, the presence of large clumps of apparently degenerating *S. gallinarum* in the MLN and the observation that *S. gallinarum* is recovered in lower numbers than *S. dublin* within a gentamicin-protected environment could suggest pathogen elimination by the host. Whether this is due to an inability of *S. gallinarum* to survive or replicate, possibly due to a lack of expression of virulence plasmid or SPI-2 encoded genes, or a greater susceptibility to killing than *S. dublin* is currently unknown.

While *Salmonella* Spp. are typically classified as facultative intracellular pathogens, their exact location during different stages of pathogenesis remains largely unknown. Salmonellas have been observed to interact with PMNs, macrophages and dendritic cells (DC) within the lamina propria of many species including calves (Watson *et al.*, 1995; Frost *et al.*, 1997; Hopkins and Kraehenbuhl, 1997; Rescigno *et al.*, 2001). As such, these cells have been speculated to translocate *Salmonella* to the regional MLN and systemic sites. In addition, the ability to survive within murine macrophages is frequently cited as being important in *Salmonella* pathogenesis (Buchmeier and Heffron, 1989; Richter-Dahlfors *et al.*, 1997; Matsui *et al.*, 2000a) and bacterial survival within cells of the reticuloendothelial system is believed to be instrumental in determining the host specific phenotype (Barrow *et al.*, 1994). Clearly, despite the predominantly cell-free niche within the mucosa and MLN, there was some bacterial association with host cells. In addition, there was an increase in leucocytes of the monocyte/macrophage lineage, in response to *Salmonella* infection, giving good reason to speculate that bacteria may be associated with cells of this phenotype within the MLN. However, while it appeared that a small proportion of *S. dublin* and *S. gallinarum* were associated with $CD14^+$ cells, clearly other leucocytes were interacting with *Salmonella* during this stage of the infection process.

It is possible that DC, due to their location and physical properties (section 1.9.1), can be implicated as important candidates for interacting with salmonellas in the intestinal mucosa, translocating bacteria to the regional MLN and contributing to the immune response to infection. In addition, intestinal afferent lymph veiled cells, from *S. typhimurium*-infected rats, have been observed to contain intracellular *Salmonella* (Mayrhofer *et al.*, 1986) and DC of murine Peyer's patches have been shown to colocalise with avirulent *S. typhimurium* in the

subepithelial dome (Hopkins and Kraehenbuhl, 1997). Furthermore, it has recently been reported that DC are able to sample salmonellas within the intestinal lumen by the direct opening of tight junctions between epithelial cells (Rescigno *et al.*, 2001). It is clear that further studies are necessary to define the precise role of DC during *Salmonella* pathogenesis both in respect to specific interactions with different serotypes in the mucosa and of their importance during the dissemination process.

After passing through the MLN, translocation to systemic tissues predominantly occurs via the efferent lymphatics. As the dissemination of pathogenic bacteria in a target species has received little study, the evidence to support a role for either the venous or lymphatic routes is somewhat indirect (section 1.9.1). The observations that *S. enteritidis* can be recovered from the MLN draining infected ileum and caecum, but not uninfected areas of the intestine 48 hours after oral inoculation of mice (Carter and Collins, 1974) supports the results obtained in this study that salmonellas disseminate predominantly via the draining lymphatics.

Interestingly, as in the mucosa and MLN, most of the bacteria within efferent lymph were in an extracellular niche. However this is perhaps not a surprising observation as two or three hours after infection of bovine ligated ileal loops, and quantification of bacteria within the mucosa, more than 90% of the initial inocula are gentamicin sensitive (Watson *et al.*, 1995). In addition, the ability to resist the bactericidal activities of complement *in vitro* does not appear to represent a barrier to survival of different serotypes including *S. gallinarum* or *S. dublin* (S. Paulin, unpublished observations; P. Watson, personal communication; Wallis *et al.*, 1997; Uzzau *et al.*, 2000a). These results suggest that *Salmonella* may be able to survive free within serum or lymph during pathogenesis.

Despite both serotypes reaching the MLN in similar numbers, *S. dublin* appeared to be able to pass more efficiently through the node and as such translocated in efferent lymph in higher numbers than *S. gallinarum*. Consistent with this, *S. gallinarum* was recovered in lower numbers than *S. dublin* from the liver and spleen 12 hours after infection of ligated ileal loops. These observations implicate the MLN in having an important role in determining the nature of subsequent infection and provide the first evidence of a correlation with the host specific phenotype in calves, although the reasons for this remain unknown and will be discussed further in Chapter 8.

The recovery of a cell-associated bacterial population within the mucosa and MLN, despite the relatively cell-free translocation in efferent lymph, could imply that salmonellas are able to induce cell lysis at different stages of pathogenesis in order to escape the intracellular environment and translocate extracellularly.

Several studies have reported that *Salmonella* Spp. are cytotoxic for infected host cells both *in vitro* and *in vivo* (section 1.9.7). Currently the precise mechanisms by which host cell death contributes to pathogenesis however remain highly controversial, particularly as this process would appear to contradict the requirement of *Salmonella* for proliferation within an intracellular niche. Several reports propose that cell death is a result of apoptosis (Chen *et al.*, 1996; Lindgren *et al.*, 1996; Monack *et al.*, 1996; Richter-Dahlfors *et al.*, 1997; Libby *et al.*, 2000), and it has been suggested that this will initiate an inflammatory response, leading to tissue damage and bacterial spread, by the activation of caspase 1 dependent IL-1 (Zychlinsky *et al.*, 1997). However, this hypothesis is in contrast to the general view that apoptosis does not induce an inflammatory response (Savill, 1997). Furthermore, interpretation of data by some studies and the choice of method used for monitoring cell death does not entirely support a role for apoptosis in this process (Chen *et al.*, 1996; Lindgren *et al.*, 1996; Monack *et al.*, 1996; Richter-Dahlfors *et al.*, 1997; section 1.9.7).

In contrast, more recent work has proposed that macrophages are killed *in vitro* by a caspase 1-dependent form of necrosis (Brennan and Cookson, 2000; Watson *et* al., 2000b) which appears to be a more feasible explanation when the pathogenesis of *Salmonella* is considered *in vivo*. For example, neither the ability of *S. dublin* nor *S. gallinarum* to induce cell death appeared to contribute to pathogenesis in calves, which is not really surprising when the infection process in this host is considered. For example, both serotypes are able to induce a potent inflammatory response within the intestinal mucosa, apparently independent of cell death. Furthermore within the intestine, and during all stages of translocation, it is clear that both serotypes can survive, and resist the bactericidal activities of serum. As such, these observations appear to negate the bacterial requirement to induce apoptosis in host cells as either a means of escape or to evade immune responses to infection. While cell lysis may be host and/or serotype dependent (Wallis *et al.*, 1997; Watson *et al.*, 2000b; Watson *et al.*, 2000c), and may contribute to specific stages of pathogenesis, many studies do not consider the possibility that in addition to survival within host cells, *Salmonella* may also favour an extracellular niche *in vivo*.

While the use of continuous anaesthesia made it impractical to assess immune responses to infection beyond 12 hours, phenotypic and microscopic analysis of eukaryotic cells demonstrated that there were *Salmonella*-induced changes associated with the MLN. These included an influx of PMNs and an up regulation of phagocytic cell markers, both of which were consistent with a rapid increased inflammatory response to infection, and an increase in MHC II expression consistent with up-take, processing and presentation of antigen

by APC. In addition, there was a reduction in the expression of T cell surface markers, which could be a result of either the down regulation of T cells, or a reduction in the expression of T cell receptors in response to *Salmonella* infection. Phenotypic changes within the bovine MLN in respect of host specificity were not directly compared in the current study. However, reports in sheep defining immune responses to a *S. abortusovis* vaccine strain (Rv6), that has a lower capacity for multiplication and dissemination (Lantier *et al.*, 1981; Pardon *et al.*, 1990), and the wild type *S. abortusovis* have shown that both strains induced a massive infiltration of PMNs into infected lymph nodes (Fontaine *et al.*, 1994). Furthermore, while the lymph node draining the inoculation site represented an efficient barrier limiting the spread of Rv6, both strains elicited a strong immune response to infection within the lymph node (Fontaine *et al.*, 1994; Doucet and Bernard, 1997; Gohin *et al.*, 1997). Taken together, these results add to the hypothesis that the ability to replicate within host tissues contributes to the virulence phenotype of particular *Salmonella* strains or serotypes.

In summary, the development of a novel venous and efferent lymphatic cannulation model has provided a unique opportunity to investigate several aspects of *Salmonella* pathogenesis in calves, that have to date remained relatively unstudied. Both *S. dublin* and *S. gallinarum* appear to interact with the intestinal mucosa and translocate to the MLN in similar numbers. *S. dublin* is able to pass more efficiently through the MLN than *S. gallinarum* and as such is able to infect systemic tissues in higher numbers. These results suggest that *Salmonella*/host interactions in the MLN are fundamental in determining the outcome of infection and therefore host specificity. Whether these serotype differences relate to an inability of *S. gallinarum* to survive and replicate or resist host killing remains to be determined. In this study it was found that bacterial or host-induced cell lysis did not appear to contribute to the pathogenesis of either serotype. Furthermore, while both serotypes translocate via the efferent lymph in a predominantly cell-free environment, association with eukaryotic cells does occur, however $CD14^+$ cells do not appear to be the main niche occupied by *Salmonella*.

# CHAPTER 8

# DISCUSSION

The aim of this study was to increase our understanding of the role of factors influencing *Salmonella* serotype-host specificity using infection of cattle as a target species. The specific serotype characteristics that contribute to the development of severe systemic and/or enteric salmonellosis in a limited range of hosts, and the reasons why the incidence of particular serotypes change constantly over time remains largely undefined. As such, increasing our knowledge of host specificity might help to evaluate whether host range determining factors could be utilised for control of the disease caused by different *Salmonella* serotypes. Cattle are a particularly biologically relevant model with which to study different aspects of pathogenesis contributing to *Salmonella* serotype-host specificity as they are naturally susceptible to both the enteric and systemic forms of the disease (Wray and Davies, 2000). The work performed during the generation of this thesis therefore provides one of the first investigations using a natural target host to study the interactions of different *Salmonella* serotypes *in vivo*.

Much of the current understanding of *Salmonella* pathogenesis has been derived from *in vitro* studies using immortalised cultured cell lines or *in vivo* studies using the murine typhoid fever model of infection. While both of these approaches have greatly increased our knowledge of different stages of the disease process, neither model is entirely suitable for the study of factors contributing to host specificity. For example, there is a lack of availability of appropriate cultured cell lines from different animal species, and the use of these cells in isolation does not reproduce the complex interactions that occur between bacteria and host *in vivo*. On the other hand, mice do not provide an accurate model with which to study the enteric form of the disease caused by different *Salmonella* serotypes (Tsolis *et al.*, 1999a; Wallis and Galyov, 2000) and as such are of limited use in understanding the mechanism of host specificity in different animal species. In addition, mice with an *Nramp*$^{s}$ background, or macrophages derived from these animals, are known to be highly susceptible to *S. typhimurium* and may elicit responses to infection that differ considerably from naturally resistant animals (Hormaeche, 1979; Harrington and Hormaeche, 1986; Hormaeche *et al.*, 1995).

Host specificity is likely to be influenced by both the probability of natural exposure to a particular *Salmonella* serotype and genetic serotype differences that contribute to the virulence for a specific host. In this study *S. dublin*, but not *S. gallinarum* or *S. abortusovis*, resulted in severe salmonellosis when administered orally to calves, suggesting that unique interactions between serotype and host contribute to the outcome of disease. *S. choleraesuis*

was also virulent following oral inoculation of calves. While this serotype has been shown to infect a diverse range of hosts following oral inoculation (Smith and Halls, 1968; Barrow *et al.*, 1994; Watson *et al.*, 2000c), *S. choleraesuis* is mainly associated with natural infection of pigs (Sojka and Field, 1970; Sojka *et al.*, 1977). As such it can be speculated that environmental exposure is likely to contribute to the host specificity of this serotype. The reasons why *S. choleraesuis* does not infect cattle naturally are unknown. However, it could be suggested that this serotype might not be able to circulate or gain access to the bovine population in sufficient numbers to cause disease. Alternatively establishment of the carrier state, which is important for maintenance of the disease within a herd (Kovota *et al.*, 1988), may act as a continuous source of infection for cattle. If *S. choleraesuis* is unable to establish its own carrier state in cattle, it may limit the prolongation of this serotype within the bovine population.

In this and previous studies it has been shown that the ability of *S. dublin* and *S. typhimurium* to invade the bovine intestinal mucosa, via both M cells and enterocytes, and induce enteropathogenic responses in ligated ileal loops appears to be important in mediating the outcome of disease in calves (Wallis *et al.*, 1995; Watson *et al.*, 1998; Watson *et al.*, 1999). Likewise, the relative inability of *S. abortusovis* to invade the bovine intestinal mucosa or induce inflammatory and secretory responses correlates with poor virulence following oral inoculation of this host. With other serotypes however, these stages of pathogenesis do not appear to correlate with either virulence or host specificity. For example, the calf avirulent serotype *S. gallinarum* was surprisingly highly invasive and enteropathogenic while *S. choleraesuis*, which was virulent in calves following oral inoculation, was poorly invasive and poorly enteropathogenic. These observations warrant careful consideration as they imply that initial interactions with the intestinal mucosa are insufficient to determine the host specific phenotype. Prior to the elimination of *S. gallinarum* from U. K. poultry flocks this serotype was occasionally associated with enteric disease in mammalian hosts (Müller, 1933; Kauffamnn, 1934; Sojka and Field, 1970; Gupta and Verma, 1993). This implicates that while *S. gallinarum* is clearly able to induce enteritis or enteropathogenic responses, additional host and/or serotype factors contribute to the limitation of this serotype to systemic disease in fowl only.

In pigs, as well as calves, *S. choleraesuis* is poorly invasive and poorly enteropathogenic (S. Paulin, unpublished observations; Bolton *et al.*, 1999b), despite the virulence of this serotype associated with experimental infection of both calves (this study) and pigs (Watson *et al.*, 2000c). These initial intestinal interactions are analogous to the situation in sheep (Uzzau *et al.*, 2001), chickens (Henderson *et al.*, 1999) and humans

(McCormick *et al.*, 1995b; Everest *et al.*, 2001) following infection with *S. abortusovis*, *S. gallinarum* and *S. typhi* respectively, where virulence in the natural host species appears to be related to an inability to recruit inflammatory cells following invasion. This strategy of 'stealth' may consequently facilitate the systemic spread of certain serotypes in the relative absence of an intestinal immune response. In accordance with this theory, it has been shown that following *in vitro* invasion of primary chick kidney cells the host-specific serotype *S. gallinarum* results in a reduction in the release of IL-1β and IL-6 compared with *S. typhimurium* or *S. enteritidis* (Kaiser *et al.*, 2000). These results correlate with the poor inflammatory response observed following oral inoculation with *S. pullorum*, but not *S. typhimurium* (Henderson *et al.*, 1999). Similarly, following subcutaneous infection of sheep with *S. abortusovis* or *S. dublin*, the levels of Th1 cytokines (IL-12 and IFN-γ) and IL-1β were higher in the *S. dublin*-infected animals (S. Bernard, personal communication). This result implies that, as in chickens, there appears to be a differential serotype-specific induction of the acute phase inflammatory response potentially limiting some serotypes to the intestine while facilitating the systemic translocation of others

The recent sequencing of the *S. typhi* (http://www.sanger.ac.uk/Projects/S_typhi/) and *S. typhimurium* (http://www:genome.wustl.edv/gsc/Projects/S.typhimurium/) genomes has provided a unique opportunity to begin to understand how gene loss or acquisition may contribute to this strategy of 'stealth'. For example, analysis of the *S. typhi* genome has shown that this serotype encodes more than 200 pseudogenes of which approximately 75% are present as intact genes in *S. typhimurium* (Parkhill *et al.*, 2001). Many of these pseudogenes correspond to genes known to contribute to virulence in other serotypes. For example, *S. typhi* possesses mutations in genes encoding secreted or surface-associated proteins including SopE2, required for invasion (Bakshi *et al.*, 2000) and SopA, required for *S. dublin*-induced enteropathogenic responses in calves (Wood *et al.*, 2000). Together these observations suggest that loss of genetic material may have contributed to the limited host range of *S. typhi* for humans and primates. It could be speculated that this serotype, possibly through inactivation of genes required for enteritis, does not induce a strong initial cellular response of the host and as such is able to translocate to target systemc tissues. As the complete genome sequences of other serotypes begin to be elucidated so our knowledge of genes that contribute to the phenotypic characteristics of individual serotypes will help to identify factor(s) responsible for *Salmonella* serotype-host specificity.

To define the contribution of systemic tissues in influencing host specificity, calves were inoculated with different *Salmonella* serotypes by the intravenous route. These results suggest that interactions with systemic sites contribute to the host specific phenotype as,

regardless of whether the gut was bypassed, the virulence of *S. dublin*, *S. gallinarum* or *S. abortusovis* was similar to that observed following oral inoculation. A surprising observation was the bi-phasic pattern of infection that occurred following intravenous inoculation with *S. choleraesuis*, which potentially correlates with the 'stealth' strategy as a reduced ability to re-seed the gastrointestinal tract may result in a prolonged period prior to the establishment of enteric infection.

The ability to survive and multiply within the liver and spleen of different hosts has been correlated with *Salmonella*-serotype host specificity (Barrow *et al.*, 1994). As *Salmonella* are classified as facultative intracellular pathogens survival within host cells, particularly macrophages, is believed to contribute to virulence (Fields *et al.*, 1986; Richter-Dahlfors *et al.*, 1997; Hensel *et al.*, 1998). As such, several studies have attempted to model bacterial persistence *in vivo* by infection of macrophages *in vitro*. Evidence suggests that uptake and persistence of *S. typhi* and *S. typhimurium* within human and murine primary macrophages respectively correlates with virulence (Lissner *et al.*, 1985; Vladoianu *et al.*, 1990; Alpuche-Aranda *et al.*, 1995; Ishibashi and Arai, 1996; Schwan *et al.*, 2000). However, as previously discussed variations in experimental techniques, the use of bacterial strains of undefined virulence and the source of cells used make some of these studies difficult to interpret (section 1.9.2.1). In addition, it has been demonstrated that *S. typhimurium* is able to survive within macrophages derived from several hosts (Henderson *et al.*, 1999; Watson *et al.*, 2000b; Watson *et al.*, 2000c) which potentially correlates with the ubiquitous nature of this serotype. Both *S. typhimurium* and *S. dublin* rapidly lyse bovine alveolar macrophages (Wallis *et al.*, 1997; Watson *et al.*, 2000c). As such, the ability of these serotypes to persist within calves cannot be assessed *in vitro* however, persistence of different serotypes within porcine alveolar macrophages does not appear to correlate with host specificity (Wallis *et al.*, 1997; Watson *et al.*, 2000b).

In calves, serotype-host specificity cannot be explained in terms of intestinal invasion, induction of enteropathogenic responses *in vivo* or interaction with macrophages *in vitro*. Therefore, to address the question of whether other stages of pathogenesis might contribute to subsequent disease, host/bacterial interactions were investigated further using the calf virulent serotype *S. dublin* and the invasive, enteropathogenic yet calf avirulent serotype *S. gallinarum*. A novel bovine intestinal cannulation model was developed to assess the route and kinetics of bacterial translocation, from the infected lumen or regional MLN, to systemic sites. In addition, use of this model aimed to more fully characterise specific serotype and host responses contributing to infection. While there were obvious limitations, including the effect

of continuous anaesthesia on the host responses, this is the first time that such an *in vivo* model system has been used in the context of host specificity.

Several fundamental conclusions that contribute to our understanding of *Salmonella* pathogenicity in calves can be made from these studies. First, salmonellas predominantly transolcated to systemic sites via the lymphatic system, rather than via the blood. Second, quite surprisingly, both *S. dublin* and *S. gallinarum* appeared to occupy a predominantly gentamicin susceptible niche within the efferent lymph and mucosa in particular. These observations demonstrate that at key stages of the infectious process, salmonellas potentially reside in a cell-free environment in calves. Third, in contrast to studies in mice (Richter-Dahlfors *et al.*, 1997; Monack *et al.*, 2000), *Salmonella*-induced cell death does not appear to contribute to pathogenesis in calves, at least in the early stages of the disease. Fourth, *S. dublin* appears to be able to pass through the MLN in greater numbers than *S. gallinarum*, implicating the importance of this tissue in retaining and possibly contributing to the elimination of particular serotypes. In accordance with this observation, infection kinetics 24 hours after oral challenge of calves clearly indicated that *S. gallinarum* was capable of invading bovine intestinal mucosa. However, in contrast to *S. dublin*, this serotype was recovered in lower numbers from the majority of intestinal sites and only intermittently recovered from systemic tissues. Taken together, these observations suggest that *Salmonella* interactions with host tissues, in particular within the MLN, are fundamental in determining the outcome of infection and as such the host specific phenotype.

Further investigations are required to define the precise nature of the interactions of different serotypes with host tissues. At this stage it is unknown whether *S. gallinarum* is unable to survive and replicate within a particular niche or whether this serotype is more susceptible to host killing than *S. dublin*. Comparison of the net growth rates of *Salmonella* serotypes, within different tissues *in vivo* would clearly contribute to a greater understanding of host specificity. Such investigations could be exploited by the use of temperature sensitive segregation plasmids such as pHSG422 (Gulig and Doyle, 1993; Gulig *et al.*, 1997; Shea *et al.*, 1999). Additionally, the phenotypic analysis of specific host cells occupied by *Salmonella* would further define their contribution to different stages of pathogenesis. While macrophages appear to have a minor role in harbouring *Salmonella* within the bovine MLN, identification of the cell type(s) with which different serotypes interact and determination of the fate of these cells *in vivo* would greatly enhance our understanding of *Salmonella* pathogenesis in calves.

From these studies it is clear that factors influencing *Salmonella* serotype-host specificity are likely to depend on specific interactions between both the infecting serotype

and the infected host. For example, all of the serotypes that were virulent in calves predominantly resulted in the enteric form of the disease suggesting that interactions with the intestinal mucosa are pivotal in influencing infection in this host. In contrast, following infection with *S. choleraesuis*, the disease in pigs more typically manifests in systemic salmonellosis with high bacterial recoveries from these tissues (Wilcock and Schwartz, 1992; Watson *et al.*, 2000c). Furthermore adult sheep infected with *S. abortusovis* are typically asymptomatic, whereas the disease manifests as abortion of pregnant ewes and subsequent infection of unborn lambs (Jack, 1968; Jack, 1971; Uzzau *et al.*, 2001). Together, these results highlight the importance of not extrapolating results from one serotype or host combination to another. In addition, the careful choice of target species together with a requirement for the use of strains of defined virulence is clearly important in the study of host specificity.

In summary, work performed for the generation of this thesis has led to a wider understanding of factors that contribute to *Salmonella* serotype-host specificity in calves and has provided a solid background for further work in this area. Of fundamental importance in determining the host specific phenotype appears to be the ability of the pathogen to survive within the target host species. Defining the specific nature of the interactions with host tissues that facilitate the elimination of certain serotypes and the persistence of others would seem a logical progression to this project. It may then be possible to identify specific genes or loci that contribute to the pathogenesis of different serotype-host combinations.

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