DOCTOR OF PHILOSOPHY

Salmonella in companion animals

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SALMONELLA IN COMPANION ANIMALS

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ANIMALS
TYPHIMURIUM
ZOONOSES
NEWPORT
ADHESION
CANINES
SEROTYPE
ZOONOTIC
PHENOTYPIC
ARIZONAE
ANTHROPONOTIC
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CHARACTERISATION
MICROBIAL
EPIDEMIOLOGY
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PROFILES
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Summary- *Salmonella* in Companion Animals

In an increasingly hygiene concerned society, a major barrier to pet ownership is the perceived role of companion animals in contributing to the risk of exposure to zoonotic bacterial pathogens, such as *Salmonella*. Manifestations of *Salmonella* can range from acute gastroenteritis to perfuse enteric fever, in both humans and dogs. Dogs are heavily associated with asymptomatic carriage of *Salmonella* as the microorganism can persist in the lower intestines of this host which can be then excreted into the environment. Studies in to the asymptomatic carriage of *Salmonella* in dogs are somewhat dated and there is limited UK data. The current UK carriage rate in dogs was investigated in a randomised dog population and it was revealed that the carriage rate in this population was very low with only one household dog positive for the carriage of *Salmonella enterica arizonae* (0.2%), out of 490 dogs sampled. *Salmonella* serotypes share phenotypic and genotypic similarities which are captured in epidemiological typing methods. Therefore, in parallel to the epidemiological investigations, a panel of clinical canine (VLA, UK) and human (Aston University, UK) *Salmonella* isolates were profiled based on their phenotypic and genotypic characteristics; using API 20E, Biolog Microbial ID System, antibiotic sensitivity testing and PFGE, respectively. Antibiotic sensitivity testing revealed a significant difference between the canine and human isolates with the canine group demonstrating a higher resistance to the panel of antibiotics tested. Further metabolic capabilities of the strains were tested using the Biolog Microbial ID System, which reveal no clear association between the two host groups. However, coupled with Principle Component Analysis two canine isolates were discriminated from the entire population on the basis of a high up-regulation of two carbohydrates. API 20E testing revealed no association between the two host groups. A PFGE harmonised protocol was used to genotypically profile the strains. A dendrogram depicting PFGE profiles of the panel of *Salmonella* isolates was performed where similarities were calculated by Dice coefficient and represented by UPGMA clustering. Clustering of the profiles from canine isolates and human isolates (HPA, UK) was diverse representing a natural heterogeneity of the genus, additionally, no clear clustering of the isolates was observed between host groups. Clustering was observed with isolates from the same serotype, independent of host origin. Host adaption is a common phenomenon in certain *Salmonella* serotypes, for example *S.* Typhi in humans and *S.* Dublin in cattle. It was of interest to investigate potential host adaptive or restricted strains for canine host by performing adhesion and invasion assays on Dog Intestinal Epithelial Cells (DIECs) (WALTHAM®, UK) and human CaCo-2 (HPA, UK) cell lines. *Salmonella arizonae* and Enteritidis from an asymptomatic dog and clinical isolate, respectively, demonstrated a significantly high proportion of invasion in DIEC in comparison to human CaCo-2 cells and other tested *Salmonella* serotypes. This may be suggestive of a potential host restrictive strain as their ability to invade the CaCo-2 cell line was significantly lower than the other serotypes. In conclusion to this thesis the investigations carried out suggest that asymptomatic carriage of *Salmonella* in UK dogs is low however the microorganism remains as a zoonotic and anthroponotic pathogen based on phenotypic and genotypic characterisation however there may be potential for particular serotype to become host restricted as observed in invasion assays.

Key words: *Salmonella*, Dogs, Canine, Epidemiology, Phenotypic, Genotypic, Invasion

For Mum & Dad

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Ego Successio!

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Abbreviation

A	Absorbance
AAI	Animal-Assisted Interventions
AB	AntiBiotics
ADS	Antibiotic Disc Susceptibility
AHVLA	Animal Health and Veterinary Laboratory Agency
API	Analytical Profile Index
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
Aw	Water activity
BARF	Bone And Raw Food
BGA	Brilliant Green Agar
BHI	Brain Heart Infusion
Bp	Base pairs
BPW	Buffer Peptone Water
BSA	Bismuth Sulphite Agar
BSAC	British Society for Antimicrobial Chemotherapy
CHEF	Contour clamped homogenous electrophoresis
CaCo-2	Caucasian colon adenocarcinoma
DCA	Desoxycholate Citrate Agar
DIEC	Dog Intestinal Epithelial Cell
DNA	DeoxyriboNucleic Acid
EDTA	EthyleneDiamine-Tetraacetic Acid

GPA	Gentamicin Protection Assays
H	Flagella
HAI	Human-Animal Interventions
HCl	Hydrochloric acid
HEA	Hektoen Enteric Agar
HPA	Health Protection Agency
Kb	Kilo-base
KCl	Potassium chloride
kDa	Kilo Daltons
LB	Lactose Broth
LPS	Lipopolysaccharide
M9	Minimal 9
MAPK	Mitogen-Activated Protein Kinases
Mbp	Mega-base pairs
$MgCl_2$,	Magnesium chloride
MIC	Minimum Inhibitory Concentration
MOI	Multiplicity Of Infection
MSSRV	Modified Semi-Solid Rappaport Vassiliadis
NA	Nutrient Agar
NaCl	Sodium chloride
NAG	N-AcetylGlucosamine
NB	Nutrient Broth
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
O	Somatic

OD	Optical Density
PCR	Polymerase Chain Reaction
pef	the fimbriae operon
PFGE	Pulsed-Field Gel Electrophoresis
PMs	Phenotype Microarrays'
PT	Phage Type
RAM	Rambach agar
RAPD	Random Amplification of Polymorphic DNA
RV	Rappaport Vassiliadis
SDW	Sterile Distilled Water
SPI	*Salmonella* Pathogenicity Island
Spv	*Salmonella* Plasmid Virulence
SS	*Salmonella Shigella* agar
TBE	Tris, Boric acid, EDTA
TE	Tris, EDTA
Tris	Tris [hydroxymethyl] aminomethane
TSA	Tryptone Soya Agar
TSIA	Triple Sugar Iron Agar
UPGMA	Un-weighted Pair Groups using Mathematical Average
UV	Ultra-Violet
Vi	Capsule
VLA	Veterinary Laboratory Agency
WCPN	Waltham Centre for Pet Nutrition
WHO	World Health Organisation
XLD	Xylose Lysine Desoxycholate agar

XLT-4 Xylose Lysine Tergitol 4 agar

Introduction

1.1 *Salmonella*

1.1.1 History of *Salmonella*

In his role as company director, Daniel Elmer Salmon undertook research alongside his assistant Theobald Smith at the Bureau of Animal Industry, Washington D.C. USA. In 1885 it was the latter who discovered the microorganism *Salmonella.* He honoured his superior by naming the microorganism after him (Candy & Stephen 1989). Smith isolated the microorganism from a swine host and correctly suspected it to be the causative agent in swine fever (Grimont *et al.*, 2000). More importantly, the typhoid bacillus was later discovered to be the causative organism in human typhoid fever (Kauffmann, 1978). Serum agglutination tests revealed that the typhoid bacillus agglutinated with serum from typhoid patients previously immunised with the typhoid bacillus (Grimont *et al.*, 2000).

1.1.2 *Salmonella* morphology and taxonomy

*Salmonella*e are Gram-negative, facultatively anaerobic, rod shaped bacteria, typically motile by possession of peritrichous flagella. They belong to the Enterobacteriaceae family which also includes pathogens such as *Escherichia coli*, *Shigella* and *Klebsiella* (Philippon *et al.*, 1989). Ubiquitous in nature they are capable of survival in a diverse range of environments including water, soil, animal hosts and faeces. Furthermore, they can also grow in broad temperature ranges between 5.5 to 45.6^{o}C (Angelotti *et al.*, 1961; Matches & Liston, 1968) and interestingly, some atypical strains have

demonstrated growth at temperatures as low at 3.5^{o}C (Morey & Singh, 2012). These organisms are neutrophiles and thrive in a pH range of 4.1 - 9.0 (Silliker, 1982) and require a minimal water activity (Aw) for growth at 0.95 (Sperber, 1983). Typically they inhabit the harsh nutrient-limiting conditions of the lower intestinal tract of animal hosts. The capacity to utilise scarce nutrients effectively and efficiently relies on the embedded proteins in the outer membranes where they play a role in transportation of such nutrients (Rycroft, 2000).

The genus comprises of two species; *S. enterica* and *S. bongori. Salmonella enterica* is associated with causing disease in warm blooded animals including human hosts, whilst *S. bongori*, is frequently associated with the intestines of cold-blooded animals including snakes and lizards and is not commonly associated with warm blooded animals (Braz, 2001). *S. enterica* can be further sub divided into six subspecies: *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae* and VI, *S. enterica* subsp. *Indica* (Dieckmann *et al.*, 2008).

Kauffmann and White (1926) proposed the concept of a 'one-serotype-one-species' naming system based on serologic identification of three antigens. Notably, the genus contains around 2500 different serotypes which are serologically identified by antigenic variation in the O (Lipopolysaccharide), H (Flagella) and Vi (Capsular) antigens (Brenner *et al.*, 2000; Madigan *et al.*, 1997). Serotypes belonging to the *S. enterica* species post-1966 are generally named on the geographical location of isolation (Brenner *et al.*, 2000). The nomenclature for the genus is still evolving and remains complex, comprising different naming systems which are inconsistently combined.

1.2 Pathogenesis and virulence

The word pathogenesis is derived from the Greek work "*pathos"* and "*genesis*" which translates into "*disease creation*". Virulence is the degree of this pathogenesis and is derived from the Latin word "*Viulentus"* meaning "*poisoned wound*". *Salmonella* are capable of causing disease in humans and animals and their pathogenicity is dependent on possession of phenotypic and genotypic virulence factors. Furthermore, the immune state of the target host plays an important role in the severity of disease. *Salmonella*'s virulence factors are required for attachment, colonisation, invasion of the intestinal mucosa and avoidance of the host immune response; a complex strategy shared by other bacterial pathogens.

1.2.1 Virulence

The ability of *Salmonella* to overcome the low pH of the stomach and survive changes in temperature and oxygen levels is a pre-requisite for passage through the host gut. Moreover the ability to then migrate, attach, colonise, express virulence factors and cause disease is essential for successful infection. *Salmonella* virulence is an intricate expression of virulence factors and virulence genes, contributing to successful pathogenesis. These traits can be exploited in phenotyping and genotyping identification methods, such as API tests and Pulse Field Gel Electrophoresis (PFGE).

As the pathogen enters the gut it is initially exposed to a very low pH environment in the stomach and then later to a higher pH level further down the gut. Typically, the initial acidic environment in the stomach, pH 1-2, is bactericidal and many potentially pathogenic bacteria are eliminated here. However, some strains of *Salmonella* are capable of overcoming this harsh condition inside the stomach by the possession of acid

survival effector proteins and regulatory proteins *(*Bang *et al.*, 2002). This ability to survive in a range of pH environment is an adaption called Acid Tolerance Response (ATR) (Foster *et al.*, 1991). Transient adaption occurs within the first 20 minutes of low pH exposure and sustained adaption has been shown to be around 60 minutes (Foster *et al.*, 1991).

As a facultative anaerobe, *Salmonella* is able to overcome the decrease in oxygen levels presented inside the intestine as they move deeper into the gut by the expression of the regulatory system proteins. These proteins react to subtle changes of oxygen concentration in the *Salmonella* cytoplasm to allow the bacterium to overcome this host defence (Alexeeva *et al.*, 2003).

The motile nature of a strain has been shown to contribute to virulence and is established by the possession of flagella, allowing the migration to targeted sites inside and outside of a host (Lillard, 1986). The role of flagella has been studied extensively *in vitro* and demonstrates that virulence is improved in mutants containing flagella (Lockman & Curtiss, 1990). Additionally, flagella play a key role in the attachment and invasion of cells contributing to the microorganism's virulence (Dibb-Fuller, 1999). Simple single assay tests such as hanging drop preparations can provide a fast and cheap method to determine the motility of a bacteria. Once the *Salmonella* invade the intestinal epithelial cells, the protein flagellin produces a pro-inflammatory cell response. The flagellum filament contains around 20,000–100,000 subunits of 50 kDa flagellin that can have diverse biotechnological applications such as vaccine adjuvant and cellular protector during chemo- and radiotherapy (Oliveira *et al.*, 2011). Conversely, it has been reported that some non-motile pathogenic strains have caused

clinical infection which suggests their ability to cause infection relies on the strength of other virulence factors (Lockman *et al.*, 1990; Paiva *et al.*, 2009).

Fimbriae are rod-shaped extra-cellular structures that also allow attachment to host cells. There are various types of fimbriae that differ in size and properties; for example type 2 fimbriae are unable to agglutinate erythrocytes as seen with the serotype *S.* Gallinarum (Throns 1995). Additionally, some serotypes are able to express many fimbriae which are highly hydrophobic thus protecting the bacteria from the harsh changes water osmolality found in different environments (Humphrey, 1996).

1.2.2 Virulence plasmids

Salmonella has a number of genes that are essential to overcome host defence mechanisms. Virulence genes are contained within the chromosome as well as in low copy virulence plasmids; which are associated with triggering a systemic disease (Rotger & Casadesus, 1999). These plasmids can range in size from 50 Kb to 90Kb however are homogenous for the presence of a 7.8 Kb region called the *Salmonella* Virulence Plasmid *(spv)* which has been associated with multiplication of the bacterium. Studies have employed techniques for the identification of the *spv* genes in systemic strains of *Salmonella* and have demonstrated an association with *spv*-dependent cytopathology in macrophages and characteristic apoptosis of host cells (Libby *et al.*, 2000). The identification of certain serotypes can be carried out by confirmation of plasmid carriage. Certain *Salmonella* serotypes belonging to subspecies I carry a large, low-copy-number plasmid that contains virulence genes (Rotger & Casadesus, 1999).

1.2.3 *Salmonella* Pathogenicity Island encoded type III secretion system

Salmonella Pathogenicity Island 1 (SPI-1) and 2 (SPI-2) encoded type III secretion systems which are associated with triggering MAPK signalling pathways, required for pro-inflammatory responses. These secretion systems are deemed one of the most sophisticated bacterial virulence determinants (Bäumler *et al.*, 2000). The highly intricate type III secretion system, illustrated in Figure 1, injects a battery of effector proteins into the host cell (SopE, B, D, SipD, C, B, A, Spip) which allows *Salmonella* to hijack the actin regulatory machinery of the host cell to promote bacterial entry (Brown *et al.*, 2007; Cossart *et al.*, 2004). These effector proteins are able to mimic host cell activators of actin polymerisation, resulting in membrinteract with the host cells binding proteins (Cdc42, Rac, SGEF, RhoG, ELMO, Dock180) which induces membrane ruffling and micropinocytosis (host cell internalisation) (Cossart *et al.*, 2004). These effector proteins can be tagged with flag epitopes that are recognised by anti-flag antibodies which can be detected by SDS-PAGE and immunoblotting methods.

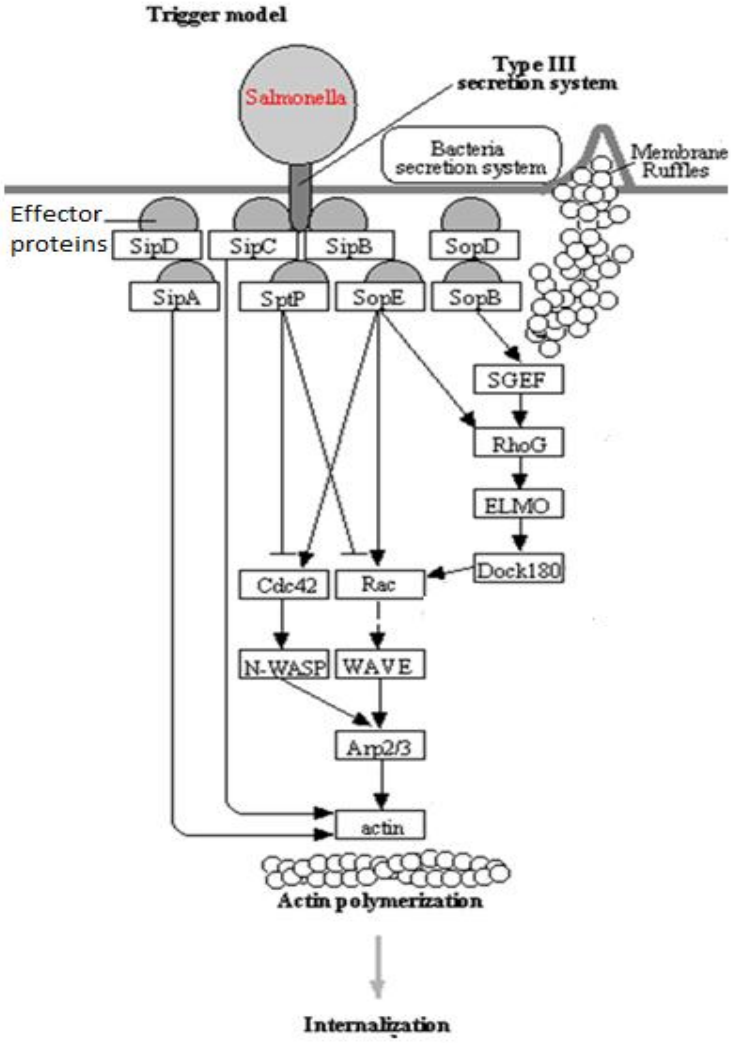


Figure 1.1 Mechanism of the *Salmonella* Type III secretory pathway. The injection of effector proteins induces a signalling pathway by interaction with host binding proteins, Cdc42 and Rac, causing micropinocytosis. Adapted from (Kaneshia Laboratories 2010).

1.2.4 Pathogenesis

Salmonella are considered ubiquitous pathogens in nature, affecting a number of hosts including humans, domestic animals, wild mammals, birds, reptiles and even insects. As one of the most important foodborne pathogens, entry into the host is typically via the oral route and generally an infective dose of 10^3 -10^7 cells is required to cause infection. However infection can still manifest with as few as 10^2 cells in contaminated foods, as the food may act as a protective layer, shielding *Salmonella* from the toxicity of the low pH conditions of the human stomach. Exposure to low pH in the stomach generally eliminates approximately 99% of *Salmonella* cells (Carter & Collins, 1974). The remaining 1 % pass into the small intestine where they are exposed to further environmental change as a consequence of secreted bile salts. Bile salts neutralise the low pH of the stomach acid and contain surfactant molecules which are broadly antimicrobial. The Enterobacteriaceae family have adapted to survive in these conditions, however, the motion of peristalsis in the small intestine maintains *Salmonella* in the gut lumen and approximately 15% of the residual *Salmonella* are retained here whereas the rest are expelled in the faeces. However, the bacteria that are able to overcome this attach to and penetrate the intestinal wall to reach the Gut Associated Lymphoid Tissue (GALT) (Carter & Collins, 1974). It is here they penetrate the submucosa and establish themselves on the lamina propria (Bäumler *et al.*, 2000). Figure 2 illustrates the stages of pathogenesis of *Salmonella* in the human intestinal gut.

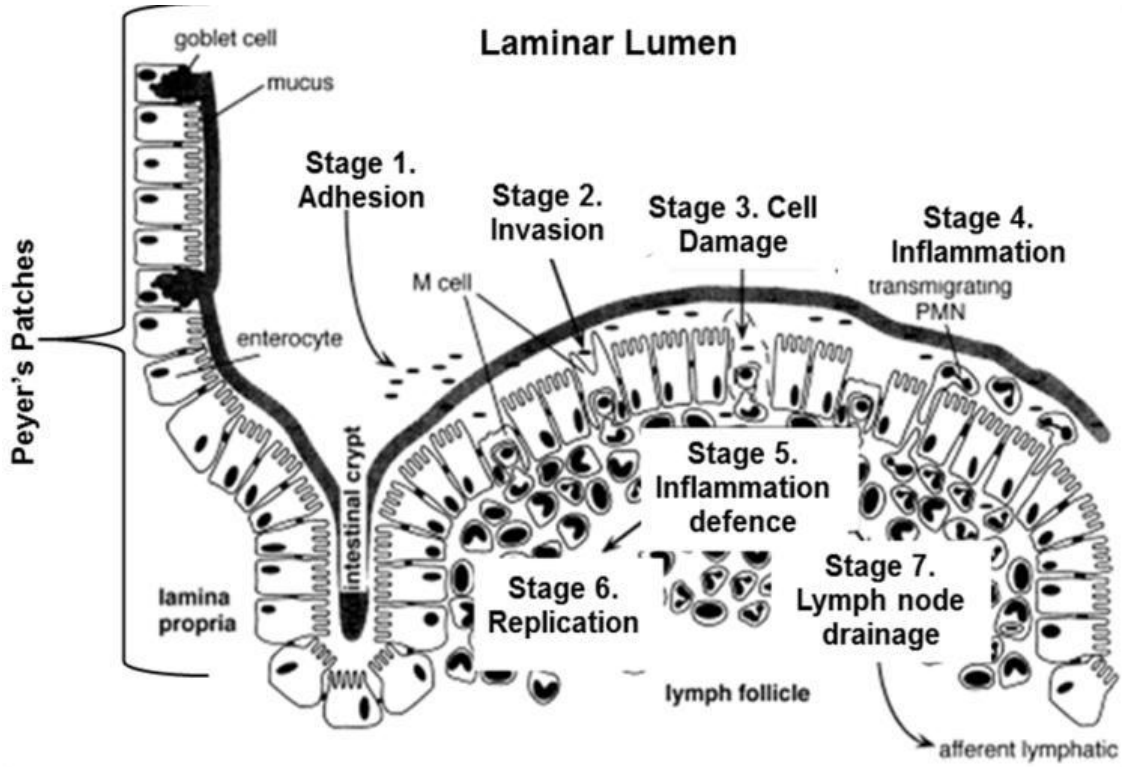


Figure 1.2 *Salmonella* adhesion (stage 1), invasion into Peyer's patches (stage 2), M cell damage (stage 3), host cell inflammation defences (stages 4 & 5), replication and for systemic infection drainage to the lymph nodes (stages 6 & 7). Adapted from (Bäumler *et al.*, 2000)

Salmonella attachment is impeded by competitive indigenous flora, a phenomenon called bacterial interference (Bäumler *et al.*, 2000). Competition for tissue adhesion sites, nutrients and metabolites, and the productions of inhibitory substances including fatty acids, cause a hostile environment for *Salmonella* pathogens. The bacteria attach to the apical epithelial surface of enterocytes initiated by polymeric protein surface organelles: flagella, apical appendages and long polar fimbriae (Bäumler *et al.*, 2000; Lillard, 1986).

Following adhesion the pathogens invade by micropincytosis and destroy M cells, located in the Peyer's patches, which triggers an inflammatory response. Peyer's patches illustrated in Figure 2, are clusters of mammalian lymph follicles and serve as a main port of entry for *Salmonella* serotypes (Bäumler *et al.*, 2000). Once inside the Peyer's patches macrophages and polymorphs are recruited to the site of infection and engulf the pathogenic cells producing intracellular superoxide radicals; which usually leads to intracellular killing. Recruitment of polymorphonuclear leukocytes (PMNs) is signalled by the lipopolysaccharide component of the bacteria binding to the Toll-like receptor 4 complexes, which activates transcription factors in the lymphocytes. Invading bacteria are then taken up by macrophages, which undergo *Salmonella*-induced caspase-1 mediated apoptosis (Bhan *et al.*, 2005).

Non-typhoidal serotypes multiply in the Peyer's patch tissue where they are drained into the mesenteric lymph nodes. It is essential for successful pathogens to overcome host phagocytes, complement, antibodies and other immunological cellular entities. If the host is unable to contain the infection then clinical gastroenteritis is presented; where the host typically presents with diarrhoea and vomiting (NHS, 2011). These clinical symptoms are due to enterotoxins produced by the bacilli and their association with proteins and genes including the SopE protein and *stn*, respectively (Chopra *et al.*, 2003; Stephen, 1985). As well as patients presenting with gastroenteritis, an immocompromised host can also develop infections such as enteric fever and bacteraemia. This occurs when the blood-barrier is breeched; the pathogen enters the bloodstream via the thoracic duct which could potentially lead to metastatic foci of intravascular lesions, oesteomyelitis and meningitis, all of which are fatal (Hilton, 1997). When this situation arises, the bacilli are able to migrate into the spleen, liver,

gall bladder, bone marrow, lymph nodes and kidneys where they survive and multiply. The Vi antigen appears important in enhancing human virulence of typhoid and molecular investigations have found that SPI-7 comprises the Vi antigen (Bhan *et al.*, 2005). This pathogenesis is different to gastroenteritis as *Salmonella* have demonstrated replication not in the lower intestines but in the cardiovascular system (Raffatellu *et al.*, 2008). The host presents with very severe and serious clinical symptoms of bacteraemia which require antibiotic treatment. The majority of reported invasive cases are from elderly and immunocompromised patients as they are more likely to be susceptible. The human host adapted serotype, *S.* Typhi is capable of causing enteric fever. Patients infected with *S.* Typhi can become asymptomatic carriers and these individuals excrete large numbers of the bacteria in their faeces, therefore, having the potential to re-infect (Ruby *et al.*, 2012). The carrier state has also been described in livestock animals and is responsible for food-borne epidemics. In the case of the cook, Typhoid Mary, asymptomatic shedding of *S.* Typhi from liver to the gallbladder was not apparent for many years and she was unaware of her role in cross contamination of the food prepared for human consumption (Mølbak *et al.*, 2006).

1.3 Salmonellosis clinical features and treatment

Salmonella are broadly competent microorganisms capable of causing disease in humans and animals. Non-typhoidal serotypes are typically responsible for causing gastroenteritis, caused by a localised infection in the lower intestines. Symptoms include vomiting, diarrhoea fever and abdominal cramps (NHS, 2011) some 6 to 48 hours after infection. Treatment is limited yet simple, rehydration with clean drinking water is usually sufficient to remove the bacteria from the site of infection. Antibiotics are not typically administered and indeed may prolong the asymptomatic carrier state

thus increasing the risk of further contamination and spread (Ebner & Mathew, 2000; Nelson *et al.*, 1980). Infections can persist for 4-7 days depending on the immune status of the host. In the UK salmonellosis is commonly caused by the non-typhoidal strains *S.* Typhimurium and *S.* Enteritidis (HPA, 2011b).

Serotypes *S.* Typhi and *S.* Paratyphi are particularly problematic in humans as they have the ability of causing life threatening systemic infections (Huang & DuPont, 2005). These serotypes are able to overcome the host's immune system and multiply inside the intestine. Successful migration into the blood stream can lead to a systemic, often deep-seated infection called typhoid fever. The infection manifests as an enlargement of the spleen and liver as the *Salmonella* are concentrated in these organs (Chalkias *et al.*, 2008). In these instances the symptoms are much more pronounced, graver and can manifest as high fever. The severity of this infection requires the patient to be treated rapidly with antibiotics, commonly: ampicillin, gentamicin, trimethoprim/sulfamethoxazole, ceftriaxone, amoxicillin, ciprofloxacin, chloramphenicol and co-trimoxazole, although there is increasing evidence of resistance to these commonly administered therapies. In particular, *S.* Typhimurium DT104 (A, C, S, Su, T) has become resistant to many of the frequently prescribed antibiotics including ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline. Resistant strains delay treatment which could lead to an exacerbated infection, dire consequences and, potentially, cause the death of the patient. In the UK, *S.* Typhi and *S.* Paratyphi are uncommon serotypes and the 569 reported cases, during 2010, were more commonly associated with international travel (HPA, 2011b).

Salmonella infection is not restricted to human hosts and is, in fact, common place with many animals, including those exploited for meat production and those kept for companionship. Meat producing animals are of particular concern as they are a major route of transmission to humans and other animals via food product vectors. Nevertheless, extensive research has been undertaken to contain such transmission and, moreover, strategies aimed at the limitation of such spread have proven successful including routine monitoring programs, disinfection and vaccination (Van Immerseel *et al.*, 2009; Wegener *et al.*, 2003).

1.4 Routes of transmission

Typically, *Salmonella* inhabit the intestinal tract of animals; however they are ubiquitous in nature and can often be found within common environments such as water and soil as a result of faecal contamination. They have also been isolated from insects, raw meats, factory surfaces and domestic kitchen utensils (Braoudaki, 2004). The vast diversity of environments that could be potential sources of *Salmonella* gives rise to comprehensive routes of transmission which contributes to the success of the pathogen. It is generally regarded that the presence of *Salmonella* within other environments can be sourced back to some prior faecal contamination.

One of the major routes of *Salmonella* infection is via contaminated human food, often meat and dairy products from farm animals (Jayarao *et al.*, 2006). The pathogenic cells may harbour in undercooked food or food that is not washed thoroughly before consumption. In addition, it is not solely direct farm animal contact that is the source of infection but also the food, water and environment they inhabit. Figure 1. 3 summarises the possible routes of transmission. The transmission route of *Salmonella* infection is

complex and difficult to control as there are many areas of exposure, including interaction with pets and animals.

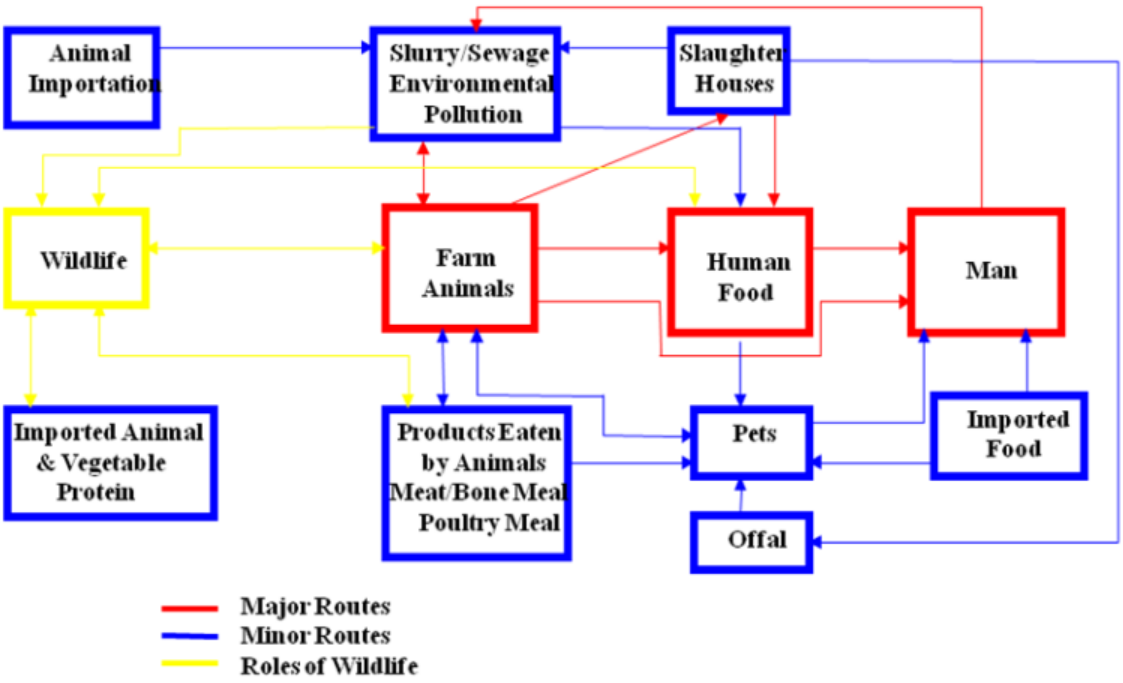


Figure 1. 3 Routes of transmission of *Salmonella*

1.5 Epidemiology of human *Salmonella* infection

1.5.1 Surveillance and notification within the UK

Surveillance of *Salmonella* outbreaks in the UK is facilitated by mandatory notification to the Consultant in Communicable Disease Control (CCDC) under the Public Health Act 1984. Incidences are anonymised and forwarded to the Office of Public Censuses and Surveys (OPCS) and used to produce the national food poisoning statistics, which are then published in the Communicable Disease Report by the HPA and the Communicable Disease Surveillance Centre (CDSC) (Hilton, 1997). In addition, *Salmonella* strains isolated from cases of infection are sent to the Laboratory of Enteric

Pathogens (LEP), the national reference laboratory, for confirmation and further identification. Post-identification, the data is sent to the CDSC and contributes to the weekly-published figures. However, as a self-limiting disease, patients tend to treat symptoms and fail to seek professional medical attention. Consequently, these cases fail to get reported to the CCDC and, ultimately, the true extent and burden of *Salmonella* food poisoning cases in the UK is highly underrepresented.

1.5.2 Incidence

The significance of *Salmonella* in human infection is recognised primarily as a foodborne pathogen as it uses food as a vehicle to spread infection. Infection by *Salmonella* causes salmonellosis, colloquially referred to as food poisoning, in humans and animals. In recent years the incidence of infections has decreased, possibly due to a combination of improved sanitation, safer water systems and improved living conditions as well as vaccination of chickens. Nevertheless, undercooked raw meats and cross-contamination within the environment are contributing factors that ensure the incidence of salmonellosis remains significant.

Despite the decrease in incidence of food poisoning due to *Salmonella*, it remains the second most common cause of food poisoning in the UK after *Campylobacter* (Little *et al.*, 2007). Infection is particularly common in hosts at the extremes of the age spectrum and is weighted heavily on the immunocompromised state of the host. Of the approximately 2500 different *Salmonella* serotypes identified only a small number are reported with significant frequency. *Salmonella* Enteritidis and *S.* Typhimurium are among the most common serotypes of non-typhoidal salmonellosis in the UK (HPA,

2011b) and are in addition the major serotypes in poultry and poultry products (Gürakan, 2008).

In the 1940s, mainly due to the war, meat and egg products were limited and were considered a delicacy. Consequently, rates of salmonellosis were low; however, post-war there was an increase in availability of these products and, as a consequence, the number of cases increased significantly. During this post-war period the serotype *S.* Enteritidis was regarded as one of minor public health significance. However, as the numbers of infections caused by this serotype increased, soon it became the dominant causative agent of human food poisoning in many parts of the world (Rodrigue *et al.*, 1990). Epidemiological data from the 1980s highlighted a concerning increase in *S.* Enteritidis Phage Type (PT) 4 in the UK and further investigations observed the principle reservoir being food containing grade A table egg shells from infected hens (Coyle, 1988). Egg contamination by penetration of the hard shell by *Salmonella* Enteritidis PT4 present in chicken faeces deposited on the outside of the egg as it passes through the cloaca (Keller *et al.*, 1995). Additionally, strains may persist for a number of months in the spleen of the egg laying hens, leading to infection of the reproductive tract and, consequently, at sexual maturity, numbers of bacteria increase and spread to the reproductive tract, which may result in vertical transmission to eggs or chicks (Shivaprasad, 2000). Infection of the ovaries followed as the strain was able to migrate from the cloacae to the reproductive organs, contributing to the re-infection cycle (Poppe, 1992). The strain PT4 had a significant advantage over other food borne pathogens. Furthermore, infected flocks were difficult to distinguish and sanitising procedures were insufficient.

National and European legislation requiring the incidence of the disease to be reported have improved hygiene and biosecurity (Barrow, 2000). Moreover, vaccination has further contributed to controlling *Salmonella* levels in poultry and subsequently, in man; but levels of infection remain significant (Thomos, 2008). Many intervention strategies have been implemented, including serological and environmental monitoring and disinfection, but vaccination remains the most significant of all the applied processes.

During the mid-1990's the introduction of the commercially available vaccine, SalenVac, demonstrated a simultaneous decline in hen and human infection from the PT4 strain (Woodward, 2002). Fortunately, the current trend in the UK demonstrates that there is a steady decline of serotype Enteritidis, including strain PT4, since 2000 to 2010 from 15,435 to 9,133 reports. This figure remains considerable and is not inclusive of the unreported cases of salmonellosis, which are likely to be significantly greater. This decline in number of cases can be seen in Figure 3 taken from the Health Protection Agency (HPA) and is also indicative of the significant role serotypes Typhimurium and Enteritidis play in human infection.

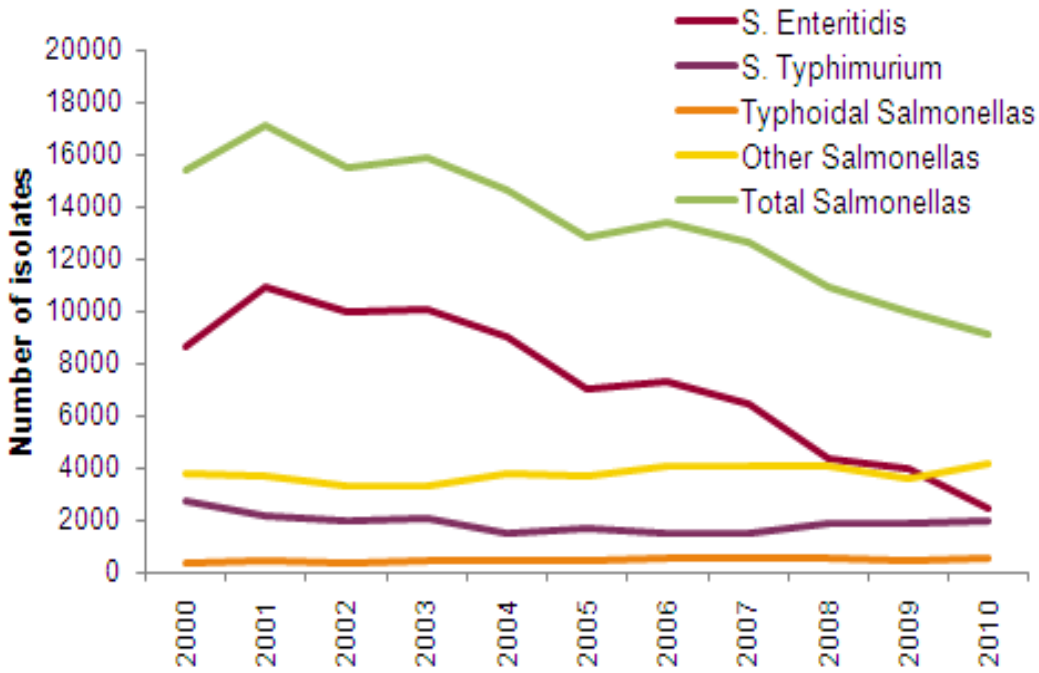


Figure 1.4 The frequency of reported cases of salmonellosis in the UK; including the common serotypes and minor serotypes which are plotted as 'other' *Salmonella* (HPA 2011b).

It is important to stress and acknowledge that although the reduction in incidence of human infection appears to be very substantial, these figures are only representative of reported cases and do not take into account the number of cases which remain unreported. A study to show the actual rates of *Salmonella* in the community observed for every *Salmonella* infection reported there were at least three unreported cases (Thomas *et al*., 2006; Wheeler *et al*., 1999).

The picture is somewhat different regarding the incidence in developing countries as numbers of cases of salmonellosis remain significantly high. The reported number of cases of *S*. Typhi and *S*. Paratyphi has reduced significantly in the past few decades in

developed countries due to improvements in sanitation and water supply, whilst numbers of cases remain high in developing countries due to poor facilities, inadequate water supplies and the circle of infection-shedding-infection amongst inhabitants. However, typhoidal cases are somewhat difficult to completely eliminate from the UK due to the ease of overseas travelling to epidemic countries and increased immigration to the UK.

1.5.3 Epidemiology of animal *Salmonella* infections in the UK

As a zoonotic pathogen some *Salmonella* strains causing infection in food-producing animals have been reported to cause infection in humans. Surveillance of *Salmonella* infection in food producing animals is vitally important as these animals are an important route of transmission into the human food-chain. Reports of infection cases are beneficial to epidemiology studies as they provide information on the current circulating serotypes and they can also indicate patterns of antibiotic resistance patterns.

The Animal Health (AH) and Veterinary Laboratory Agency (VLA) have combined (AHVLA) and are in place to "help safeguard animal health and welfare and public health, protect the economy and enhance food security through research, surveillance and inspection" (DEFRA, 2012). The CCDC, HPA, LEP, and AHVLA are analogous in their recording of salmonellosis cases, observing incidence in humans and animals, respectively. Specifically, the AHVLA record data from food producing animals including cattle, pigs, and egg laying hens and this information and epidemiological data are routinely updated and published.

Salmonella infection in cattle is not uncommon in the UK and serotypes Enteritidis, Dublin and Typhimurium are particularly prevalent. Currently there is no routine *Salmonella* monitoring of cattle in the UK, therefore the majority of isolates come from cattle with clinical disease. The number of reports is dependent on the total cattle population and the number of diagnostic submissions to Government veterinary laboratories (VLA, 2012). Figure 4 illustrate the proportion of incidences of *Salmonella* cases in cattle and demonstrate the common serotypes isolated.

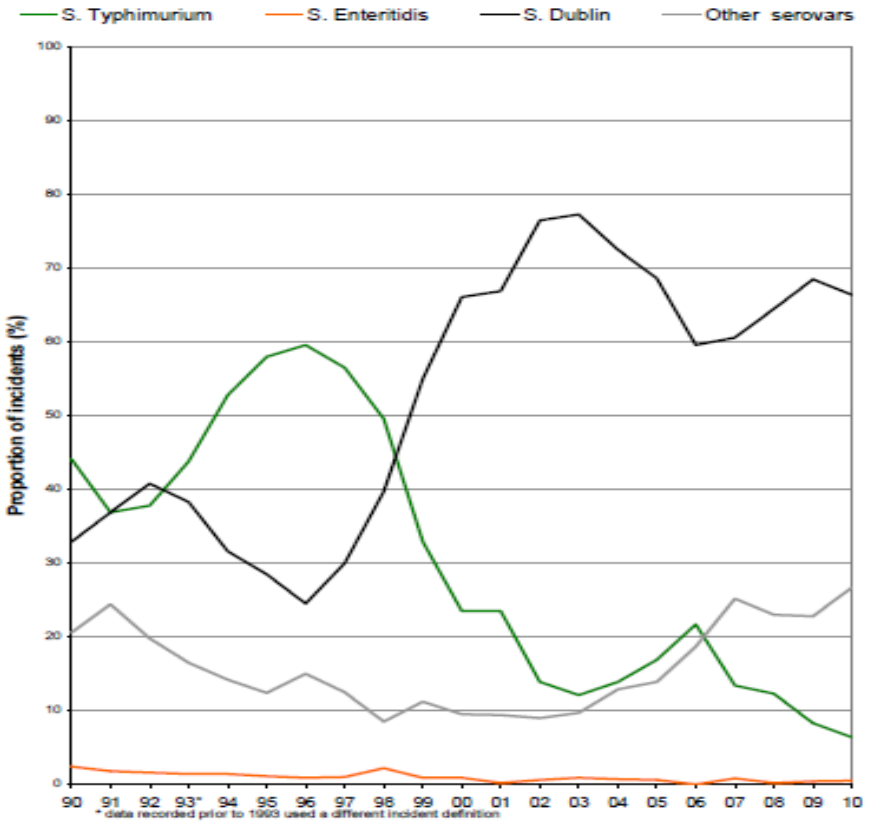


Figure 1.5 The incidence of *Salmonella* cases in clinically ill cattle in Great Britain between 1990 and 2010. Common serotypes isolated are plotted and the trend highlights the increase in *Salmonella* Dublin in cattle in recent years.

As aforementioned, poultry play a significant role in the human epidemiology of *Salmonella* infections in the UK. This close association to chickens has led to statutory *Salmonella* testing programmes in order to fulfil the requirements of EU legislation (European Council Directive 2003/99/EC, Zoonoses Regulation 2160/2003, Commission Regulation (EC) No 1003/2005 and Commission Regulation (EC) No.1168/2006). Most major commercial poultry companies carried out additional voluntary testing schemes to avoid cases of *Salmonella*-positive chickens (VLA report, 2008). Interestingly, as well as chickens, *S.* Typhimurium has been commonly isolated from wild birds. Strains DT56, DT41b, DT135, DT104 and DT40 have been isolated from UK wild birds and DT40 has also been isolated from domestic cats and dogs, demonstrating the ease of routes of transmission and cross-contamination into the environment.

Epidemiological reports have demonstrated an increasing number of cases of the uncommon human serotype *Salmonella enterica* sub species *arizonae* in the UK (HPA, 2008b). In 1998, 30 human cases of *S. arizonae* were recorded out of 23,134 non-typhoidal *Salmonella*s in England and Wales (0.13%). In 2007, 55 human *arizonae* cases were noted out of 11,943 non-typhoidal *Salmonella'*s (0.46%) (HPA, 2008b). The association of an increase in human cases of this serotype is related to the increase in reptile pet ownership. Reptiles are capable of faecal shedding of *Salmonella* and can carry it on their skin. Therefore the public health implications of this inside the home should not be underestimated as the serotype can be fatal in human hosts especially in infants (Mahajan *et al.*, 2003; Willis *et al.*, 2002).

1.6 Salmonellosis in dogs

As a successful zoonotic pathogen, *Salmonella* is capable of causing infection in humans and animals, including dogs. A dog's physiology is different to that in humans and, consequently, the clinical symptoms of salmonellosis in dogs also differ. Presentation of salmonellosis in dogs is rare as they are regarded as having a naturally high resistance and tolerance to the bacteria (Buxton, 1957). However, infection in immunocompromised and stressed dogs can manifest as acute gastroenteritis and, in severe cases, enteric fever (Hoelzer *et al.*, 2011). The majority of symptomatic cases of *Salmonella* infection are seen in puppies and very old dogs, likely due to their naive or weakened immune systems, respectively (Morley *et al.*, 2006). Symptoms in dogs typically include watery diarrhoea inducing dehydration, vomiting, fever, lethargy, abdominal cramps, endotoxemia, anorexia and depression. These symptoms can present within 3-5 days of initial contact. It has also been reported that, in young puppies infected with non-typhoidal *S.* Dublin, the infection manifested as meningoencephalitis and neurological changes (Milstein, 1975). Infected bitches can suffer from further complications including miscarriage, stillbirth, utero infections and weak offspring, but these occurrences are rare (Caldow & Graham, 1998; Carter & Quinn, 2000). Furthermore, gastroenteritis can cause acute enteritis in the intestinal tract evident from blood-stained faeces. Histological reports have demonstrated that the pathogenesis of *Salmonella* in dogs can cause mucosal erosion and infiltration of neutrophiles and macrophages into the lamina propria surrounding the Peyer's patches, as in human salmonellosis (Carter & Quinn, 2000). Infection is dependent upon many factors, specifically, infectious dose, serotype or strain, the virulence of the strain and bacterial competition, or lack of it, within the gut flora (Carter & Quinn, 2000).

Symptomatic presentations are rare, however, dogs regarded as asymptomatic carriers of *Salmonella* have demonstrated long periods of intermittent shedding through faeces for up to 6 weeks (Finley *et al.*, 2007) and this is clearly of public health concern. Non-faecal diagnosis of salmonellosis in canines is employed due to the high incidence of asymptomatic carriers; as species identified through faecal contamination could have been there prior to symptomatic presentation as an asymptomatic strain. Therefore specimens originating from blood, cerebral fluid, affected tissue, or transtracheal washing are used. Leucocytes present in faeces however are indicative of infection (Fox, 1991).

Treatment of acute infections tends to be by replenishment of water and electrolytes. Antimicrobial intervention is deemed undesirable to avoid exacerbating the issue of antibiotic resistance of bacteria. However, systemic infections, due to their severity, require antimicrobial treatment, typically: trimethoprim-sulfonamide combinations, ampicillin, flouroquinolones and third generation cephalosporins. The selection of antibiotics is based upon antimicrobial sensitivity patterns. Conversely, in some cases it has been observed that clinical signs shown by dogs are treated swiftly and blindly with a broad spectrum of antibiotics as diagnosis is sometimes considered too time consuming. This questionable prescribing practice contributes to the growing number of *Salmonella* isolates being resistant to available antibiotics.

1.6.1 Transmission of *Salmonella* in dogs

Dogs are potentially at high risk of contracting *Salmonella* as they employ indiscriminate eating habits largely associated with scavenging and hunting for small animals including rodents, wild birds and reptiles. This tendency, coupled with the

ubiquitous nature of *Salmonella*, increases the exposure of the microorganism to the host. Major sources of *Salmonella* contamination tend to be linked to contaminated water and food, associated heavily with meat products (mainly chicken), offal and meat bone meal as the frequency of contamination within these food sources is relatively high (Carter & Quinn, 2000; Finley *et al.*, 2008; Morley *et al.*, 2005). However, transmission in dogs has been reported to be largely associated with the faecal-oral route (Tanaka *et al.*, 1976b) via carrier animals and their faeces and vomit; this route of transmission is exacerbated by the copraphagic tendencies of many dogs (Carter & Quinn 2000; Finley *et al.*, 2006). The cycle of infection and risk of cross-contamination is formed by *Salmonella's* occupation of the lower intestinal tract, from where it can be excreted within faeces into the environment. Bone and Raw Food (BARF) is a regime where companion dogs are fed a diet based upon raw meat. Owners often claim the diet results in improved immune function and overall health, increased energy, improved coat and skin condition and decreased body odour (Joffe & Schlesinger, 2002). However, there are no published data to support this anecdotal evidence. In fact, published studies have shown that the 50% of dogs fed a BARF diet were positive carriers of *Salmonella* and the control group, fed a non-raw meat diet, were all negative for *Salmonella* carriage (Finley *et al.*, 2006). A further study observed that up to 80% of BARF diet dogs were positive for shedding *Salmonella*, specifically *S.* Typhimurium, *S.* Heidelberg, *S.* Kentucky, and *S.* Newport serotypes. (Lefebvre *et al.*, 2008: Morley *et al.*, 2006). This particular study also demonstrated that 30% of commercially fed dogs were also asymptomatically shedding *Salmonella.* This demonstrates that *Salmonella* can be transmitted through a number of sources and highlights elusiveness of *Salmonella* eradication (Joffe & Schlesinger, 2002).

1.6.2 Mechanisms of host adaption and restriction

The importance of studying infectious diseases in humans caused by zoonotic pathogens, particularly those associated with animals in the human food chain and companion animals, is paramount. Zoonotic pathogens exhibit a phenotype which allows them to maintain the ability to colonise and potentially cause infections in more than one host species. Conversely, some strains of pathogens, as observed within the Salmonellae, are significantly host restricted, or adapted, and are generally only able to cause disease in one host.

The analysis of the genetic material of *Salmonella* serotypes reveals that there is a common ancestor that existed 25 to 40 million years ago and the ability of this bacterium to adapt to the host environment for successful pathogenicity has led to the evolution of different *Salmonella* strains from this common ancestor (Bäumler, 1998). Genetic changes have been associated with bacterial host adaptation (Toft & Andersson, 2010). Host adaption in *Salmonella* is demonstrated at a species level, *Salmonella enterica* has adaptation to cause infection typically in warm blooded hosts whereas *Salmonella bongori* has evolved to infect cold blooded hosts; for example snakes and lizards. Strains that have a high association of isolation with a particular host causing systemic infection are considered to be host adaptive or restrictive.

Salmonella enterica serotype Typhi and Paratyphi are host adapted to humans causing a perfuse infection, typhoid fever. As these serotypes show low levels of genetic variation whole-genome sequences using 454 (Roche) and Solexa (Illumina) technologies on a panel of *S.* Typhi have been investigated and revealed that evolution in the Typhi population seems to be characterized by ongoing loss of gene function,

consistent with a small effective population size. A strong adaptive selection for mutations conferring antibiotic resistance in *S.* Typhi has been reported. Furthermore, observed patterns of genetic isolation and drift are consistent with the proposed key role of asymptomatic carriers, with a chronic infection (Holt *et al.*, 2008). For example mutations in gene *gyrA* are known to adaptively select for fluoroquinolone resistance in *S.* Typhi serotypes. These revelations in evolutionary dynamics are important for understanding and applying implications for the control of typhoid (Holt *et al.*, 2008).

The serotype *Salmonella enterica* Dublin is typically associated with infection in cattle and is considered host adaptive to this host. *Salmonella enterica* serotype Choleraesuis are typically isolated from pigs and the from the slaughter houses they are taken, which is significant in the human food transmission route (Paulin *et al.*, 2002).

Wild birds have been associated with the carriage of *Salmonella*, and studies have reported a host restricted strain of *S.* Typhimurium variant Copenhagen phage type 99 in pigeons (Pasmans *et al.*, 2003).

1.6.3 Zoonosis

Salmonella are among a number of pathogens capable of causing clinical infections in both humans and animals; these pathogens are termed zoonotic. Zoonosis is defined by The World Health Organisation (WHO) as: “any disease or infection that is naturally transmissible from vertebrate animals to humans”. Isolates from serotypes such as *S.* Typhimurium and *S.* Enteritidis predominantly retain the ability to infect more than one mammalian host, including humans and are regarded as zoonotic strains (Thorns, 2000).

Zoonotic infections can be caused by microorganisms including bacteria, viruses, fungi, and parasites. There are a number of transmission routes for zoonotic infections, often and largely associated with the ingestion of faecal-contaminated food, drink or materials from the shared environment; through to skin and mucous membrane contamination via bites, scratches or other direct animal contact. Arthropods and invertebrates, fleas, can also act as vectors for transmission, and are commonly associated with unclean animals.

Zoonotic infections thrive in individuals that are immunocompromised which include the elderly, pregnant women, neonates and adults with a predisposing disease. Nonetheless and more clinically significant, they can also cause infection in healthy hosts (Mani *et al.,* 2009). Instances of well recognised zoonotic infection include bubonic plague from fleas, Lyme disease from deer and CJD from cattle (Baum, 2008). Table 1 lists a number of zoonotic infections and their associated microorganisms.

Table 1.1 Zoonotic diseases that are common in companion animals, grouped by pathogens (Mani *et al.*, 2009).

Type of disease	Zoonotic disease	Causative microorganism/ agent
Bacterial	Campylobacteriosis	*Campylobacter spp*
	Salmonellosis	*Salmonella*
	Bordetellosis	*Bordetella bronchiseptica*
	Bartonellosis	*Bartonella spp*
	Capnocytophagosis	*Capnocytophaga spp*
	Pasteurellosis	*Pasteurella multocida*
	Yersiniosis	*Yersinia pestis*
	Tularenia	*Francisella tularensis*
	Leptospirosis	*Leptospira spp.*
	Methicillin-Resistant *Staphylococcus* Aureus (MRSA)	*Staphylococcus aureus*
	Canine Brucellosis	*Brucella canis*
	Mycobacteriosis	*Mycobacteria*
	Chlamydiosis	*Chlamydophila psittaci*
	Coxiellosis (Q-fever)	*Coxiella burnetii*
Viral	Lymphocytic Choriomeningitis	
	Canine influenza	Influenza virus
	Monkeypox	Orthopoxivirus
	Cowpox	Eurasian Orthopoxvirus
Helminths	Intestional nematodes (Roundworms)	*Toxacara* spp.
	Echinococcosis	*Echinococcus* spp.
	Dipylidiasis	*Dipylidium caninum*
Protozoa	Cryptosporidiosis	*Cryptosporidium spp.*
	Giardiasis	*Giardia spp*
	Toxoplasmosis	*Toxoplasma gondii*
Fungal	Systemic mycoses	*Blastomyces dermatiditis, Histoplsma capsulatum, Coccidioides immitis*
	Sporotrichosis	*Sporothrix schenckii*
	Dermatophytosis	*Microsporum*

Cryptococcosis	*Cryptococcus neoformans*
Malassezia	*Malassezia pachydermatis*

The majority of emerging infectious diseases are zoonotic and it is of interest to understand and be aware of them as they are less controllable than human to human diseases (Baum, 2008). Additionally, zoonotic disease awareness is increasing due to the intense and growing exposure of Human-Animal Interaction (HAI), particularly in the now globally connected world. More so than ever there are an increasing number of pet owners; about 50% of all households in the Western world are home to a companion animal, primarily cats and dogs (PFMA, 2011).

As *Salmonella* is ubiquitous in nature its prevalence in the environment means that there is an increased risk of humans and animals being exposed to this organism which is a concerning given its zoonotic potential. In addition, many animals are reservoirs of *Salmonella* as they harbour the bacteria in the intestines and can also asymptomatically shed the organism into the environment.

1.6.4 Anthroponosis

An anthroponotic infection is when an agent causing disease in humans is capable of symptomatic infection in animals. It is presumed that many diseases are the result of an animal reservoir which transmits the disease to humans and surrounding environments, however, there are reports of humans transmitting disease to animals. Diseases such as Methicillin Resistant *Staphylococcus aureus* (MRSA) infection from humans have been reported in dogs (Duquette & Nuttell, 2004; Manian, 2003) and is of particular concern due to the fact these bacteria are resistant to many available antibiotics. A human host with a zoonotic disease could potentially infect other animal hosts, where both animal and human hosts act as reservoirs for the microorganism and the disease is considered zoonotic and anthroponotic.

1.7 Human-Animal-Interactions

Human-Animal-Interactions (HAI) is considered as a relatively young discipline, however the importance of HAI are becoming much more apparent and will be explored later in this section. This field embraces people's association with all kinds of animals including companion pets, wildlife, therapy, agricultural, zoo and laboratory animals (McCune & Serpell, 2012). Nowadays, interaction with companion animals is more intimate as a large proportion of households keep them indoors and share their living area, including bedrooms. Smaller households due to factors such as family breakdown and separation, and a growing aged population, means companion animals play an important role in healthy child development and healthy ageing (McCune & Serpell, 2012).

The Pet Food Manufacturer's Association (PFMA) have recorded numbers of companion animal ownership since 1965 and have demonstrated an increase in companion dog owners from 4.8 million to 8 million (PFMA, 2011). Their role in society has also changed dramatically from labour intensive roles to a more intimate companionship role (Owen *et al.*, 1983). It is estimated that 50% of UK households have at least one companion animal, which is equivalent to approximately 13 million, and of these 8 million households (23%) own a dog and 8 million (19%) own a cat. A large number of UK households own small pets too which include, fish (20-25 million), rabbits (1 million), caged birds (1 million), lizards (300,000), snakes (200,000) and many more (PFMA, 2011). The popularity of small and exotic pet ownership is growing (Redrobe *et al.*, 2010). Having a companion pet has often been considered a modern practice associated with a Western affluence and materialism, however,

historical findings suggest that pet keeping stems from neither and that it has been practised continuously throughout human history (Serpell, 2011).

The study of HAI and child development is expanding as advantageous associations have been shown. An increase in cognitive and language development has been demonstrated in children with an association with companion animals. Furthermore, an association with an enhanced immune system is coupled with a decrease in the likelihood of developing certain kind of allergies (McNicholas *et al.*, 2004). Companionship with animals has shown an encouraging trend in health benefit, including promoting physical and mental well-being, in all ages of the population. People whom share their living environment with a companion animal have shown to have healthier physiological responses to stress indicated by a lower level of cortisol in the blood, self-reported anxiety, lower baseline heart rate and blood pressure. In addition, people demonstrate less cardiovascular reactivity to, and a faster recovery, from mild stressors (Allen *et al.*, 2001; Allen *et al.*, 2002).

As the ageing population increases so does the concern for a healthy ageing process allowing the individual to retain independence and quality of life by preserving physical, mental and social well-being (McCune & Serpell, 2012). Reports in older adults have demonstrated those having a strong attachment to a companion animal showed a lower rate of having a recent illness than those who were less attached (Garrity *et al.*, 1989). Furthermore, a physical improvement is seen with companion dog owners which are due to the walking and chores that are associated with caring for the dog (Thrope *et al.*, 2011).

The use of companion animals in a therapeutic setting is also increasing especially with children with physical and mental disabilities (Allen & Blascovich 1996; Martin & Farnum, 2002). Animal-Assisted Intervention (AAI) is a term describing a situation where an animal is brought into a therapeutic setting. These interventions are currently adopted in hospital, nursing homes, schools, universities, work places, prisons, community centres and mental care settings. Studies have shown that animal assisted visitations contribute to human welfare and the prevention of many diseases including cardiovascular diseases, stress reduction and emotional strengthening (Mani & Maguire, 2009). The benefits given by AAI are presumed to be derived from their ability to demonstrate appropriate behaviour and behavioural consequences, and by providing opportunities for participants to learn and practise alternative behaviours without the fear of criticism or rejection that can occur in human-human interactions (McCune & Serpell, 2012).

The interactions between the human body's immune system, nervous system and psychological process have demonstrated a relationship and the study of these interactions is called Psychoneuroimmunology; it is regarded as one of the most popular Complementary and Alternative Medical (CAM) techniques. Studies have demonstrated that during stressful periods the body induces the sympathetic nervous system along with the endocrine system which in turn produces a negative effect on the immune system (Chrousos & Gold, 1992; Glaser & Kiecolt-Glaser, 1994). Moreover, studies have also demonstrated the positive effects on the immune system when there has been a positive psychological effect on the patient. These positive effects are associated with HAI's and the physical benefits of having a companion animal are linked with the enjoyment of a companion animal (Barker *et al.*, 2008; Jorgenson,

1997). The inter-relationship with companion animals however is also a growing public health concern regarding animal related injuries, including animal bites and zoonotic diseases which can impact on health conditions especially in immunocompromised individuals (McCardle *et al.*, 2010).

The contemporary scientific and lay literatures underscore the important role pets play in the lives of people and within society as a whole (Melson, 2003). Many studies have demonstrated health benefits of pet ownership (McCardle *et al.*, 2010) although there is still a need for further research including zoonotic and anthroponotic diseases such as *Salmonella* infection (Esposito *et al.*, 2011; Herzog, 2011). The promise of these studies needs to be balanced by the potential challenges and risks of pet ownership, most of all of which can be addressed through responsible pet ownership to enable a healthy, happy relationship (Haverkos *et al.*, 2010).

1.8 Isolation and characterisation of *Salmonella*

Humans in contact with *Salmonella* infected animals are at risk of contracting the pathogen and may present with acute gastroenteritis or even systemic enteric fever. As mentioned previously gastroenteritis is a self-limited disease however can be fatal in immunocompromised individuals, therefore it is paramount that the causative agent is isolated and identified for the appropriate antibiotic treatment to be applied. In a clinical setting a rapid diagnosis using serological testing is favoured for very young children and the immunocompromised. However there are many specialist media available for the isolation of *Salmonella* from food, environment and faecal specimens as discussed in the following paragraphs.

1.8.1 *Salmonella* culture media and recovery of sub-lethally damaged cells

The ubiquitous nature of *Salmonella* has led to the development of a variety of culture media and methodologies each with a particular focus on isolating organisms from clinical, environmental or food matrices (Bauwens *et al.*, 2006; Hendriksen, 2003;Silliker *et al.*, 1964). Specimens presented for analysis are wide ranging including complex bacterial communities in faecal material and environmental swabs and sub-lethally damaged organisms following food processing. The method selected for isolation and enumeration of *Salmonella* must therefore be appropriate to inhibit competing microflora whilst encouraging the recovery of injured *Salmonella* cells. Failure to inhibit competitors can lead to overgrowth and too selective an environment may kill sub-lethally damaged organisms leading to false-negative results. In some cases isolation is as simple as direct culturing whereby the sample taken is from a sterile source of *Salmonella*.

Standardised protocols for the isolation of sub-lethally damaged *Salmonella* from complex environments have been described and widely implemented in routine diagnostic laboratories (HPA, 2008a & 2011b). Whilst some variability in the detail of the methodology may be observed, the generalised protocol follows a stage of pre-enrichment, followed by selective-enrichments and finally isolation of recovered *Salmonella* onto selective agars. The role of each of these stages is described below.

1.8.1.1 Pre-enrichment

The viability state of *Salmonella* is dependent on the specimen presented for analysis, which are typically from hostile environments. Bacteria may be feeble or sub-lethally injured and will require a pre-enrichment stage for successful recovery. The isolation of these damaged pathogens is valuable as they may still have the capacity to elicit an infection under optimal conditions (Corry *et al.*, 1969).

Lactose broth (LB) is a widely employed non-selective pre-enrichment broth used for the detection of coliform bacteria in water, foods, and dairy products as per standard methods. The broth is composed of lactose, peptone and vegetable or beef extract. Beef extract and peptone provide carbon and nitrogen sources for general growth requirements, while lactose provides a carbohydrate source, which *Salmonella* cannot ferment. The use of large quantities of peptone is employed in microbiological enrichment media as it serves as a highly nutritious base. Buffered Peptone Water (BPW) was designed as a modification of LB as lactose fermentation created a potentially detrimental acidic environment. Studies demonstrated that BPW was superior to the LB media using artificially inoculated food specimens (Edel & Kampelmacher, 1973; Hilker, 1975). Additionally, M9 pre-enrichment media, composed of ammonium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium chloride has a similar buffered effect on the pH of the media during exponential growth of *Salmonella*; however, BPW is still the established media of choice.

A common non selective enrichment broth which is widely used in the isolation of *Salmonella* is selenite F. It also comprises of lactose as a carbohydrate which bacteria,

such as enterococci and coliforms ferment, producing and maintaining an acidic environment. However there are many disadvantages of using this media including reduced self-life and producing toxic metals that can cause infertility.

1.8.1.2 Selective enrichment

Following pre-enrichment a selective enrichment stage is typically employed. The role of selective enrichment media is to manipulate unique metabolic capabilities of *Salmonella* and to exploit the capacity to withstand biochemicals, suppressing competitors within the sample. During the last 10 years Rappaport-Vassiliadis broth has established itself as a reliable enrichment medium (Busse, 1995) and is habitually coupled with the use of BPW. The original formulation described was specifically developed to exploit the four characteristics of *Salmonella* species when compared with other Enterobacteriaceae (Konforti, 1956). The RV broth manipulates distinctive characteristics of *Salmonella*: survival in relatively high osmotic pressure, growth in low pH, relatively resistant to malachite green and to survive in nutrient limiting condition. Tetrathionate is another established selective broth and is dependent on the combination of thiosulphate and tetrathionate to suppress commensal coliform organisms. *Salmonella* and *Proteus* species are positive for the possession of the enzyme tetrathionate reductase therefore allowing growth in the medium*; Escherichia coli* and *Shigella* are negative for the enzymes and fail to growth (Pollock & Knox 1944).

1.8.1.3 Selective agar

Culturing of *Salmonella* onto plating media allows for isolation and quantification. Plating media employ a selective role for the target bacteria and an inhibition of competitor bacteria. Selective media are designed to differentiate *Salmonella* from

other species according to their appearances on the agar. Selective plating media is a comprehensive area and choice of selection is typically dependent on cost and specimen sample. Each agar is designed with a specific mode of action and modifications of established agars are constantly being made for better and reliable confirmation.

A well-recognised agar for *Salmonella* identification is Xylose Lysine Desoxycholate agar (XLD), which was initially designed for the isolation of *Shigellae* from faecal specimens (Taylor, 1965). The agar exploits xylose fermentation, lysine decarboxylation and production of hydrogen sulphide (H_2S) which can be used also for the discrimination of *Salmonella* from other bacteria. The lysine and hydrogen sulphide inhibit the growth of *Shigella* and coliforms are inhibited by the addition of sodium desoxycholate. Inhibition of non-pathogenic bacteria is carried by a low pH level produced by the fermentation of lactose and sucrose which cannot be counteracted by the alkaline products of lysine metabolism by unwanted bacteria. Another agar which shares the same mode of action is Xylose Lysine Tergitol-4 agar (XLT-4) where XLD has been modified by the addition of tergitol 4 (niaproof), an ionic surfactant, which has an inhibitory effect against *Proteus, Pseudomonas and Providencia* (Miller *et al.*, 1991). The appearance of *Salmonella* colonies in XLD and XLT-4 is black on a red phenol agar base. The phenol agar is pH sensitive which results in the colour changes observed. *Salmonella Shigella* agar (*SS*) is a pink-red media where *Salmonella* colonies appear colourless with a black centre. Exploitation of the hydrogen sulphide (H_2S) production of *Salmonella* allows for growth, however, *Proteus* spp have been reported to give false positives results (Dixon, 1961).

The use of high quantities of peptone as a source of nutrient for bacterial growth and is considered a mode of action of Hektoen Enteric agar (HE). As well as high peptone content, an antibiotic novobiocin is added for inhibition of competitor microorganisms. Bile salts and the dyes bromthymol blue and acid fuchsin inhibit the growth of most Gram positive organisms. Lactose, sucrose, and salicin provide fermentable carbohydrates to encourage the growth and differentiation of microorganisms. Sodium thiosulphate provides a source of sulphur. Ferric ammonium citrate provides a source of iron to allow production of hydrogen sulphide from sodium thiosulphate, which provides a source of sulphur. Ferric ammonium citrate also allows the visualisation of hydrogen sulphide production by reacting with hydrogen sulphide gas to form a black precipitate. *Salmonella* present as dark green-blue colonies against a green agar base after 18-24 hour incubation (Microbugs, 2012). The only exception is for serotype Typhi, which is a weak H_2S producer.

Brilliant Green Agar (BGA) is well-established and has been selected due to its ability to isolate *Salmonella* from a diverse range of food and faeces specimens (Koyuncu & Haggblom, 2009). *Salmonella* present as red colonies after an 18-24 hour incubation period. However, identification of *S.* Typhi cannot be performed on this media. Another form of BGA is Mannitol Lysine Crystal Violet Brilliant Green Agar (MLCBA) which is also designed for faecal and food specimens. The morphology of *Salmonella* is unique on this agar and is described as a mauve colony with a black centre which is dependent on the concentration of magnesium ions (Cassar & Cuschier, 2007). Conversely, MLCBA has a relatively weak selectivity as the property of the agar can be negatively affected by heavily contaminated samples containing inhibitory bacterial species, therefore prior use of selective pre-enrichment is necessary and MLCBA is

coupled with RV broth. Hydrogen sulphide production is manipulated again which gives rise to the dark purple colonies and the metabolism of mannitol fabricates black centres. The agar was designed to selectively isolate non-lactose fermenting *Salmonella*, such as *S. arizonae* however false positives may occur if *Citrobacter* are also present in the sample. Bismuth sulphite agar is a selective agar where *Salmonella* present as black or green colonies following an 18 hour incubation period. Furthermore, this agar has the capacity to isolate the atypical *S.* Typhi, where they present as black "rabbit-eye" colonies. The selective agent inhibits unwanted coliform growth by the active bismuth sulphite together with brilliant green as a selective agent. The black morphology of *Salmonella* is due to the metallic salts in the medium that stain the colony in the presence of sulphide production. This agar is suitable for samples heavily contaminated with other bacterial species as the media has a potent inhibitory action however atypical colonies could potentially appear if organic matter in the sample is overpowering (Cook, 1952).

Presumptive confirmation is carried out for quick identification of *Salmonella* and Triple Sugar Iron Agar (TSA) and Modified Semi-solid Rappaport Vassiliadis (MSSRV) agar are routinely used for this purpose. However as colonies are only presumed to be *Salmonella* they are typically plated onto other agars for confirmation. The TSA works on the ability of *Salmonella* to ferment lactose, sucrose and glucose, and to produce hydrogen sulphide.

1.8.1.4 Chromogenic selective agars

Brilliance Salmonella chromogenic agar uses an Inhibigen™ compound comprised of two components, combined together by a bond that can only be cleaved by a specific

enzyme. *Escherichia coli* produce this enzyme intracelluarly and the cleaving of this compound is toxic, causing cell lysis. *Proteus* spp. and *Pseudomonas* spp. inhibition is carried out by the addition of the antibiotic novobiocin and cefsulodin. *Salmonella* colonies are identified as purple against the white agar and this colour change is achieved by the action of two chromogens that also target specific enzymes: caprylate esterase and ß-glycosidase. Caprylate esterase is an enzyme present in all *Salmonella*e as well as some species of *Klebsiella, Enterobacter* and *Proteus* (Cooke *et al.*, 1999). Organisms possessing caprylate esterase cleave the chromogens to release an insoluble purple chromophore. Chromophore accumulates as the cells grow which produces the purple colouring. A blue colouring is seen for bacteria that possess ß-glycosidase.

Novel *Salmonella* agars incorporating chromogenic substrates have become commercially available and are a popular choice for bacterial identification (Cooke *et al.*, 1999). Rambach agar (RAM) was designed to exploit *Salmonella* ability to produce acid from propylene glycol and the selective nature is derived from the presence of bile salts. Positive colonies appear crimson-red and other Enterobacteriaceae appear as blue-green as a result of the chromogenic substance 5-bromo-4-chloro-3-indole-β-D-galacttosidase. Typhoid causing serotypes *S.* Typhi and *S.* Paratyphi do not produce the acid for the colour change and appear colourless on the agar which makes them difficult to identify (Kühn *et al.*, 1994; Pignato *et al.*, 1995).

1.9 Typing methods

As well as identification by media preparations *Salmonella* are also identified by phenotypic and genotypic methods. Infections caused by *Salmonella* in animals and humans can be controlled and are typically isolated incidences, however outbreaks can

occur which require further investigations to isolate the source of the infection and introduce measures to control the situation. Epidemiological investigation centres have been adopted by many nations and have established extensive surveillance systems to track *Salmonella* infections and disrupt epidemic spread (Winokur, 2003). If outbreaks, largely foodborne, occur then distinguishing of the epidemic strain from the other strains occurring in the food vehicle or its source is necessary and this is done by phenotypic and genotypic techniques. There are numerous phenotypic and DNA profiling techniques that are used to characterise and identify causative *Salmonella* strains which differ in their discriminatory ability.

1.9.1 Phenotypic methods

Phenotyping is a technique used to discriminate gene product expression including physical characteristic such as the possession of flagella or cell capsule. The discriminatory capacity of phenotypic techniques can be limited, however novel technologies are emerging and detailed profiles can be harvested from simple phenotypic tests.

1.9.1.1 Serotyping

Serotyping is where extracellular structures called antigens on the cell surface of Salmonellae are recognised by specific antibodies. There are three antigens used to serotype *Salmonella*, O type (somatic), H type (flagella) and Vi (capsular) type; the different configurations of antigen-antibody associations gives rise to the identification of the strain and this method is widely used in epidemiological investigations. Serotyping is carried out using slide agglutination which was first described in 1896 by

Widal who observed the phenomenon with *S.* Typhi (Ley *et al.*, 2010). More recently Enzyme-Linked ImmunoSorbent Assay (ELISA), using rabbit antisera has been produced to test a number of variables in a single assay. Commercial kits are available including the *Salmonella* test kit (Remel) and the Wellcolex colour *Salmonella* kit (Remel). *Salmonella* test kit is a rapid latex agglutination test for the presumptive identification of *Salmonella* and has been designed for rapid screening of clinical samples. The principles behind the test kit are that it uses polyvalent antibodies prepared on latex particles that are designed to agglutinate with a range of *Salmonella* flagella antigens. Association of latex particles to corresponding antigens is demonstrated by clumping of the bacteria and no agglutination is seen with other Enterobacteriaceae. However, test kits are designed for a presumptive identification and therefore confirmation should be carried out using selective agar plating (Oxoid *Salmonella* kit manual). The Wellcolex colour *Salmonella* kit is designed to give a presumptive identification of *Salmonella* at a serotype level. The kit consists of two test reagents containing three antibody latex particles, red, and blue and green which are specific for different *Salmonella* serogroups. Agglutination and colour change will occur when a homologous association between antigen and antibody occurs (Remel Manual). Detailed serotyping is typically undertaken by reference laboratories such as the National Reference Centre for *Salmonella*, the LEP at Colindale U.K. They receive approximately 120 strains per year serodiagnosis which can be restrictive due to the cost of sera (Chart *et al.*, 2007).

1.9.1.2 Phage typing

Phage typing of enteric pathogens, including *Salmonella* has been successfully used to characterise disease-causing agents in epidemiological investigations and for

surveillance. It is a fast and reliable method but depends on the experience of the individual laboratory and on support from the reference centre that coordinates the maintenance of phages and the updating of the system (Baggesen *et al.*, 2010; Demczuk *et al.*, 2003). The principle behind this phenotypic method is that *Salmonella* are typically susceptible to the lysis of bacteriophages producing lysis pattern which are unique to groups of strains. Homologous lysis patterns allow for the identification of strains and this method has been successful with epidemiological investigations in *Salmonella* outbreaks. Phage typing is also typically employed in reference laboratories such as LEP at Colindale for identification of *Salmonella* strains.

1.9.1.3 Biotyping

Biotyping is a technique adopted to identify metabolic characteristics, environmental tolerances and colony morphology. This technique has advantages including good reproducibility and being an easily performed technique that can be practised without a need for expensive equipment. Investigations have shown that using biotyping to discriminate between strains within a serotype gives a poor discriminatory power (Barker & Old, 1989). However, biotyping is a technique commonly used in the clinical microbiology laboratories as many of the microorganisms concerned belong to the Enterobacteriaceae and rapid diagnosis is essential. Historically single tube assay were performed, including investigating pH tolerance to carbohydrate fermentation; however more recently "prepared identification reagent kits", such as the Remel agglutination kits and API test have taken centre stage, providing a 24 hour turn over diagnosis.

1.9.1.3.1Analytical Profile Index API

Salmonella have the ability to utilise a profile of certain carbohydrates that is distinctive from other microorganisms. These differences are exploited in "prepared identification reagent kits" such as, Analytical Profile Index (API) test; which are designed for the identification of different bacteria typically at the Genus level. The identification of *Salmonella* is performed on a 20E API strip which contains 20 biochemical reactions which are listed in Table 2 (bioMérieux®, France).

Table 1.2 The panel of biochemical reagents on an API 20E tests with the corresponding reaction/enzymes being investigated.

Active Component	Reaction/enzymes
2-nitrophenyl-βD- galactopyranoside	B-galactosidase (ortho NitroPhenyl-βD-galactopyranosidase
L-arginine	Arginine DiHydrolase
L-lysine	Lysine DeCarboxylase
L-ornithine	Ornithine DeCarboxylase
Trisodium citrate	CITrate utilization
Sodium thiosulfate	H_2S production
Urea	UREase
L-tryptophane	Tryptophane DeAminase
L-tryptophane	INDole production
Sodium pyruvate	Acetoin production
Gelatin	GELatinase
D-glucose	Fermentation/ oxidation (Glucose)
D-mannitol	Fermentation/ oxidation (Mannitol)
Inositol	Fermentation/ oxidation (Inositol)
D-sorbitol	Fermentation/ oxidation (Sorbitol)
L-rhamnose	Fermentation/ oxidation (Rhamnose)
D-sucrose	Fermentation/ oxidation (Saccharose)
D-melibiose	Fermentation/ oxidation (Melibiose)
Amygdalin	Fermentation/ oxidation (Amygdalin)
L-arabinose	Fermentation/ oxidation (Arabinose)

Novel biochemical identification kits are becoming popular as they are designed to give rapid diagnosis and are more comprehensive than the conventional API kits. The Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ (Technopath, Ireland) is a novel rapid identification biotyping system capable of producing Phenotypic Microarrays (PM). It has the capacity to identify over 2,500 species of aerobic and anaerobic bacteria, yeasts and fungi. The system uses a simple rapid protocol and results are very reliable. They provide multiple numbers of biochemical reactions on one simple panel for both Gram- positive and Gram-negative bacteria (Biolog 2012). This form of phenotyping employs a wide range of biochemical reactions including the utilisation of amino acids, salts, carboxylic acids, esters, fatty acids, hexose acid, hexose phosphates and reducing agents. This system has proven its reliability and accuracy with foodborne pathogens, although performing multiple tests could be expensive (Odumeru, 1999). Phenotypic Microarrays have the potential to evaluate nearly 2000 phenotypes of a microbial cell and make it possible to quantitatively measure thousands of cellular phenotypes simultaneously. According to the manufacturer's claims, the assay can provide cell's metabolic and chemical sensitivity properties characterise cell phenotypes for taxonomic or epidemiological studies and even allow for the discovery of new targets for antimicrobial compounds (Biolog, 2012). The vast amount of physiological information collated from these plates is a powerful approach to studying cells because it seeks to understand and enumerate the various subsystems that function within cells (Bochner, 2009).

1.9.1.3.2 Susceptibility testing

Antimicrobial susceptibility testing (AST) is a well-established method for phenotypic profiling of microorganisms. Sensitivity patterns produced are called antibiograms and

primarily allow for correct dosage dispensing and selection of antibiotics for the successful treatment of infections. The technique is highly favoured as it is easy to perform, inexpensive, rapid and highly quality controlled. However, for epidemiological purposes, AST has poor discriminatory capacity. As antibiogram profiles tend to change over time, as microorganisms are sensitive to environmental pressures, this limits the application of AST as a powerful epidemiological typing methodology.

Minimum Inhibitory Concentration (MIC) is an example of an AST technique. It is considered the "gold standard" for the determination of bacterial susceptibility to antimicrobials and is also used as a benchmark to determine the performance of all other methods of susceptibility testing (Andrews, 2001). There are two main techniques of MIC determination; agar dilution plates and broth dilution. The microorganism is investigated for visible growth on agar plates or growth in broth containing antimicrobial agents, after an 18-24 hours incubation period (EUCAST, 2000). Following incubation the lowest concentration at which growth of the microorganism is inhibited is termed the MIC. The Minimum Bactericidal Concentration (MBC) can also be determined using the dilution technique and is the concentration of antimicrobial agent that demonstrates no detectable survivors following a specified contact time, typically suggesting a 99.9% bactericidal effect (Gilbert & McBain, 2003). In addition to determining antibiotic sensitivity of bacterial isolates, MIC is a widely employed technique in epidemiological investigations. Harmonised published protocols including, British Society for Antimicrobial Chemotherapy (BSAC, UK), European Committee on Antimicrobial Susceptibility Testing (EUCAST), and Clinical Laboratory and Standards Institute (CLSI, USA), allow for sensitivity patterns to be produced from

isolates. Additionally, these patterns or profiles are then compared with outbreak suspected isolates to identify indistinguishable profiles (Lawson *et al.*, 2004; Murphy *et al.*, 2001; Thurm & Gericke 1994).

Antibiotic Disc Susceptibility (ADS) is another AST technique, which uses sterile paper discs impregnated with known concentrations of antibiotic. The antibiotic discs are applied to a lawn of bacteria on an agar plate. The customary level of density for bacterial suspension is at 0.5 McFarland standard as this has been demonstrated to bereliable (Andrews, 2001b). Too heavy an inoculum produces varied reproducibility and too light produces errors. Following incubation for 24 hours inhibition caused by the antibiotic can be observed by clear zones around the discs indicating bacterial lysis. These zones are measured and analysed using the BSAC breakpoint tables (Andrews, 2001b). Zones of inhibition can vary with discs from different manufacturers due to differences in the testing methods adopted by manufacturers in various countries (Brown & Kothari 1975). Therefore, continuity of disc type is essential for data to be comparable. Frequent checks for method updates are required as methodology changes, breakpoint changes and the introduction of new antibiotics can have a significant impact on the interpretation of the results obtained. Antibiotic sensitivity testing has clinical significance in the treatment of individual patients and it is an essential test for monitoring levels of resistance and is an employed technique used for epidemiological investigation. These data influence prescribing practices and also contributes to knowledge for the development of novel antimicrobial agents.

1.9.2 Genotypic methods

Advances in molecular biology have allowed the detailed comparison of *Salmonella* strains at a genotypic level and have found major application during epidemiological investigation of outbreaks. Established techniques for *Salmonella* are based on restriction endonuclease digestion of DNA (Enter-net/Salm-gene, 2012), plasmid profiling (Morshed & Peighambari, 2010) and amplification using PCR-based methods (Cohen *et al.*, 1996). DNA isolation as a precursor for all these techniques and is key but different methods employ different strategies of DNA preparation (Cohen *et al.*, 1996; Kawasaki *et al.*, 2005). Following DNA preparation the genome can be manipulated through digestion or amplification to provide comparative banding patterns.

1.9.2.1 Pulse Field Gel Electrophoresis

Restriction endonuclease digestion requires the isolation of DNA following cell lysis of the microorganism, which is then digested to form large DNA fragments and subsequently separated by electrophoresis techniques. A gold standard genotypic method for *Salmonella* is Pulsed-Field Gel Electrophoresis (PFGE) as it is a reliable method for the identification of epidemiologically related strains (Zou *et al.*, 2012). Digestion of DNA isolated from *Salmonella* with the restriction endonuclease *Xba*I produces a few large fragments (approximately 1.2 megabases in size) due to the relatively low number of recognition sites (Schwartz *et al.*, 1983). Prior to the restriction digestion *Salmonella* is embedded in an agarose gel matrix and subjected to cell lysis by the aid of Proteinase K, Sarkosyl, Tris-HCL and EDTA. Expelled DNA remains suspended in the gel and is then vulnerable to *Xba*I digestion; complete digestion is essential for the production of clear discriminatory banding patterns.

Agarose slices of DNA fragments are then added to an agarose gel ready for separation by an interval switching current. Electrophoresis is performed in a Contour Clamp Homologous Electrophoresis (CHEF) cell which is fabricated with a hexagonal electrode allowing current to flow in three directions. Migration of fragments is carried out by the short pulses and continual shift in direction of current throughout the electrophoresis run. Visualising of fragments requires a DNA stain which is revealed under UV illumination. Banding pattern analysis is some what complex however guide lines for the interpretation of banding patterns have been produced as well as software systems, such as GelCompar and Bionumerics (Bionumerics, Belgium) are widely used for standardisation (Tenover *et al.*, 1995). The PFGE technique is regarded as highly discriminatory and reproducible due to the harmonised protocol which enables worldwide comparisons of PFGE profiles (Enter-net/Salm-gene, 2012). The PFGE technique however is time consuming with some protocols taking up to six days (Matushek *et al.*, 1996). Specialist equipment including the CHEF cell and reagents are additionally expensive (Rollason, 2007).

1.9.2.2 Plasmid extraction and profiling

Plasmid profiling is a well-established typing method for *Salmonella* and is used alongside phenotypic methods including phage typing and biotyping. Plasmids typically range from 2-150Kb in size, are autonomously replicating extra chromosomal DNA and considered the best understood mobile elements in bacteria. Plasmid analysis differentiates strains by the number and size of harboured plasmids which are visualised through gel electrophoresis separation (Weller *et al.*, 2000). Profiling is relatively inexpensive and rapid with profile generation within two hours from simple overnight cultures (Navia *et al.*, 1999). Some serotypes can carry different plasmid

DNA however if size and frequency are the same then they may present with an indistinguishable profile. Additional strain differentiation may be provided by performing a restriction endonuclease step following plasmid extraction (Singh *et al.*, 2006). Since the prevalence of plasmids in *Salmonella* species was reported over four decades ago there have been many reports that plasmid profile analysis is useful as an epidemiological tool in outbreaks of salmonellosis. Plasmid profile analysis of *S.* Typhimurium was thought to be at least as reliable as phage typing in identifying related or unrelated isolates from outbreaks (Nakamura *et al.*, 1986). Plasmid analysis has been important and successful in identifying epidemic strains of *S.* Enteritidis and *Escherichia coli* O157:H7 (Wachsmuth *et al.*, 1991).

1.10 *In vitro* assays

A standard approach for the investigation into cell permeability, transcytosis mechanisms, adhesion and invasion of bacteria into eukaryotic cells is using an *in vitro* assay (Friis *et al.*, 2005). Investigations into the nature of host-bacterial interactions are also carried out using *in vivo* animal models to demonstrate host-restriction in particular host cells (Graves *et al.*, 2012) however *in vitro* model investigations have contributed much more knowledge of this interaction (Friis *et al.*, 2005).

Infections by zoonotic *Salmonella* in human and animal hosts are associated with invasion into the Peyer's patches and necrosis of M-cell epithelia in the lower intestinal tract. *In vivo* the epithelial layers are highly polarised with the basolateral membrane interfacing with cells in the lamina propria; apical membranes are organised facing the luminal contents. Highly polarised cells are difficult to establish *in vitro* however they provide more information on the complex interaction of host and bacteria cells during

invasion. *In vitro* cultures however are non-polarised; fortunately the function of the membrane is not dependant on the polarity of the cell and transport and cellular localisation of surface components such as Toll like receptors (Backhed & Hornet, 2003; Gewirtz *et al.*, 2001; Monstov *et al.*, 2000). *In vitro* models also reduce the use of animal models.

The Gentamicin Protection Assay (GPA) (invasion assay) is an established *in vitro* method used for the investigation of bacterial invasion into eukaryotic cells (Byrne *et al.*, 2007; Cossart & Sansonetti 2004; Flentie *et al.*, 2008). Typically *Salmonella* are sensitive to the antibiotic gentamicin and it also has a limited penetration of eukaryotic cells. Therefore when investigating bacterial invasion, gentamicin is used following incubation of bacteria with the eukaryotic cells to kill non-invading, attached and extra cellular, bacteria in the suspension whilst avoiding inhibition of invaded bacteria which are protected inside the eukaryotic cells. Enumeration of invaded bacteria is carried out following lysis of eukaryotic cells using detergent or water and the subsequent use of standard bacterial plating techniques (Friis *et al.*, 2005). Invasion assays have been exploited to investigate intracellular survival and replication (Friis *et al.*, 2005). Modifications of the method are employed depending on aspects of the hypothesis under investigation. In some investigations the adhesion properties of the bacterium are omitted by a centrifugation step which physical pulls and associates the bacterium to the cells in the monolayer (Friis *et al.*, 2005).

Typically, the eukaryotic cell of choice for invasion assays is the human colon adenocarcinoma (CaCo-2) cell line as they mimic *in-vivo* conditions best (MacCallum *et al.*, 2005). They are an established continuous line of heterogeneous cells that have

the same cylindrical polarized morphology and properties as enterocytes, expressing microvilli, transporters, enzymes and tight junctions. They were developed by the Sloan-Kettering Institute for Cancer Research through research conducted by Dr. Jorgen Fogh and were isolated from colon carcinoma enterocytes (Fog & Trempe, 1975). Different cell lines originating from different organs and animals can be established and manipulated to mimic their functions *in vivo*. For example, Dog Intestinal Epithelial Cells (DIEC) originating from the small intestine of adult beagles created by Andrea Quaroni, Cornell University, New York are a good cell line for investigation of canine gut cells (Personal communication with WALTHAM® Centre for Pet Nutrition). Primary cultures of DIEC are established and subsequently immortalised using a temperatures sensitive mutant of the simian virus 40 large tumour antigen for the use in these assays (SV40 T-Ag**;** these cells were provided by the WALTHAM® Centre for Pet Nutrition)**.**

Confluent monolayers of cells are established onto an adherent surfaces and a known concentration of bacteria is inoculated onto the monolayer. The ratio of bacteria to eukaryotic cells is an important variable, referred to as the Multiplicity Of Infection (MOI), and studies have shown differences in invasion with different numbers of inoculated bacteria up to a maximum (Kusters *et al.*, 1993). Studies in *Campylobacter* models have shown that invasion efficiency is higher at lower MOI's and this efficiency decreases with increasing MOI (Friis *et al.*, 2005). However, two or more MOI's are established for invasion assays as there is variability in invasion capabilities depending on strain and cell monolayer type. Invasion of epithelial cells by *Salmonella*e has been studied extensively (Altmeyer *et al.*, 1993; Elsinghorst *et al.*, 1989; Finiay & Falkow 1989; Lee & Falkow, 1990; Schiemann & Shope, 1991).

Although these studies have increased insights into the invasion mechanisms used by *Salmonella*e, some contradictory observations have been reported and essential questions remain unanswered (Kusters *et al.*, 1993).

Adhesion of bacteria to the eukaryotic cell surface can also be investigated by similar methods. Bacteria cells are washed with buffered reagents following an incubation period and non- associated bacteria are removed. However the gentamicin or selected antibiotic step is omitted and associated bacteria that have attached and invaded are counted following eukaryotic cell lysis (Friis *et al.*, 2005; Kusters *et al.*, 1993). There are many variables that can be targeted to investigate different aspects of bacteria host interactions including incubation time (Finlay *et al.*, 1989), bacteria load concentration (Francis & Thomas, 1996), eukaryotic cell type and supplementing antibiotics use (Kusters *et al.*, 1993; Mroczenski-Wildey *et al.*, 1989). These assays can be used to investigate different capacities in invasion and adhesion of bacterial strains in different host cell lines to reveal any host restricted tendencies.

Aims and Objectives

Historical data has shown that 0-43% (McElrath, 1952), 1-35% (Finley *et al*., 2007), 1.2% (Gorham & Garner, 1951), 4.4% (Galton *et al*., 1952) and 15% (Shimi *et al*., 1976) of non UK canines asymptomatically carry or shed non-typhoid *Salmonella* species. More recently epidemiological investigation of *Salmonella* outbreaks has revealed zoonotic transfer of *Salmonella* isolates from dogs to their owners (CDC, 2008). This highlighted the concern regarding the potential increase of zoonotic infections from companion animals to their owners especially with approximately 50% of western households owning a companion animal with cats and dogs being among the most popular (PFMA, 2011). However, there are limited data on the asymptomatic carriage of *Salmonella* in canines in the UK with the available data being from non-UK sources that are some what dated. During this time the relationship between humans and animals has changed to a much more intimate role and HAI and AAI's are becoming much more popular due to their positive benefits on individuals. Therefore, the first objective of this study was to estimate the current incidence of asymptomatic carriage of *Salmonella* in UK companion dogs.

Recent studies have identified *Salmonella* strains with a limited host specificity suggesting host restricted characteristics (Pasmans *et al.*, 2008). It is currently not known if isolates from canines demonstrate any host specificity or are entirely zoonotic to humans. For these reasons, *Salmonella* strains that cause clinical infections in humans and canines were investigated for zoonotic potential and host restrictive characteristics. In human hosts typhoid fever or enteric fever is caused by a host adaptive *Salmonella* Typhi and it is of interest to establish a potential host adaptive

serotype for a canine host, if there are or is. This study therefore sought to determine if any host adaptive strains of *Salmonella* exist by profiling isolates from canine and human hosts using genotypic and phenotypic methods which were then analysed and compared to reveal any significant associations.

The ultimate aim of this study was to investigate asymptomatic carriage of *Salmonella* in dogs and to investigate their potential for zoonotic infection of humans.

More specifically the aims were to:

- Devise a sampling protocol for the microbiological analysis of *Salmonella* in canine faeces including culture media, sensitivity of detection, storage parameters and timescales for analysis.
- Determine the prevalence of asymptomatic carriage of *Salmonella* species in a selection of UK canines by faecal culture using a validated isolation protocol.
- Genotypically characterise *Salmonella* isolated from canines presenting with symptomatic disease using the HPA harmonised protocol (PulseNet), Pulsed-Field Gel Electrophoresis (PFGE).
- Compare canine PFGE patterns with human clinical PFGE profiles by the generation of dendrograms.
- Characterise a panel of clinical canine and clinical human *Salmonella* isolates using phenotypic microarrays, API 20E, antibiograms and motility confirmation.
- Analyse complex phenotypic microarray data with Principal Component Analysis (PCA), to determine if differences exist between clinical canine and human isolates in their biochemical profiles.
- Determine any potential host adaptive, restrictive or competitive advantage of *Salmonella* strains isolated from canines by conducting adhesion and invasion

assays using CaCo-2 and DIEC, human and dog intestinal epithelial cell lines, respectively.

Chapter 2 The stability of *Salmonella* in canine faeces and development of isolation methodology

2.1 Introduction

The ubiquitous nature of *Salmonella* has led to the development of a variety of culture media and methodologies each with a particular focus on isolating organisms from clinical, environmental or food matrices (Dusch & Altwegg, 1995; Pignato *et al.*, 1995; Ruiz *et al.*, 1996; Schrank *et al.*, 2001). A wide range of specimens are presented for analysis including; complex bacterial communities in faecal material and environmental swabs and sub-lethally damaged organisms following food processing. The method selected for isolation and enumeration of *Salmonella* must therefore be appropriate to inhibit competing microflora whilst encouraging the recovery of injured *Salmonella* cells. Failure to inhibit competitors can lead to overgrowth and too selective an environment may kill sub-lethally damaged organisms leading to false-negative results.

The culture-based method for isolation of *Salmonella* from environmental, faecal samples and processed food samples is well established (HPA, 2012a) and has been widely adopted for many years (Bager *et al.*, 1991; Davies *et al.*, 2000; De Boer *et al.*, 1998; HPA, 2008a, 2011b & 2012a). The first stage is pre-enrichment and is typically in a non-selective, generally supportive broth such as Nutrient Broth (NB) or Buffered Peptone Water (BPW). Buffered Peptone Water contains peptone, sodium chloride, disodium phosphate, and potassium dihydrogen phosphate (Pietzsch *et al.*, 1975). The

use of large quantities of peptone is employed in microbiological enrichment media as it serves as a highly nutritious base. Sodium chloride and disodium phosphate are both salts where the former is considered an essential nutrient used in a wide variety of biochemical applications including the use as diluents to increase ionic strength in buffers or culture media (Sigma, 2012a). Potassium dihydrogen phosphate is a naturally occurring simple inorganic compound which has been employed for its buffer and neutralizing properties; further it is as a nutritional base for yeast (Milling & Richardson, 1995). Inoculated BPW requires incubation at 37^{o}C for 18–24 hours to allow the recovery, growth and multiplicity of sub-lethally damaged bacterial cells. As the media is non-selective, any other bacteria present and capable of growing will also increase in number. This necessitates the second stage in the isolation of *Salmonella* which is to use the pre-enriched sample as inocula for a selective-enrichment broth to continue the preferential growth and multiplication of *Salmonella* whilst inhibiting competitors. Several broths are in routine use with Rappaport-Vassiliadis (RV; Hammack, 1999; Vassiliadis, 1981; Vassiliadis 1983) and Tetrathionate Broth (De Zutter 1991; Hammack 1999; Harvey, 1981) being amongst the most common. The selective nature of the broths is provided by the inclusion of inhibitory components to which *Salmonella* is resistant, such as malachite green in the case of RV broth (Sigma, 2012b).

Plating media for *Salmonella* is comprehensive however is an essential step for *Salmonella* isolation following the pre-enrichment stages. As with selective enrichment broths plating media are designed on *Salmonella* selectivity and non-*Salmonella*

pathogen differentiation. There are a number of commonly employed media including Xylose Lysine Deoxycholate (XLD), Brilliant green, Rambach, Hektoen enteric (HE), and *Salmonella-Shigella*. Modification of selective agars is on-going and a surge of chromogenic based agars are becoming a popular choice. *Brilliance™ Salmonella* (BS) is a chromogenic based agar designed for the isolation of *Salmonella* from food and faecal specimens (Oxoid, 2008a). The agar comprises a two component compound, Inhibigen, that when cleaved by enzymes found intracelluarly in *E. coli*, is toxic and disrupts cell wall integrity. The addition of antibiotics novobiocin and cefsulodin inhibit the growth of other competing flora such as *Proteus* spp. and *Pseudomonas* spp. Differentiation of *Salmonella* from the other organisms that grow on BS agar is achieved through the inclusion of two chromogens that also target specific enzymes: i) caprylate esterase is an enzyme that cleave the chromogen to release an insoluble purple chromophore present in all Salmonellae as well as some species of *Klebsiella*, *Enterobacter* and *Proteus.* ii) ß-glucosidase enzyme that some Enterobacteriaceae, including *Klebsiella* and *Enterobacter* have but not *Salmonella*. If these organisms grow, they will form blue or dark blue colonies, even if they are esterase positive, which make them easy to differentiate from purple *Salmonella* colonies (Yabuuchi, 2001; Cooke, 1999; Oxoid 2008a).

The time period from faecal specimen collection to processing in a laboratory, for the confirmation of a microbiological infection is typically within 48 hours (CDC, 2012), as this limits exposure time of the organism to the external environment hence reducing damage. Processing is carried out in safety cabinets due to the risk of infectious

aerosols as the faecal specimen may contain hazardous group 3 organisms such as *S.* Typhi. The processing of faecal samples, for the confirmation of a microbiological infection, can take up to four days if enrichment stages are utilised. Therefore the faecal samples are typically stored in sterile conditions and held at <-15^{o}C with or without a preserving medium; Cary Blair medium is commonly used for transportation of microbiological specimens (HPA, 2012a).

The aim of part of this chapter was to establish a suitable pre-enrichment, selective enrichment and selective plating media method to be taken forward in this thesis for the isolation of *Salmonella* from canine faecal samples. The knowledge of the stability and characteristics of *Salmonella* survival in faeces is very limited and in this chapter the freezing at -80^{o}C of faecal samples artificially contaminated with *Salmonella* was investigated to demonstrate if this is a suitable storage method for the successful recovery of *Salmonella* from faeces. It was also of interest to investigate the resilience of *Salmonella* in canine faeces in the environment. The reasons being that dogs tend to share living areas with their owners and therefore there is a risk of infection due to contamination of the home environment with faeces; *Salmonella* grow well at room temperatures (24-26^{o}C) and thus are able to increase in numbers in contaminated objects (Chengappa *et al.*, 1993). In addition, dogs require frequent outdoor exercise and many owners walk their dogs on public footpaths and public parks. Unfortunately faecal defecation from dogs is known in these shared public spaces and potential cross contamination through direct or indirect contact may be possible to members of the public and surrounding wildlife.

2.2 Materials and Methods

2.2.1 Microbial Cultures

National Collection of Type Culture (NCTC) strain *Salmonella* Typhimurium 74 was selected from the Aston University culture collection. The microorganism was stored on microbank cryobeads (Microbank™, Pro-Lab Diagnostics, Canada) at -80°C until required.

2.2.2 Microbiological media

Nutrient Agar (NA), Nutrient Broth (NB), Buffered Peptone Water (BPW), Rappaport Vassiliadis (RV), Hektoen Enteric (HE) agar and Xylose Lysine Deoxycholate agar (XLD), and Brilliance™ *Salmonella* agar (BS) were purchased from Oxoid (Basingstoke, UK) and prepared as per manufacturer's instructions and sterilised at 121°C for 15 minutes, with exceptions to XLD, BS and HE which were gently heated allowing the dried powered to dissolve. Agars were cooled in a water bath to 50°C before pouring and broths were stored at 4°C until required.

2.2.3 Faecal samples

Faecal samples for the studies in this chapter originated from either four different breeds of dogs, Labrador retriever ID L1664, miniature schnauzer ID MS104, miniature poodle ID MP4 and cocker spaniel ID COS43 housed at WALTHAM® Centre for Pet

Nutrition (WCPN; Waltham-on-the-Wolds, UK). Faecal samples used for positive and negative controls were taken from dog ID L1664. For the outdoor environmental study a faecal sample was taken from a household companion border collie dog from the Coventry region.

2.2.4 *Salmonella* Typhimurium Calibration curve

A calibration curve was prepared using *S.* Typhimurium (NCTC 74) from the Aston University collection. The isolate was resurrected from -80^{o}C storage by inoculating a NA plate. Following incubation of the NA plate at 37^{o}C for 24 hours a single colony was inoculated into 50mL of NB and incubated at 37^{o}C in a shaking incubator (Orbital shaking incubator, Gallenkamp, UK) at 200rpm. A 100µL sample was taken every 0.5 hours and dilution was performed on the sample (10^{-1}-10^{-8}) in SDW as required. A volume of 100uL of each dilution including the neat was inoculated onto NA. Following incubation of the plates at 37^{o}C for 24 hours the resulting colonies were counted and corrections for dilution and volume was applied to calculate the colony forming units per mL (cfu/mL). Simultaneously, a 1mL sample of the suspension every 0.5 hours was used to measure the Optical Density (OD) at 600nm in a Novaspec LKB spectrophotometer (Pharmacia-Biotech Pty. Ltd.); samples were placed back into the original vessel aseptically. Readings were performed in triplicate of the OD and cfu/mL and presented as Log cfu/mL against OD at 600nm.

2.2.5 Preparation of positive and negative controls

A faecal sample was determined negative for the presence of *Salmonella.* This involved plating a loop full (~10 µL) of the faeces onto selective agars XLD, HE and BS following direct culturing; additionally samples were subjected to enrichment culturing as described in section 2.2.6. Following incubation at 37^{o}C for 24 hours any colonies present were observed for typical *Salmonella* morphology. Absence of *Salmonella*-like colonies on all plates indicated that the faecal sample was negative for *Salmonella*. The remaining faecal sample was placed into a sterile stomacher bag (Sterlin, UK) and stored at -80^{o}C for use as a negative control in future experiments.

A *Salmonella*-positive faecal sample was prepared by introducing 1mL of a NB suspension containing *S.* Typhimurium (NCTC 74) at a concentration of 10^3 cfu/mL. The concentration was determined and altered by measuring the absorbance of the suspension at A_{600} using the calibration curve performed in section 2.2.4. The known suspension was then added to the canine faeces (25g) resulting in a final concentration of 40 cfu/g of faeces. Direct and enrichment culture were used to confirm the presence of *Salmonella*-like colonies and the remaining faecal sample was placed in a sterile stomacher bag and stored at -80^{o}C for use as a positive control in future experiments.

2.2.6 Confirmation of isolation method using pre-enrichment, selective broth and selective agars for the recovery of *Salmonella* in canine faeces

Pre-enrichment using BPW was selected as it is a well-established pre-enrichment medium for faecal and food specimens, which involved inoculating 22.5mL of BPW

with 2.5 g of *Salmonella* positive faecal sample described in section 2.2.5. The sample was manually homogenised in a stomacher bag (Sterlin, UK) by gentle agitation for approximately 30 seconds and subsequently incubated at 37^{o}C for 24 hours. Following incubation a 9.9mL volume of RV broth, was inoculated with 0.1mL of the pre-enriched BPW suspension. The RV broth was incubated at 42^{o}C for 24 hours. Following selective enrichment a 10µL loop of RV suspension was inoculated onto the surface of three selective agars: XLD, HE and BS. Following incubation at 37^{o}C for 24 hours the plates were analysed for colony morphology typical of *Salmonella*. Random colonies were tested using *Salmonella* agglutination (Oxoid, UK) and Wellcolex® Colour *Salmonella* Rapid Latex agglutination test (Oxoid, UK) for serotype confirmation and to confirm the credibility of these identification methods. Colonies were analysed according to the manufacturer's instructions and positive colonies were maintained frozen on microbank beads at -80^{o}C (Microbank^{TM} Pro-Lab Diagnostics, Canada) for future reference.

2.2.7 Viability of *Salmonella* Typhimurium in canine faeces after exposure to -80^{o}C temperature

A 25g sample of fresh canine faeces was obtained from four dogs, (Labrador retriever ID L1664, miniature schnauzer ID MS104, miniature poodle ID MP4 and cocker spaniel ID COS43), and were confirmed negative for the presence of *Salmonella*. In parallel the samples were separately homogenised manually in a stomacher bag and a 1g aliquot was placed into 9mL of BPW, for confirmation of negative possession of *Salmonella* in the faecal samples (negative control). The faecal sample was vortexed

and ten-fold serial dilutions were prepared from the neat suspension until a dilution of 10^{-6} was attained. A 0.1mL volume of the serial dilutions, at concentrations of $10^{-2,}$ 10^{-4} and 10^{-6}, was then inoculated onto the three agars mentioned in section 2.2.6 and incubated aerobically at 37^{o}C for 24 hours. The remaining 24g faecal sample was inoculated with 1ml of 10^{7} cfu/mL *S.* Typhimurium (NCTC 74) to give an estimated faecal *Salmonella* concentration of approximately 4.16 x 10^{5} cfu g^{-1}. The sample was homogenised with the bacteria for approximately 30 seconds in a sterile stomacher bag. A 1g aliquot of the inoculated faecal samples was subjected to the pre-enrichment and selective enrichment method detailed in section 2.2.6. This sample represented faeces inoculated with *Salmonella* without freezing. The reminder of the inoculated faecal sample was dispensed into 1g aliquots and frozen at -80^{o}C. Viable counts of the *Salmonella* present in the faecal sample were determined at time points, 2 hours, 6 days, 4 weeks, and 6 months, 1 and 2 years, using pre-enrichment and selective enrichment methods detailed in section 2.2.6. Positive recovery of *Salmonella* was determined by observation of colonies with typical morphology on the three agars. Colonies were confirmed as *Salmonella* using *Salmonella* agglutination (Oxoid, UK) and Wellcolex® Colour *Salmonella* Rapid Latex agglutination test (Oxoid, UK). Colonies were analysed according to the manufacturer's instructions and positive colonies were maintained frozen on microbank beads at -80^{o}C for future references (Microbank^{TM} Pro-Lab Diagnostics, Canada).

2.2.8 Viability of *Salmonella* Typhimurium in canine faeces at room temperature

A 25g sample of fresh canine faeces was taken from three dogs housed at WALTHAM® (Labrador retriever ID L1664, miniature schnauzer ID MS104 and miniature poodle ID MP4) and were confirmed negative for the presence of *Salmonella*, using a 1g aliquot sample. The remaining 24g faecal samples were inoculated with 1ml of 10^7 cfu/mL *S.* Typhimurium (NCTC 74) resulting in a faecal *Salmonella* concentration of approximately 4.16 x 10^5 cfu g^{-1}. The sample was dispensed into 1g aliquots and incubated at room temperature for 0, 2, 24, and 48 hours. To determine the resilience of *Salmonella* at room temperature, a 1g aliquot for each of the three samples at each time point was used to inoculate 9mL of NB and vortexed to produce a homogeneous suspension. *Salmonella* were enumerated from a derived dilution series, 10^{-2} to 10^{-8}, prepared in NB. A 0.1mL volume of the dilutions were then used to inoculate a XLD, HE and BS agar plate. Following incubation of all agar plates at 37^{o}C for 24 hours, the number of colonies with *Salmonella* morphology was determined.

2.2.9 Viability of *Salmonella* Typhimurium in canine faeces subjected to outdoor conditions

The method described in section 2.2.8 was employed however aliquots of faecal sample were retained in a sterile plastic tub with a loosely placed transparent lid and exposed to the outdoors during a 6 month period from January 2009 to June 2009 in a private garden away from the general public. Samples were taken once a week during this period of time and a record of the daily local temperature and precipitation was made as

recorded by the metrological office (MetOffice, 2010). Viability of *Salmonella* was determined as described in section 2.2.6.

2.3 Results

2.3.1 *Salmonella* Typhimurium Calibration curve

The generation of a calibration curve was required to provide a fast and reliable method for determining the concentration of *Salmonella* in a suspension. A calibration curve for the growth of *S.* Typhimurium (NCTC 74) in NB was calculated by plotting the OD at 600nm against viable log cfu/mL, represented in Figure 2.1. The calibration curve gave an R^2 value of 0.9598 and the regression line was in close proximity to the data points confirming the accuracy of the calibration curve. Calibration curves are typically used for the enumeration of bacterial cell counts and this data supports the use of OD_{600} as a quick and reliable method determining the concentration of *Salmonella* suspensions for future experiments.

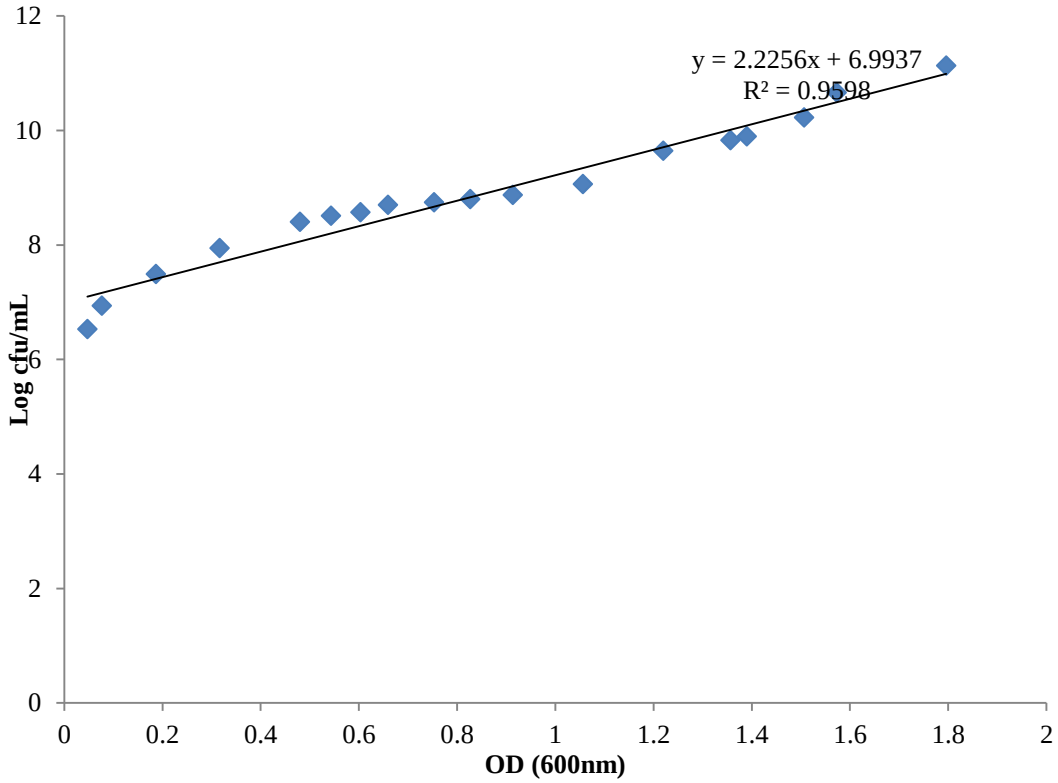


Figure 2.1 Calibration curve of *S*. Typhimurium (NCTC 74) grown in NB. The mean of three replicates represents the data points.

2.3.2 Selection of isolation methods

A *Salmonella*-positive faecal sample, prepared as described in section 2.2.5, was used to confirm the ability of pre-enrichment and selective enrichment methods to isolate *Salmonella* from canine faecal samples. Buffered Peptone Water pre-enrichment coupled with the RV broth selective media gave positive results in the selection of *Salmonella* when cultured onto selective plates, XLD, HE and BS. The XLD agar gave rise to black colonies against the red agar base making it clear to differentiate *Salmonella's* from other species of bacteria. The black colonies were as a result of

hydrogen sulphide production which also gave a foul egg odour which was distinctive. Additionally, *E.coli* was also found to be viable in the faecal samples and was successfully isolated on XLD agar presenting as yellow colonies as a result of this species lowering the pH levels of the media. *Salmonella* presented as dark green/ black centred colonies on the green coloured HE agar. Coliforms, also present in the faecal matter, grew as bright orange/pink colonies which were clearly differentiated from the *Salmonella* colonies. The chromogenic BS agar was also selective for *Salmonella* and gave colonies with a distinctive pink colour against the opaque white agar base. This agar was superior to XLD and HE due to its ability to inhibit the growth of *E.coli*. All three selective agars, XLD, HE and BS, were chosen for their ability to isolate *Salmonella* from a faecal source due to their high discriminatory power.

Direct culturing allowed for a rapid confirmation that was quantifiable whereas pre-enrichment of faecal specimens required a three day time length however was not quantifiable. Pre-enrichment however allows for low numbers of *Salmonella* bacteria present in faecal samples that would be overlooked in direct culturing.

2.3.3 Viability of *Salmonella* Typhimurium in canine faeces after storage at -80^{o}C temperature

The viability of *Salmonella* Typhimurium after storage at -80^{o}C was determined by inoculating four canine faecal samples with *Salmonella* at a concentration of approximately 4.16 x 10^{5} cfu g^{-1} and subjecting them to -80^{o}C for between 2 hours and 2 years. The concentration and confirmation of *Salmonella* was determined using pre-

enrichment, as described in section 2.2.6. The data revealed that there was positive recovery of the test strain *S.* Typhimurium after a two year period in canine faecal samples. The samples were tested three times and the standard error was calculated (data not shown).

2.3.4 Viability of *Salmonella* Typhimurium in canine faeces at room temperature

The viability of *S.* Typhimurium in canine faeces at an ambient room temperature (24-26^{o}C) was determined by inoculating three *Salmonella* negative canine faeces with *S.* Typhimurium (NCTC 74) at 10^{7}cfu/mL, as described in section 2.2.8. The concentration of *Salmonella* was determined over a period of 48 hours using direct and pre-enrichment techniques described in section 2.2.6.

The data collected from this investigation demonstrates a slight log increase in viability after 2 hours incubation at room temperature. Samples L1664 and MS104 demonstrated a 2 log increase after 24 hours however MP4 remained stable as illustrated in Figure 2.2. Following 48 hours, samples L1664 and MS104 demonstrated a slight log decline but again there was little change with respect to sample MP4 which remained stable. Three samples were taken for each faecal sample at each time and standard error was calculated.

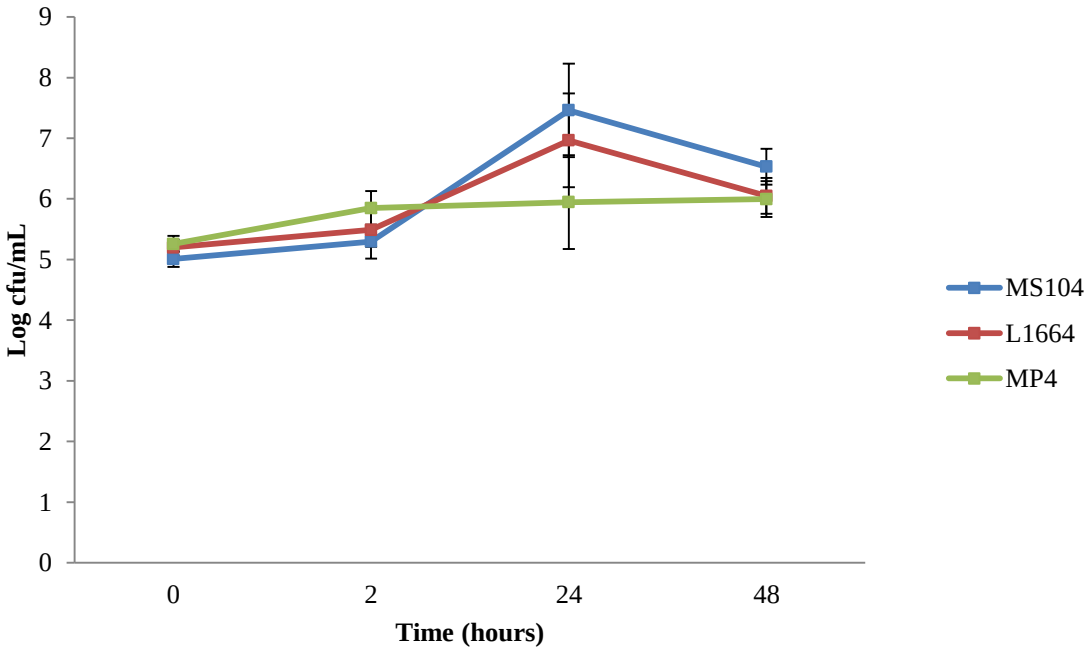


Figure 2.2 Viability of *S.* Typhimurium (NCTC 74) in three samples (MS104, L1164, MP4) of canine faeces subjected to room temperature (20-25^{o}C) conditions for various periods of time. Standard error of each sample was calculated from the three technical replicates.

2.3.5 Viability of *Salmonella* Typhimurium in canine faeces subjected to the outdoor environment

The viability of *S.* Typhimurium exposed to an outdoor environment over a 6 month period was determined. There was an inverse correlation in the viability of *S.* Typhimurium (NCTC 74) as the temperature recorded increased with time due to the changing of seasons, which is illustrated in Figure 2.3. After 6 months there was a 7 log decline in the number of *Salmonella* isolated, however viable cells still remain present

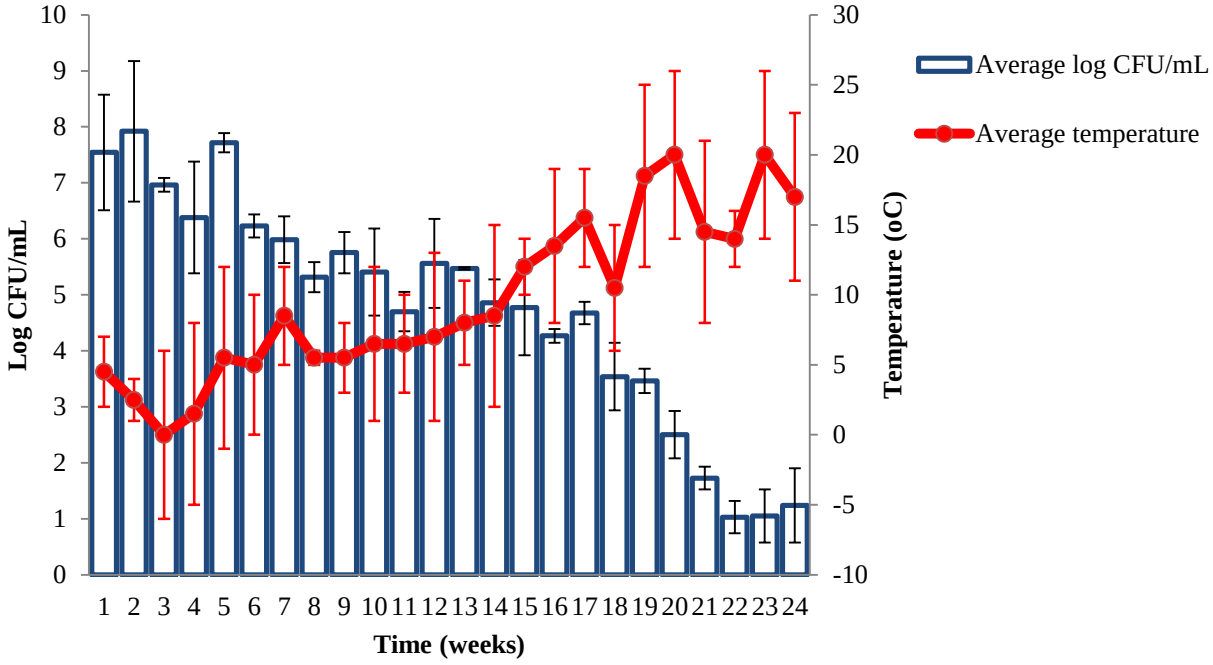


Figure 2.3 Viability of *S*. Typhimurium (NCTC 74) in canine faeces over a 6 month period from January 2009 to June 2009 subjected to outdoor conditions. Standard error of each sample was calculated from the three technical replicates. Average temperature recorded (right-hand axis) over the 6 months is depicted as a red line graph, error bars indicate the maximum and minimum temperatures for each day.

2.4 Discussion

Salmonella isolation requires a comprehensive range of selective and non-selective culture media depending on the source of the sample. Consideration of the sensitivity of bacteriological methods in the design of studies based on faecal culture is important in defining experimental parameters, as studies have shown that sensitivity of recovery can vary depending on the method employed (Davies *et al.*, 2000). Therefore, the aim of this chapter was to select and validate a reliable media and experimental approach for the routine isolation of *Salmonella* from canine faecal samples. Additionally, as samples sometimes required storage prior to analysis, this chapter investigated the effect of storing faeces at -80^{o}C for up to two years on the viability of *Salmonella*. The stability and viability of *Salmonella* in canine faeces was also investigated under a number of different conditions that mimicked household and outdoor conditions.

2.4.1 Selection of isolation methods

Relatively few investigations have evaluated *Salmonella* isolation from canine faecal samples but there are a number of published studies describing the isolation of *Salmonella* from food samples. Previous studies evaluating isolation of bacterial species from faecal samples demonstrated the lack of heterogeneity of *Salmonella* distribution in faeces (Davies *et al.*, 2000; Funk *et al.*, 2000; O'Carroll *et al.*, 1999); reflecting the generally unreliable nature of bacteriological culture of individual faecal

samples. Therefore, it was essential to ensure complete homogenisation of the faecal sample and to select the optimum isolation media for epidemiological investigations.

Faecal matter is eliminated waste excreted from the body and consists of 67% undigested food and 33-34% of liquids. Resident "good" bacteria within the gut breakdown contents of the food which creates organic compounds rich in nitrogen, sulphur and gases such as hydrogen sulphide that produces an environment suitable for *Salmonella* survival. However, it is only after excretion and exposure to the external environment that bacterial species may become sub-lethally damaged, giving reason to a quick processing time for bacterial analysis of faecal samples (CDC, 2012b; HPA, 2008a; HPA 2012a). Buffered Peptone Water (BPW) is established as a pre-enrichment media and is widely used in *Salmonella* isolation and was therefore chosen as a pre-enrichment broth. A study demonstrated that the use of BPW pre-enrichment for the isolation of *Salmonella* from dry specimens resulted in a 25% increase in recovery (Thomason *et al.*, 1977). Studies into *Salmonella* isolation in dogs have used BPW pre-enrichment as part of the isolation methodology and have successfully isolated non-typhoidal *Salmonella* serotypes (Finely *et al.*, 2007; Lenz *et al.*, 2009; Leonard *et al.*, 2011). Typically, pre-enrichment using BPW is coupled with the selective enrichment media, Rappaport Vassiliadis (RV) and has been demonstrated as an effective method for the isolation of *Salmonella* (Harvey *et al.*, 1981; Rall *et al.*, 2005). Enrichment in RV was carried out at 42°C as studies have demonstrated that at this temperature recovery of *Salmonella* was superior than at 37°C (Davies *et al.*, 2000). Recovery was successful using the pre-enrichment method BPW followed by the selective enrichment of RV broth has been proven as effective for the isolation of *Salmonella* from naturally

infected human faeces (HPA, 2008a; HPA, 2011b) and naturally infected pig faeces (Davies *et al.*, 2000). In this chapter a canine faecal sample was artificially inoculated with *S.* Typhimurium as a positive control. Recovery of *Salmonella* employing the standard BPW and RV media coupled with the use of the three selective agars, XLD, HE, BS, was successful in culturing the *S.* Typhimurium from the canine faeces. The chromogenic BS agar was very successful in the isolation of *Salmonella* from canine faeces and demonstrated an excellent recovery of *Salmonella* coupled with an inhibition of unwanted microorganisms present in the sample. The agars XLD and HE presented *Salmonella* colonies that were distinct from the other organism present in the sample. Salmonellae are typically H_2S producers and XLD is highly specific for the growth of these microorganisms, indeed this is key for the black colony morphology produced on the agar. Conversely, black colonies can only be presumptively positive for *Salmonella* as *Edwardsiella* also present as red colonies with black centres on XLD. Furthermore, *Proteus* and *Pseudomonas* species present as red colonies and are less distinctive then yellow colonies produced by *Escherichia*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Proteus*, *Serratia* (Oxoid, 2008a). Although colonies of the correct morphology were obtained it is essential to confirm their identity as *Salmonella* by further analysis.

The use of BPW and RV increased the number of *E. coli* because XLD and HE are negative for the inhibition of this organism and in some incidences produced a heavy lawn on neat samples, making identification of the colonies difficult. Therefore prior to agar plate inoculation, samples were diluted to reduce the number of competitor

organisms. This method was successful and taken forward for the isolation of *Salmonella* from canine faeces throughout the thesis.

The use of two or more selective media is recommended to improve the isolation rate and to remove any overlooked atypical serotypes within the sample (Persaud & Eykyn, 1994). Therefore the simultaneous use of HE agar was implemented to recover any atypical strains that XLD cannot culture. Additionally a chromogenic, BS agar was also implemented. The use of chromogenic agars is highly recommended for isolation of *Salmonella* from faecal samples, on the basis they allow for enhanced sensitivity after enrichment and enhance specificity before and after enrichment in comparison to non-chromogenic agars (Cassar *et al.,* 2003). This agar possesses an Inhibigen™ component that inhibits the growth of *E.coli*. The agar comprises of two chromogen enzymes: caprylate esterase and ß-glucosidase. Organisms *Klebsiella*, *Enterobacter* and *Proteus* are positive for caprylate esterase, capable of cleaving the chromogen to release an insoluble purple chromophore which when built up presents as a blue colony on the white opaque agar. The lack of ß-glucosidase in *Salmonella* however allows the colonies to remain purple in colour, differentiating colonies by colour. Many selective agars comprise of antibiotics that target unwanted inhibitors that may be present in the specimen. Novobiocin and cefsulodin are present in BS agar to inhibit the growth of competing flora such as *Proteus* spp. and *Pseudomonas* spp. As *Proteus* spp can present colonies similar to *Salmonella* on XLD agar it was beneficial to use BS for its inhibiting power against the bacteria and to rule out any false positives that may have

been seen on the XLD. Nevertheless as with all agars, BS has limitations including the inability to culture the serotypes *S.* Typhi and *S.* Paratyphi.

In this study all three selective plating agars were successful in recovery by direct culture of the *S.* Typhimurium positive control. Coupled with the BPW and RV enrichment the method demonstrated a robust isolation and enumeration method for use in this study. The use of BPW and RV has its limitations as it rules out quantitative count on the agars as these stages increase numbers of *Salmonella* present in the original specimen. All three agars were successful for the isolation of *Salmonella* however BS was a better selective agar due to the inhibition of other organisms that are able to grow on the agar. Therefore all three agars were used throughout this thesis and the use of multiple selective agars would eliminate any possible atypical colonies that would have been missed if only one agar was implemented.

2.4.2 Viability of *Salmonella* Typhimurium in canine faeces after storage at -80^{o}C temperature

Faecal specimens required for sampling are collected and processed in a laboratory within 48 hours of defecation, prior to antibiotic use (CDC, 2012b, HPA, 2008, HPA 2012a). The collection, transport and storage protocol to maximise maintenance of *Salmonella* viability prior to analysis is important as environmental conditions can cause sub-lethal damage to the pathogenic bacteria excreted in the faeces. Following sampling the remaining faeces are stored at temperatures below <-15^{o}C, however, theses samples are only for antigen or PCR testing due any lethal damage freezing may

cause on the bacteria present in the faeces (CDC, 2012b). In this study fresh or faecal samples no older than 24 hours old were collected inside sterile collection bags and processed for the presence of *Salmonella* within this time period. Following confirmation of *Salmonella* absence in the faecal sample, *S.* Typhimurium was used to artificially inoculate the sample. It was of interest to determine a suitable storage method, for future experiments involving canine faecal sampling where potentially large quantities of faecal samples could be given at one time and analysis of these samples could be unmanageable. More importantly, the investigation was to consider the stability and viability of the artificially inoculated *Salmonella* when subjected to -80^{o}C storage conditions over a range of time periods.

Each of the four faecal samples was processed by a pre-enrichment and selective enrichment stage, followed by culturing onto XLD, HE and BS agar. The use of pre-enrichment and selective enrichment removes the quantitative measurement of the colonies and the enrichment stages increase the number of *Salmonella* present in the original sample. This phenomenon is demonstrated by the 1 log increase in the colony forming units calculated in the samples. All samples gave a positive count for *Salmonella* recovery and no sample was negative for the presence of *Salmonella*, suggesting that freezing is a suitable storage for faeces. Conversely, as a pre-enrichment and selective enrichment stage was performed, sub-lethally damaged cells could successfully grow, eliminating any detrimental damage caused by freezing. Studies into freezing of bacterial cells demonstrate that a slight loss of viability is typically associated with re-nucleation and irreversible mechanical damage of cell membranes (Cressy *et al.*, 2003; Elhanafi *et al.*, 2004). The frequent thawing and

freezing of bacteria could potentially cause further cell damage and freezing without exogenously supplied cryoprotective agent, such as glycerol, severely decreases their viability. Studies into *E. coli* have demonstrated that loss of viability is proportional to the number of freeze-thaw cycles that cells experience. This suggested that the length of time a sample is stored for influences the bacterial cell damage less than if the samples were subjected to a cycle of freezing and thawing (Sleight, 2006). This study and the supporting literature suggest that storage of faecal samples at -80^{o}C is sufficient, with a pre-enrichment and selective enrichment that are non-quantifiable, however, only for a single thawing process.

2.4.3 Viability of *Salmonella* Typhimurium in canine faeces stored at room temperature

Direct culture of *Salmonella* from artificially inoculated faecal samples was performed after incubation at room temperature for 0, 2, 24 and 48 hours. Enumeration of colonies following incubation demonstrated that after 2 hours there was a 5.7% (L1664) and, 5.4% (MP4) and 11.04 % (MS104) increase in the number of *Salmonella* colonies (cfu/g) isolated from the three faecal samples. The largest increase in colony count was demonstrated after 24 hours where there was a 48% (MS104), 33.9% (L1664) and 12.9% (MP4) increase in the number of colonies isolated from time zero. Following 48 hours of incubation at ambient temperatures there was a slight decline in cfu/mL in all three samples.

A study into broiler chicken faeces reported that there was a marked increase in number of *Salmonella* spp. (cfu/g) present in faeces stored for 1 week under ambient room temperature and humidity conditions of the laboratory as compared to the number present in freshly passed faeces (Traub-Dargatz *et al.*, 2006). This finding together with the result from this study implies that the number of pathogenic *Salmonella* can increase in faeces under environmental conditions similar to those in a household situation. The decline in numbers of *Salmonella* at 48 hours observed in this study is similar to the findings of another study that showed a decline in cfu/ml after 3 weeks of incubation at room temperature (Traub-Dargatz *et al.*, 2006). However, different strains of *Salmonella* were used and lower inoculum concentration (cfu/g) was used in the Traub-Dargatz *et al*. study and therefore direct comparisons cannot be made. Previous investigations of *Salmonella* viability in rat faeces under indoor conditions demonstrated a 7 log reduction after 84 days which is a much faster rate than in this investigation (Hilton *et al.*, 2002).

Reports suggest that many companion pets share living environments with their owners (Walther *et al.*, 2012) and as the number of companion dog owners increase so do the risks of zoonotic infection from exposure to dog faeces. The majority of cases of non-typhoidal *Salmonella* infection in humans are foodborne and a significant number of cases appear to be acquired from households contaminated with *Salmonella enterica* (Haddock *et al.*, 1994; Schutze, 1999). Sources and sites of contamination other than household members with clinical disease and pets with sub-clinical infection, include contaminated items brought into the home, toilet bowls, floors including carpets,

refrigerators, kitchen sinks and counter top (Rice *et al.*, 2003). If faeces or contaminated objects are not effectively removed or left in the home environment then there may be a potential risk of cross contamination. Studies have demonstrated that households that are associated with livestock or occupations associated with *Salmonella* exposure show no isolation in their household (Rice *et al.*, 2003). However, as dogs are considered asymptomatic carriers and shed *Salmonella* spp then this could be a source of *Salmonella* in the household.

2.4.4 Viability of *Salmonella* Typhimurium in canine faeces subjected to outdoor environment.

Artificially inoculated canine faeces was left outside over a 6 month period and routinely sampled on a weekly basis for the recovery of *Salmonella*. The data demonstrates that there was a maximum seven log reduction in the number of *Salmonella* isolated after 6 months compared to the initial inoculums. Meteorology data collected at the same time demonstrated a gradual increase in temperature coinciding with dry periods throughout the trial from January to June. This temperature increase and dry conditions could be a contributing factor for the reduced survival of *Salmonella* after 6 months. Variability in recovery rates correspond to difference in biotic and abiotic factors as well as variability in temperature moisture and different environmental exposure to the faeces.

The HPA provide data on incidence of *Salmonella* cases through the year (see appendix 9.3). The increase of incidence of *Salmonella* correlates with the increase in

environmental temperatures throughout the British seasons. Although, seasonal temperature variation is a well-known phenomenon in the epidemiology of enteric infections the increase in incidences has also been attributed to increases in replication cycles of foodborne pathogens at high ambient temperatures; in the summer months there is an increase in outdoor prepared and cooked food and therefore the opportunity for food handling errors increases.

This study demonstrates that if infected faeces were left in the environment then *Salmonella* remains viable in the faeces for up to 6 months, which increases the potential for cross-contamination to the surrounding environment. This highlights the importance of hygiene and removal of canine faeces from the public environment. It is estimated that 900 tonnes of dog faeces is produced every day and the Department of the Environment research indicates that half of all owners never dispose of their dog's faeces in bins provided in public parks. In a city survey conducted in 1996, seven per cent of street sites were found to be fouled in London (Unison, 2012). The survival of *Salmonella* observed in this investigation highlights the importance of reducing the numbers of dog faeces in the public environment and the enforcement of punishment to owners should be increased.

2.5 Conclusion

Pre-enrichment (BPW), selective enrichment (RV) and the use of a range of selective agars (XLD, HE and BS) have shown to be repeatable and reproducible methods for the isolation of *Salmonella* from canine faeces. The storage of faecal samples at -80^{o}C is adequate for the stability of *Salmonella* infected faecal samples however; potential damage to *Salmonella* could be caused by multiple freeze-thawing of faecal samples. Viability of *Salmonella* in faeces left at room temperature and outdoor over a period of time has been demonstrated which highlights the need for proper disposal of faeces to remove the exposure and potential for cross contamination to the surrounding environment.

Chapter 3 Epidemiology of asymptomatic carriage of *Salmonella* in UK canines and their environment

3.1 Introduction

Salmonella is the second most common bacterial cause of human foodborne infection within the UK, responsible for 9133 reported cases in England & Wales in 2010 which is predicted to be much lower than the true incidence of disease (HPA, 2011a). Of the currently >2500 serotypes of *Salmonella* that have been identified only approximately 200 are associated with human clinical infections. The major route by which humans become infected is by the consumption of contaminated food. Reports have demonstrated that approximately 30% of all chicken on sale are contaminated with *Salmonella* (Harrison *et al.*, 2001). Furthermore, other non-typhoidal *Salmonella* species have been frequently reported in food-producing animals and reptile pets (Foley & Lynne, 2008). Epidemiological investigations into *Salmonella* in food-producing cattle, sheep and pigs revealed that serotypes *S.* Dublin and *S.* Typhimurium (phage type 4,5,12:I and 4,12:I) were the most common serotypes to cause clinical infection (Lewerin *et al.*, 2011). *Salmonella* Typhimurium and *S.* Enteritidis are commonly associated with chickens however as a consequence of statutory monitoring and control introduced by The Poultry Breeding Flocks and Hatcheries Orders Act 1993, infection caused by these serotypes have decreased in breed flocks (Defra, 2012). On-going monitoring and recording of human salmonellosis in the UK reveals that serotypes *S.* Typhimurium and *S.* Enteritidis are the predominant serotypes and that the current number of reported cases has dramatically decreased from those in the 1990s which is

associated with the decreased incidence in chickens and eggs as a consequence of the Salnvac® and Salnvac®T vaccination programme (Oostenbach, 2012). Conversely, there has been a slight increase in reported cases of the sub species *S. arizonae*; in 1998 0.13% of total cases were caused by *S. arizonae* which has increased to 0.46% in 2007. This increase may be a reflection of the increased popularity of reptiles as pets in which this sub-species is known to harbour (HPA, 2008b).

Vets and farmers are also considered to be particularly at risk of infection, due to their close interaction with animals, and their increased exposure to asymptomatic animals shedding *Salmonella*. However, although trends show that *Salmonella* infection in human and food-producing animals has reduced, particularly in the UK, there is still a growing concern for the high volume of cases that are reported worldwide. Millions of human cases are reported worldwide every year and the disease results in thousands of deaths. It constitutes a major public health burden and represents a significant cost in many countries (WHO, 2005).

Current trends indicate that there are an increasing number of pet owners, with approximately 57% of UK households reported to own pets; primarily cats and dogs (Murray, 2010). In parallel there have also been reports of an emerging number of zoonotic diseases (Jones *et al.*, 2008; Taylor *et al.*, 2001). Symptomatic presentation of *Salmonella* infection in dogs is rare (Finley *et al.*, 2007), however dogs are regarded as one of the more important asymptomatic carriers of *Salmonella*, as they can harbour high numbers of the organism in the intestines and mesenteric lymph nodes (Morse *et al.*, 1976), which can be shed in their faeces without symptomatic presentation (Buxton, 1957; Carter *et al.*, 2000; Finley *et al.*, 2007; Greene, 1998; Hoelzer *et al.*, 2011). Investigations have also been conducted to show that naturally occurring salmonellosis

in dogs can result in a shedding period of up to 6 weeks and experimental investigations have seen asymptomatic shedding for up to 117 days (Morse *et al.*, 1976). This could be of significant importance to public health as dogs have frequent and close contact with family members in households.

Previous studies undertaken in the early 1950s reported the prevalence of *Salmonella* in healthy dogs to be 0-43% however; these data were obtained from non-UK animals and are now somewhat dated (McElrath *et al.*, 1952). More recently, a study estimated the prevalence of *Salmonella* isolation from clinically healthy dogs in Canada to be between 1% and 35% (Finley *et al.*, 2007). Both these studies demonstrate a wide variability in asymptomatic carriage, in particular percentages in the range of 35% and 43% are cause for concern. With the different attitudes in companion animal care and hygiene policies in different countries it is of interest to investigate the current asymptomatic carriage in UK dogs; a country typically regarded as a 'nation of animal lovers'.

There are many routes by which dogs can be exposed to infection with *Salmonella* including: ingestion of contaminated food and water, materials from the environment, through the skin and mucous membrane via bites, scratches or other direct animal contact including invertebrate vectors. Dogs have also been known to be copraphagic and are likely to scavenge or hunt for food, which ultimately increases their risk of exposure to *Salmonella* and consequently infection and re-infection. Populations of canines demonstrate variability in their susceptibility to infection, which is increased by crowding, unsanitary conditions, stress, surgery and immunosuppression (Calvert, 1985). This highlights the importance of good hygiene practice following interaction with pets. However many pet owners and animal workers may be unaware of the

potential risk of infection with communicable diseases, transmitted from the animals with which they interact. Most *Salmonella* infections are apparent in and mild in canines, and as such they are not detected. Surveys of canine populations confirm that salmonellosis is no rarity and it is estimated that 10 per cent of the dogs have experienced infection during their lifetimes (Morse *et al.*, 1976).

The aim of this investigation therefore was to gain a current perspective of the role of canines as potential reservoirs of *Salmonella* by determining the incidence of asymptomatic carriage of *Salmonella* in domestic canines. This chapter describes a cross-sectional study, using a randomised design, where the prevalence of *Salmonella* in faeces was determined from a diverse range of healthy dogs, located in Central England. Additional sampling was performed in the dog's environment to expose environmental reservoirs of *Salmonella* which could lead to subsequent exposure or infection. It has been observed that the prevalence of salmonellosis in dogs is greater during autumn and early-winter (Caraway *et al.*, 1959; Stucker *et al.*, 1952). These periods coincide with the breeding or estral season for the dog and therefore this study also investigated intermittent or seasonality of *Salmonella* shedding by repeated sampling of a defined cohort of dogs over a nine month period (Morse *et al.*,1976).

3.2 Materials and Methods

3.2.1 Microbial cultures

Salmonella Typhimurium (NCTC 74) was used in this chapter as a positive control and preparation and storage is as in section 2.2.1

3.2.2 Microbiological media

Agars and broths were as stated in 2.2.2

3.2.3 Prevalence of asymptomatic carriage of *Salmonella* in canines from the UK.

Canine faecal samples were obtained from West Midlands and Leicestershire and were received on a voluntary basis. Samples were acquired from household dogs, the WALTHAM® Centre for Pet Nutrition (WCPN; Waltham-on-the-Wolds, UK), BlueCross rescue centre (Bromsgroves, UK), and Perry Barr retired greyhound trust (Birmingham, UK). Companion dog owners completed a consent form and a questionnaire detailing the dog's age, sex, breed, weight, diet, medical history, origin of dog and eating habits (see Appendix 9.1 and 9.2). A positive control for the *Salmonella* isolation method was prepared as described in section 2.2.5 resulting in artificially inoculated faeces with a concentration of approximately 10^5cfu/g faeces. Canine faecal samples were received fresh or no older than 24 hours and were manually homogenised inside a sterile collection bag for 30 seconds. A 2.5g aliquot was used to inoculate 22.5mL BPW. The samples were mixed thoroughly by agitation for one minute. Ten-fold serial dilutions were prepared from the neat faecal suspension down to 10^{-6} in NB.

A 0.1ml volume of the dilution, at a concentration of $10^{-2,}$ 10^{-4} and 10^{-6}, was inoculated onto XLD, HE and BS agar and incubated at 37^{o}C for 24 hours aerobically, to determine the presence of *Salmonella* by direct culturing. The remainder of the BPW suspension was incubated at 37^{o}C for 24 hours. A 0.1mL volume of the pre-enriched suspension was used to inoculate 9.9mL RV broth and incubated at 42^{o}C for 24 hours. Ten-fold serial dilutions were made from the neat RV broth suspension to 10^{-8} in NB and 0.1mL, of concentrations of 10^{-4}, 10^{-6} and $10^{-8,}$ were plated onto the three selective agar plates. To aid recovery of any slow growing strains of *Salmonella*, BS plates were incubated at 37^{o}C for 24 to 48 hours. Following incubation, plates were observed for typical *Salmonella* morphology and any suspect colonies were confirmed using API 20E (bioMérieux, France) *Salmonella* agglutination and Wellcolex® Colour *Salmonella* Rapid Latex agglutination test according to the manufacturer's instructions.

3.2.4 Environmental sampling

The sampled surface areas measured approximately 40cm x 40cm and were swabbed using a BPW soaked sterile sponge (Fisher, UK). The sponges were then placed into sterile bags, which were provided with the sponge. Samples were stored at ambient temperature for no more than 2 hours prior to analysis. Upon receipt in the laboratory the BPW was compressed out of the sponge and the recovered BPW incubated at 37^{o}C for 24 hours. Following incubation, a 0.1ml aliquot of each sample was inoculated into 9.9mL RV broth and incubated for a further 24 hours at 42^{o}C. A 0.1mL aliquot of each sample was then used to inoculate XLD, HE and BS agar for the confirmation of *Salmonella* presence in that environmental sample area. Agar plates were incubated at 37^{o}C for 24 hours and presence of *Salmonella* was determined as described in section 3.2.3.

3.2.5 Repeat sampling of individual dogs

A repeat sampling of individual dogs was established to investigate potential intermittent shedding of faecal *Salmonella* throughout an extensive period of time. Ten companion animals in a domestic environment were recruited on a voluntary basis and sampled repeatedly over nine months, from February to October 2010, incorporating all the seasons in the UK. Each companion dog owner completed a survey and signed a consent form (see Appendix 9.1 and 9.2). The dogs were recruited from six different households and were sampled on three consecutive days in each month resulting in a total of 270 faecal samples throughout the period of the study.

3.3 Results

3.3.1 Prevalence of asymptomatic carriage of *Salmonella* in canines located in Central England

A total of 490 faecal samples were obtained from canines in the West Midlands and Leicestershire regions of the UK from a number of different breeds of dogs (Table 3.1). Information collated from the questionnaires completed by the owners is tabulated in Table 3.1, Figure 3.1 and Table 3.2.

Table 3.1 Summary information collected from epidemiological study volunteer dogs.

Sex	Age Range	Weight Range	No. of dogs on medication
F=236 M=254	0.3 months -16years	2Kg-48Kg	Advocate (1), Benazecare (1), Cosequin (3), Clomicalm (3), Dorwest Cod Liver Oil (2), Duphamox (1), Glucosamine (8), Chondroitin manganese (1), Type II Collagen (1), Green lipped mussel powder (Seatone) (1), Loxicom, (2), Meloxidyl (1), Multivitamin (33), Pheromone (3), Prednoleucotropin (1), Vetmedin (1), Vitamin C (1)

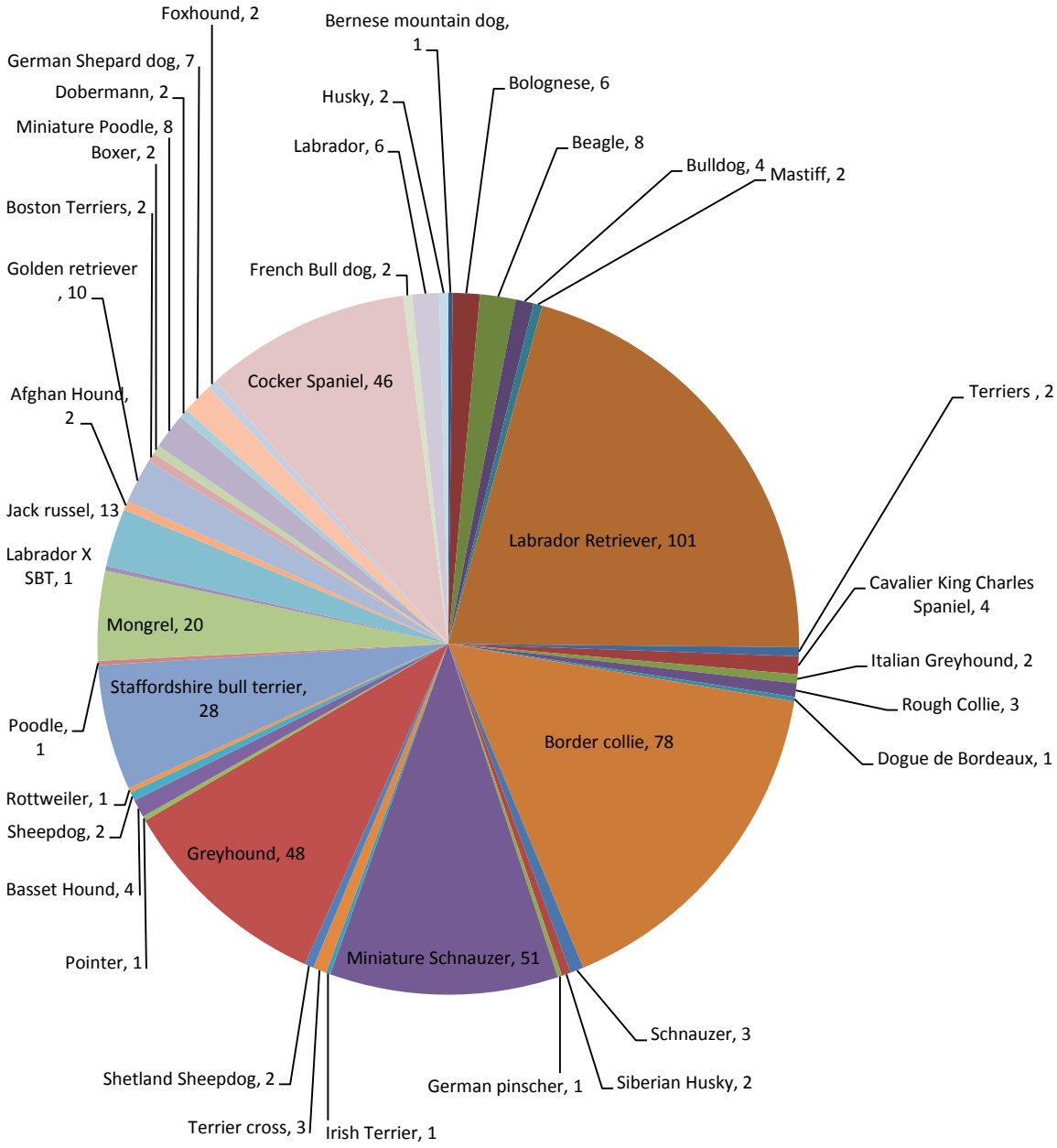


Figure 3.1 The frequency of dog breeds from epidemiological sampling.

Table 3.2 Summary of *Salmonella* isolation from different population groups.

Location/ population	**No. of dogs**	**No. of faecal samples**	**No. of positive samples for *Salmonella***	**Serotype isolated**
WCPN	148	148	0	-
Retirement/boarding kennels	43	80	0	-
Rescue centres	87	87	0	-
Households	126	126	1 (0.8%)	*S. arizonae*
Greyhounds	39	91	0	-
Annual cycle population [a]	10	270	0	-

[a] Dogs were sampled over a 9 month period from February to October 2010; three consecutive faecal samples were collected from each dog each month.

Each faecal sample received was tested for the presence of *Salmonella* by direct culture and selective enrichment culture as described in section 3.2.3. Of the 490 faecal samples, only one (0.20%) faecal sample was found to be positive for the presence of *Salmonella*. This was identified using selective enrichment culture and originated from a household companion dog in the West Midlands, as detailed in Table 3.2.The dog was a West Highland white terrier breed, aged 2 years, was not neutered, and weighed approximately 10Kg. The dog had a diet of wet pouches and dry complete food, DENTASTIX® and dog biscuits as treats. It was noted that the dog had no medical history of gastrointestinal problems and was not on any form of medication during the sampling period. It was also highlighted that this dog had shown copraphagic

tendencies however it did not practice the habit at the time; additionally the dog had a habit of chewing on furniture, soft toys, wood, plastics and shoes.

The isolated strain from the household dog presented with typical morphology on the BS agar following the 24 hour incubation. A single colony derived from the plate was inoculated onto NA and following incubation at 37^{o}C for 24 hours, the resulting colonies were maintained frozen on microbank beads at -80^{o}C. The isolate was confirmed as *Salmonella* using *Salmonella* latex agglutination. Wellcolex® Colour Latex Test confirmed that the isolate was *Salmonella enterica* and the 20E API test revealed the species to be *Salmonella enterica* sub species *arizonae.*

As *Salmonella* is typically a food associated bacterium, it was of interest to collate information on gastroenteritis problems of the faecal donor dogs through specific questions. The results indicated that three dogs had diarrhoea or loose stools, one dog had tapeworm, two dogs were sensitive to diet changes and one dog had previous gastroenteritis, however confirmation of causative microorganism was not known. The information of dietary habits of participating dogs including snack and unusual eating habits as they can be key in finding sources of contamination and is summaried in Table 3.3.

Table 3.3 Summary information of eating habits of volunteering dogs, including main diet, additional snacks and unusual eating habits.

Main diet (No. of dogs fed diet)	Snacks (No. of dogs consuming snacks)	Unusual habits (No. of dogs with unusual eating habits)
Beta large breed adult dry mix (2)	Biscuits (7)	Bedding (1)
Burns dry food (39)	Bone marrow rolls (2)	Books (2)
Supagrey (39)	Animal bones (139)	Bottles (1)
Canned meat and dry (32)	Cheese (1)	Grass (1)
Canned meat (1)	Chew strips (2)	Newspaper/paper/cash notes (12)
Canned meat and dry complete (2)	Dentastix (203)	Plastic pegs (1)
Canned Meat wet/dry (114)	Dried turkey neck (1)	Rug (4)
Canned meat, dry (2)	Earls snack time (1)	Slippers (1)
Canned meat, meal biscuits, home prepared (1)	Eggs (1)	Socks (1)
Chappie and home scraps (1)	Fruit (1)	Toilet roll (1)
Dry complete (13)	Gravy bones (120)	Faeces (including dog, cat, bird, fox , horse, rabbit, duck) (76)
Dry complete, little meat (2)	Green lipped mussels (1)	
Dry complete/home prepared (1)	Jambone (118)	
Dry James wellbeloved (2)	Joint care stick (115)	
Hills dry (12)	Leftover veg/ Scrap (2)	
Hills puppy (4)	Markies (1)	
Home prepared (Cooked meat) (25)	Milk (1)	
Home prepared/raw (2)	Munchie rolls (1)	
Iams and pedigree chum (38)	Pig ears/ pig ear	

	strips (140)
James well beloved/turkey and rice adult dry (7)	Puppy marko treats (1)
Peanut butter sandwiches, complete dry (2)	Raw hides (2)
Pedigree senior dry/ patches (20)	Schmackos (118)
Puppy complete (8)	Toast (3)
Puppy small (2)	Treats of all varieties (22)
Salters dry food complete (1)	Tripe sticks (1)
senior active maturity complete (9)	Hind meat (47)
WALTHAM® trial Diet (148)	

3.3.2 Positive controls

All positive controls used throughout the microbiological analysis returned *Salmonella* colonies of expected morphology on all selective agars. A positive control was performed for each day samples were analysed.

3.3.3 Environmental sampling

Environmental samples taken in the office areas (floors, carpet, work benches), pens, gardens, dog drinking bowls and water and dog toys were swabbed. They all revealed that none of the areas sampled at WALTHAM® harboured *Salmonella* spp.

3.3.4 Repeat sampling of individual dogs

All of the 270 faecal samples tested from the ten dogs were negative for the presence of *Salmonella*. During the nine month period there was positive isolation of coliforms from every dog which included the bacteria *Escherichia*, *Enterobacter*, *Klebsiella pneumoniae*, *Citrobacter*, *Proteus*, *Serratia* and *Edwardsiella.* Three of the ten dogs had copraphagic tendencies however none of the participants had any underlying gastroenteritis problems or were on medication. Table 3.4 contains the details of each participating dog.

Table 3.4 Summary of details of participating companion animal for the longitudinal study.

Dog ID	Breed	Sex (F/ M)	Age (Years)	Weight (Kg)	Neutered/ Entire	Medical history	Rescue dog (Y/N)
1	Mongrel	F	6	6	N	None	N
2	Border Collie	F	6	25	N	None	N
3	Border Collie cross	F	2	14	N	Previous case of tapeworms	N
4	Rough Collie	M	2	28	N	None	N
5	Mixed breed terrier	F	6	16	N	None	Y
6	Staffordshire bull terrier	F	4	18	E	None	N
7	Staffordshire bull terrier	M	1	18	E	None	N
8	Staffordshire bull terrier	M	2	16	E	None	N
9	Greyhound	M	5	30	N	None	Y
10	Greyhound	F	3	22	N	None	Y

Diet and snacks/habits (including copraphagic tendencies) were also recorded for each dog. All canines were feed a non-raw meat based diet including dry complete, canned meat, home-cooked meat, and dogs ID 9 and 10 were additionally fed peanut butter sandwiches daily. All dogs received snacks, including dog chews, dentastixs, cheese, toast, marrow filled bones, biscuits and other commercially available dog treats. Dogs ID 7 and 8 demonstrated copraphagic tendencies.

3.4 Discussion

3.4.1 Prevalence of asymptomatic carriage of *Salmonella* in canines located in the UK

Previously, dogs have been regarded as one of the most important asymptomatic carriers of *Salmonella* (Buxton, 1957; McElrath *et al.*, 1952; Morse *et al.*, 1976), however the number of dogs that actually present with clinical symptoms is very small (Finely *et al.*, 2007). If indeed dogs are as prevalent asymptomatic carriers of *Salmonella* as previously described the increase in companion animal ownership could pose an increased risk of zoonosis. Therefore it is important to determine the current prevalence of *Salmonella* in the dog population to allow the zoonotic risk to be more accurately quantified.

The aim of this chapter was to gain a current perspective of asymptomatic carriage of *Salmonella* in canines from the UK. Different dog populations were sampled to represent diverse populations including rescue centres, boarding kennels, household, retired greyhounds and dogs housed at WCPN®. This study demonstrated that the prevalence of asymptomatic carriage of *Salmonella* in dogs was very low at *ca.* 0.20%. Consequently the current risk of *Salmonella* zoonoses from canines in the UK is very low. In this study only one dog from the household population was positive (0.8%) for the asymptomatic carriage and shedding of *Salmonella* sub species *arizonae*. No association of *Salmonella* carriage with dog breed, gender or age was observed.

Previous studies into *Salmonella* shedding in domestic canines from different countries demonstrated that dogs homed in Hawke's bay, New Zealand had an absence of carriage (Timbs *et al.*, 1975), Washington had a 1.2% carriage rate (Gorham & Garner, 1951), Iran had a 4.4% carriage rate (Shimi *et al.*, 1976) and the highest carriage rate was reported in Florida at 15% (Galton *et al.*, 1952). Many of the dogs sampled in the Florida cohort were racing greyhounds which were fed offal and raw meat which are potential sources of *Salmonella* and might explain the high carriage rate observed in these dogs. The epidemiological data gained from this study revealed that the current carriage rate of asymptomatic *Salmonella* in domestic household canines in the UK is 0.8%, which is lower than the levels previously reported for other countries with the exception of New Zealand. Studies carried out in 2007-2008 by the World Society for the Protection of Animals (WSPA) constructed a comprehensive worldwide survey into dog and cat ownership from different parts of the world (Batson, 2008). Countries including the USA, UK and New Zealand have welfare laws in place however, that was not the case in Iran. This may explain the lower levels of *Salmonella* observed in USA, UK and New Zealand compared to Iran. However, registration laws were not in place during the period of the study. Dogs were shown in many countries to be kept primarily as companion dogs, the other roles of the dogs were as guard dogs, a food source or as herd dogs. In Europe the basic husbandry of dogs was to sleep inside and play outside, whereas in North America the majority of dogs were kept in or solely in the yard and in Asia they were in the yard and were allowed to sleep inside and play outside. This study highlights the variety of dog keeping practices adopted by the various countries which again may explain the geographical differences in the prevalence of *Salmonella* observed.

Salmonella is a common foodborne causing infection and therefore the feeding regimes of different countries also need to be taken into consideration. In Europe 50% of dogs were fed a home cooked diet and the remaining 50% were fed a commercial pet food (dry, wet and mixed feeding). The North America region the dogs are predominantly fed a commercial dry pet food (60%) and the remaining 40% are fed table scraps. A diverse range of feeding regimes are adopted in Asia including in order of popularity commercial dry pet food, table scraps, home cooked and even scavenged food. Interestingly, in the Oceania region 50% of dogs are fed a home cooked diet and 50% are fed table scraps. The data collected from these studies above and published studies into *Salmonella* carriage in dog are somewhat dated, ranging from the 1950's to the mid 1970's. During the 1930's the birth of the pet food industry began after James Spratt of Cincinnati, Ohio observed the relaxed eating habits of dogs in the UK. It was from then the development of dog biscuits began and the broadening knowledge about companion animal nutrition and food technology enabled the industry to develop and diversify in leaps and bounds (PFMA, 2012). Over the years, the pet population has increased, in parallel pet food sales have consistently increased, however, not all pet owners were able to afford pet food therefore dogs were still feed raw scraps from the butchers or left to forage on wildlife. These changes in feeding practice and the distinct feeding regimes adopted by the different countries might explain the high prevalence of asymptomatic carriage of *Salmonella* observed in the majority of studies published to date. Commercial pet foods provide balanced nutrition for the pet and tend to be sterile, reducing the chances of foodborne infections such as salmonellosis.

Only one variant of *Salmonella* was identified in this study, *S. enterica* subspecies *arizonae.* The isolation of this subspecies was unusual as it is predominately found in reptiles, such as, snakes, lizards and terrapins (Butt & Morris, 1952.; Edwards *et al.*, 1959; Finely *et al.*, 2007); however isolation of this same subspecies has been previously reported in dogs however there is very little published data regarding this association (Hoag *et al.*, 2005). In addition, *S. arizonae* has been isolated from dog treats including dried pig ears, which may act as a potential source of contamination for dogs (Personal communication- Hilton, 2011). The *S. arizonae* was isolated following pre-enrichment suggesting it was present in low numbers. *Salmonella arizonae* is uncommon in human (Di Bella *et al.*, 2012) and canine infection. Nonetheless, there has been an increase in the number of incidences of infection with this strain as the popularity of reptile ownership increases (Butt & Morris, 1952). *Salmonella arizonae* has also been reported to manifest as salmonellosis in humans that have consumed contaminated snake meat or ingested medicines with traces of snake (Di Bella *et al.*, 2011; Hoag *et al.*, 2005). Typically, reported human cases are from vulnerable groups including immunocompromised or very young children (Di Bella *et al.*, 2011; Edwards *et al.*, 1959; Grupka *et al.*, 2006). No evidence of any repeated or intermittent shedding was found in the positive *S. arizonae* household dog beyond that of the single positive sample suggesting a brief transient asymptomatic carriage of *Salmonella*. Analysis of the questionnaire revealed that the canine was the only pet in the household, was fed a diet of commercially processed wet and dry pet food and was inclined to scavenge in the local environment. Therefore, it is most likely that the transient *S. arizonae* was acquired from the environment as a result of scavenging behaviour.

In this chapter no shedding was observed in the other location groups which was surprising considering boarding kennels and rescue centres are considered to be stressful environments. Dogs housed in kennels for long periods of time have been observed displaying behavioural frustration and depression which coincides with a high cortisol level, which is an indicator of stress (Beerda *et al.*, 1999; Beerda *et al.*, 2000). It has been reported that these events occur due to a deprivation of adequate opportunities to engage in species-specific behaviours such as a social interaction with other dogs and humans (Hetts *et al.*, 1992; Hubrecht, 1992; Stephen *et al.*, 2005). Veterinary hospitals are also considered stressful environments and tend to house immocompromised animal which is largely associated with asymptomatic shedding of *Salmonella* (Carter *et al.*, 2000; Burgess *et al.*, 2004; Schotte *et al.*, 2007). Studies conducted outside the UK have reported that asymptomatic shedding in dogs housed in boarding kennels was 16.6 % in the USA (McElrath *et al.*, 1952) and 15.5% in Iran (Shimi *et al.*, 1976). In addition, previous studies that have been conducted in the USA have investigated *Salmonella* shedding in greyhounds and reported a high prevalence, figures include; 36% (Galton *et al.*, 1952), 43%, (Stucker *et al.*, 1952) and 44% (Chengappa *et al.*, 1993). These high levels of asymptomatic shedding in greyhounds have been associated with the raw meat and offal diet typically provided to these animals. Generally when infected with *Salmonella*, racing greyhounds tend to present with symptoms and greyhound puppies often present with high morbidity rates of up to 100% and mortality rates are approximately 40%. A study investigating *Salmonella* infection in humans associated with greyhounds revealed that the source of the infection was from the greyhound faeces (Chengappa *et al.*, 1993). The fact that none of the kenneled dogs in this study were positive for the asymptomatic carriage of *Salmonella* may simply represent the absence of *Salmonella* in the canine gut or

reduced shedding due to the low stress levels in dogs sampled as a consequence of good animal husbandry practices. In addition, the questionnaires revealed that the UK dogs were not fed raw meat or offal, which was associated with the high levels of asymptomatic shedding, observed in the USA greyhounds. Analysis of the data obtained from the questionnaire revealed that only one dog in this study was fed a raw meat diet. This dog was negative for the presence of *Salmonella*. The low prevalence of *Salmonella* coupled with the minimal number of dogs fed a raw meat diet meant that it was not possible within this study to investigate the association of *Salmonella* with the consumption of raw meat diets. The bones and raw food diet (BARF) is being championed by many owners that practice homeopathy "holistic" life styles. They advertise that benefits include improved immune function and overall health, increased energy, improved coat and skin condition, and decreased body odour (Joffe & Schlesinger, 2002). However studies revealed that 80% of BARF food samples contained *Salmonella* and 30% of dogs fed on a BARF diet shed *Salmonella* spp (Joffe & Schlesinger, 2002). Therefore dogs fed BARF diets are a potential public health risk and the feeding of raw diets should be discouraged to reduce exposure of dogs and humans to harmful zoonotic pathogens such as *Salmonella*.

Copraphagic tendencies were also investigated as part of this study to identify a potential link in asymptomatic shedding and re-infection caused by ingesting contaminated faeces (Eldredge *et al.*, 2007). A total of 76 dogs were found to be copraphagic and some were reported to consume a diversity of animal faeces including duck, bird, cat and fox faeces, however all these dogs were negative for *Salmonella* shedding.

Symptomatic presentation including diarrhoea is one of a number of *Salmonella* gastroenteritis manifestations. A total of three dogs had diarrhoea in this study and they were all negative for the shedding of *Salmonella*. This is in agreement with previous studies which also showed no association between *Salmonella* spp isolation and dogs presenting with diarrhoea whether it is watery, mucous, or bloody (Cantor *et al.*, 1997). However, another study showed the prevalence of *Salmonella* in diarrheic dogs to range from 0 to 3.5% (Marks *et al.*, 2011).

3.4.2 Environmental sampling

Cross-contamination is a well-established route of transmission for spreading diseases such as *Salmonella*; whereby the source can contaminate the surrounding environment by direct contact or through secondary physical transmission. The majority of outbreaks caused by cross contamination arise from meat products contaminated in slaughter houses whereby *Salmonella* carried in the faeces and gut of the food stock animal is the source of contamination (Chengappa *et al.*, 1993; Plummer *et al.*, 2005). In this study the potential cross contamination from dog faeces, or other potential sources, to the local environment at WALTHAM® was investigated. *Salmonella* was undetectable in environmental swabs taken from office areas, pens, gardens and the dog's drinking water. The inability to detect *Salmonella* in the surrounding environment demonstrates that in this context the environment can be regarded as a low risk for exposure. This is almost certainly linked to the lack of excretion of *Salmonella* from the dogs interacting with the environment. The typically ubiquitous nature of *Salmonella* is due to its ability to withstand harsh environments and survive in water limited conditions. A study into non-typhoidal salmonellosis found that transmission was not only caused by food itself

but was attributable to a range of other routes including person-to-person transmission (32%), waterborne transmission (3%) and zoonotic transmission (2%) (King *et al.*, 2011). Studies have shown that effective cleaning regimes reduce colonisations of *Salmonella* and *Campylobacter* (Slader *et al.*, 2002).

3.4.3 Repeat sampling of individual dogs

To investigate the seasonality of *Salmonella* shedding, as demonstrated in other studies (Caraway *et al.*, 1959; Foley *et al.*, 2007; Stucker *et al.*, 1952; Tanaka *et al.*, 1976), a nine month longitudinal study was implemented. Intermittent shedding in canines has been shown to be greater in the spring and summer seasons which coincidently is the same for human cases (HPA, 2011c). During the spring and summer months the climate changes to a warmer temperature and this increase in environmental temperature has also been associated with *Salmonella* shedding in swine (Pires *et al.*, 2009). However limited data are available for the canine host and this association has not been widely explored. Therefore, intermittent shedding of dogs was investigated by sampling three consecutively excreted faecal samples every month for a total of nine months. *Salmonella* was not isolated from any of the ten dogs during this 9 months sampling period. This supports the epidemiological study method of sampling one faecal sample per dog and suggests that *Salmonella* was truly absent from the samples and not missed as a consequence of point sampling. Furthermore, if present the *Salmonella* would have been successfully isolated by direct or pre-enrichment methods as demonstrated by the positive control.

The low incidence of asymptomatic shedding of *Salmonella* observed in this study correlates well with the decreasing incidence of human salmonellosis observed in the UK over the past ten years as recorded by the Health Protection Agency (HPA). This decrease is primarily attributed to the introduction of vaccination of egg laying hens. Concurrently, there has been a rise in the use of commercial pet foods as they provide the balanced nutrients for the pet and tend to be processed to such an extent as to reduce the presence of bacterial pathogens. Conversely, there are still many companion animal owners who feed their pets with raw meat meals which could potentially be a route for *Salmonella* into the cycle of re-infection (Lenz *et al.*, 2009). Nowadays companion animals hold a more intimate relationship with their owners as they are considered part of the family setting and tend to share communal areas together. Whilst canines have been implicated in zoonotic diseases, good hygiene practice along with regular worming and vaccinations can contribute to the prevention of dog-related zoonotic diseases and are deemed to be 'responsible dog ownership' practices (Mani *et al.*, 2009).

3.5 Conclusion

In conclusion, the current perspective of asymptomatic faecal shedding of *Salmonella* in UK canines is *ca.* 0.2%. Where present it is shown to be a transient carriage and likely to have been picked-up from the local environment. From this study companion dogs do not represent a significant zoonotic risk for salmonellosis in humans eliminating one of the potential barriers to dog ownership.

Chapter 4 Phenotypic characteristics and antibiotic sensitivity of clinical human and canine *Salmonella* isolates

4.1 Introduction

Non-typhoidal zoonotic *Salmonella* serotypes, including the common disease-causing serotypes Enteritidis and Typhimurium, are widely distributed foodborne pathogens and one of the most common causes of bacterial foodborne illnesses in both humans and animals (WHO, 2005). In addition to serotype classification, *Salmonella* can also be classified based on their ability to cause disease in different hosts. *Salmonella* has been classified into host-restricted, host adapted and unrestricted serotypes according to the degree of host specificity, or into typhoidal and non-typhoidal serotypes, according to clinical presentation of systemic disease in humans (Eswarappa *et al.*, 2008; Lawson *et al.*, 2011; Pasmans *et al.*, 2003). Host adaption and restriction demonstrated in *Salmonella* strains is well-documented (Rabsch *et al.*, 2002; Shah *et al.*, 2005), furthermore cases of emerging strains demonstrating host restriction and adaption have been reported (Lawson *et al.*, 2011; Paulin *et al.*, 2002). This form of host restriction is typically revealed by an increased frequency of association of a particular strain with a defined host, and commensurate shift in metabolic capacity to allow the organism to exploit a competitive advantage within the new host. Such examples of host adaptation are *Salmonella enterica* subsp. *enterica* serotype Dublin associated with cattle and *Salmonella enterica* subsp. *enterica* serotype Typhimurium Variant Copenhagen Phage Type 99 associated with pigeons (Pasmans *et al.*, 2003; Pullinger *et al.*, 2010). Host adapted bacteria have also been observed to acquire novel properties through horizontal gene transfer resulting in the rapid acquisition of new capabilities. Studies have

revealed that the evolution of virulence in *Salmonella* is driven by horizontal gene transfer and has resulted in highly flexible pathogens that are able to colonize new niches and extend their host range (Bäumler, 1997). For example it is suggested that the *Salmonella* Pathogenicity Islands (SPIs) 1 and 2 were acquired by horizontal gene transfer. These SPI's house genes associated with the Type III Secretion System (T_3SS) allowing the transfer of effector proteins into the host cytosol via the syringe-like projections formed on the Gram-negative pathogen (Dieye *et al.,* 2009; Galan, 2001; Waterman *et al.,* 2003). It has also been documented that addition and deletion events and sequence divergence by point mutation also contribute to changes in the host ranges of *S. enterica* serotypes. Point mutations in particular have been seen in avian host restricted *S.* Gallinarium and *S.* Pullorum whereby they have lost their motile capacity and their ability to mediate Mannose-Sensitive Haemagglutination (MSHA) (Bäumler *et al.,* 1998; Crichton *et al.,* 1989; Li *et al.,* 1993). These phenotypic differences are therefore a useful tool for the identification and characterisation of host restricted; host adapted and unrestricted *Salmonella* strains.

Phenotyping is an established method for the identification of microorganisms and is routinely employed in clinical diagnostic laboratories (Koneman *et al.,* 1979; Nucera *et al.,* 2006; O'Hara *et al.,* 1992; Peele *et al.,* 1997). Phenotypic methods characterise expressed or physical properties of microorganisms ranging from the presence of flagella, and cell wall morphology revealed by Gram-stain, to metabolic rates and virulence factor production. Each organism is capable of utilising certain biochemicals and this results in a unique biochemical/metabolic profile; this metabolic profile is exploited in phenotypic identification systems such as the Analytical Profile Index (API) (BioMérieux, France). More recently, comprehensive phenotypic micro-arrays

have become possible through the development of systems such as the Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ (Technopath, Ireland).

The Biolog system is a 96-well plate format which allows simultaneous determination of a variety of metabolic characteristics. This form of phenotyping employs a wide diverse range of biochemical reactions including the utilisation of amino acids, salts, carboxylic acids, esters, fatty acids, hexose acid, hexose phosphates and reducing agents. Carbohydrate utilisation in particular is frequently used to differentiate bacterial strains and uses the concept that bacteria require an environmental energy source in order to produce ATP; an essential component required for biosynthesis, maintenance and reproduction. This concept can be used to investigate differences in bacterial strains as each bacterium has its own collection of enzymes which oxidise energy sources (Johnson &Schwarz, 1944). The Biolog system works on the basis that after oxidation by-products are produced and pH indicators added to the biochemicals to detect the degree of metabolic acids produced. With respect to the API 20 E tests, some reactions require the addition of reagents after incubation and these reagents react with the bi-products or metabolic pathway intermediates to indicate the level of utilisation of energy source.

Patterns in the profiles obtained from micro-array investigations can be difficult to determine by simple observations due to the complexity and volume of data generated. Exploratory statistical approaches such as Principal Component Analysis (PCA) have proven invaluable in revealing differences in metabolic profiles otherwise hidden within complex data (Hughes *et al.*, 2012). The PCA approach is a multivariate

statistical method which analyses observations from several intercorrelated quantitative dependent variables. The principal components are a set of orthogonal variables which enable the observations to be displayed as a pattern of similarity as points in maps (Abdi & Williams, 2010).

Additionally to metabolic profiling, bacterial strains can be characterised by their antibiotic sensitivity, with Minimum Inhibitory Concentration (MIC) and Antibiotic Disc Susceptibility (ADS) being amongst the most widely used methods (Andrews, 2001a & 2001b). Antibiotic resistance within veterinary isolates of zoonotic pathogens is of particular concern as clinical infections are showing an ever increasing resistance to antibiotics used to treat them (Addis *et al.*, 2011; Breuil *et al.*, 2000; Collignon, 2012; Poppe *et al.*, 2006).

The objective of work described in this chapter was to perform well-established phenotypic identification/ characterisation methods including, API 20 E test, Biolog Inc. Microbial Identification Systems GEN III MicroPlate™, MIC and ADS test to produce profiles of *Salmonella* isolated from symptomatic canine and human hosts within the UK. The relatedness of human and canine *Salmonella* isolates was determined by comparing profiles produced by API 20 E and measuring their similarity using Dice coefficients. These coefficients were represented by UPGMA (Un-weighted Pair Group Method with Arithmetic Mean) clustering and a dendrogram produced using GelCompar software (Bionumerics, Belgium). The comprehensive data from Biolog ID plates was analysed using PCA (Statistica, data analysis software system,

Version 10, Tulsa, USA) to reveal differences in profiles between the *Salmonella* isolates. Antibiotic sensitivity testing was determined using break points established by the British Society for Antimicrobial Chemotherapy (BSAC). Indistinguishable human and canine *Salmonella* phenotypic profiles may be suggestive of zoonotic or anthroponotic strains. In contrast, distinct profiles significantly restricted to one host may indicate that the isolates are moving towards limited host specificity.

4.2 Materials and Methods

4.2.1 Microbial cultures

One hundred and seventy five isolates of *Salmonella* were investigated in this chapter: Eighty five were canine isolates provided by VLA, UK, seventy nine were clinical human isolates from Aston University, UK, ten were current clinical human isolates from Queen Elizabeth (QE) Hospital, Birmingham, UK and a single isolate, *Salmonella enteric* subspp *arizonae*, isolated from an asymptomatic household dog (see Chapter 3). Isolates were stored on microbank beads at -80°C until required, where they were plated onto NA and grown at 37^{o}C in aerobic conditions for 24 hours. An *E.coli* NCTC 12241 was purchased from the HPA, UK, for the control strain in the antibiotic sensitivity profile methods. Table 4.1 List the serotypes of isolates used in this chapter otherwise stated differently in the text.

Table 4.1 *Salmonella* serotypes investigated for metabolic analysis using Biolog Inc. Microbial Identification Systems GEN III MicroPlate™.

Canine isolated *Salmonella* serotypes and ID number	**Clinical isolated *Salmonella* serotypes and ID number**
Agama - 1, 2, 68	Agona – 1
Amsterdam – 3	Anatum – 2
Anatum – 4, 69	Arizonea – 3
Bovismorbifican - 5	Atlanta – 4
Carmel – 6	Banana – 5
Derby – 7, 8	Bedford – 6
Dublin – 9, 72, 73	Berta – 7
Grumpensis – 16	Binza – 8
Hadar – 17	Bispebjerg – 9
Havana – 18	Brandenburg – 10
Infantis 20, 77, 78	Brookfield – 11
Isangi – 21	Clairbonei – 12
Javiana – 22	Corvallis – 13
Kisarawe- 23	Driffield – 14
Livingstone – 24, 25, 79, 80	Ealing – 15, 16
London – 26, 27	Eastborne – 17
Montevideo – 28, 29, 81	Enteritidis – 18, 19, 64, 65, 83, 107, 109, 110
Newport – 30, 31, 82	
Oranienburg – 32	Ferlac – 20
Orion – 33	Frintop – 21
Schwarzengrund – 34, 35, 85	Heidelberg – 23
Senftenberg – 37	Infantis – 24
Stourbridge – 38	Kedougou – 25
Typhimurium- 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 63, 64, 65, 66, 93, 94, 95, 96, 97, 98	Kubacha – 26
	Malawi – 27
	Manchester – 28
Brandenburg – 70	Mbandaka – 30
Cerro – 71	Montevideo – 31, 82
Durham – 74	Muechen – 32

Enteritidis – 75	Napoli – 33
Rissen – 83	Norwich – 35
Telaviv – 87	Saintpaul – 38
Tennessee – 88	Stanley – 39
arizonae – 99	Thompson – 40
	Lille – 49, 50
	Typhimurium – 52, 53, 54, 59, 61
	Rubislaw – 75
	Havana – 77
	Cambridge – 78
	Worthington -79
	Virchow – 80, 103
	Albuquerque – 81

4.2.2 Microbiological media

Agars and broths were as stated in section 2.2.2. In addition Tryptone Soya Agar (TSA) and Brain Heart Infusion broth (BHI) were purchased from Oxoid (Basingstoke, UK) and sterile Phosphate Buffer Saline (PBS) was purchased from Fisher Scientific, UK. The aforementioned media were prepared as per manufacturer's instructions and sterilised at 121^{o}C for 15 minutes. Agars BHI and TSA were cooled to 50^{o}C before pouring and PBS was stored at 4^{o}C until required.

4.2.3 Biochemical profiling using API 20E

The API 20E (BioMérieux, France) was used in accordance with the manufacturer's recommendations as a confirmatory test for the positive identification of *Salmonella* and also to identify any differences in biotypes between the different isolates. Suspensions of each isolate, listed in Table 4.1 were prepared in 5mL volumes of sterile 0.85% ($^{w}/_{v}$) saline to a 0.5 MacFarland standard. Inoculation and analysis of API 20E strips was carried out according to manufacturer's instructions. Each cupule (miniaturised test tubes) was filled with the bacterial suspension taking care not to introduce air bubbles. Anaerobic reactions were prepared by adding a drop of mineral oil overlay into the cupule. Strips were then placed into sterile incubation boxes containing a few drops of sterile water to maintain humidity. Following incubation at 37^{o}C for 18-24 hours the strips were read and interpreted using the guidance provided by the manufacturer and reactions were denoted positive or negative depending on the appropriate colour change. A negative control was performed by introducing inoculated saline into the API 20E strip.

4.2.4 Phenotypic microarrays using the Biolog Microbial ID plate

All strains in Table 4.1 were resurrected onto TSA and incubated at 37^{o}C for 24 hours. The Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ and Inoculating Fluid A (IFA) (Technopath, Ireland) were removed from 4^{o}C storage and allowed to reach room temperature before use. Following incubation of the TSA plates, a single colony was used to inoculate the prepared IFA to produce a homologous emulsion, according to manufacturer's instructions. A volume of 150µL of the IFA suspension was dispensed into each of the 96 wells in the Microbial ID plate; the plates were covered with their lids and incubated at 37^{o}C for 24 hours. Following incubation the Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ were analysed for end-point data by reading the absorbance of each well, containing redox dye tetrazolium, at 590nm in a Biolog plate spectrophotometer (Biotek Synergy HT, UK) coupled to a PC running KC4 data analysis software (Biotek, UK).

4.2.5 Motility Test

All *Salmonella* isolates from Table 4.1 were characterised as motile or non-motile by performing a motility test. Isolates were resurrected from -80^{o}C storage and inoculated onto NA plates and incubated at 37^{o}C for 24 hours. Sloppy agar was prepared using BHI with 0.5% ($^{w}/_{v}$) agar (Oxoid, UK) and 7mL aliquots were dispensed into sterile test-tubes. A single colony, taken from the NA plate, was used to inoculate a single test-tube containing the sloppy agar by stabbing the agar in a single vertical motion. Inoculated test-tubes were incubated at 37^{o}C for 24 hours. Isolates were analysed for motility by observing any horizontally dispersed growth into the sloppy agar.

4.2.6 Antibiotic sensitivity profiles

4.2.6.1 Preparation of antimicrobial agents

Antibiotics ampicillin, amoxicillin chloramphenicol, gentamicin, tetracycline, and trimethoprim were purchased from Sigma, Poole, UK. All the antimicrobials were purchased as laboratory standard powders with the exception of gentamicin which was purchased as a 50mg/mL solution. Each antibiotic was dissolved according to the manufacturer's instructions at a stock concentration of 256µg/mL. Powdered antimicrobial ampicillin, chloramphenicol, trimethoprim and tetracycline, were reconstituted in Sterile Distilled Water (SDW). Amoxicillin was reconstituted in SDW adjusted to pH 8 using 1M ammonium hydroxide (Fisher Scientific, UK). The 1M ammonium hydroxide solution was also tested to ensure it did not contribute to any observed antimicrobial effects. Antibiotic solutions were subsequently filter sterilised by passing through a 0.2µm cellulose syringe filter (Nalgene, Leicester, UK). Each stock solution was stored at 4^{o}C for 24 hours and subsequently at -20^{o}C to prevent degradation.

4.2.6.2 Determination of the Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentrations (MIC) were determined producing a profile for each isolate by performing the standard microbroth dilution method as published by BSAC (Andrews, 2001a). Antibiotic stock solutions (shown on figure 4.1) were prepared in sterile universals from the 256µg/mL antibiotic stocks stated in section 4.2.6.1. The antibiotics were double the concentrations as stated in Figure 4.1 (128µg/mL to 0.0625µg/mL), however once the inoculum is was added this concentration was halved; giving the correct concentration to be tested. For gentamicin

and trimethroprim a further dilution of 0.03µg/mL was also prepared. A 75µL aliquot of each antibiotic concentration was dispensed into sterile 96 well round bottom microtitre plates (Sterlin, UK), illustrated in Figure 4.1. *Salmonella* isolates were resurrected from -80^{o}C by plating on XLD selective agar and incubating at 37^{o}C for 24 hours. Following incubation a single colony was removed and used to inoculate a NA plate and again incubated at 37^{o}C for 24 hours. A single colony from the NA plate was used to inoculate 10mL NB which was incubated for 24 hours at 37^{o}C in an orbital shaker (Orbital shaking incubator, Gallenkamp) set at 200rpm. The OD of bacterial cultures was measured at A_{600} and adjusted to 5 x10^{5} cfu/mL and 75 µL of the suspension was added to each of the wells. Controls included *E.coli* NCTC 12241as a positive control, antibiotic free inoculum as a negative control and three wells containing media only to give background levels. Plates were incubated at 37^{o}C for 18-20 hours. The MIC was determined as the lowest concentration of the antimicrobial agent required to inhibit the growth. Published breakpoints provided by BSAC were used to determine resistance characteristics (Andrews, 2001a).

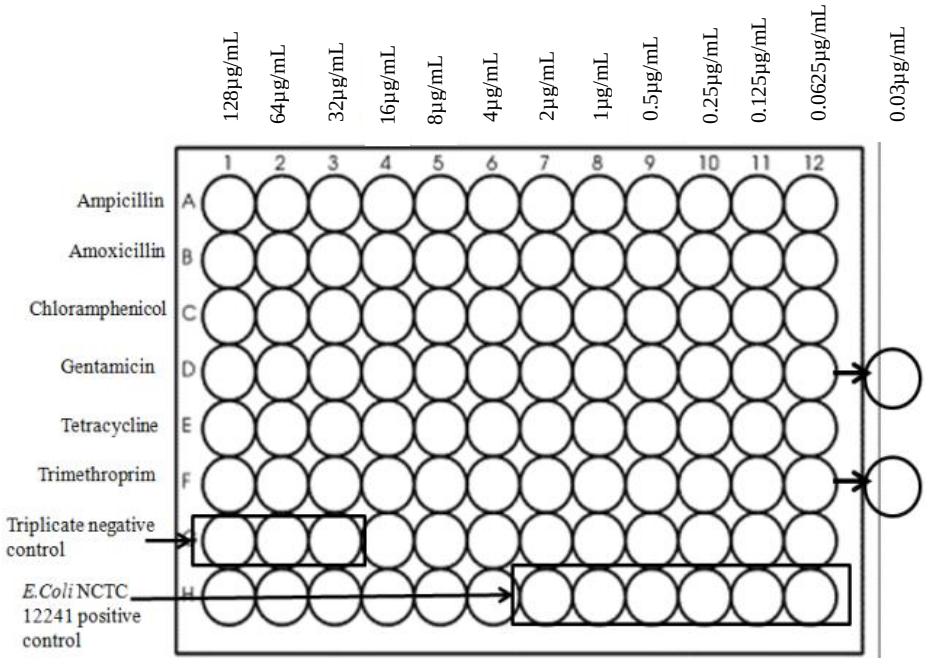


Figure 4.1 Schematic layout of MIC 96 well plate.

4.2.6.3 Antibiotic Disc Susceptibility testing

The standard method published by BSAC for ADS was performed on all *Salmonella* strains listed in Table 4.1 (Andrews, 2001b) to produce an antibiotic sensitivity profile per isolate. Suspensions of *Salmonella* isolates were prepared in 5mL volumes of PBS to a concentration/turbidity of 0.5 MacFarland standard. Fifty microlitres of bacterial suspension were spread onto NA using a sterile spreader to create a lawn of growth. Antibiotic discs (Oxoid, UK) were applied to the lawn using a disc dispenser (Thermo Scientific, UK). A sensitive strain *E. coli* NCTC 12241 was used as a sensitive control. Following incubation at 37^{o}C for 24 hours all inoculated plates were assessed for resistance or sensitivity to the six antibiotics by measuring the diameter of the zone of inhibition in millimetres around the antibiotic disk.

Table 4.2 Antibiotic concentration ranges for Minimum Inhibitory Concentration (MIC) and Antibiotic Disc Sensitivity (ADS) assays.

Antibiotic	Concentration range (µg/mL) for microdilution assay	Disc concentration (µg/disc)
Ampicillin	0.25- 128	10
Amoxicillin	0.25- 128	10
Chloramphenicol	0.25- 128	30
Gentamicin	0.03-128	120
Tetracycline	0.25- 128	30
Trimethroprim	0.03-128	5

4.2.7 Data analysis

The API profiles for each of the *Salmonella* isolates were analysed using the internet identification tool ApiWeb database (BioMérieux, France). The database allows for the identification of bacteria at a genus and species level following a 24 hour incubation time. The 20 biochemical reactions on each API strip, following incubation with each *Salmonella* isolate, were denoted black or white depending on whether the reaction was positive or negative respectively, thus generating a binary profile representing the pattern of biochemical activity. These profiles were scanned and normalised and uploaded to GelCompar software (Bionumerics, Belgium) where relatedness was calculated by UPGMA and a dendrogram produced. UPGMA is a simple hierarchical clustering method used in bioinformatics.

The Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ data (absorbance values) were exported from the plate reader software to a Microsoft Excel spreadsheet. The data was organised appropriately, where the ID and serotype of each isolate was added in a column and the affiliated 96 absorbance values for each isolate was added adjacent in rows. This sheet of data was exported into the statistical software Statistica 10 (StatSoft, Inc. (2011). STATISTICA (data analysis software system), version 10, USA) and analysed using the multifactor analysis, PCA. Score plots (projections of the samples in the space explained by all Biolog tests) of the components were investigated for correlations with serotype, host, and metabolic absorbance values of biochemicals presented on the Biolog Inc. Microbial Identification Systems GEN III MicroPlates™.

MIC data were analysed using chi-square once resistance or sensitivity had been established using the BSAC breakpoints. Chi-square investigates whether distribution of categorical variables differs from one another by comparing numerical counts in each category. With respect to antibiotic resistance, chi-square analysis was used to determine if there was a significant association between antibiogram (sensitivity of an isolate to different antibiotics) sensitivity profiles gained from *Salmonella* isolated from canine and human origin. Significant associations were determined using the chi square equation below:

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Where *O* is the observed frequency with which resistance occurs within the population and *E* is the expected frequency with which resistance would occur within a population.

In chi-square analysis, the observed numerical differences between two data set populations are measured against a null-hypothesis which states that the expected frequencies will match the observed frequencies in a normal distribution. If the observed results obtained are sufficiently different (P value is <0.05) to the expected results then the null-hypothesis will be rejected and a statistically significant relationship will be concluded to exist within the data.

The observed frequencies are tabulated against the chosen variable parameters of a 2 x 6 contingency table; where two represents the two host groups canine and human, and where six represents the number of isolates resistant to a number of antibiotics, R<5,

R<4, R<3, R<2, R<1 and none. The observed frequencies used in this chapter were the number of isolates that were resistance to a number of antibiotics. Resistance of the isolates to the antibiotics tested was determined by breakpoints established by BSAC (Andrews, 2001a), listed in Table 4.3

Table 4.3 Investigated antibiotics and associated breakpoints; defined by British Society of Antimicrobial Chemotherapy (BSAC).

Antibiotic	**MIC breakpoint (µg/mL)**			**Interpretation of zone diameters (mm)**		
	R >	**I**	**<S**	**< R**	**I**	**S>**
Ampicillin	8	-	8	14	-	15
Amoxicillin	8	-	8	14	-	15
Chloramphenicol	8	-	8	20	-	21
Gentamicin	4	-	4	19	-	20
Tetracycline	2	2	1	19	20-23	24
Trimethoprim	4	4	2	13	14-16	17

R= Resistance, I= Intermediate, S= Sensitive

4.3 Results

4.3.1 API Test 20E

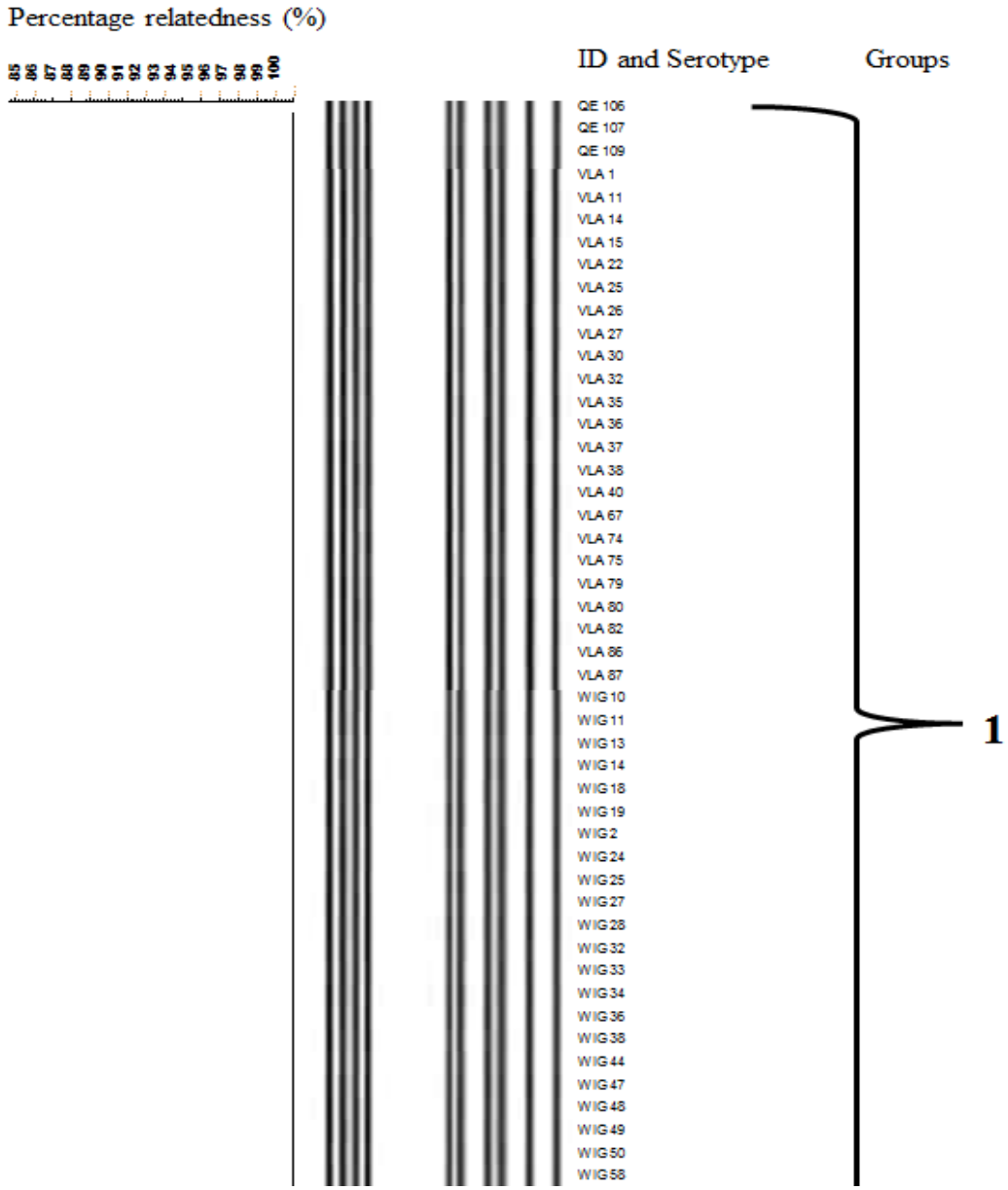
API profiles were obtained for *Salmonella* isolates using the ApiWeb database. The *Salmonella* strains gave profiles ranging from Excellent to Unacceptable Identification for *Salmonella* spp. Group 1, illustrated on the dendrogram in Figure 4.2 gave Excellent Identification to genus at 89.4% (6704552), Group 2 gave Excellent Identification at 99% (6704752), Group 3 gave Doubtful Identification at 99% (6705752), and Group 4 gave another Doubtful Identification at 89.4% (6705552). The dendrogram in Figure 4.2 produced by GelCompar software revealed that in Group 1 a total of 23 isolates shared API profiles at 85% relatedness. Group 1 were isolates that were negative for inositol fermentation and Voges Proskauer (VP) production. Group 2 demonstrated a negative reaction to inositol however a positive reaction to VP. Group 3 demonstrated positive reactions to both inositol and VP. Group 4 demonstrated positive reaction to inositol however a negative reaction to VP.

The remaining profiles ranged from Good, Doubtful to Unacceptable Identification, relating the profiles to *Salmonella* spp and for the later identification to *Serratia marcescens.* A total of seventeen isolates shared profiles with one or two other isolates and eleven isolates gave unique profiles. Of the unique profiles, four were canine isolates; *S.* Dublin (isolate number 73), *S.* Kisarawe (isolate number 23), *S.* Anatum (isolate number 69) and *S.* Agama (isolate number 68) and seven isolates were from the human population; *S.* Rubislaw (isolate number 75), *S.* Worthington (isolate number 79), *S.* Albuquerque (isolate number 81), *S.* Ferlac (isolate number 20), *S.* Typhimurium (isolate number 55), *S.* Norwich (isolate number 35), *S.* Gallinarum

(isolate number 22). Some isolates gave unusual results associated with environmental contamination and therefore were removed from the analysis to avoid the error in the analysis.

Eight isolates were positive for the presence of Tryptophan Deaminase (TDA), which is atypical and these profiles gave an unacceptable identification; however the rest of the profile was typical for *Salmonella* identification, which was determined by the ApiWeb analysis. There was no clear association between *Salmonella* serotype and API 20E profiles from this investigation. In addition, there was no clear grouping of canine and human isolates based on their API profiles.

The negative control demonstrated no bacterial growth and biochemicals remained un-reacted after incubation.


Percentage relatedness (%)
ID and Serotype
Groups
QE 106
QE 107
QE 109
VLA 1
VLA 11
VLA 14
VLA 15
VLA 22
VLA 25
VLA 26
VLA 27
VLA 30
VLA 32
VLA 35
VLA 36
VLA 37
VLA 38
VLA 40
VLA 67
VLA 74
VLA 75
VLA 79
VLA 80
VLA 82
VLA 86
VLA 87
WIG 10
WIG 11
WIG 13
WIG 14
WIG 18
WIG 19
WIG 2
WIG 24
WIG 25
WIG 27
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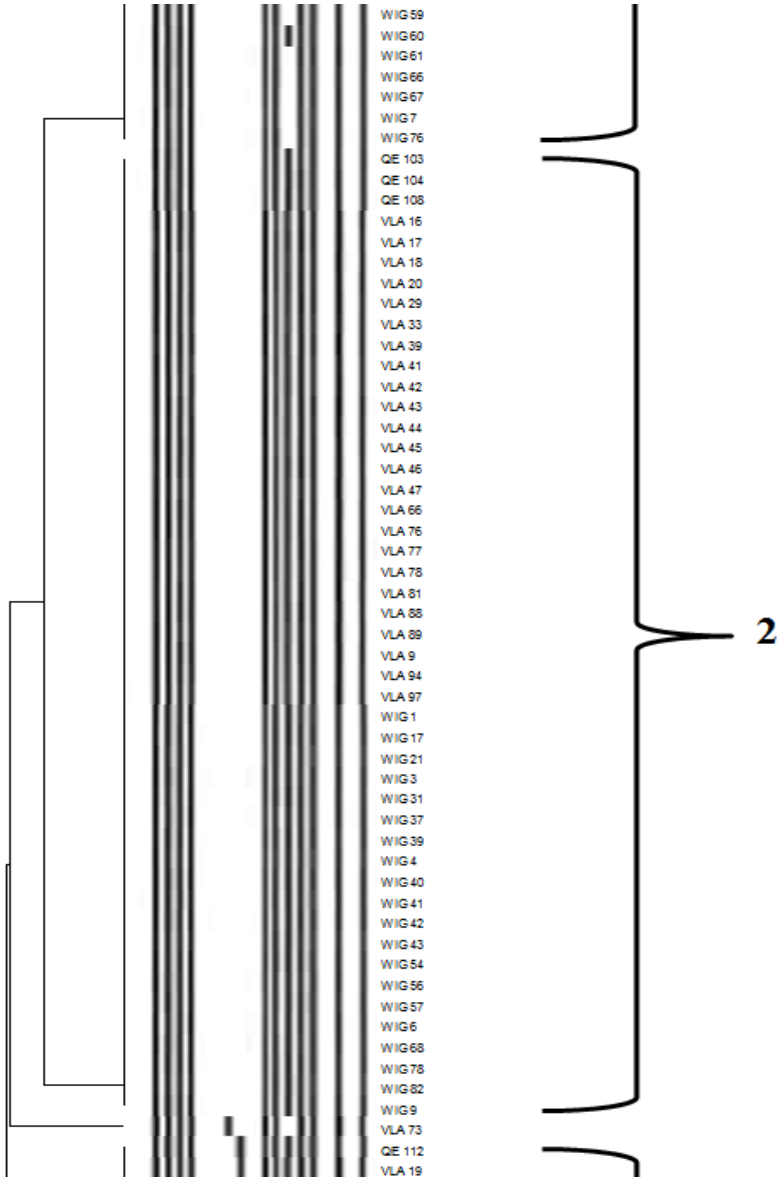
WIG 47
WIG 48
WIG 49
WIG 50
WIG 58
1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WIG 60
WIG 61
WIG 66
WIG 67
WIG 7
WIG 76
QE 103
QE 104
QE 108
VLA 16
VLA 17
VLA 18
VLA 20
VLA 29
VLA 33
VLA 39
VLA 41
VLA 42
VLA 43
VLA 44
VLA 45
VLA 46
VLA 47
VLA 66
VLA 76
VLA 77
VLA 78
VLA 81
VLA 88
VLA 89
VLA 9
VLA 94
VLA 97
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WIG 3
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WIG 4
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WIG 41
WIG 42
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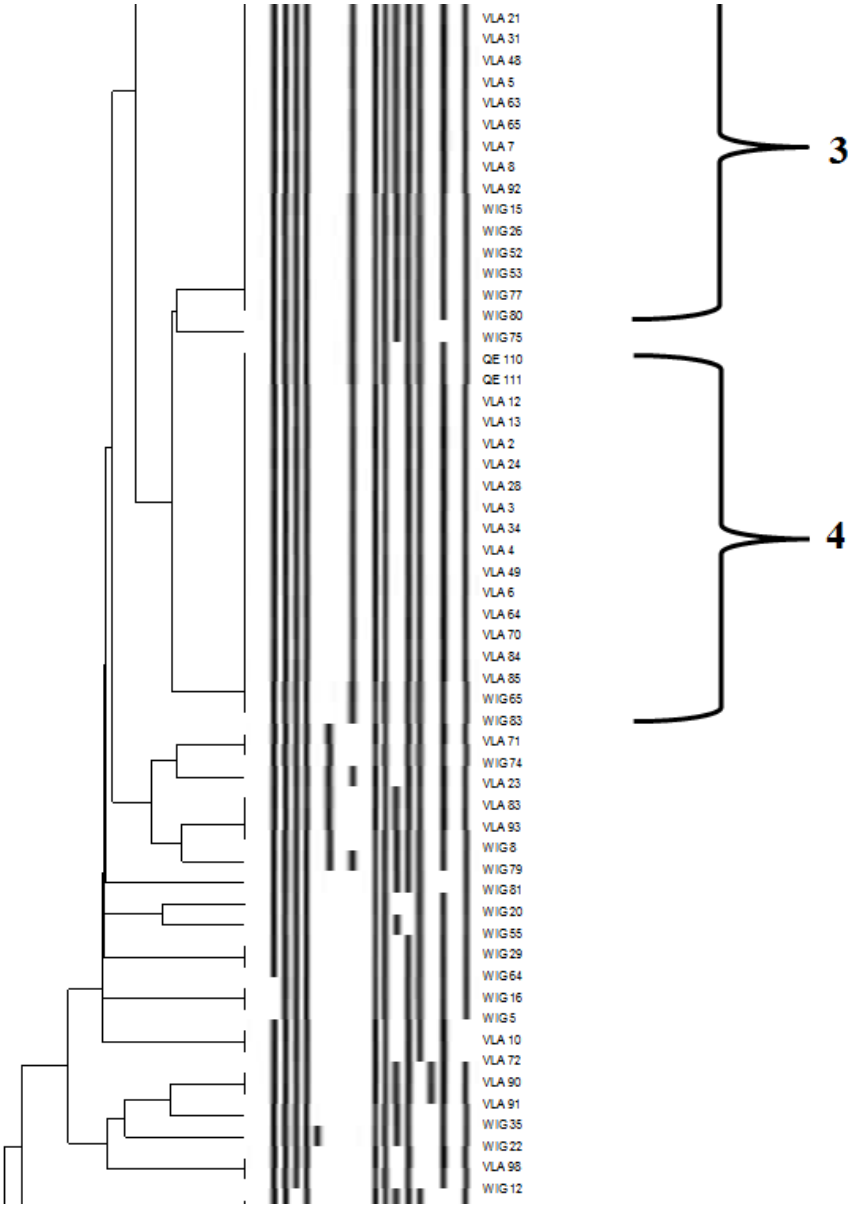
WIG 82
WIG 9
VLA 73
QE 112
VLA 19
2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VLA 31
VLA 48
VLA 5
VLA 63
VLA 65
VLA 7
VLA 8
VLA 92
WIG 15
WIG 26
WIG 52
WIG 53
WIG 77
WIG 80
WIG 75
QE 110
QE 111
VLA 12
VLA 13
VLA 2
VLA 24
VLA 28
VLA 3
VLA 34
VLA 4
VLA 49
VLA 6
VLA 64
VLA 70
VLA 84
VLA 85
WIG 65
WIG 83
VLA 71
WIG 74
VLA 23
VLA 83
VLA 93
WIG 8
WIG 79
WIG 81
WIG 20
WIG 55
WIG 29
WIG 64
WIG 16
WIG 5
VLA 10
VLA 72
VLA 90
VLA 91
WIG 35
WIG 22
VLA 98
WIG 12
3
4

Figure 4.2 API 20E profiles obtained from canine (VLA) and human (WIG & QE) *Salmonella* isolates with isolate number and serotype depicted. Percentage relatedness is shown represented by UPGMA clustering. The binary profiles start with biochemical reactions left to right on an API 20E strip and are listed in Table 1.2 in Chapter 1.

4.3.2 Microbial Identification Systems GEN III MicroPlate assays.

All of the *Salmonella* isolates were investigated for their ability to metabolise a number of different carbon sources including carboxylic acids, esters, fatty acids, hexose acids, amino acids, hexose phosphates, sugars, reducing power reagents and salts. The absorbance (590nm) measurements, after 24 hour incubation of the isolates with each biochemical test, ranged from zero to four. The PCA showed that the biochemical profiles of the majority of *Salmonella* isolates were similar with the exception of two canine isolates *S.* Typhimurium and *S.* Newport, illustrated in Figure 4.3.

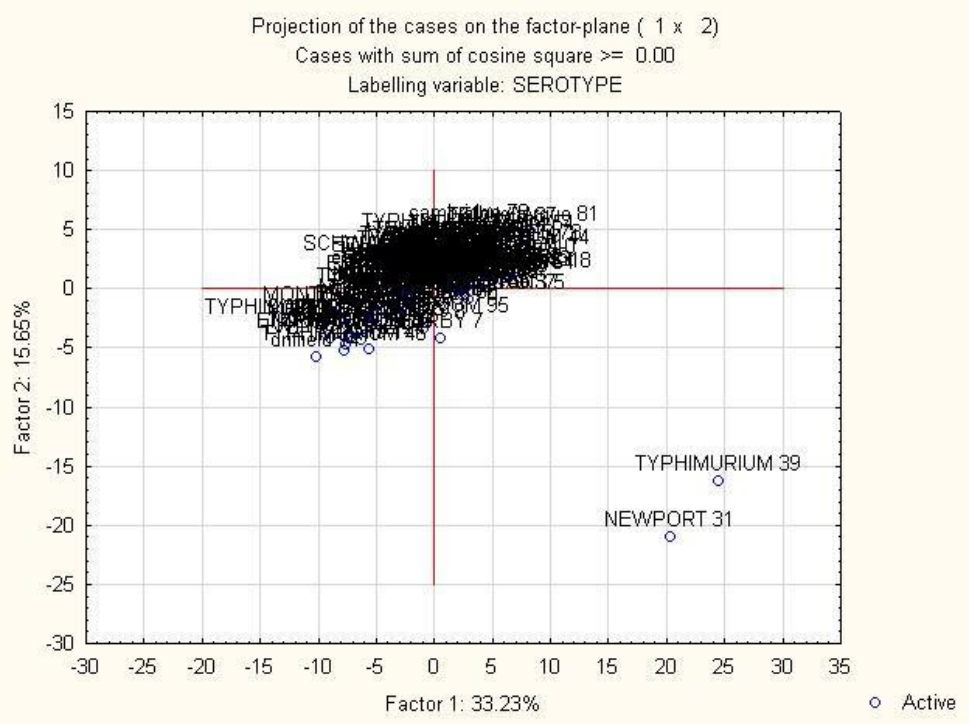


Figure 4.3 PCA of Microbial Identification Systems GEN III MicroPlate absorbance values from canine and human isolates of *Salmonella*, whereby the components are serotypes, factor 1 by factor 2.

Further analysis of the metabolic profile of these two outlying strains was undertaken to determine the combination of reactions giving rise to their discrimination. Absorbance values for each of the biochemical tests for the two selected isolates were compared by investigating which reactions showed a two-fold greater or two fold lower than average absorbance values. The data revealed that the utilisation of stachyose and N-Acetyl-D-Glucosamine (NADG) or commonly known as N-Acetyl Glucosamine (NAG) biochemical tests had a two-fold greater than average value suggesting that up-regulation was occurring in these isolates. Single tube assays were performed using the two distinct isolates and ten randomly selected isolates to confirm the high metabolism of the biochemicals stachyose (Sigma, UK) and NAG (Sigma, UK). This confirmed that the isolates *S.* Newport (31) and *S.* Typhimurium (39) had higher than average metabolism for these two biochemicals when compared with ten randomly selected isolates as listed in Table 4.4.

Table 4.4 Absorbance values of *S*. Typhimurium (39) and *S*. Newport (31) along with ten randomly selected isolates and their absorbance values in the biochemicals NAG and stachyose wells.

Isolate serotype and ID	Absorbance value for NAG	Absorbance value for stachyose
Newport 31	0.300	0.710
Typhimurium 39	0.317	0.717
Bispebjerg 9	0.361	0.028
Kedougou 25	0.158	0.09
Typhimurium 42	0.051	0.103
Rubislaw 75	0.278	0.075
Enteritidis 110	0.265	0.05
Dublin 10	0.077	0.275
Typhimurium 63	0.004	0.386
Enteritidis 75	0.243	0.7
Schwarzengrund 85	0.11	0.639
Typhimurium 95	0.179	0.292

4.3.3 Motility Test

Each isolate was confirmed positive for the presence of functionally working flagella as the isolates were able to grow horizontally from the point of inoculation.

4.3.4 Antibiotic sensitivity tests

Antibiotic sensitivity determined using the MIC method showed that there were varying degrees of antibiotic resistance among all the strains tested. A total of five canine isolates, *S.* Typhimurium (VLA 44 and 97), *S.* Roodenpoort (VLA 84), *S.* Schwarzengrund (VLA 35), and *S.* Infantis (VLA 78) were resistant to all antibiotics investigated. None of the human isolates demonstrated resistance to the whole panel of antibiotics. The ADS method revealed that three canine isolates, *S.* Havana (VLA 76), *S.* Newport (VLA 82) and *S.* Typhimurium (VLA 89), showed resistance to all the antibiotic discs tested whereas, only one human isolate *S.* Enteritidis (WIG 18) demonstrated resistance to all the antibiotics. Table 4.8 summarises the results for the MIC and the ADS methods.

Chi squared (χ^2) analysis of the MIC data demonstrated a significant association between a number of the canine isolates and resistance to 5 antibiotics in the test panel. Conversely, there was no significant association between the two groups canine and human isolates when a χ^2 analysis was performed using the ADS method. Each isolate was denoted either resistant (R) or sensitive (S) depending on their MIC and the associated break point for each antibiotic. The resulting antibiogram data is detailed in Table 4.5.

Table 4.5 The number and percentage of isolates including frequency of resistance (R) to test antibiotics using MIC data.

Resistance (No. of antibiotics)	**Percentage of canine isolates (n=83)**	**Percentage of human isolates (n=80)**
R= 5	5/83 =6.2%	0%
R≥4	9/83= 10.84%	0%
R≥3	27/83= 32.53%	10/80= 12.5%
R≥2	40/83=48.19%	28/80= 35%
R≥1	63/83= 75.90%	58/80= 72.5%
Non	20/83= 24.09%	22/80= 27.5%

The ADS testing revealed that 59 canine isolates and 56 human isolates were resistant to one or more of the antibiotics discs tested. The number of isolates that demonstrated resistance to the antibiotics are listed in Table 4.6.

Table 4.6 The number and percentage of isolates including frequency of resistance to test antibiotics using ADS data.

Resistance (No. of antibiotics)	Percentage of canine isolates (n=83)	Percentage of human isolates (n=80)
R= 5	3/83= 3.61%	1/80= 1.25%
R≥4	16/83=19.27%	6/80= 7.5%
R≥3	28/83= 33.73%	16/80= 20%
R≥2	41/83= 49.39%	30/80= 37.5%
R≥1	59/83= 71.88%	56/80= 70%
Non	27/83= 3253%	24/83= 28.91%

R = resistant and the numbers adjacent indicate how many of the antibiotics the isolates where resistant to.

4.3.4.1Chi-square analysis

Analysis of the data using chi-square ($\chi 2$) revealed a significant association between isolate host group and the level of antibiotic susceptibility from the MIC and ADS tests. Table 4.7 summaries the $\chi 2$ critical levels and the significant association of isolate origin and the number of isolates resistant to the panel of test AB's. Table 4.7 summarises the number and percentage of isolates from both human and canine host populations that demonstrated resistances to the panel of test AB's. No significant association was demonstrated between the percentages of isolates that were resistant to each AB and host group using MIC data. However a significant association was observed at 1% (15.86%) using the ADS test, where there was a higher level of resistance to antibiotics in the canine group in comparison to the human group.

Table 4.7 Summary of significant associations using χ2 analysis of resistance patterns of canine and human *Salmonella* isolates.

No. of resistant isolates	χ2 critical level		Significant association		Summary
	MIC	AB Disc	MIC	AB Disc	
=5	3.841	-	0.005	No	Significant association between canine isolates and resistance to 5 AB in the test panel at 5% using MIC data.
≥4	5.412	3.841	0.002	0.005	Significant association between canine isolates and resistance to ≥4 AB in the test panel at 2% and 5% using MIC and AB Disc data, respectively.
≥3	6.625	3.841	0.001	0.005	Significant association between canine isolates and resistance to ≥3 AB in the test panel at 1% and 5% using MIC and AB Disc data, respectively.
≥2	-	-	No	No	No significant association
≥1	-	3.841	No	0.005	Significant association between canine isolates and resistance to ≥1 AB in the test panel at 5% using AB Disc data.
None	-	-	No	No	No significant

Table 4.8 Summary of resistance patterns of canine and human isolates against six antibiotics.

Antibiotic	**MIC**		**Disc Diffusion**	
	Canine	**Human**	**Canine**	**Human**
Ampicillin	39 (46%)	31 (38.8%)	34 (40.9%)	35 (43%)
Amoxicillin	36 (43%)	27 (33.8%)	31 (37%)	12 (15%)
Chloramphenicol	40 (48%)	37 (46%)	27 (32%)	15 (18%)
Gentamicin	13 (20%)	9 (11%)	22 (26%)	7 (8.7%)
Tetracycline	17 (20%)	7 (8.7%)	39 (46%)	28 (35%)
Trimethroprim	15 (20%)	7 (8.7%)	22 (32%)	6 (7.5%)

4.4 Discussion

The aim of this chapter was to phenotypically profile a panel of clinical canine and human *Salmonella* isolates using biotyping and antibiogram testing methods including, API 20E, Microbial ID plates, Motility tests, MIC and ADS tests. Strains of *Salmonella* have been shown to differ in their metabolic characteristics and these can be utilised to reveal differences in strains (Lewis & Stocker, 1971); comparison of phenotypic characteristics may indicate potential for zoonotic or host adapted canine and human strains. If characteristics are indistinguishable in both groups then this would suggest zoonotic potential however if there is a prominent difference in characteristics in the two group then this may be suggestive of host restriction of the isolates.

4.4.1 API Test 20E

No clear discrimination was observed in profiles from either host groups based on their API 20E tests, suggesting a similar metabolic strategy adopted by all the strains. Two major groups demonstrated inositol fermentation/oxidation. Another two groups were Voges Proskauer (VP) negative and a cluster of eight isolates were found to be positive for TDA.

Inositol is one of the most abundant carbohydrates in freshwater and land ecosystems (Turner *et al.*, 2002); and in some bacteria it is utilised as an antioxidant, osmolyte, cell membrane component, and as a carbon storage substrate. In Gram-negative bacteria, genes involved in inositol metabolism are more dispersed across the chromosome and if present then fermentation of inositol is possible. Studies into different strains of *S.* Typhimurium revealed that the majority of the strains were negative for fermentation of

inositol and the remaining strains demonstrated a weak fermentation (Lewis & Stocker, 1971). This negative observation of inositol fermentation is employed as one of a number of established tests for the identification of *Salmonella* species. The positive tests in this study therefore are uncommon for *Salmonella* species and maybe a result of weak fermentation as demonstrated in previous studies (Lewis & Stocker, 1971).

Two groups containing a total of 35 isolates demonstrated positive for the VP test and three individual isolates with unique profiles also demonstrated this trait. The reaction is dependent upon the production of acetoin, a non-volatile reducing substance (Werkman, 1930). Typically VP positive organisms include *Enterobacter*, *Klebsiella*, *Serratia marcescens*, *Hafnia alvei*, *Vibrio damsela*, however *Vibrio alginolyticus* and *Salmonella* should typically yield a negative reaction (Garibaldi & Bayne, 1970). However, a study into the production of acetoin in isolates from the genus *Salmonella* demonstrated that under certain nutritional conditions *Salmonella* produce abundant quantities of acetoin (Garibaldi & Bayne 1970). Considering these observations the API 20E results should be treated with scepticism.

In this investigation a total of eight strains isolated from both host groups were positive for the presence of Tryptophan Deaminase (TDA). The substrate L-Tryptophan is metabolised by TDA into Indole pyruvic acid in the presence of ferric iron and hydrochloric acid and this reaction is an employed test for the positive identification of *Proteus* spp. These profiles resulted in 'Doubtful' identification to *Salmonella* at 99.9% to 'Unacceptable' profiles when analysed using the ApiWeb tool analysis. The API system is not fully reliable and it has been criticised that reliance on a single identification method can lead to misidentification in a clinical setting (Janda Abbot,

2002; Liguori *et al.*, 2010; Nucera *et al.*, 2006). A correspondence in *Clinical Infectious Diseases* highlighted problems concerning the potential misidentification of bacteria when commercial identification systems are used (Frederiksen & Tenning, 2001). However for the purpose of profiling individual isolates carried out in this thesis the API 20E system allowed for 20 biochemical test to be performed in 24 hours and gave either a positive or negative results; therefore a simple profile pattern of individual isolates could be made and analysed for similarities and differences. Phenotypic systems such as the API test are still the most common approach used in clinical laboratories to identify bacteria however as with all bacterial identification systems it has several drawbacks in misidentification of bacteria and should be either used alongside another phenotypic method or a genotypic method should be employed (Janda & Abbot, 2002). In this study identification of the isolates was confirmed prior to phenotypic analysis therefore these irregularities observed maybe due to the rogue contaminates or the poor discriminatory power of the API system.

4.4.2 Microbial Identification Systems GEN III MicroPlate

Principle Component Analysis (PCA) of the Biolog data demonstrated that the majority of the isolates shared similar metabolic capabilities but there were two distinct isolates which did not cluster with the main group. These two isolates were serotypes *S.* Typhimurium (39) and *S.* Newport (31) and both interestingly were of canine origin. The biochemical reactions driving the differences between the major group of strains and outlying two strains were stachyose and NAG. The outliers demonstrated an enhanced rate of metabolism within the 24 hour test period compared to the rate observed with the comparative group.

Stachyose is a precursors of D-galactose; an important surface lipopolysaccharide of *Salmonella* (Christian *et al.*, 2007). In addition NAG to also a surface lipopolysaccharide. The surface lipopolysaccharide forms the outermost components of Gram-negative bacteria cells and they are the primary components to interact with the external environment. *Salmonella* is a robust bacterium encountering and surviving varieties of environmental pressures including low pH in the animal stomach through to the surrounding Nitric oxide (NO) inside the macrophages (Bäumler *et al.*, 2000). Lipopolysaccharide is made from three segments, the O side-chain, core (inner and outer) and finally Lipid A and it is D-galactose and NAG that are required to formed the outer core (Bäumler *et al.*, 2000). These strains demonstrated a higher rate of growth in the environment abundant with NAG and stachyose; which could become a competitive advantage in an environment containing these components. Stachyose is a naturally found water soluble saccharide produced in the cytoplasm of plants and vegetables (Martínez-Villaluenga *et al.*, 2008) therefore if the host were nurtured on a stachyose rich diet then it might by hypothesised that these strains could have a competitive advantage over other microbes in the gut environment. This compound is part of the galactose metabolism pathway where it is hydrolysed to produce constituents for D-galactose synthesis; lactose hydrolysis constituents can also synthesis D-galactose. D-galactose is required for essential pathways including amino acid and nucleotide sugar metabolism, bacteria chemotaxis, carbohydrate digestion and absorption and mineral absorption. N-Acetyl-Glucosamine is also part of the amino sugar and nucleotide metabolism, peptidoglycan biosynthesis and phosphotransferase system as demonstrated on the Kegg pathway encyclopaedia (Kanehisa Laboratories, 2011). The change that has been observed in this study may be an early indication of genetic changes including point mutation, deletion or horizontal gene transfer, in

Salmonella strains causing an infection in canine host by altering the metabolic capacity to utilise stachyose and NAG and their associated pathways.

An advantage of *Salmonella* spp. to cause infection in a host is that the host will become a chronic carrier which will have the potential to transmit the strain (Bäumler, 1998). New variants of microbial strains typically emerge through the selection pressures of their microenvironments as they can gain or lose large numbers of genes by point mutation and deletions to adapt to the harsh condition in their host (Gilsdorf *et al.*, 2004). They can co-evolve within their host by adopting strategies to change surface antigens in-turn avoiding the host immune system without impeding their ability to colonize and replicate (Gilsdorf *et al.*, 2004). Canine diets and intestinal environments are different to humans and this could be the reason for a selective change in these strains.

4.4.3 Motility Test

In this study the possession of flagella was investigated as an additional phenotypic difference between the strains. Motility is a virulence factor of many pathogenic bacteria which allows strains to interact and adhere with the host's intestinal epithelial cells (Allen-Vercoe *et al.*, 1999; Jonson *et al.*, 2005; Nakae *et al.*, 1975). From this study all isolates from each group were positive for the possession of flagella and demonstrated motility. Whilst motility is an important virulence factor, studies have revealed that several non-motile strains demonstrate virulence and those flagella are a major contributor to *Salmonella* locomotion and are therefore not required for virulence *per se* (Carsiotis *et al.*, 1984). The importance of flagella and motility of *S.* Typhimurium and *S.* Dublin in models of extra animal survival has been investigated

and has demonstrated a strong association that motility and chemotaxis are important during the spread of *Salmonella* from one animal to the next through the external environment. Motility is a characteristic required for external-host survival as it is important in the full infection cycle for zoonotic Salmonellae (Olsen *et al.*, 2012).

4.4.4 Antibiotic sensitivity profiles.

A somewhat different approach to identifying phenotypic differences in isolates was investigating their antibiotic sensitivity patterns, using the well-established methods of MIC and ADS tests. Sensitivity patterns were used to reveal any association between the resistance pattern of an isolate to antibiotics and where it was isolated from, either canine or human host. The association between the resistance profiles of an isolate to antibiotics and origin, being human or canine host was determined using the panel of antibiotics, ampicillin, amoxicillin, chloramphenicol, gentamicin, tetracycline and trimethoprim, by performing the gold standard method, MIC and ADS on all strains listed in Table 4.1. Antibiotic resistance can also be regarded as another phenotypic characteristic heavily influenced by exposure to antibiotics and environmental pressures. It was of interest to investigate any relationships between resistance profiles of the isolates and its origins. The antibiotics in this study were selected as they are commonly used for the treatment of *Salmonella.*

Antibiogram profiling revealed that canine isolates yielded a higher percentage of resistance in comparison to the human isolates with the tested antibiotics. A total of nine canine isolates showed resistance to four or five of the antibiotics tested whereas none of the human isolates demonstrated this level of resistance. This evidently

suggests that the canine isolates tested are resistant to more antibiotics than human strains and statistical analysis using χ^2 confirmed that there was a significant association between the groups with respect to their resistance profiles. This result is not unexpected as the prescribing practice of antibiotics in the veterinary setting is much more relaxed than the human clinical settings (Hughes *et al.*, 2012). This implies that bacterial infections in companion canine animals may be treated improperly which contributes to the increase in antibiotic resistance as demonstrated in the canine isolates from this study (Prescott *et al.*, 2002). Consequently it could be suggested from the results that *Salmonella* isolated from canine hosts are phenotypically different to the human isolates because some are able to withstand the effects of antibiotics and this could potentially give rise in the future to host restricted *Salmonella* strains in canines. Table 4.8 details the numbers of isolates from each group that are resistant to each antibiotic. The data shows that in both groups more isolates demonstrated resistance to tetracycline and ampicillin. A study investigating antibiotic resistance in *Salmonella* strains isolated, from 1959 to 1970, from humans and animals revealed an increase in resistance to tetracycline, of which one third of these isolates were also resistant to ampicillin (Manten *et al.*, 1971). Antibiotic sensitivity testing is paramount in the current times as antibiotic resistance in bacteria and in humans host infections is on the rise. Prudent use of antimicrobial drugs is integral to reduce the resistance which is being observed in this current time. Analysis of ADS demonstrated a significant association between canine *Salmonella* isolates and being resistant to four or less of the tested antibiotics. Standard organisations including the National Committee for Clinical Laboratory Standards (NCCLS) or British Society for Antimicrobial Chemotherapy (BSAC) define zone diameter and interpret using published data. As with many protocols, there are limitations with using ADS, as zone diameters can vary depending

on the methods used, for example medium used, depth of agar and inoculum density can all impact the results.

The treatment of *Salmonella* in symptomatic dogs is a controversial topic, with many microbiologists and infectious disease specialists arguing that antibiotics are not advocated for treating animals with uncomplicated *Salmonella* gastroenteritis. Simultaneously, in humans antibiotic therapy has no positive clinical effect on the treatment of diarrhoea caused by *Salmonella* in healthy children and adults with non-severe diarrhoea (Sirinavin & Garner, 2009). Instead, antibiotics should be reserved for animals and humans with concurrent signs of systemic infection or a history of immunosuppression. A study conducted using human hosts demonstrated that antibiotic therapy had apparent effect on stool cultures only in the early stages. Antibiotic regimens of 1-14 days did not decrease the positive rates of intestinal *Salmonella* after 2-3 weeks therefore faecal shedding can be prolonged with the injudicious administration of antibiotics; this could be also the case in canine host (Hughes *et al.*, 2012). Infecting organisms may acquire transferable resistance via horizontal transfer of plasmids and selective pressure will occur especially with frequent use of antibiotics (Hughes *et al.*, 2012). They are not useful for intestinal *Salmonella* eradication and should not be recommended (Sirinavin & Garner, 2009). For septic dogs, antibiotics reported to be effective against *Salmonella* include fluoroquinolones, chloramphenicol, trimethoprim-sulfonamide, and amoxicillin. Determination of a susceptibility profile is recommended for selection of optimal antimicrobials (WSAVA, 2007). The profiling of *Salmonella* isolates using antibiotic sensitivity has been demonstrated in this chapter to discriminate isolates that are phenotypically different in their ability to resist commonly used antibiotics; the results demonstrate a trend of higher resistance of canine isolates

of *Salmonella* which could contribute to the suggestions of canine host adaptive isolates.

4.4.5 Phenotypic characterisation as a tool for revealing host restricted or adapted strains in canines

The simple method to phenotypically characteris bacterial isolates is to use biotypes and antibiogram profiles. Similarities and differences could be early indications of zoonotic and host adaptive strains, respectively. There is an increasing concern for the potential of zoonotic transfer of *Salmonella* from companion dogs to humans and *vice versa*. Companion animal ownership is expanding in the western world; 13 million UK households owning a pet (PFMA, 2011). Simultaneously the number of cases of zoonotic infections is on the rise with one of the factors being an increase in pet ownership and the intimate interactions between the owners and companion animals (Mani, 2009). The similarities observed in the metabolic profiles of the isolates infer an equal capability to exploit the host environment, suggesting zoonotic potential between the two hosts. Zoonotic infections of *Salmonella* have been reported between canines and humans whereby the *Salmonella* was contracted through exposure to pet food and a shared home environment in the USA (CDC, 2008).

4. 5 Conclusion

In conclusion the majority of *Salmonella* strains isolated from symptomatic canines shared common phenotypic characteristics with human clinical strains. API profiles of *Salmonella* from both host groups were generally homogenous, only demonstrating differences in one or two reactions provided on the test strip. The Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ has proven to be a useful tool for an in-depth metabolic profiling of strains. Absorbance data coupled with PCA analysis allowed for the discrimination of two canine strains from all of the other isolates based on differences in their ability to metabolise stachyose and NADG. Antibiotic sensitivity testing proved valuable in revealing resistance profiles in isolates of *Salmonella* from canine and human hosts. Canine isolates revealed significantly higher levels of resistance in comparison to the human isolates. Overall the phenotypic profiles produced from *Salmonella* isolates from canine and human host demonstrated a lack of distinction, suggesting a zoonotic and anthroponotic capacity to cause infection in both dogs and humans. There were only two isolates that demonstrated potential host restricted characteristics based on the Biolog Inc. Microbial Identification Systems GEN III MicroPlates™. Phenotypic methods may lack discrimination therefore genotypic typing methods to investigate any genetic differences was investigated in the following chapter.

Chapter 5 Genotypic characteristic of clinical and canine *Salmonella* isolates

5.1 Introduction

Salmonella is recognised as one of the most significant causes of bacterial foodborne infection in developed countries, second to *Campylobacter* (Hilton *et al.*, 1996; Louis *et al.*, 2005). In England and Wales in 2000, 1.3 million cases and 480 deaths were due to indigenous food-borne diseases; where enteric bacterial pathogens including *Campylobacter* and *Salmonella* accounted for the greatest disease burden (Lukinmaa *et al.*, 2004). Common serotypes associated with clinical infection have recognised phenotypic and genotypic characteristics, and it is these characteristics that are exploited in traditional phenotypic tests such as API biotyping, phage typing, serological methods and antibiograms (Liebana *et al.*, 2001b). These methods are relatively inexpensive however can lack discrimination in more commonly encountered types which may be revealed by genotypic characterisation methods which have become more frequently employed in recent years. Pulse-Field Gel Electrophoresis (PFGE) of *Salmonella* species is amongst the most well-established genotypic tool, producing reproducible banding profiles or molecular fingerprints, characteristic of different bacterial strains. Profiles of strains can be normalised and compared to determine their relatedness; profiles of banding patterns that are indistinguishable to each other are considered to be highly related if indeed not the same strain.

Genotypic methods are preferred for outbreak investigations as they allow epidemiologically related isolates to be identified from non-related isolates by targeting

molecular differences that are clonally unique to the outbreak strain (Liebana *et al.*, 2001b). Molecular PFGE fingerprints can be used to identify the source or route of infection and assist in identifying strategies to control the outbreak. Pulse-Field Gel Electrophoresis is largely regarded as the "gold standard" for molecular typing of *Salmonella* as it is highly reproducible, owing to a harmonised protocol and interpretation criteria established by the EnterNet and PulseNet database (Enter-net/Salm-gene, 2012; Liebana, *et al.*, 2001b). The method is also routinely used in epidemiological investigations of *Salmonella* outbreaks by the Centres for Disease Control and Prevention (CDC) and Laboratories for Enteric Pathogens (LEP) and is widely used in health departments in the United States, as well as in Canada, Latin America, Asia, and Europe; coupled with the International PulseNet and EnterNet database systems as it has proven to be a reliable comparative method (Soyer *et al.*, 2010; Swaminathan *et al.*, 2001, Swaminathan *et al.*, 2006). These databases play a vital role in monitoring foodborne infection outbreaks and surveillance.

Banding patterns obtained by PFGE arise from digested chromosomal DNA fragments produced by restriction endonucleases. These fragments are large pieces of DNA ranging in size from 5Kb-6Mb. The DNA fragments are resolved by alternating the electrical field with a pulse angle of 120^{o}, between spatially distinct pairs of electrodes which allows the DNA to re-orientate and move at different speeds through the pores in the agarose gel (BioRad, UK). The restriction endonuclease *Xba*1 gives rise to discriminatory *Salmonella* profiles which can be compared with other *Salmonella* profiles that have been manipulated using the same harmonised protocol (Enter-net/Salm-gene, 2012).

Although considerable data on the diversity of *Salmonella* PFGE types and the discriminatory ability of PFGE to characterise isolates from human clinical cases are available (Agasan *et al.*, 2002; Camps *et al.*, 2005; CDC, 2008; Grandesso *et al.*, 2008; Lomonaco *et al.*, 2008), evaluation of *Salmonella* PFGE type diversity among dog isolates is rare. However, studies investigating *Salmonella* contamination of pet-food products have demonstrated the zoonotic association with infection from companion dog feed products and the food-handling owner (CDC, 2008; Clark *et al.*, 2001; Crum-Cianflone, 2008; Finley *et al.*, 2006; Pitout *et al.*, 2003). Using PFGE to compare strains allows for the potential sources and reservoirs of zoonotic and foodborne disease to be identified. Additionally, analysis of PFGE profiles is critical to identify potentially host-restricted subtypes (Soyer *et al.*, 2010). A pilot study to further characterize *S.* Typhimurium isolates from passerine mortality incidents collected from northern England, in 2005 to 2006, using PFGE found minimal variation among isolates, some of which appeared to be clonal, indicating that the bacterial strains may be host adapted to wild bird populations (Hughes *et al.*, 2008).

This chapter describes application of the harmonised PFGE protocol (Enter-net/Salm-gene, 2012) to the genotypic characterisation of 95 clinical canine *Salmonella* isolates (VLA, UK) with a diverse range of serotypes in comparison to PFGE profiles of human clinical *Salmonella* isolates (HPA, UK). It was of interest to investigate the incidence of canine salmonellosis and common associated serotypes with the human population by comparison to the HPA PFGE database; a form of genotypic characterisation. Therefore PFGE profile relatedness from both host groups was determined by Dice coefficient and represented by a UPGMA dendrogram produced in Bionumerics

software (Bionumerics, Belgium). Profiles from homologous serotypes indistinguishable by PFGE from both human and dog hosts may be suggestive of zoonotic or anthroponotic transmission. Clustering of canine PFGE profiles in a restricted branch demonstrating minimal variation of the dendrogram may also be suggestive of host restricted characteristics.

5.2 Materials and Methods

5.2.1 Microbial cultures

Ninety five of the isolates used in this chapter were donated by the Veterinary Laboratory Agency, Surrey, UK (VLA) and were isolated from UK dogs over a period of time approximately 10 years ago from dogs symptomatic for *Salmonella* infection. Ten isolates originated from Queen Elizabeth Hospital (QE), Birmingham, UK, these were isolated from patients presenting with infection during 2010 – 2011. During the investigation described in chapter 3, one *Salmonella enterica* subspecies *arizonae* strain was isolated from an asymptomatic companion dog and this isolate was also employed in this study. The reference strain *S. enterica* serovar Braenderup H9812 was purchased from the HPA as described in the harmonised method for PFGE typing of *Salmonella* isolates (Enter-net/Salm-gene, 2012). Isolates were stored on microbank beads at -80^{o}C until required.

5.2.2 Microbiological media

Tryptone Soya Agar (TSA) (Oxoid Ltd, Basingstoke) was used to resurrect the isolates as stated in section 4.2.2

5.2.3 Pulse Field Gel Electrophoresis (PFGE)

A total of 95 *Salmonella* isolates originating from symptomatic canines provided by the VLA were prepared for PFGE analysis using the protocol published by Enter-net proposed harmonised-protocol for PFGE (Health Protection Agency, 2012). Serotyping

of the strains was performed by the VLA prior to PFGE profiling. Overnight cultures harvested from TSA were re-suspended in Cell Suspension Buffer (CBS; 100 mM Tris, 100 mM EDTA, pH 8.0) at an absorbance of 0.38-0.44 at 450 nm (McFarland No. 5). A volume of 0.5ml of chromosomal grade agarose (2.0%; Bio-Rad Chromosomal, UK) in 0.5ml TE buffer (10 mM Tris, 1.0 mM EDTA pH 8.0) with 1% of sodium dodecyl sulphate (SDS) was prepared and cooled down to 50^{o}C. Proteinase K (Sigma, UK; 0.5 mg/ml final concentration) was added to the cell suspension and then mixed in a 1:1 ($^{v}/_{v}$) ratio with the agarose, dispensed into block plug moulds (BioRad, UK) and allowed to solidify.

Solidified plugs were added to lysis buffer (50 mM Tris, 50 mM EDTA, 1 % ($^{w}/_{v}$) Sarkosyl, 0.1 mg/mL Proteinase K, pH 8.0; Sigma, UK) and incubated for a minimum of 4 hours in a water bath at 54^{o}C. Plugs were then washed twice in a minimum volume of 5 mL SDW, and then washed 2 to 3 times in TE buffer at 50^{o}C in a shaking water bath. The blocks were then stored at 4^{o}C until further required. Three millimetre slices cut from the plugs were added to 50-100 µL buffer with the addition of 0.8 U/µL *Xba*I (Promega, UK) and incubated at 37^{o}C for 6 hours for digestion. A 1.2 % agarose (Bio-Rad, Pulsed Field Cert.) gel was prepared with 0.5 x TBE (50mM Tris, 50mM boric acid, 0.5mM EDTA, pH8) buffer. Digested slices were loaded and sealed with agarose, into the wells and digested slices of molecular reference marker strain CDC (PulseNet) *S. enterica* serotype Braenderup H9812 were placed in wells at either end of the gel. A CHEF DR III cell (Geneflow, UK) was set at standardised conditions of 200V, switch time 2s-64s, run time 20 hours at 14^{o}C in 2.5 litres of 0.5 x TBE buffer.

Following electrophoresis the gels were stained for 40-45 minutes in 10mg/mL ethidium bromide (Sigma, UK) and de-stained in SDW for 40-45 minutes. GeneSnap and Genetools (Syngene Cambridge, UK) was used to visualise and record gels under UV-illumination.

5.2.4 Non-typeable strains

Strains that did not give clear banding patterns therefore considered non-typeable were repeated with the addition of a 50µM of thiourea (Sigma, UK) in the running buffer prior to electrophoresis which allowed for typeability. The gels were visualised as above using ethidium bromide and GeneSnap and Genetools. Resulting banding patterns were considered acceptable for analysis as they were consistent with the typeable strains and molecular reference marker strain *S. enterica* serotype Braenderup H9812 (Enter-net/Salm-gene, 2012) which was placed in wells at either end of the gel.

5.2.5 Analysis of profiles

Banding patterns were considered acceptable for analysis if they were consistent with the quality of banding style produced by the molecular reference marker strain *S. enterica* serotype Braenderup H9812 which was included on every gel. The images were uploaded onto the HPA's BioNumerics (Applied Maths, Belgium) software package and profiles were normalised using the reference strain *S. enterica* serovar Braenderup H9812 to allow direct comparison to the HPA PFGE human *Salmonella* profiles. Normalisation involved each gel to be lined together against the reference marker strain allowing profiles to be compared. Once normalised, profiles were added to the vast database of human isolated *Salmonella* from the UK within a ten year period

recorded by the HPA. Canine isolates were annotated with serotype and VLA ID number. The isolates from the HPA database were also annotated with serotype and labelled HPA. Banding pattern similarities between isolates from canine and human host were calculated by the Dice co-efficient (tolerance of 1%) and UPGMA dendrogram.

5.3 Results

5.3.1 Canine PFGE profiles using harmonised protocol

All serotypes were typeable with the exception of serotypes *S.* Livingstone (isolates 24 & 25) and *S.* Oranienburg (isolate 32) which with the addition of thiourea in the running buffer allowed for typeability. Figure 5.1 illustrates the non-typeable strains and following manipulation Figure 5.2.3 gel 10 demonstrates the capability of thiourea to produce well-presented banding profiles of these strains.

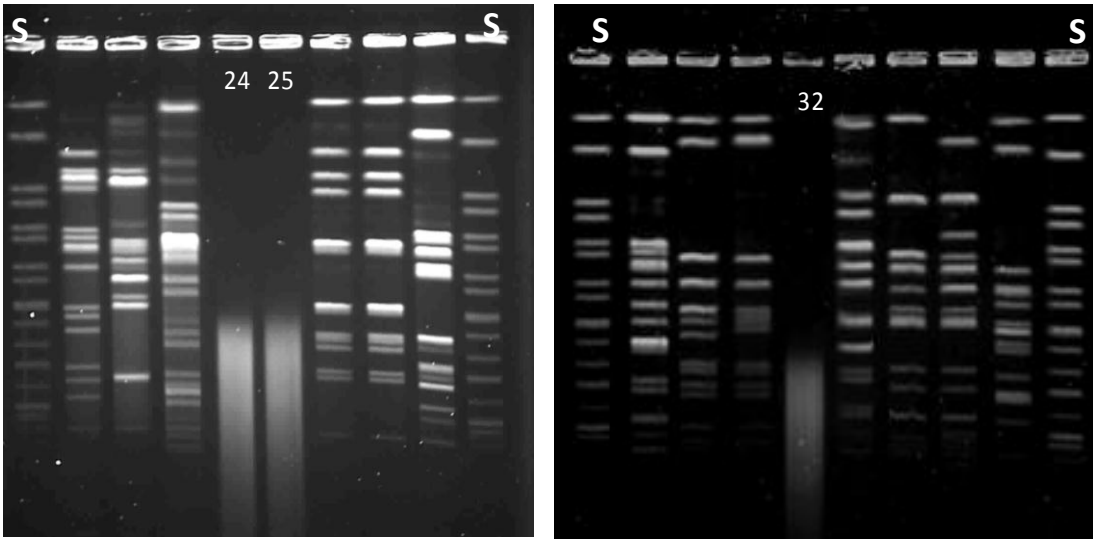
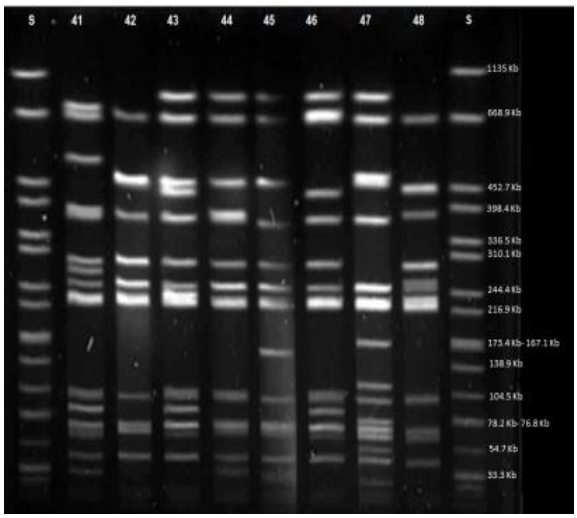
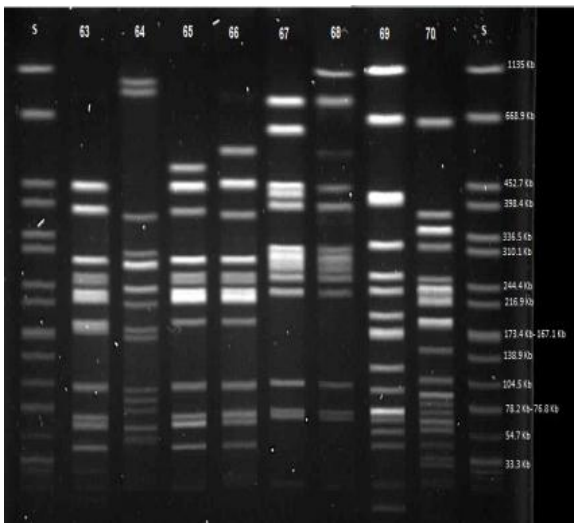


Figure 5.1 Serotype *S.* Livingstone (24 & 25) and canine isolate serotype *S.* Oranienburg (32), before the use of thiourea into the electrophoresis buffer. Both outside lanes (S) contain the control strain *S*. Braenderup H9812.

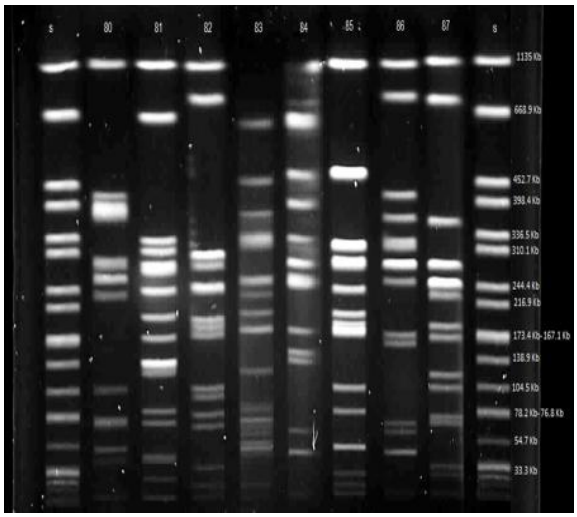
Pulse-Field Gel Electrophoresis following *Xba*1 restriction endonucleases digestion of *Salmonella* isolated from canines was performed and clear banding patterns were visulised using UV illumination, illustrated in Figure 5.2 (Synergen, UK).



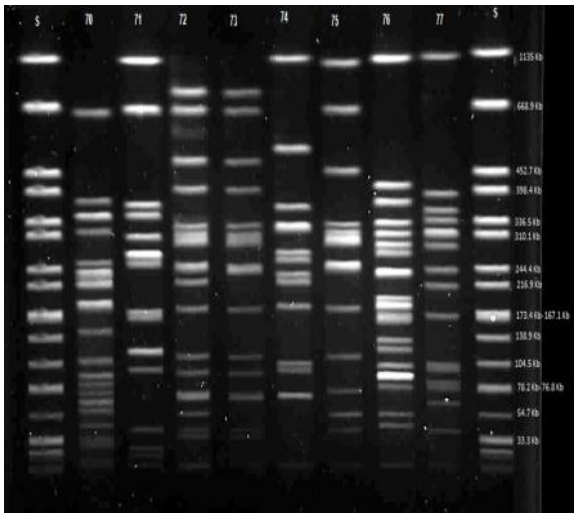
Gel 1



Gel 2

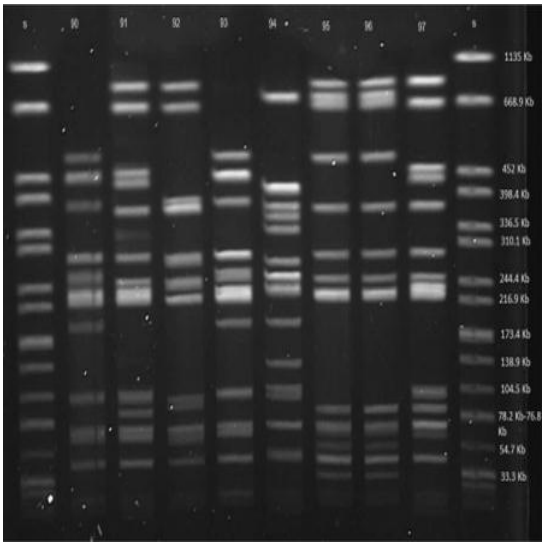


Gel 3

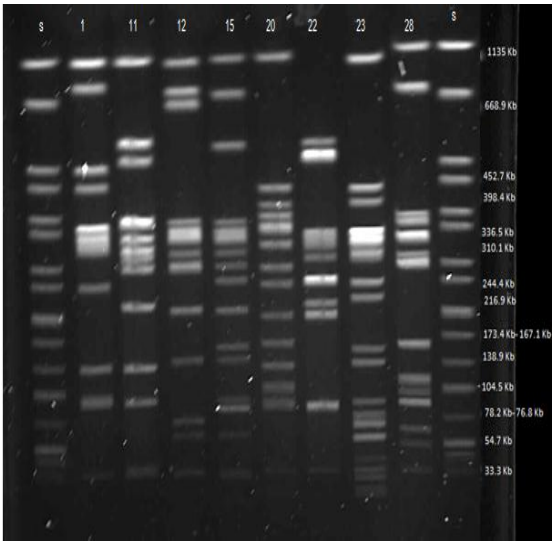


Gel 4

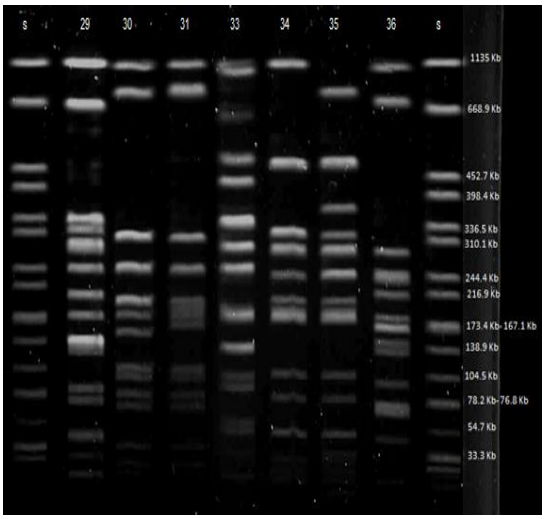
Figure 5.2.1 PFGE profiles of *Salmonella* isolates from clinical canine hosts. Each gel proceeds and ends with the reference strain *S.* Braenderup H9812. The images show typical banding patterns for Gel 1) *S.* Typhimurium isolates 41, 42, 43, 44, 45, 46, 47, 48 (left to right), Gel 2) *S.* Typhimurium (63-66), *S.* Agama (67-68), *S.* Anatum (69) and *S.* Brandenburg (70), Gel 3) *S.*Cerro (71), *S.* Dublin (72-73), *S.* Durham (74), *S.* Enteritidis (75), *S.* Havana (76) and *S.* Infantis (77), Gel 4) *S.* Livingstone (80), *S.* Montevideo (81), *S.* Newport (82), *S.* Rissen (83), *S.* Roodeport (84), *S.* Schwarzengrund (85), *S.* Senftenberg (86) and *S.* Telaviv.



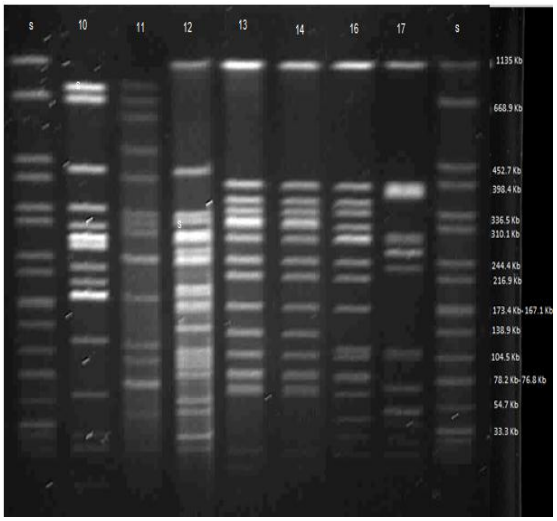
Gel 5



Gel 6

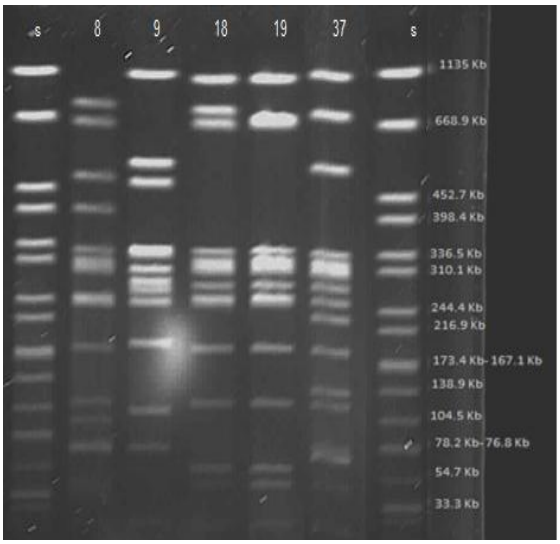


Gel 7

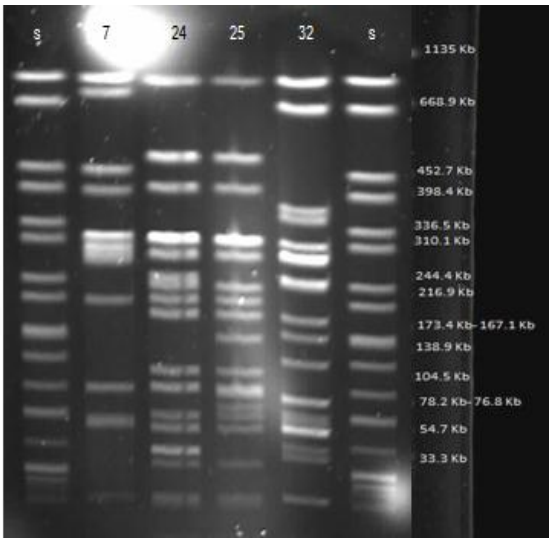


Gel 8

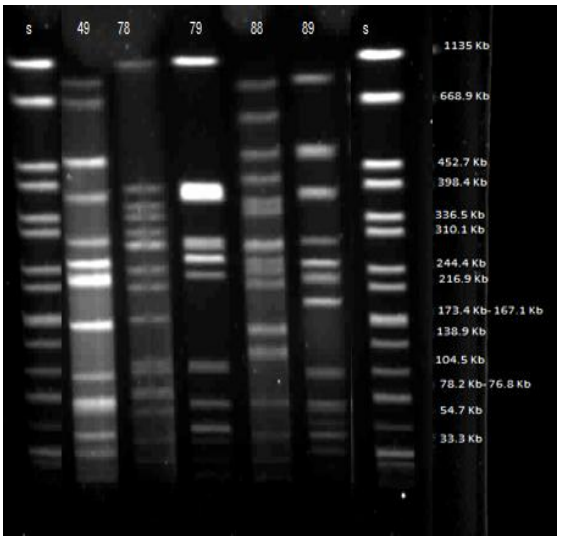
Figure 5.2.2 PFGE profiles of *Salmonella* isolates from clinical canine hosts. Each gel proceeds and ends with the reference strain *S.* Braenderup H9812. The images show typical banding patterns for Gel 5) *S.* Typhimurium (90-97). Gel 6) *S.* Agama (1), *S.* Durham (11), *S.* Enteritidis (12, 15), *S.* Infantis (20), *S.* Javiana (22), *S.* Kisarawe (23) and *S.* Montevideo (28). Gel 7) *S.* Montevideo (29), *S.* Newport (30-31), *S.* Orion (33), *S.* Schwarzengrund (34-35) and *S.* Senftenberg (36). Gel 8) *S.* Dublin (10), *S.* Enteritidis (11-14), *S.* Grumpensis (16) and *S.* Hadar (17).



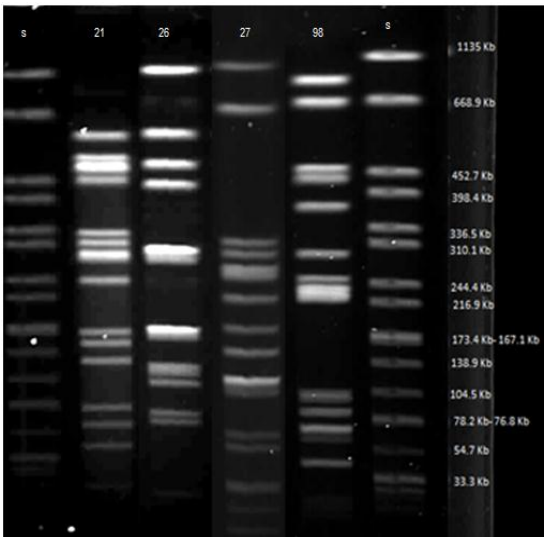
Gel 9



Gel 10

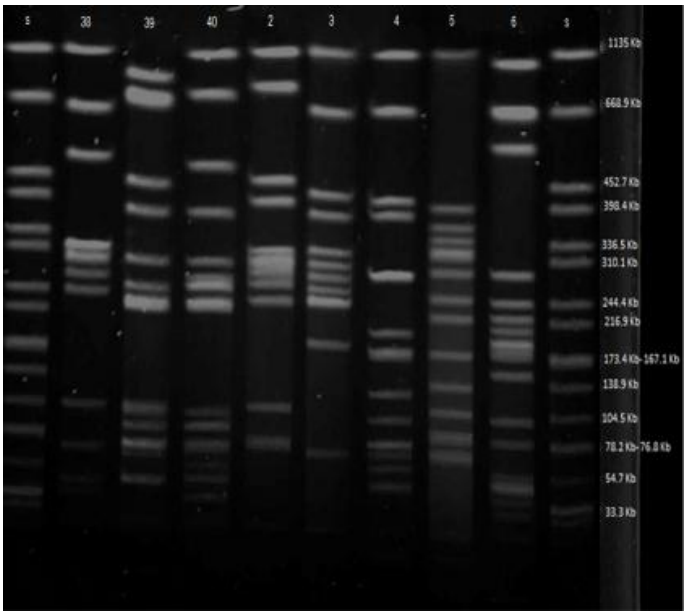


Gel 11



Gel 12

Figure 5.2.3 PFGE profiles of *Salmonella* isolates from clinical canine hosts. Each gel proceeds and ends with the reference strain *S.* Braenderup H9812. The images show typical banding patterns for Gel 9) *S.* Derby (8), *S.* Dublin (9), *S.* Havana (18), *S.* Infantis (19) and *S.* Senftenberg (37). Gel 10) *S.* Derby (7), *S.* Livingstone (24-25) and *S.* Oranienburg (32). Gel 11) *S.* Typhimurium (49), *S.* Infantis (78), *S.* Livingstone (79), *S.* Tennessee (88) and *S.* Typhimurium (89). Gel 12) *S.* Isangi (21), *S.* London (26-27) and *S.* Typhimurium (98).

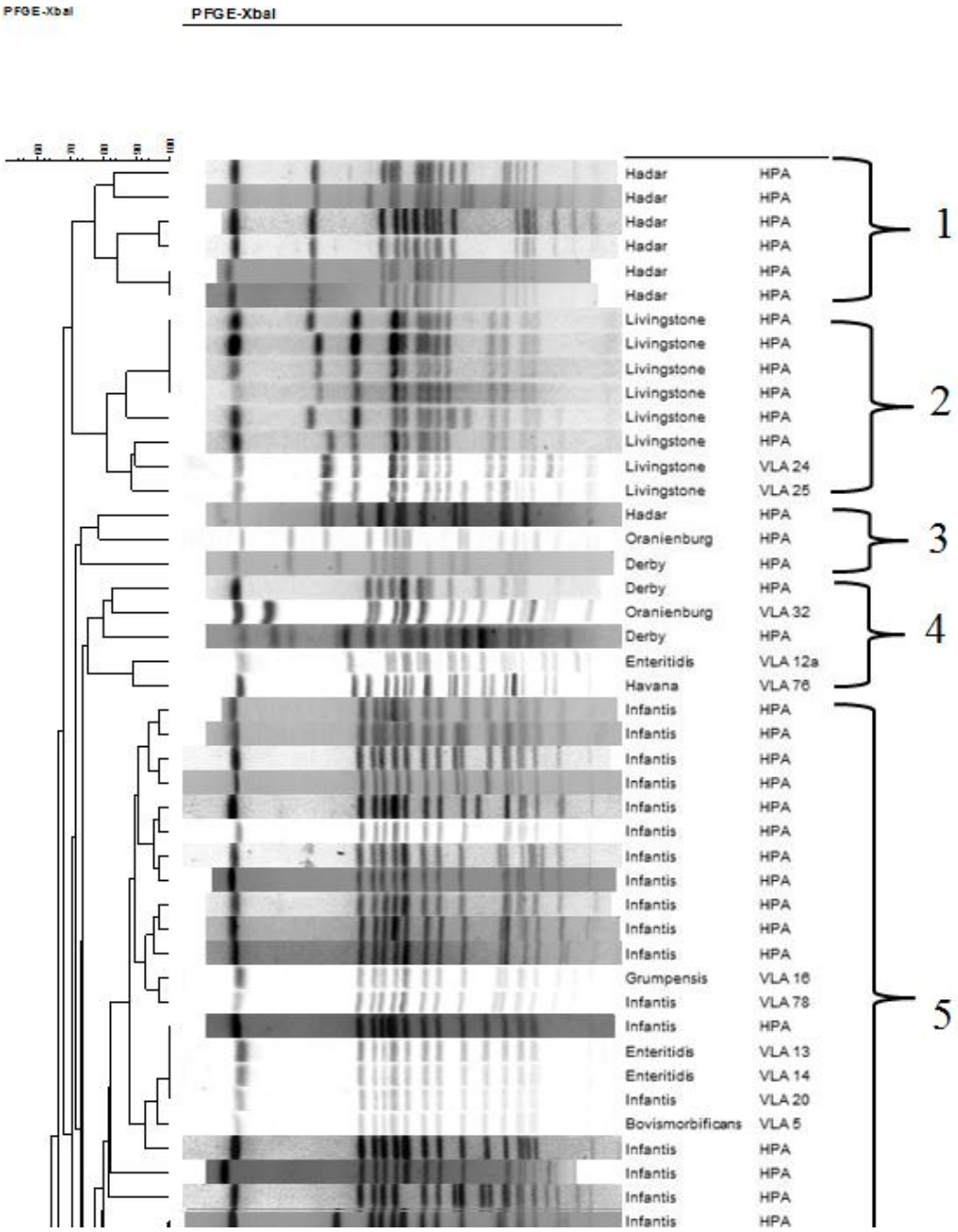


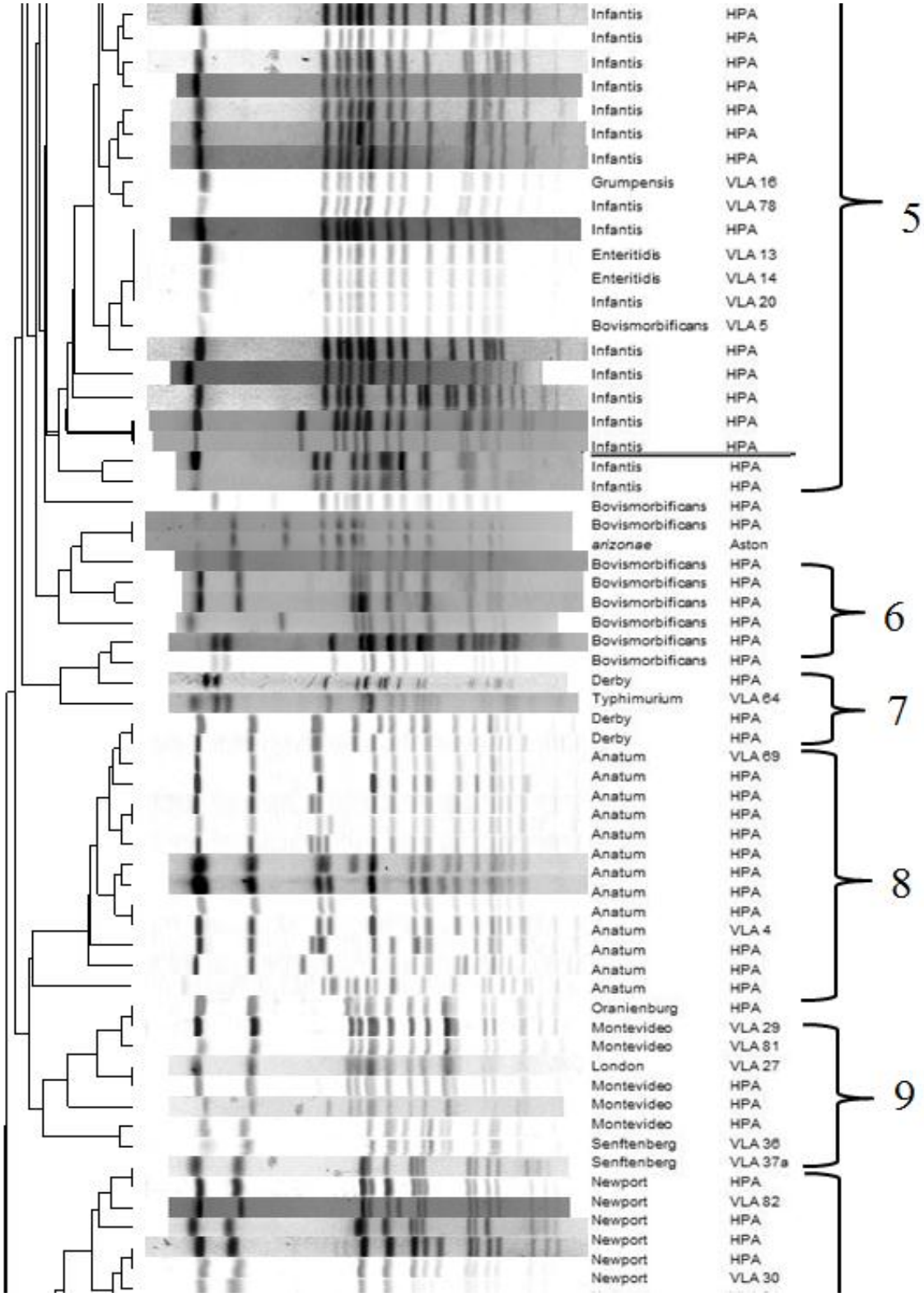
Gel 13

Figure 5.2.4 PFGE profiles of *Salmonella* isolates from clinical canine hosts. Each gel proceeds and ends with the reference strain *Salmonella* Braenderup H9812. The images show typical banding patterns for Gel 13) *S*. Stourbridge (38), *S.* Typhimurium (39-40), *S.* Agama (2), *S.* Amsterdam (3), *S.* Anatum (4), *S.* Bovismorbificans (5) and *S.* Carmel (6).

5.3.2 The comparison of PFGE profiles of clinical human and canine isolates of *Salmonella*

Pulse-Field Gel Electrophoresis profiles of *Salmonella* isolates from canine and human hosts were analysed to determine their percentage relatedness, as illustrated in Figure 5.3.




Infantis HPA
Infantis HPA
Infantis HPA
Infantis HPA
Infantis HPA
Infantis HPA
Infantis HPA
Grumpensis VLA 16
Infantis VLA 78
Infantis HPA
Enteritidis VLA 13
Enteritidis VLA 14
Infantis VLA 20
Bovismorbificans VLA 5
Infantis HPA
Infantis HPA
Infantis HPA
Infantis HPA
Infantis HPA
Infantis HPA
Infantis HPA
Bovismorbificans HPA
Bovismorbificans HPA
arizonae Aston
Bovismorbificans HPA
Bovismorbificans HPA
Bovismorbificans HPA
Bovismorbificans HPA
Bovismorbificans HPA
Bovismorbificans HPA
Derby HPA
Typhimurium VLA 64
Derby HPA
Derby HPA
Anatum VLA 69
Anatum HPA
Anatum HPA
Anatum HPA
Anatum HPA
Anatum HPA
Anatum HPA
Anatum HPA
Anatum HPA
Anatum VLA 4
Anatum HPA
Anatum HPA
Anatum HPA
Oranienburg HPA
Montevideo VLA 29
Montevideo VLA 81
London VLA 27
Montevideo HPA
Montevideo HPA
Montevideo HPA
Senftenberg VLA 36
Senftenberg VLA 37a
Newport HPA
Newport VLA 82
Newport HPA
Newport HPA
Newport HPA
Newport VLA 30
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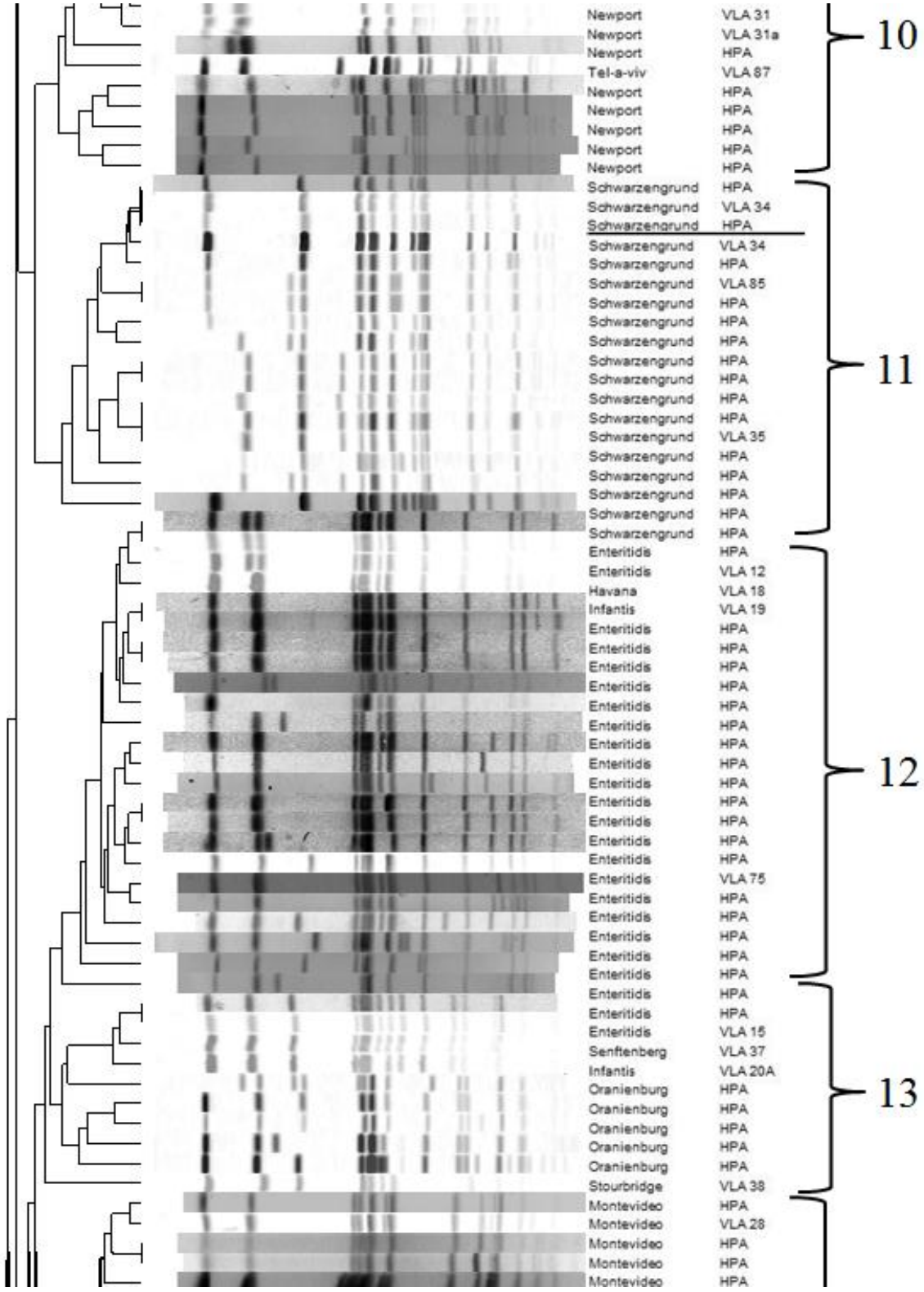
6
7
8
9


Newport VLA 31
Newport VLA 31a
Newport HPA
Tel-a-viv VLA 87
Newport HPA
Newport HPA
Newport HPA
Newport HPA
Newport HPA
Schwarzengrund HPA
Schwarzengrund VLA 34
Schwarzengrund HPA
Schwarzengrund VLA 34
Schwarzengrund HPA
Schwarzengrund VLA 85
Schwarzengrund HPA
Schwarzengrund HPA
Schwarzengrund HPA
Schwarzengrund HPA
Schwarzengrund HPA
Schwarzengrund HPA
Schwarzengrund HPA
Schwarzengrund VLA 35
Schwarzengrund HPA
Schwarzengrund HPA
Schwarzengrund HPA
Schwarzengrund HPA
Schwarzengrund HPA
Enteritidis HPA
Enteritidis VLA 12
Havana VLA 18
Infantis VLA 19
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis VLA 75
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis HPA
Enteritidis VLA 15
Senftenberg VLA 37
Infantis VLA 20A
Oranienburg HPA
Oranienburg HPA
Oranienburg HPA
Oranienburg HPA
Oranienburg HPA
Stourbridge VLA 38
Montevideo HPA
Montevideo VLA 28
Montevideo HPA
Montevideo HPA
Montevideo HPA
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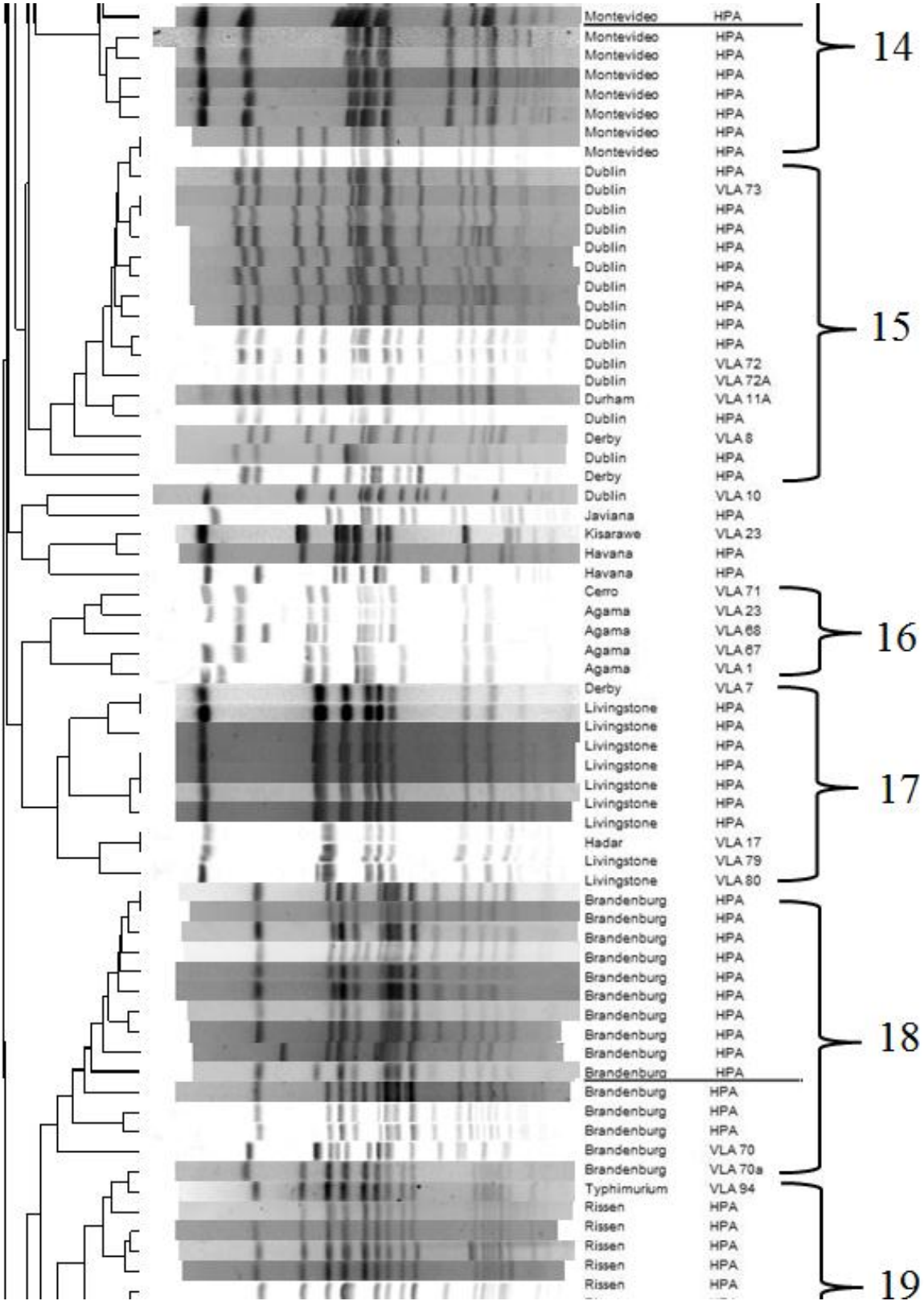
12
13


Montevideo HPA
Montevideo HPA
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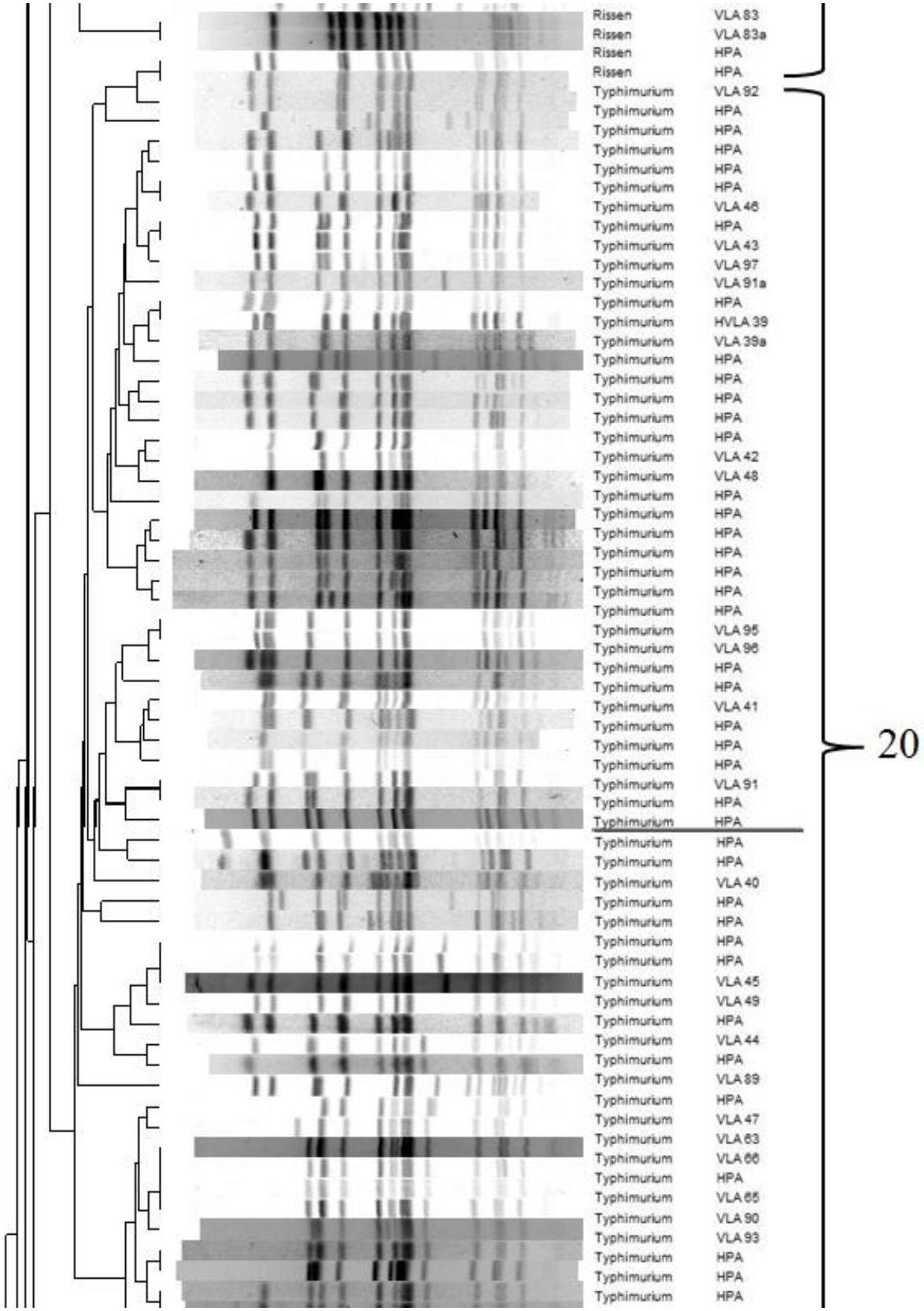
Montevideo HPA
14
Dublin HPA
Dublin VLA 73
Dublin HPA
Dublin HPA
Dublin HPA
Dublin HPA
Dublin HPA
Dublin HPA
Dublin HPA
Dublin HPA
Dublin HPA
Dublin VLA 72
Dublin VLA 72A
Durham VLA 11A
Dublin HPA
Derby VLA 8
Dublin HPA
Derby HPA
15
Dublin VLA 10
Javiana HPA
Kisarawe VLA 23
Havana HPA
Havana HPA
Cerro VLA 71
Agama VLA 23
Agama VLA 68
Agama VLA 67
Agama VLA 1
16
Derby VLA 7
Livingstone HPA
Livingstone HPA
Livingstone HPA
Livingstone HPA
Livingstone HPA
Livingstone HPA
Livingstone HPA
Hadar VLA 17
Livingstone VLA 79
Livingstone VLA 80
17
Brandenburg HPA
Brandenburg HPA
Brandenburg HPA
Brandenburg HPA
Brandenburg HPA
Brandenburg HPA
Brandenburg HPA
Brandenburg HPA
Brandenburg HPA
Brandenburg HPA
Brandenburg HPA
Brandenburg HPA
Brandenburg HPA
Brandenburg VLA 70
Brandenburg VLA 70a
18
Typhimurium VLA 94
Rissen HPA
Rissen HPA
Rissen HPA
Rissen HPA
Rissen HPA
19


Rissen VLA 83
Rissen VLA 83a
Rissen HPA
Rissen HPA
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Typhimurium HPA
Typhimurium HPA
Typhimurium VLA 46
Typhimurium HPA
Typhimurium VLA 43
Typhimurium VLA 97
Typhimurium VLA 91a
Typhimurium HPA
Typhimurium HVLA 39
Typhimurium VLA 39a
Typhimurium HPA
Typhimurium HPA
Typhimurium HPA
Typhimurium HPA
Typhimurium HPA
Typhimurium VLA 42
Typhimurium VLA 48
Typhimurium HPA
Typhimurium HPA
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Typhimurium HPA
Typhimurium HPA
Typhimurium HPA
Typhimurium VLA 95
Typhimurium VLA 96
Typhimurium HPA
Typhimurium HPA
Typhimurium VLA 41
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Typhimurium HPA
Typhimurium VLA 91
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Typhimurium VLA 40
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Typhimurium VLA 49
Typhimurium HPA
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Typhimurium VLA 89
Typhimurium HPA
Typhimurium VLA 47
Typhimurium VLA 63
Typhimurium VLA 66
Typhimurium HPA
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20

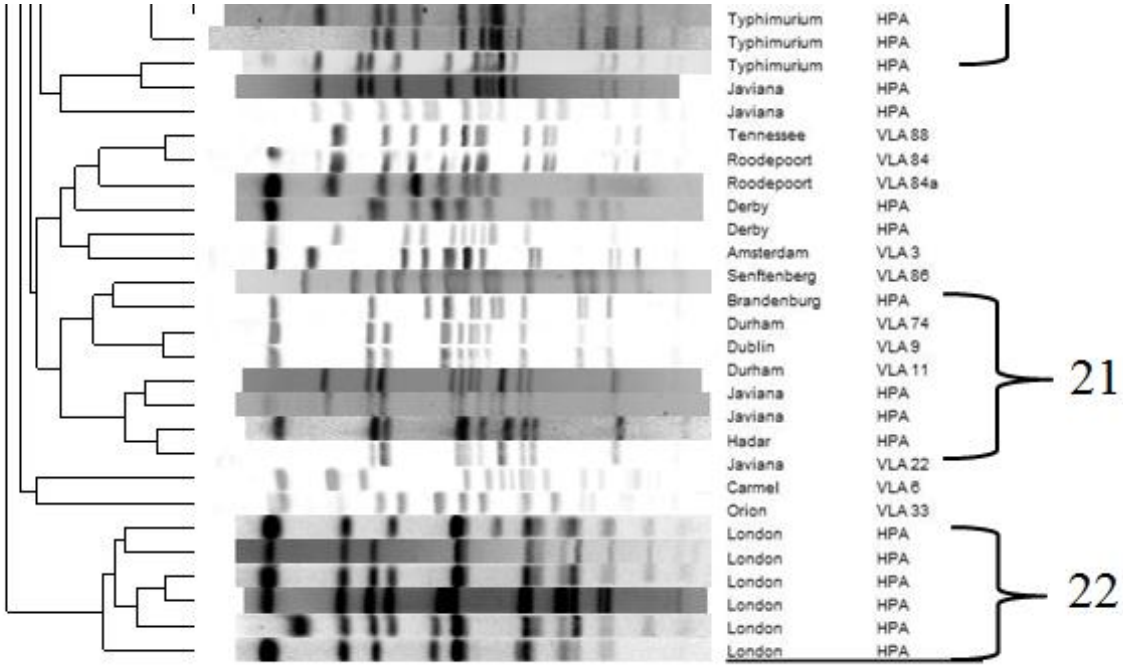

Figure 5.3 Dendrographic representation of the diverse range of PFGE profiles obtained from the canine population (VLA, UK) and profiles obtained from the human population (HPA, UK). Relatedness was calculated by Dice coefficient and represented by UPGMA clustering. Isolates originating from the humans are named HPA and those from canine are named VLA followed by strain ID number. Serotype of each isolate is adjacent to each profile.

Analysis of the PFGE profiles of *Salmonella* from both human and canine hosts revealed 243 unique profiles from a total of 306 isolates; however many shared similar profiles with few band differences. In total 31 different serotypes and one different species of *Salmonella* were identified with *S.* Agama, *S.* Roodenpoort, *S.* Stourbridge, *S.* Grumpensis, *S.* Senftenberg, *S.* Tel-a-viv, *S.* Kisarawe, *S.* Cerro, *S.* Tennessee, *S.* Amsterdam, *S.* Durham, *S.* Carmel, *S.* Orion and *S. arizonae* only being represented in the UK canine population, as detailed in Table 5.1. Serotypes Typhimurium, a total of 39 in human host and 5 in canine host, and Enteritidis, a total of 21 in human host and 23 in canine host were amongst the frequently identified serotypes in both populations.

The dendrogram revealed a total of 22 major clustering groups of which 4 groups contained human isolates only, consisting of two groups of serotype Hadar, one group of Bovismorbificans and one group of London.

Table 5.1 Summary of serotypes identified following PFGE analysis of human and canine isolates.

Serotype	Host	
	Canine	Human
Agama	4	0
Amsterdam	1	0
Anatum	1	11
Arizonae	1	0
Bovismorbificans	1	8
Brandenburg	1	13
Carmel	1	0
Cerro	1	0
Derby	2	9
Dublin	4	10
Durham	2	0
Enteritidis	5	21
Grumpensis	1	0
Hadar	1	8
Havana	2	2
Infantis	4	16
Javiana	1	5
Kisarawe	1	0
Livingstone	4	13
London	1	6
Montevideo	3	12
Newport	3	10
Oranienburg	1	7
Orion	1	0
Rissen	1	8
Roodenpoort	1	0

Schwarzengrund	3	14
Senftenberg	3	0
Stourbridge	1	0
Tel-a-viv	1	0
Tennessee	1	0
Typhimurium	23	39

Total number of isolates = 306

Total number of serotypes = 32

Total number of canine only serotypes = 14

Table 5.2 Summary of clusters and percentage relatedness as determined from the dendrogram. Table includes the number of isolates from the human and canine samples in each cluster along with the serotypes represented by each cluster.

Group	Percentage relatedness (%)	No. of isolates	Serotype represented
1	78	HPA = 6	Hadar
2	82	HPA = 6	Livingstone
		VLA = 2	
3	72	HPA = 3	Hadar, Oranienburg, Derby
4	72	HPA = 1	Derby, Oranienburg, Enteritidis, Havana
		VLA = 3	
5	80	HPA = 19	Infantis, Grumpensis, Enteritidis
		VLA = 6	
6	78	HPA = 6	Bovismorbificans
7	80	HPA = 3	Derby, Typhimurium
		VLA = 1	
8	86	HPA = 11	Anatum
		VLA = 2	
9	72	HPA = 3	Montevideo, London, Senftenberg
		VLA = 4	
10	80	HPA = 9	Newport, Tel-a-viv
		VLA = 5	
11	76	HPA = 13	Schwarzengrund
		VLA = 3	
12	74	HPA = 19	Enteritidis
		VLA = 4	
13	76	HPA = 6	Enteritidis, Senftenberg, Infantis,
		VLA = 3	Oranienburg
14	88	HPA = 9	Montevideo
		VLA = 1	
15	72	HPA = 11	Dublin, Derby, Durham
		VLA = 1	
16	72	VLA = 5	Agama, Cerro
17	76	HPA = 7	Livingstone, Hadar, Derby
		VLA = 4	
18	80	HPA = 11	Brandenburg
		VLA = 2	
19	74	HPA = 8	Rissen
		VLA = 2	
20	74	HPA = 39	Typhimurium

		VLA = 24	
21	64	HPA = 4	Brandenburg, Durham, Dublin, Javiana,
		VLA = 4	Hadar
22	76	HPA = 6	London

The dendrogram revealed that a number of serotypes were closely related as detailed in Table 5.2. In addition, the numbers of human and canine isolates in each of the clusters formed at between 64% - 88% relatedness which is also detailed in Table 5.2. Clustering of different serotypes can be seen with lower percentage relatedness, for example *S.* Hadar and *S.* Livingstone cluster together at 70%.

5. 4 Discussion

The aim of this chapter was to characterise *Salmonella* isolates using PFGE as a form of profiling and to determine if *Salmonella* genotypes isolated from canine hosts were represented with an indistinguishable banding pattern to the human isolates in the HPA PFGE database. Absence of representation may suggest host restriction in canine isolated *Salmonella* strains; similarly, profiles characterising isolates from both human and canine origin suggests zoonotic potential.

A set of guidelines for the interpretation of PFGE banding patterns have been comprehensively determined and published (Tenover *et al.*, 1995) which details the criteria that may be used to differentiate strains within a serotype. The software Bionumerics (Bionumerics, Belgium) used in this chapter allows for these relationships to be seen by clustering profiles based on their banding profiles. To interpret and compare PFGE banding profiles it is necessary to understand the random genetic events that may alter these patterns. A single band difference between two strain-types is likely to arise from one of two possible genetic events: i) Point mutations causing loss or gain of a restriction site; ii) insertion or deletion of DNA between two restriction sites. Multiple band differences between two strain-types are likely to arise from multiple genetic events and therefore the strains may be considered less closely related. Strains that were once genetically indistinguishable are more likely to demonstrate a greater number of differences in their PFGE banding patterns after an extended period of time due to natural genetic divergences (Tenover *et al.*, 1995). Moreover, host adaptive or restricted strains demonstrate a number of genetic differences from their

common ancestor which is represented by a multiple number of banding differences. For this chapter PFGE profiles were normalised against the reference strain *S. enterica* serotype Braenderup H9812 allowing comparison of all human and canine isolate profiles used in this analysis. Clustering of profiles was performed by the software system Bionumerics (Bionumerics, Belgium) which performed clustering using Dice coefficient and represented by UPGMA. The 22 major groups obtained were selected as isolates within each group shared only a few band differences. Published criteria along with Bionumerics software (Tenover *et al.*, 1995) were applied to interpret the major groups of banding patterns produced from the canine and human isolates: Within each group some profiles where considered indistinguishable however the majority were considered closely related. The published criteria are listed below.

- Strains that had the same number of bands and corresponding bands of the same apparent size were considered indistinguishable.
- Strains that differed by one genetic difference, typically two or three band differences, were considered closely related.
- Strains that differed by two genetic differences, typically four to six band differences were considered possibly related.
- Strains that differ by three or more genetic differences, typically seven or more band differences, were considered unrelated.

Non-typeable strains *S.* Livingstone (isolates 24, 25) and *S.* Oranienburg (isolate 32) were successfully profiled with the addition of thiourea in the running buffer in parallel to the harmonised protocol. Thiourea is thought to neutralize a nucleolytic peracid derivative of Tris that is formed at the anode during electrophoresis (Ray *et al.*, 1995).

This neutralisation allowed for clearer banding patterns to be obtained for the strains aforementioned.

5.4.1 Analysis of PFGE profile

A total of 306 isolates were profiled from the human and canine host groups and a total of 32 serotypes were found to be represented in this population. A total of 14 serotypes were unique to the canine host group: Of these, 4 and 3 isolates were serotypes Agama and Senftenberg respectively, whereas, the remaining 12 serotypes were each represented by a single isolate. The dendrogram also highlighted that there were 22 major clusters; 4 of the clusters contained human isolates only and where represented by the majority serotypes Hadar (two clusters of this serotype), Bovismorbificans and London. There were 12 serotypes that clustered with profiles from both the canine and human population: Anatum, Brandenburg, Dublin, Enteritidis, Infantis, Livingstone, Montevideo (two separate clusters), Newport, Rissen, Schwarzengrund and Typhimurium. Interpretation of these clusters demonstrates that PFGE profile types were represented in both the human and canine population which could be suggestive of a zoonotic association.

Salmonella Typhimurium was highly represented in both host groups clearly demonstrating its pathogenic capacity. Apart from two isolates, a total of 62 of which 23 and 39 isolates were from the canine and human hosts respectively, were clustered together at 74% relatedness. This pattern of infection supports evidence in the literature that this serotype is one of the most common zoonotic serotypes, appearing ubiquitous in the environment, colonising and causing infection in a variety of animal hosts and environmental niches (Thorns, 2000). Reports reveal that *S.* Typhimurium accounted

for 21% of total salmonellosis cases in the UK which was the second highest to *S.* Enteritidis in 2010 (HPA, 2011a). More concerning is that there is an association of antimicrobial resistance with this serotype, especially among with DT104 phage type (Casin *et al.*, 1999; Gebreyes & Altier, 2002; Ridley *et al.*, 1998). A total of eight strains from the canine population were indistinguishable to human isolates, isolates ID VLA 45, 46, 49, 65, 90, 91, 92, 93. Largely profiles from this serotype were not indistinguishable but were found to be closely related suggesting some genetic diversity. This diversity in banding patterns in each strain suggests that within the approximate 10 year period in which the strains were gathered, genetic differences may have evolved producing slight changes in PFGE banding patterns.

Salmonellosis caused by *S.* Enteritidis remains an important public health problem in Europe and other parts of the world (Janmohamed *et al.*, 2011). Outbreaks caused by *S.* Enteritidis infection are closely associated with eggs and egg products (Ejidokun *et al.*, 2000; Gantois *et al.*, 2009). The HPA reported 221 cases of human *S.* Enteritidis PT 14b infection since the beginning of this year, the majority of cases being in North West England (104 cases), the West Midlands (36 cases) and the East Midlands (26 cases). It was revealed that the cause of this infection rooted from the consumption of imported Spanish eggs (HPA, 2012c). From this chapter Enteritidis was the second most represented serotype in both host groups supporting the current epidemiological data regarding cases of infection caused by *S.* Enteritidis. A total of two canine strains were indistinguishable (isolates ID VLA 12 and 15) from human strains of *S.* Enteritidis. However, a further two strains were indistinguishable with the serotype Infantis, suggesting either an error in serotyping or analysis of designating the correct

serotype to the isolate. As each serotype contains unique DNA content and the digestion carried out in PFGE by *Xba*1 typically results in producing the banding profile. Therefore if an indistinguishable profile is produced from two different serotypes then this is much more likely to have occurred through human error of miss-naming the correct serotype to the isolate.

In this chapter the dendrogram revealed two canine isolates that were indistinguishable with human *S.* Schwarzengrund isolates; one canine isolate was also considered to be closely related. This demonstrates that these strains from the same serotype are genotypically related and therefore could potentially cause infection in both hosts. The serotype *S.* Schwarzengrund was of particular interest during 2007 as it was associated with the multistate outbreak causing infection in companion dogs and their owners in the U.S (CDC, 2008). PFGE patterns were electronically submitted to PulseNet, the national molecular subtyping network for foodborne disease surveillance (Gerner-Smidt *et al.*, 2006). The outbreak strain of *S.* Schwarzengrund was isolated from faecal specimens from two dogs that ate dry pet food in the homes of two of the ill persons; their isolate PFGE profiles were indistinguishable, therefore revealing a zoonotic capability of this serotype (Behravesh *et al.*, 2010).

A total of three *S.* Newport isolates were in the canine population, a relatively low number in comparison to the number of dog foods, including BARF diets and treats, reported to be contaminated with this serotype (Finley *et al.*, 2006; Hoelzer *et al.*, 2011). The dendrogram illustrated that the canine isolates and human isolates grouped closely together with at least 80% relatedness, demonstrating a close association with

slight diversity of genotypic profiles. Again, the evidence points towards a zoonotic association. *Salmonella* Newport has recently been implicated in many outbreaks of salmonellosis in both humans and animals (Dallap Schaer *et al.*, 2010; HPA 2012b; Steneroden *et al.*, 2010; Zhoa *et al.*, 2003). The HPA reported an outbreak of a strain of *S.* Newport which caused infection among 30 people in England, Wales and Northern Ireland since the beginning of December 2011. The isolates were confirmed as sharing the same PFGE profile to those that caused infection in Scotland, Ireland and Germany (HPA, 2012d). Additionally, reports of *S.* Newport isolation in dairy cattle are increasing and these strains are demonstrating multidrug resistance patterns (Poppe, 2006). These isolates have also been reported to be the causative agents of human infections where there was one to one contact with the cattle and the feed (Karon *et al.*, 2007).

Analysis of the dendrogram reveals that isolates from the serotype Infantis from both humans and dogs were closely related. A recent outbreak reported in the U.S in April 2012, has linked dry dog food and *S.* Infantis infection using PFGE. The outbreak strain was isolated from one clinical dog and one asymptomatic dog by the Ohio Department of Agriculture; both dogs consumed recalled products. A total of 49 further cases (47 cases in 20 states, and two cases in Canada) of human infections with the outbreak strain were reported. A previous study reported that the incidence of *S.* Infantis infections in Alberta rose dramatically and subsequent laboratory and epidemiological investigations established that an outbreak of human disease caused by this organism was occurring across Canada and was associated with pet treats for dogs produced from processed pig ears (Clark *et al.*, 2001). These reports demonstrate the significance to public health of this serotype in contaminated food and highlight the ease of

transmission as a zoonotic disease. Additionally, the reliability of PFGE typing of strains and the PulseNet database are demonstrated valuable in these outbreak situations. From this chapter, isolates from serotype Infantis were among the largest groups in both human and canine hosts suggesting the zoonotic and anthroponotic potential.

The dendrogram revealed clustering of canine and human *S.* Montevideo serotype isolates. There are two clusters of this serotype with 4 or more band differences therefore using the interpretation criteria this would suggest that on the basis of PFGE profiling these strains are possibly related. A *S.* Montevideo outbreak related to contaminated cooked chicken has previously been reported in England (Threlfall *et al.*, 1999). The study demonstrated the importance of microbial strain discrimination in outbreak investigations and illustrated the value of close liaison between microbiologists, epidemiologists, and environmental health officers in the control of *Salmonella* outbreaks. *Salmonella* Montevideo has also been linked to outbreaks associated with consumption of sesame seed based products (Unicomb *et al.*, 2005) and tomatoes (Hedberg *et al.*, 1999) elsewhere in the world. A large outbreak of asymptomatic canine salmonellosis in military watch dogs due to *S.* Montevideo was recognized only through a monitoring program, implemented by the military forces. *Salmonella* Montevideo isolated from suspected dog feeds and faecal samples were profiled using PFGE and comparison of the profiles confirmed that commercial dehydrated dog feeds were the source of contamination. This outbreak demonstrates that *Salmonella* infections in dogs can occur without clinical symptoms and highlights the particular risk for zoonotic transfer of disease from an asymptomatic dog (Schotte *et al.*, 2007).

The remaining serotypes Livingstone, Anatum, Dublin, Brandenburg and Rissen also demonstrated a possibility of zoonotic and anthroponotic transmission based on their PFGE characteristics as profiles were indistinguishable with homologous serotypes in both dogs and humans.

Salmonella serotypes: Senftenberg, Stourbridge, Tel-a-viv, Tennessee, Orion, Kisarawe, Grumpensis, Durham, Cerro, Carmel, Amsterdam and Agama where only present in the canine population. Their profiles within the dendrogram demonstrated at least 60% relatedness with the total population of *Salmonella* serotypes. *Salmonella* Agama was represented by four isolates which is interesting as it may be considered a frequent occurrence in comparison to the total number of canine isolates. A study investigating *Salmonella* carriage in household dogs in Nsukka, Southeast Nigeria, isolated *Salmonella* from 6% of the canine population in a randomised sampling; where *S.* Agama was one of seven serotypes isolated (Chah *et al.*, 2001). This serotype has also been reported to cause infection in sheep and badgers (AFBI, 2010; VLA 2012). *Salmonella* Agama and Stourbridge have also been isolated from UK sheep as reported by DEFRA in 2004. Therefore the association of this serotype to canines and the environment is not uncommon and maybe suggestive of their presences in the canine population.

Salmonella Senftenberg has been frequently reported recently in poultry production and isolated from hatcheries. Studies have shown that some strains of this serotype are able

to persist throughout the rearing period in farmed poultry, thus representing a potential risk for consumers (Boumart *et al.*, 2012). This health hazard is strengthened by the existence of several intestinal and hospital-borne infections in humans linked to *S.* Senftenberg. The serotype to be circulating in the canine population may be associated with working farm dogs or dogs that are fed a raw poultry diet.

The remaining serotypes are not commonly isolated serotypes from human infection cases however reports have shown that they are circulating in the human and animal population (Fegan *et al.*, 2004; Komitopoulou & Peñaloza, 2009; Schwille-Kiuntke *et al.*, 2011; Sojka *et al.*, 1977). These isolates although only seen in the canine population cannot be attributed to host restriction as the frequency of each serotype is very low at only one isolate per serotype within the canine *Salmonella* isolates. Ideally, if host adaptation were to be a factor a much higher incidence within the preferred host would be anticipated. As with *Salmonella enterica* serotype Typhimurium variant Copenhagen it has been shown to be frequently isolated from pigeons and has therefore been termed a host restricted pathogen (Parsmans *et al.*, 2004). *Salmonella* Dublin is another serotype which has a high frequency in isolation from cattle and again is known as a host restricted serotype (Brackelsberg *et al.*, 1997).

Phage typing is the initial choice of discrimination in epidemiological studies however more than 75% of the Salmonellae isolated during an outbreak belong to a single phage type therefore PFGE typing is carried out to discriminate isolates further (Walker *et al.*, 1999). Conversely, a study into the ability of PFGE to differentiate *S.* Enteritidis strains observed a close genetic similarity between epidemiologically unrelated and outbreak-related isolates. This suggests that PFGE typing is of limited value in the

epidemiological analysis of particular isolates, possibly because of the highly clonal nature of pathogenic strains of *S.* Enteritidis (Thong *et al.*, 1995). In contrast, the application of PFGE has demonstrated to provide a stable and highly discriminatory analysis of bacterial isolates including *Salmonella*, *E.coli* and MRSA (Avery *et al.*, 2002; Bosch *et al.*, 2010; Sandt *et al.*, 2006).

5.5 Conclusion

Cluster analysis observed by phylogenetic relationship of the PFGE *Salmonella* profiles from canine and human hosts indicated that isolates from the same serotypes cluster closely together demonstrated by a similarity in banding patterns. There was no clustering of isolates from the canine host suggesting that clinical human and canine strains are equally represented in both populations and have the capacity to cause infection in both hosts. There was no serotype that was solely found in dogs at such a frequency as to suggest host preference and therefore no indication of a host restricted strain or serotype. Serotypes Typhimurium and Enteritidis were the most frequently represented serotypes in both humans and dogs supporting the zoonotic association with these common infection causing serotypes.

Chapter 6 Invasion and adhesion of *Salmonella* isolates in CaCo-2 and DIEC cell lines

6.1. Introduction

Salmonella constitutes a genus of zoonotic bacteria of worldwide economic and health importance (Uzzau *et al.*, 2000). Typically *Salmonella* serotypes from the subspecies *Salmonella enterica* are associated with warm-blooded vertebrates and are usually transmitted by ingestion of food or water contaminated by faeces. Zoonotic and anthroponotic infections display a promiscuous phenotype in that they maintain the ability to colonize and potentially cause infections in more than one host species. These infections of *Salmonella* include the most established human and animal infection causing serotypes, Typhimurium and Enteritidis (Threlfall, 2006). In contrast, some pathogenic agents are significantly host restricted, or adapted, and are normally only able to cause disease in one host (Thomson *et al.*, 2008*). Salmonella* Typhi and Paratyphi C are host adapted to cause typhoid fever in humans. These organisms are endemic in regions of the world where sanitation is poor; in the UK, most cases reported are associated with foreign travel (HPA, 2012b). Host adaption of *Salmonella* serotypes has also been seen in food producing animals, including *S.* Dublin in cattle and *S.* Gallinarum in chickens (Chadfield *et al.*, 2003; Fitzgerald *et al.*, 2003; Thomson *et al.*, 2008) which can lead to detrimental outbreaks within farming areas. *Salmonella* can also cause infection in dogs; a host adapted or restricted serotype has not yet been established in this host. Symptomatic infection in dogs is rare however they are regarded as being asymptomatic carriers of *Salmonella* in their lower intestines.

Recent outbreaks of *S.* Schwarzengrund in pet food have reported symptomatic infection caused by the same strain in companion dogs and companion dog owners (CDC, 2008). Phenotyping and genotyping methods are typically established to confirm the identification of the strain causing infection. A number of Salmonellae isolated from symptomatic canine and human hosts have been shown to share indistiguishable PFGE profiles which is suggestive of a zoonotic relationship (see Chapter 5). Phenotypic characteristics were also investigated using Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ which revealed two canine *Salmonella* isolates which were outliers from the rest of the population using PCA (see Chapter 4). These two isolates differed from the other canine and human *Salmonella* isolates as they were capable of metabolising stachyose and NAG at a higher rate. These two isolates where genetically indistingushable with their PFGE profiles nestling within the human PFGE profile cluster. Therefore it was of interest to use an established method for adhesion and invasion of bacterial cells on human and dog cell lines to demonstrate any host restriction characteristics of the two selected strains based on their phenotypic differences.

Motility is an important virulence determinant and is typically conferred by the possesion of functional flagella. Flagella and fimbrae also function in adhesion and invasion in intestinal host cells (Allen-Vercoe *et al.,* 1999; Jonson *et al.,* 2005; Nakae *et al.,* 1975). The majority of pathogenic strains of *Salmonella* are positive for motility therefore confirmation for the presence of functional flagella in isolates allows invasion and adhesion studies to be performed. The confirmation of positive motility of isolates used in this chapter was revealed in chapter 4. *Salmonella* contains at least 13 putative

fimbrial operons and they have been associated with adhesion on host cells (Pan *et al.*, 2009). Studies performing genetic and functional analysis of the mannose-specific type 1 fimbrial adhesins from a variety of serotypes of *Salmonella enterica* demonstrated that specific mutant variants of FimH are common in host-adapted strains associated with systemic invasion (Kisiela *et al.*, 2012; Pan *et al.*, 2009).

A routinely used method to investigate the mechanisms of bacterial invasion of eukaryotic cells is an invasion assay (Friis *et al.*, 2005). This assay involves incubating suspensions of bacteria with eukaryotic cells. Subsequently, the unattached bacteria are washed away to eliminate them from the assay. Adhered bacteria are then killed by the addition of gentamicin, an aminoglycoside antibiotic which is known to have restricted penetration into eukaryotic cells. Bacteria that have successfully invaded the eukaryotic cells are therefore protected and these bacteria can be enumerated following cell lysis. Attachment of bacteria can also be determined by a modified version of this assay whereby gentamicin introduction is eliminated and the surface-attached bacteria plus the successfully invaded bacteria can again be enumerated following cell lysis. The use of such *in vitro* assays for the study of bacterial pathogenic mechanisms eliminates the need for animal models. Little information is available on the *in vivo* interactions of *Salmonella* and host cells, however it is well known that adhesion of *Salmonella* bacteria to host cells triggers a complex set of signalling events. Invasion assays have been used to demonstrate the roles of different *Salmonella* pathogenicity islands which have provided insights into the mechanisms of *Salmonella* invasion, establishment and pathogenicity in eukaryotic cells (Wisner, 2011). *Salmonella* can trigger a complex set of signalling events which changes the morphology of the host cell allowing the

invasion of the *Salmonella*; without this step *in vitro* assays have shown a negative response in the host cell (Suárez & Russmann, 1998).

The increasing concern over the number of new emerging diseases, the majority of which are zoonotic infections, prompted this study into the potential of *Salmonella* to be transmitted from canines to humans and *vice versa*. In addition, investigations into the potential for host restricted strains of *Salmonella* were also of interest. *Salmonella* serotypes belonging to subspecies I carry a large, low copy-number plasmid that contains virulence genes (Rotger & Casadesús, 1999). Plasmids of *Salmonella enterica* vary in size from 2 to more than 200 kb. The best described group of plasmids are the virulence plasmids (50–100 kb in size) present in serotypes Enteritidis, Typhimurium, Dublin, Cholerae-suis, Gallinarum, Pullorum and Abortus-ovis (Libby *et al.*, 2002; Rychlik *et al.*, 2006). The presence of a virulence plasmid greatly enhances the virulence of those *Salmonella* serotypes in experimental infections (Fierer, 2001). Analysis has shown that the virulence plasmids from different serotypes are homologous for a 7.8-kb virulence region, the *spv* operon which contains a cluster of genes, which includes five open reading frames designated *spvR*, *spvA*, *spvB*, *spvC*, and *spvD* (Boyd *et al.*, 1998). The genes known to be contained on the large virulence plasmid are important in invasion determinants. Additionally, there are many more genes contained in the plasmids that are associated with infection. As invasion is a key stage in causing infection the presence of *Salmonella* plasmid may indicate successful invasion, which is paramount for the isolates investigated for invasion capabilities. Plasmid extraction is a well-established method used in epidemiological investigation

into outbreak causing strains (Lukinmaa *et al.*, 1999; Morosini *et al.*, 1996; Nakamura *et al.*, 1986).

This chapter reports the use of the invasion assay on selected strains of *Salmonella* to investigate differences in the rate of invasion. The assay was performed on dog intestinal epithelical cells (DIEC) and a human caucasian colon adenocarcinoma cells (CaCo-2). Prior to the assays the presence of plasmids was confirmed as invasion is typically dependent on genes located on plasmid DNA.

6.2. Materials and Methods

6.2.1. Microbial cultures

Transformed *E. coli* PRS426 was designed and evolved at Aston University by Dr Stephane Gross. *Salmonella* Newport (VLA 31), *S.* Typhimurium (VLA 39) were selected from the canine culture collection donated by the VLA. *Salmonella* Enteritidis (ID 18) is a human clinical isolate from the Queen Elizabeth Hospital, Birmingham, UK. *Salmonella arizonae* was isolated from an asymptomatic household dog (see Chapter 3). The American Type Culture Collection (ATCC) *Escherichia coli* 10536 was selected for the negative control in the invasion assay. The positive control for invasion of cells was *S.* Typhimurium NCTC 74. Microorganisms were stored on microbank beads at -80^{o}C until required.

6.2.2. Plasmid Isolations

The ability of *Salmonella* to invade host cells is dependent on genes that are typically located on plasmids. Therefore before the invasion assay was performed the presence of plasmids was investigated.

6.2.2.1. Plasmid DNA purification using the QIAprep

Plasmid DNA was obtained using the QIAprep® Spin Miniprep Kit (Qiagen, UK) according to manufacturer's instructions. Transformed *E. coli* PRS426 was used as a positive control for plasmid recovery and undigested *S.* Typhimurium NCTC 74 was used for a negative control for *Xba*1 digestion. Plasmid DNA extractions were performed on the canine isolates *S.* Newport (VLA 31) and *S.* Typhimurium (VLA 39).

Cultures were resurrected from -80^{o}C storage and inoculated onto NA. The transformed *E. coli* was grown on NA containing 1% ampicillin (150µL/mL) to select for plasmid DNA production. Following incubation at 37^{o}C for 24 hours plates were removed and a single colony from each isolate was used to inoculate sterile 100ml bottles containing 5mL of Luria Bertani (LB) medium of which 1% ampicillin was added into the *E. coli* broth for the further enhancement of plasmid production. Inoculated flask where incubated at 37^{o}C for 16 hours at 200rpm in a shaking incubator (Orbital shaking incubator, Gallenkamp).

Bacterial cells were harvested following incubation by centrifugation at 8000 rpm (6800 x g) in a table-top microcentrifuge (Spectrofuge, Labnet international Inc, USA) for 3 minutes at room temperature (15–25°C). The supernatant was discarded by inverting the open centrifuge tube until all the supernatant media was drained into an appropriate collecting vessel leaving a cream coloured bacterial pellet in the bottom of the microcentrifuge tube. The bacterial pellet was re-suspended into 250µL Buffer P1, containing RNases A, making sure there were no visible clumps before the solution was transferred to a sterile microcentrifuge tube. Re-suspension of the pellet was facilitated by vortexing to remove any remaining clumps. A volume of 250µL of Buffer P2 was added and the tube was inverted 4–6 times to thoroughly but gently mix the contents but avoid shearing of the bacterial DNA. Subsequently 350µL of Buffer N3 was added and the tube again immediately inverted 4-6 times to allow for thorough mixing to avoid localised precipitation of the DNA. The solution became cloudy demonstrating that SDS precipitation had been effective. The solution was then centrifuged for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge, resulting in a compact white pellet containing unwanted proteins. The supernatant was carefully decanted into

a sterile QIAprep spin column and centrifuged for 60 seconds at 13,000 rpm. The flow through was discarded and then 0.5mL Buffer PB was added to wash the remaining residue out of the column. Following centrifugation for 60 seconds at 13,000 rpm the flow through was discarded and a second step was carried out by adding 0.75 mL Buffer PE and centrifuging for 60 seconds. Again, the flow-through was discarded and the column centrifuged for an additional 1 minute to remove any residual wash buffer. Elution of the plasmid was carried out by placing the column in a sterile microcentrifuge tube and adding 50µL Buffer EB to the centre of the QIAprep spin column. The column was allowed to stand for one minute and then centrifuged for one minute at 13,000rpm, to release the harvested plasmid DNA from the column.

6.2.2.2. Determination of plasmid DNA quantity and quality

The quality and quantity of extracted plasmid DNA was determined using a UV spectrophotometer (Jenway, UK). A 5µL aliquot of plasmid DNA was added to 995µL of SDW in a quartz crystal cuvette and the A_{260} and A_{280} was measured. An optical density of 1 at 260nm wavelength is equal to 50µg/mL DNA. Ratios of $A_{260:}A_{280:}$ of 1.7-1.9 indicate DNA of high purity.

6.2.2.3. Restriction endonuclease *Xba*1 analysis of plasmid DNA

Restriction endonuclease *Xba*1 (Promega, UK) was used to linearise the supercoiled *Salmonella* plasmid DNA according to manufacturer's instructions. The restriction digest was carried out in a 20µL total volume containing 16.3µL SDW, 2µL of 10 x RE buffer, 0.2µL BSA (10µL/µL), 1 µL plasmid DNA (1µg/µL) and 0.5µL of the *Xba*1 restriction endonuclease enzyme (10 units/µL). All components were mixed thoroughly

before use and the master mix was again mixed thoroughly before digestion. Digestion was carried out in a water-bath at 37^{o}C for 16 hours. Following incubation, a 10µL aliquot of resulting digested plasmid DNA was combined with 2 µL of 6 x loading buffer (0.25% $^{w}/_{v}$) bromophenol blue, and 30% ($^{v}/_{v}$) glycerol (Fisher Scientific, UK) and 5µL was loaded into separate wells on a 2% agarose gel containing 1µg/mL ethidium bromide (Sigma, UK). Both preceding wells were loaded with 5µL of the standard ladder 300-5000bp (Geneflow, UK). Electrophoresis was carried out in a standard electrophoresis tank (Geneflow, UK) containing 1 x TAE buffer (40mM Tris-HCL, 1mM EDTA and 0.1% ($^{v}/_{v}$) glacial acetic acid) at 65 volts for 80 minutes (Caddick, 2005). The resulting gel was visualised for the presence or absence of plasmid DNA in each well using UV illumination (Syngene, UK) and the image was photographed and edited using the software GeneGenius Bio imaging System (Syngene, UK).

6.2.3. Cell culture

To reduce the risk of contamination all cell culture work was carried out in a sterile laminar flow cabinet (MicroFlow, Advance Bio safety Cabinet Class 2 hood) using standard aseptic techniques.

6.2.3.1. Cell Lines

For the invasion and adhesion assays, CaCo-2 were purchased from the HPA culture collections. These cells are adherent colon epithelial cells isolated from a primary colonic tumour in a 72-year-old Caucasian male using the explants culture technique (Fogh *et al.*, 1977). Additionally, DIEC used in this study were created by Andrea

Quaroni, Cornell University, New York; and used under agreement with WCPN®. Primary cultures from the small intestine of adult beagles were established using methods similar to those described in previous studies (Quaroni & Beaulieu, 1997). These primary cultures were then immortalised using a temperatures sensitive mutant of the simian virus 40 large tumour antigen (SV40 T-Ag). Cells were established and grown as per previous studies (Golaz *et al.*, 2007).

6.2.3.2 Culture of CaCo-2 cells

CaCo-2 cells (Health Protection Agency, UK) were rapidly thawed in a water bath at 37^{o}C and transferred into a sterile 10mL centrifuge tube (Sterlin, UK). An 8mL volume of complete media, DMEM (PAA, UK) supplemented with 2mM Glutamine (PAA, UK), 1% Non-Essential Amino Acids (NEAA, PAA, UK) and 10% Foetal Bovine Serum (FBS, PAA, UK), was gently added to the cells. Subsequently, the cells were pelleted by centrifuging for 5 minutes at 1000rpm. Following centrifugation the supernatant was aspirated off and the cells re-suspended in 7mL fresh complete media. The cells were then split between three T75 flasks (Fisher Scientific, UK) and an additional 8mL of complete media was added to each flask. Flasks were incubated at 5% CO_2 and 37^{o}C for 24 hours. Following incubation the medium was aspirated off and replaced with fresh complete media, which had been pre-warmed to 37^{o}C, and the cells were again incubated at 5% CO_2 at 37^{o}C. Culture vessels were viewed under a light microscope to ensure no contamination had occurred and to verify the cells had reached 80-90% confluency. The cells were then sub-cultured by removing the media and washing twice with sterile calcium free PBS (PAA, UK). Subsequently, the cells were dislodged from the surface of the flask by the addition of 5mL trypsin (PAA, UK) and incubated at 37°C for 5-10 minutes. Cell detachment was confirmed by viewing under a

light microscope and if required the flask was gently tapped to ensure complete removal of the cells and then a 5mL volume of complete media was added to deactivate the trypsin. The cells were transferred into a 50mL sterile centrifuge tube (Sterlin, UK) and pelleted by centrifugation at 1500 rpm for 5 minutes. The supernatant was aspirated off and 5mL fresh complete media was added to re-suspend the cells. A volume of 10mL of fresh complete media was added to a new flask and 1ml of the re-suspended cells was introduced. The flask was labelled appropriately and again incubated at 37^{o}C at 5% CO_2 for cell growth and attachment.

6.2.3.3 Culture of DIEC

Vials of DIEC were provided by the WALTHAM® Centre for Pet Nutrition. Each Vial contained approximately 1 x 10^6 cells, and was stored in liquid nitrogen on receipt. Cells were rapidly thawed in a water bath at 37^{o}C and aspirated into a sterile 10ml centrifuge tube. DIEC growth medium (8mL), detailed in Table 6.1 was added to the cells and the cells pelleted by centrifugation at 1000rpm for 5 minutes. After centrifugation the process detailed in section 6.2.3.2 was followed except incubation was carried out 32^{o}C, 6% CO_2 and DIEC growth media was used which was supplemented with 50μg/ml streptomycin for routine culture. For the invasion and adhesion assays cells were grown in antibiotic free media. In addition, a media change was performed 24 hours after seeding once the cells had attached to the surface of the flask. Culture vessels were viewed under a light microscope to ensure no contamination had occurred and to verify the cells had reached 75% confluency, which was indicative of cells requiring sub culturing. Sub-culturing of cells was carried out as described in section 6.2.3.2 with the exception that cells were trypsonised and grown at 32°C rather than 37^{o}C.

Table 6.1 Preparation of 100ml of DIEC growth medium (antibiotic-free)

Ingredient (PAA, Laboratories GmbH, UK)	**Final Concentration in Medium**
92 ml OptiMEM reduced serum medium liquid	n/a
1 ml HEPES buffer	10mM
1 ml l-glutamine	2mM
1 ml GlutaMAX1	2mM
4 ml FBS	4%
20µl EGF	20ng/ml
1 ml insulin	10µg/ml
100µl hydrocortisone	0.075µg/ml

6.2.3.4. Enumeration of eukaryotic cells

The number of DIEC and $CaCO_2$ cells was calculated using a haemocytometer (Fisher Scientific, UK). This involved removing the attached cells from the culture flask by trypsonisation as described in section 6.2.3.2. A 20µL sample of the cell suspension was added to 20µL of Trypan blue dye (Sigma, UK) in a sterile eppendorf tube and mixed gently. A 10µL sample of the suspension was then added to a haemocytometer and only cells stained blue by the Trypan blue dye were counted, as stated according to manufacturer's instructions, as this was indicative of live cells.

6.2.4. Invasion Assay

6.2.4.1. Culture and enumeration of bacterial strains

Isolates, *S.* Typhimurium (canine isolate 39), *S.* Newport (canine isolate 31), *S. arizonae* (isolated from an asymptomatic dog), *S.* Enteritidis (clinical isolate 18), *S.* Typhimurium (positive control) and *E. coli* (negative control) were resurrected from -80^{o}C and inoculated onto NA. Following incubation at 37^{o}C for 24 hours uniformly sized single colonies were obtained. A heavy loopful of the overnight culture was removed from the NA plate and used to inoculate 2mL of DIEC growth medium (see Table 6.2). The suspension was then vortexed for 1 minute to dissipate the bacteria. Dilutions at 1:10 and 1:100 of the bacterial suspension were created using a solution from 500µL of 100% sterile glycerol (pre-autoclaved) and the number of bacteria present in the original stock was calculated per mL using a haemocytometer; enumeration and calculation was performed according to manufacturer's instruction (Fisher Scientific, UK). The DPBS and sterile glycerol were added to reduce motility of the bacteria in the haemocytometer, allowing accurate counting of the bacteria.

6.2.4.2. Calculating Multiplicity of Infection (MOI)

The MOI is the ratio of infectious agents (*Salmonella*) to infection targets (DIEC & CaCo-2). Bacterial MOI's of 50 and 500 were targeted which were calculated using the following equation:-

$$\text{Bacterial MOI} = \frac{\text{Number of bacteria (CFU/mL)}}{\text{Number of DIEC or CaCo-2 per well}}$$

Invasion and adhesion assay were performed in 24-well plates (Fisher Scientific, UK) which had a surface area of 200mm^2 and cells were seeded at a density of 5 x 10^4

cells/mL. Plates were incubated at 37^{o}C in 5% CO_2 for CaCo-2 and 32^{o}C in 6% CO_2 for DIEC for 24 hours to establish confluent monolayers. Following incubation the media was gently aspirated off and the cells washed three times with sterile DPBS. A 200µl volume of trypsin was added to the wells and the plates incubated for 3-5 minutes to allow detachment of the cells from the well surface. The trypsin was neutralised by the addition of 3mLs of antibiotic free growth medium and the cells enumerated using a haemocytometer. The number of cells per well was determined by averaging the cell counts from the three wells.

The bacterial suspensions were adjusted appropriately, using sterile NB, to obtain an MOI 50 or an MOI of 500. A viable count was performed from the adjusted suspensions at dilution 10^{-2} to 10^{-10} in NB and a 1mL volume was used to inoculate NA plates. Following incubation at 37^{o}C for 24 hours confirmation of the "actual" number of bacteria in each suspension was calculated.

6.2.4.3. Adhesion and Invasion assay

Adhesion and invasion assays were performed in 24-well plates as illustrated in Figures 6.2 and 6.3. Following 24 hour's incubation, the antibiotic free DIEC growth media and CaCo-2 complete media was aspirated from each well. The cells were washed gently three times with 500µl DPBS and a volume of 500µl of the calculated MOI 50 and MOI 500 suspensions was added into the appropriate wells. Subsequently, the plates were incubated for 20 minutes at 32^{o}C for DIEC and 37^{o}C for CaCO-2 in 6% and 5%

CO_2 respectively Following incubation with the bacteria, all wells were washed a further three times with 500µl of sterile DPBS.

For the adhesion assay a 1ml volume of ambient temperature sterile distilled water (SDW) was added to the monolayer and the plates incubated at 37^{o}C for 20 minutes to lyse the cells. Figure 6.1 illustrates a summary of the procedure. The numbers of bacteria within the cell lysates were enumerated by appropriate serial dilution and plating on NA. Following incubation at 37^{o}C for 24 hours plates were analysed for the number of adhering *Salmonella*.

For the invasion assay 500µL fresh pre-warmed cell culture media, appropriate to each cell line, containing 500µg/mL gentamicin (Sigma, UK) was added to the cell monolayers and the plates incubated for 90mins under the appropriate cell culture conditions as prepared in section 6.2.3.2 and section 6.2.3.3 for CaCo-2 cell line and DIEC cell lines, respectively. Subsequently, the antibiotic containing media was gently aspirated off and the cells washed 3 times with 500µL DPBS to remove any residual antibiotics. The cells were then lysed and again the number of internalised bacteria determined by inoculating the cell lysates onto NA using appropriate dilutions and incubating at 37^{o}C for 24 hours plates. The adhesion and invasion assays were performed in triplicate as Figure 6.2 and 6.3 illustrates and also included a positive and negative control.

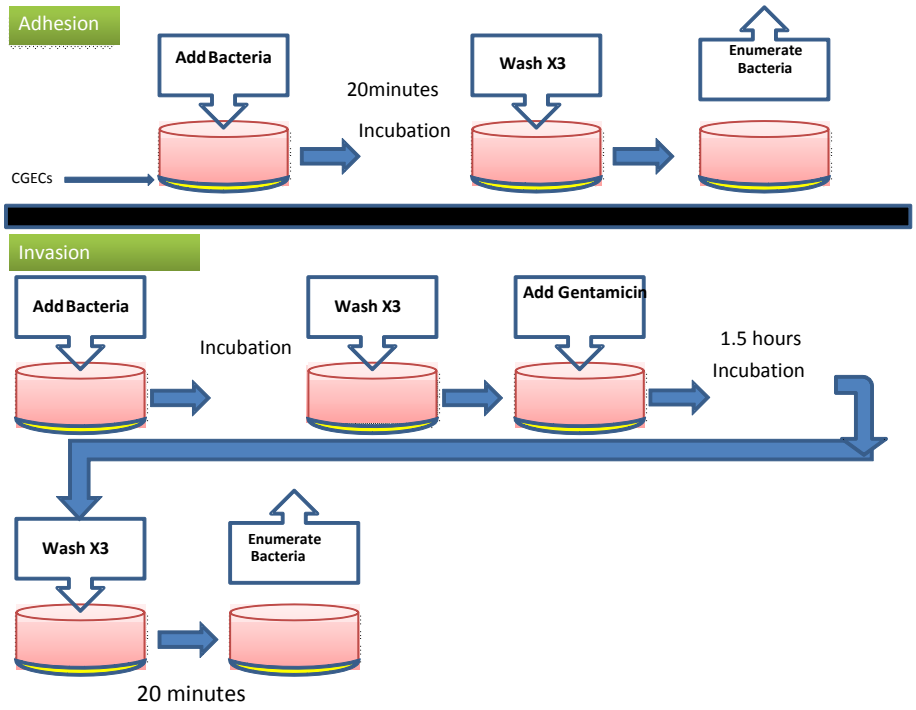

Figure 6.1 Schematic of adhesion and invasion assay (Adopted from Niran Patel, WCPN, 2012)

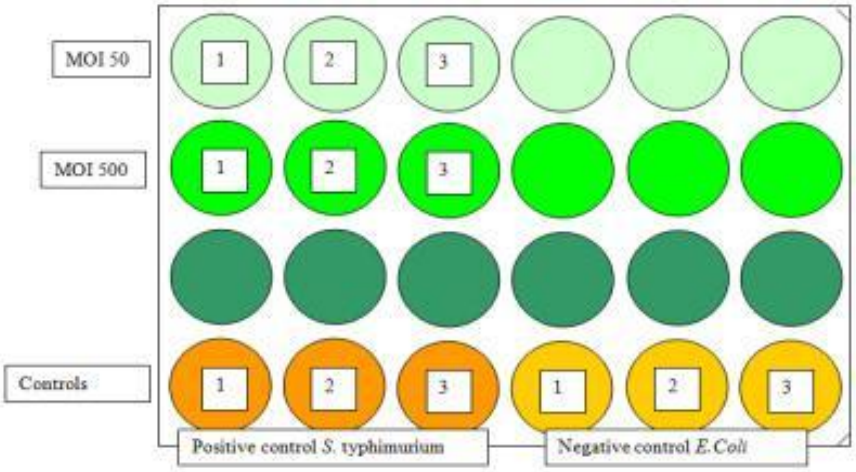

Figure 6.2 Representative plate layout for both adhesion and invasion assay (20 min time-point). All assays were performed in triplicates which are depicted above as 1, 2 and 3. Situated at the lower area of the plate are the positive and negative controls. *S.* Typhimurium (NCTC 74) and *Escherichia coli*, respectively.

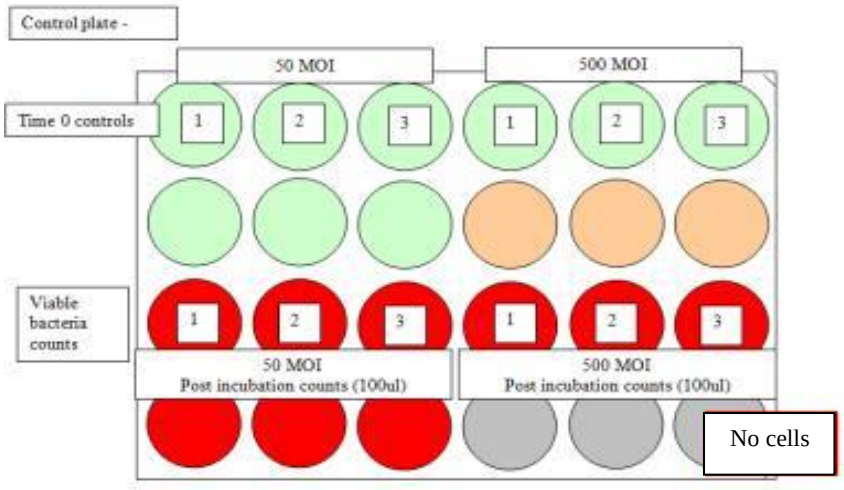

Figure 6.3 Representative plate layout for controls. Invasion and adhesion assays were also performed at time 0 to investigate the efficiency of the washing steps once. Pre and post incubation viable bacterial counts were also performed determine the actual MOI.

6.2.5 Data analysis

The data obtained from both the adhesion and invasion assay was analysed using logistic regression analysis and analysis of variance (ANOVA) on proportion of *Salmonella* invaded (Percentage of *Salmonella* invaded out of total number of bacterial adhered) using Statistica, version 10 (Statsoft, Tulsa, USA). The adhesion assay was performed to allow for a linear model to be constructed allowing *Salmonella* isolates and cell lines to be statistically compared.

An ANOVA is a statistical method for making simultaneous, comparisons between two or multiple means. For this study an ANOVA allowed for multiple comparisons between isolates and cell types. In this instance it was used to compare proportions. This test yields values that can be tested to determine whether a significant relation exists between variables. If no specific hypotheses are identified or a significant F value is obtained then a post hoc test is conducted which determines if the critical differences that have to be exceeded by a pair of group means is significant. This statistical test will identify any significant difference between isolates and their ability to invade the two cell types.

A logistic regression is a Generalised Linear Model (GLM) which analyses an outcome or dependent variable that is binomial. Binomial or binary logistic regression refers to the instance in which the observed outcome can have only two possible types which in this study is that the host cell was invaded or non-invaded. This model has the ability to estimate the probability that a particular subject of categorical data (serotype or cell line

type) will develop the outcome (Hosmer *et al.*, 1991). The model can predict variables with various types of probability distributions by fitting a linear predictor function of the above form to some sort of arbitrary transformation of the expected value of the variable. Logistic regression was performed on statistical software packages within Statistica.

6.3. Results

6.3.1 Isolation of plasmid DNA

Plasmid DNA was isolated from *Salmonella*, and digested with the restriction endonuclease *Xba*1, to investigate if the presence of plasmid DNA is associated with the invasion ability of *Salmonella*. Electrophoresis of digested plasmid DNA extracted from the positive controlled transformed *E. coli* pRS426 (5726bp) (Lane 2), *S.* Typhimurium (Lane 3), *S.* Newport (Lane 4) and undigested DNA (Lane 5) revealed positive possession of plasmid DNA which is a potential indicator of pathogenicity, illustrated in Figure 6.4. Plasmid extraction was not performed on the remaining isolates used in this study, as *S. arizonae* typically does not carry plasmids and *S.* Enteritidis is a well establish pathogenic serotype which typically carries plasmid DNA, therefore data not shown. *E. coli* transformed with a plasmid was used as a positive control for positive extraction.

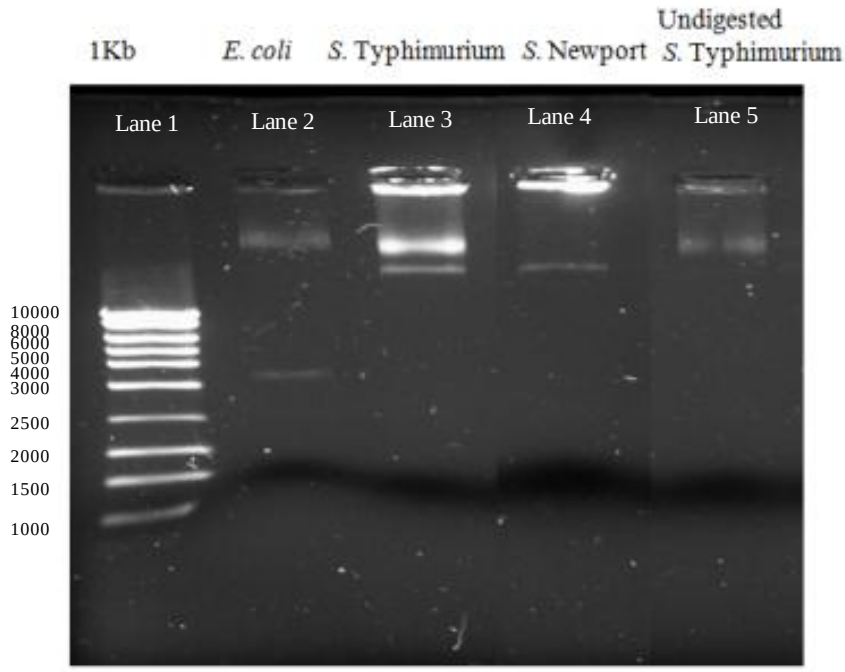

Figure 6.4 Plasmid DNA linearised with the restriction enzyme *Xba*1; *E.coli* transformed with a plasmid (2), *S*. Typhimurium (3), *S*. Newport (4) and undigested *S*. Typhimurium (5). The 1KB Molecular Weight ladder (Promega, UK) is shown in lane 1.

6.3.2 Invasion of CaCo-2 and DIEC by *Salmonella*

The ability of the *Salmonella* isolates to invade CaCo-2 and DIEC is described in this chapter. This involved determining the number of adhering and invading bacteria for each cell line. The actual number of bacterial cells associated with host cells at target MOI's of 500 and MOI 50 is summarised in Table 6.2. The number of successfully invading and adhering bacterial cells was determined by undertaking serial dilutions following lysis of eukaryotic cells and enumerating number of colonies formed on the NA plates following incubation. The data collected from the MOI 500 was taken forward for data analysis as this higher MOI was deemed to be more indicative of saturation of bacteria cells to attachment sites on the host cells. The data revealed that

the actual MOI was 10 fold greater or more than expected and ranged from 1504 to 79197, as detailed in Table 6.2.

Table 6.2 Actual number of adhering/invading bacteria for each *Salmonella* isolate in each cell line at target MOI's of 50 and 500

	No. of adhering and invading (target MOI 50)		**No. of adhering and invading (target MOI 500)**	
	CaCo-2	**DIEC**	**CaCo-2**	**DIEC**
S. Typhimurium	1919	2612	4073	4244
S. Newport	16832	66328	21641	79197
S. Enteritidis	16874	1218	7948	1504
S. arizonae	9103	1513	20711	2281
S. Typhimurium (Positive control)	7111	5351	3950	4378
E. coli (Negative control)	290	257	1733	655

The ability of the investigated *Salmonella* isolates to invade CaCo-2 and DIEC cell lines was investigated using the adhesion and invasion data at an MOI of 500 in a logistic regression and ANOVA. The negative control, *E. coli* ATCC 10536 and the positive control *S.* Typhimurium NCTC 74 were also included in the data analysis.

The linear regression analysis, depicted in Figure 6.5, compares each of the *Salmonella* isolates against each cell line and confirmed that the positive control *S.* Typhimurium

NCTC 74 was capable of invading both CaCo-2 cells and DIEC after 20 minutes and that the non-invading negative control *E. coli* ATCC 10536 demonstrated poor invasion in both cell lines. Both the controls demonstrated significant differences in invasion as their means did not overlapped. The logistic regression analysis also illustrated that the *Salmonella* isolated from the asymptomatic canine in chapter 3, *S. arizonae*, demonstrated a high number of invading bacteria in the DIEC (7.5% $^{+}$/-0.5%) in comparison to the positive control and to the other isolates investigated. In contrast, *S. arizonae* demonstrated poor invasion in the CaCo-2 cells (0.05%) and demonstrated similar invasion with the negative control. Similarly, the human clinical isolate *S.* Enteritidis demonstrated a similar lack of invasion in the CaCo-2 cells (0.4% $^{+}$/-0.2%) and a high number of invaded bacteria in the DIEC (5.5%$^{+}$/-0.5%). The two canine isolates selected for their phenotypic differences in chapter 4, *S.* Typhimurium (VLA 39) and *S.* Newport (VLA 31) demonstrated ability to invade both CaCo-2 (3.2% $^{+}$/-0.5% 1% $^{+}$/- 0.2%, respectively) and DIEC (2.5% $^{+}$/- 0.3% and 0.9%, respectively) in comparison to the negative control. The canine clinical isolate of Typhimurium (VLA 39) demonstrated a greater level of invasion than *S.* Newport and the positive control. This isolate also demonstrated better invasion in CaCo-2 cells in comparison to all isolates investigated in this study. In the DIEC the strain was on-par with the invasion ability of the positive control.

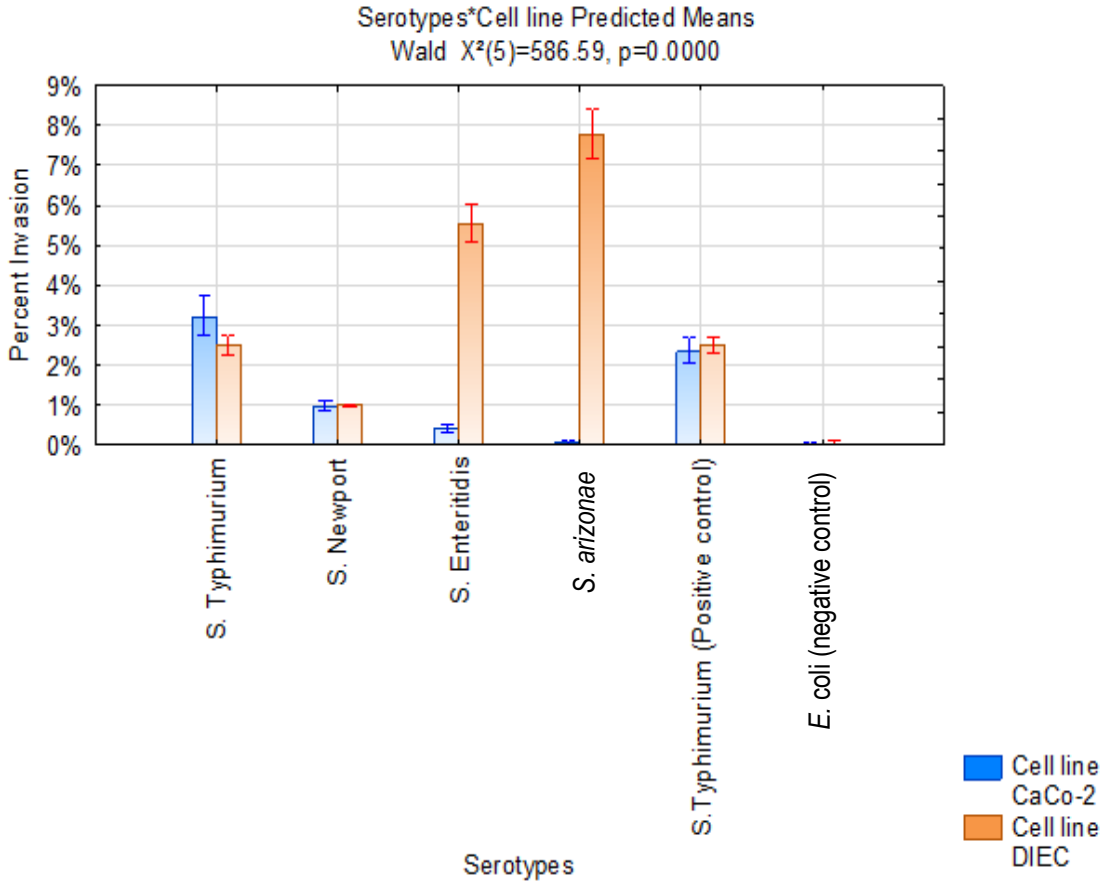

Figure 6.5 Logistic regressions on the average proportions of invaded bacteria in CaCo-2 cells and DIEC. Error bars represent ± standard error of the mean.

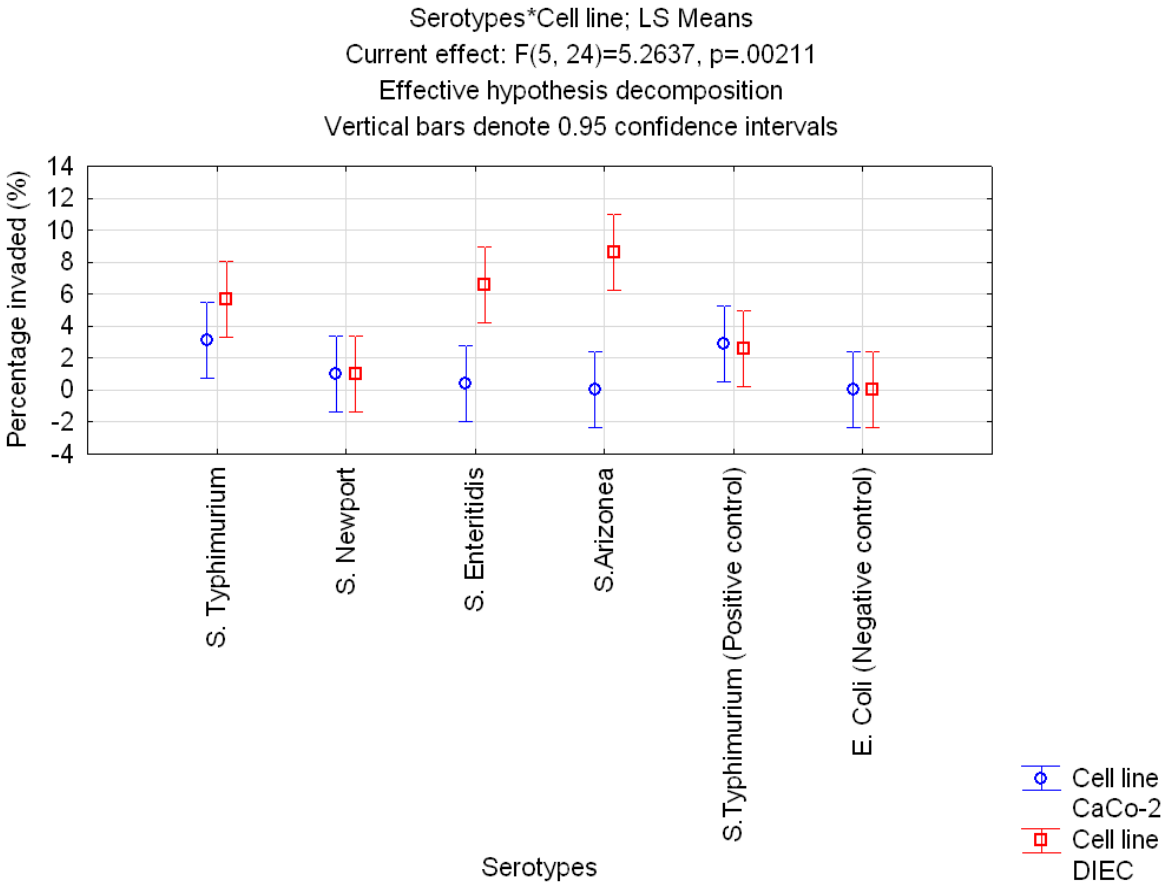

Figure 6.6 ANOVA of percentage of invaded bacteria that adhered to the cell surface. Differences in the ability of *Salmonella* isolates to invade CaCo-2 and DIEC is illustrated by the two data points with addition of 95% confidence interval bars.

The ANOVA revealed that there was no significant difference with the canine isolate, *S.* Typhimurium (p= 0.552) and the ability to invade CaCo-2 cells and DIEC (see Table 6.3). Similarly no significant difference was revealed with *S.* Newport (p= 0.99) and its ability to invade both cell lines. Conversely, *S. arizonae* (p= <0.001) and *S.* Enteritidis (p= <0.001) demonstrated significant differences in their ability to invade the two cell lines. No significant difference was observed with the controls, *S.* Typhimurium (p= 0.92) and *E.* coli (p= 0.609) and cell line type.

Table 6.3 Summary of Post Hoc analysis which are interactions of isolates and cell lines using ANOVA. Values in red represent significantly different comparisons (p value =< 0.05).

			1	2	3	4	5	6	7	8	9	10	11	12
	Isolate	**Cell Line**												
1	*S.* Typhimurium	CaCo-2		0.55210	0.03351	0.03433	0.00037	0.11861	0.00000	0.03066	0.71060	0.78295	0.00000	0.00000
2	*S.* Typhimurium	DIEC	0.55210		0.00866	0.00890	0.00008	0.32002	0.00000	0.10327	0.33755	0.38670	0.00000	0.00000
3	*S.* Newport	CaCo-2	0.03351	0.00866		0.99101	0.07282	0.00072	0.00013	0.00013	0.07233	0.05967	0.00001	0.00006
4	*S.* Newport	DIEC	0.03433	0.00890	0.99101		0.07121	0.00074	0.00013	0.00013	0.07396	0.06105	0.00001	0.00006
5	*S.* Enteritidis	CaCo-2	0.00037	0.00008	0.07282	0.07121		0.00000	0.01398	0.00000	0.00097	0.00076	0.00198	0.00692
6	*S.* Enteritidis	DIEC	0.11861	0.32002	0.00072	0.00074	0.00000		0.00000	0.50411	0.05763	0.06991	0.00000	0.00000
7	*S. arizonae*	CaCo-2	0.00000	0.00000	0.00013	0.00013	0.01398	0.00000		0.00000	0.00000	0.00000	0.42071	0.76498
8	*S .arizonae*	DIEC	0.03066	0.10327	0.00013	0.00013	0.00000	0.50411	0.00000		0.01332	0.01660	0.00000	0.00000
9	*S.* Typhimurium (Positive control)	CaCo-2	0.71060	0.33755	0.07233	0.07396	0.00097	0.05763	0.00000	0.01332		0.92360	0.00000	0.00000
10	*S.* Typhimurium (Positive control)	DIEC	0.78295	0.38670	0.05967	0.06105	0.00076	0.06991	0.00000	0.01660	0.92360		0.00000	0.00000
11	*E. Coli* (Negative control)	CaCo-2	0.00000	0.00000	0.00001	0.00001	0.00198	0.00000	0.42071	0.00000	0.00000	0.00000		0.60996
12	*E. Coli* (Negative control)	DIEC	0.00000	0.00000	0.00006	0.00006	0.00692	0.00000	0.76498	0.00000	0.00000	0.00000	0.60996	

Numbers 1-12 from left to right are associated with serotypes 1-12 from top to bottom. Individual interactions can be obtained by reading across from chosen serotype and cell type and reading down from chosen serotype. For example the interaction of *S.* Typhimurium (1) in CaCo-2 cells and *S.* Newport (3) gives a p value of 0.03351 which is red and therefore there is a significant difference between these two serotypes in the CaCo-2 cell line. Empty spaces are interactions with self therefore are dismissed.

6. 4 Discussion

Salmonella can manifest as gastroenteritis or systemic infection in animals including canines, however, as of yet no strain has been identified as canine host restricted. Zoonotic stains are capable of causing infection in a variety of host, including humans and canines, suggesting a similar mode of pathogenesis and invasion of host cells to elicit infection. The ability of *Salmonella* to invade host cells is necessary to cause infection. The aim of this chapter was to investigate the ability *Salmonella* isolates *S.* Typhimurium (canine isolate), *S.* Newport (canine isolate), *S. arizonae* (asymptomatic canine isolate), *S.* Enteritidis (human isolate) and positive and negative control to invade human CaCo-2 cells and DIEC.

6.4.1 Plasmid DNA confirmation

Plasmids are autonomously replicating extra chromosomal DNA and considered the best understood mobile elements in bacteria. Typically plasmid analysis differentiates strains by the number and size of harboured plasmids which are visualised through gel-electrophoresis separation (Weller, 2000). However for this study it was of interest to consider the presence of plasmid DNA in the isolates taken forward from Chapter 4. Typically invasion genes are located on plasmids therefore plasmid extraction and identification was a good indication that the strains will invade the cells. The method of plasmid DNA extraction used in this study coupled with visualisation using UV imaging following electrophoresis revealed a positive confirmation of plasmid DNA in the isolates *S.* Newport (VLA 31) and *S.* Typhimurium (VLA 39). This confirmation for plasmid possession (Gulag *et al.*, 1987; Jones *et al.*, 1982) is strongly associated

with virulence involving adhesion and invasion mechanisms. Plasmid DNA from prokaryotic cells is typically negatively supercoiled and the degree of supercoiling affects many cellular processes, including replication, integratation, transposition and transcription (Richardson *et al.* 1984). The plasmid DNA was linearised for gel imaging by digesting with an endonuclease. The endonuclease *Xba*1, produced by an *E. coli* strain that carries the *Xba*1 gene from *Xanthomonas badrii* (ATCC 11672), is a well-established endonuclease for the digestion of *Salmonella* DNA (Chai *et al.*, 2012; Mirza *et al.*, 2000; Rodriguez *et al.*, 2012). The gel in Figure 6.4 illustrates that the plasmid from the transformed *E.coli* pRS426 presented with one linear band of DNA, which is typically accepted from artificially modified plasmid DNA. A clear single linearised band was also obtained for two *Salmonella* isolates; *S.* Typhimurium (VLA 39) and *S.* Newport (VLA 31). The bands presented above the 1Kb Molecular Weight (MW) ladder indicating that the one piece of plasmid DNA is larger than 1Kb. The actual size therefore could not be associated to the fragment as the 1Kb MW ladder was too small. This is not uncommon as *Salmonella* plasmid DNA can range from 2- 200Kb (Libby *et al.*, 2002; Rychlik *et al.*, 2006). Studies investigating *Salmonella* plasmid DNA have demonstrated large linear pieces of DNA greater in MW than 10Kb (Giles *et al.*, 2004). Isolate *S.* Typhimurium (lane 5) undigested contained a large band which is most probably undigested plasmid. This undigested plasmid band can also be seen in the *E.coli* and *S.* Typhimurium (VLA 39) lane suggesting that not all plasmid DNA was digested. Overall both isolates contained plasmid DNA, suggesting they are potentially able to invade host cells and they were taken forward for the adhesion and invasion assays. Plasmid extraction was not performed on the *S. arizonae* isolate as studies have demonstrated that the *spv* virulence locus typically found on large plasmids in *Salmonella* subspecies I serotype associated with severe infections, was confirmed to

be located on the chromosome of *S. arizonae* (Boyd *et al.*, 1998; Libby *et al.*, 2000). Additionally the clinical human isolate *S.* Enteritidis was not confirmed for the presence of plasmid as it is a well know infection causing serotype which is a result of the possession of virulence plasmid. Therefore it was taken that this isolate would have the plasmid. (Rychlik *et al.*, 2006; Rotger & Casadesus, 1999; Morshed & Peighambari, 2010).

6.4.2 Adhesion assay

Adhesion to the host cell surface is a prerequisite for invasion of the bacterial cell subsequently initiating a signal transduction cascade (Bäumler *et al.*, 1996). Adhesion of each isolate on each cell line was of interest as it also allowed for the calculation of invasion of the isolates as a proportion of those that adhered. Following co-incubation of *Salmonella* isolates with CaCo-2 cells and DIEC, bacterial counts were determined and the number of invading bacteria as a proportion of the total number of adhered bacteria was calculated.

The ability of *Salmonella* isolates to adhere to DIEC and CaCo-2 cells was determined using a modified protocol (Benz *et al.*, 1989; Charbonneau *et al.*, 2006; Letourneau *et al.*, 2011). The general principle of the method is employed widely in investigations into bacterial adhesion mechanisms due to its simplicity and limited use of consumables therefore reducing cost. Overall all isolates in this investigation demonstrated adhesion after 20 minutes of incubation with both monolayers of DIEC and CaCo-2 cells. A study into the adhesion characteristics of *S.* Typhimurium

investigated different periods of incubation time, 10 minutes to 60 minutes, with intestinal epithelial monolayers and reported that after 20 minutes there was just fewer than 2% cell association (McCormick *et al.*, 1993). A study into *Salmonella* adhesion to different surfaces concluded that *Salmonella* adhesion is strongly strain-dependent and this can constitute to the differences in virulence observed among the numerous serotypes (Oliveira *et al.*, 2006). It has been reported that *Salmonella* serotypes initiate infection by attaching to the intestinal mucosa of the host cells and having the ability to adhere efficiently to host cells is associated with host adaptive strains (Boyd *et al.*, 1997; McCormick *et al.*, 1993). Recent reports suggest that the recognition of intestinal surfaces by adhesins may contribute to the host adaptation of *Salmonella* serotypes (Boyd *et al.*, 1997).

6.4.3 Invasion assay

This study demonstrated that *S. arizonae* isolated from an asymptomatic household dog and *S.* Enteritidis isolated from a symptomatic human host were significantly better at invading DIEC in comparison to the other isolates. Both isolates were significantly less able to invade CaCo-2 cells in comparison to the other isolates and controls. This suggests that these isolates have host restricted characteristics towards the canine host.

The process of intestinal invasion by *Salmonella* can be somewhat replicated *in vitro* assays by infecting tissue culture cells of gut epithelial origin. Studies have demonstrated using these assays that bacterial uptake is heavily associated with host cell type. Membrane ruffling is frequently observed on the surface of infected cells at the site of bacterial entry (Rosenshine *et al.*, 1994) and invasive pathogenic *Salmonella*

serotypes are able to hijack the actin regulatory machinery of the host cell to promote bacterial entry by inducing a rearrangement of network actin microfilaments (Friis *et al.*,2005). The majority of *Salmonella* molecular determinants involved in this entry process are encoded in a pathogenicity island located at the centisome 63 of the bacterial chromosome (Suarez *et al.*, 1998). Additionally the successful invasion and colonisation of *Salmonella* into the host cell occurs depending on the ability of the *Salmonella* to avoid PMN transepithelial migration and other host defence strategies. Invasion assays or Gentamicin Protection Assays (GPA) have been exploited to investigate the *in vitro* invasion characteristics of different pathogens in a variety of host cells (Chu *et al.*, 2010; Flentie *et al.*, 2008; Friis *et al.*, 2005; Shi *et al.*, 2006; Townsend *et al.*, 2008). Studies demonstrated *Salmonella* invasion-defective mutants, negative for *inv*A genes, are unable to induce a cellular response in host cells (Galán *et al.*, 1992; García *et al.*,1993; Suárez, 2008) and highlighted that invasion into host cells is paramount for a systemic infection.

In this study the ability of *Salmonella* isolates to invade CaCo-2 cells and DIEC was investigated. Following infection of cell lines, bacterial counts were determined and the proportion of invading bacteria with respect to the total number of adhered bacteria was calculated. The data analysis provided evidence that *S. arizonae* and *S.* Enteritidis demonstrated an enhanced capacity to invade DIEC; in comparison to the other strains tested in this study. However, both isolates demonstrated a restricted ability to invade CaCo-2 cells as their invasion capacity was not significantly different to the non-invasive control. This potentially controversial observation, particularly with reference to the established zoonotic pathogen *S.* Enteritidis will be explored further later.

However the invasion characteristics of *S. arizonae* will be discussed first. *Salmonella arizonae* is predominately found in cold blooded reptiles, such as, snakes, lizards and terrapins (Butt *et al.*, 1952.; Edwards *et al.*, 1959; Finely *et al.*, 2007) and is an uncommon human pathogen (Stefano *et al.*, 2012) which supports the low invasion obtained in CaCo-2 cells. However human hosts with depressed CD4-mediated T-cell immunity, such as those infected with human immunodeficiency virus, are particularly susceptible to the development of serious and persistent systemic infections with serotype *arizonae* (Libby *et al.*, 2002). The virulence determinants of serotype *arizonae* are poorly understood. However, the ability of this organism to cause extra intestinal infection in both reptiles and humans is consistent with recent evidence that this subspecies contains the *spv* virulence gene locus (Boyd *et al.*, 1998). The *spv* loci in these strains are highly homologous, consisting of the transcriptional regulator *spvR* and four structural genes, *spvABCD.* Studies concluded that the *spv* genes of serovar *arizonae* were located in the chromosome (Boyd *et al.*, 1998). However, this study did not rule out the possibility that the *spv* locus was carried on a very large plasmid. In this chapter *S. arizonae* isolated from an asymptomatic dog demonstrated significantly greater invasion in canine cells in comparison to the other isolates studied, however the host was not presenting symptoms. Published data regarding *arizonae* isolation from canines is very limited and the relationship of asymptomatic or symptomatic presentations cannot be made. Reptiles frequently harbour the sub species *arizonae* and the sub species is considered a commensal organism. This suggests that the serotype contains the appropriate genes to successfully invade particular hosts however they may lack the genes that allow them to defend themselves from the immune system. Studies have shown that within the *spv* region of plasmid DNA, the most significant differences between a*rizonae* and the subspecies I strains, associated with clinical diseases, are the

absence of *spvD* and polymorphisms in *spvA* (Libby *et al.*, 2002). However, it is possible that this gene could have a function in other hosts or environments (Libby *et al.*, 2002). Typically, *Salmonella* spp. are mainly transmitted by the faecal-oral route. However it is common to state that they are carried asymptomatically in the intestines or gall bladder of many animals, and are continuously or intermittently shed in the faeces. Therefore these bacteria are able to infect certain host asymptomatically and again this may be due to the possession of virulence genes.

Controversially, data from this study revealed a very low invasion count with *S.* Enteritidis in CaCo-2 cells. This is peculiar as this serotype is considered one of the most common causes of human salmonellosis worldwide (HPA, 2011b; Pan *et al.*, 2009). This serotype owes its prevalence to its association to chickens and eggs used in food consumption. Reports have also shown its zoonotic capability in cattle and chickens; however this serotype is largely associated with human infection (VLA, 2012). Interestingly, a study demonstrating invasion of different phage types of *S.* Enteritidis revealed that one phage type in particular, PT11, demonstrated poor invasion into CaCo-2 cells (Pan *et al.*, 2009). It was concluded that other PT that are isolated from infected chickens were better at survival and invasion whereas the PT11 strains that originated from a variety of hosts, including canines, rodents and hedgehogs (VLA Enteric Reference Unit) demonstrated poor survival and invasion in CaCo-2 cells. This observation was found to be due to a lack of plasmid-borne genes, pefA, B, C, D, srgC and rck, and four chromosomal genes encoding putative amino acid transporters (Pan *et al.*, 2009). The observations demonstrated in this chapter for this serotype are unusual and could be a result of a poor invading strain. Additionally, as only one isolate was

represented for each serotype in this study the results may be questioned in terms of representativeness for the serotype as a whole. Therefore future work would involve repeating the experiment with a larger number of isolates from the same serotype to conclude an association if there is one to be made.

A study investigating bacterial adhesion and invasion revealed *S.* Typhimurium adhered equally well to non-viable and viable human Int-407 cells, which indicates that adhesion does not require metabolically active cells. Additionally, viable and non-viable *S.* Typhimurium adhered equally well and competed with each other for cellular adhesion sites (Kusters *et al.*, 1993). The unusual low level of invasion of *S.* Enteritidis in CaCo-2 revealed in this chapter may have been attributed to a suspension consisting of many sub-lethally damaged and or non-viable bacterial cells that were able to adhere to the CaCo-2 cell surface, reducing the number of site for viable bacterial cells to invade. A previous study discovered that adhesion of *S.* Typhimurium followed saturation kinetics, with a maximum of 10 adhesive bacteria per cell. This indicates that there are a limited number of bacterial adhesion sites (receptors) available on the surface of the host cell (Kusters *et al.*, 1993).

The two strains, *S.* Typhimurium (VLA 39) and *S.* Newport (VLA 31), selected for their metabolic differences (see chapter 4) demonstrated no significant difference in invasion of the two cell types. As the second leading serotype to cause infection in human (HPA, 2011b) *S.* Typhimurium is a successful pathogen and invasion into DIEC and CaCo-2 cells is demonstrated in this chapter. Studies have shown that *S.*

Typhimurium has many virulence determinants including, TTSS-1, which is required for eliciting dihorrial disease (Watson *et al.*, 1998; Tsolis *et al.*, 1999; Barthel *et al.*, 2003). The needle like TTSS-1 apparatus injects effector proteins into host epithelial cells, thus triggering host cell invasion and pro-inflammatory responses making this serotype a successful pathogen (Sturm *et al.*, 2011). The serotype has also been isolated from canine host including kennel dogs (unpublished data, Liverpool University); household dogs in which *S.* Typhimurium was the predominate isolated serotype (33.3%) from the population sampled (Leonard *et al.*, 2010); and dog-keeping households and dogs attending four different veterinary clinics again revealing *S.* Typhimurium to be the predominate serotype isolated (Ojo *et al.*, 2009). This serotype is a well-established zoonotic pathogen which is supported by the similar levels of invasion in CaCo-2 cells and DIEC.

Salmonella Newport is consistently reported as one of the top six most frequently isolated serotypes of *Salmonella enterica* in England and Wales. Between 2000 and 2010, an average of 223 cases were reported per annum (range 124 – 662), the maximum being reported in 2004 following a national outbreak attributed to the consumption of lettuce at restaurants and takeaways. Salmonellosis caused by the serotype Newport has also been reported in humans from food sources including watermelons and lettuce (HPA, 2012). The isolate investigated in this study demonstrated a level of invasion significantly higher than *S. arizonae* and *S.* Enteritidis in the CaCo-2 cells suggesting this serotype is more pathogenic in human host. This trend also supports the literature of levels of Newport food poisoning outbreak cases in humans. The data also revealed that there was no significant difference in the ability of

S. Newport to invade and the two cell lines, suggesting that this serotype is capable of invading both hosts equally and indicates that this is a potential zoonotic serotype. The 2011VLA report of *Salmonella* isolation in livestock animals including cattle, sheep, pigs and poultry in Great Britain recorded the incidence of Newport as increasing from 26 in 2007 to 60 in 2011. Therefore this serotype has significance in both human and animal reports of *Salmonella*. Limited data is available on *S.* Newport in dogs; however, from the canine isolates donated from the VLA for this thesis, overall Newport represented 3% of the population. Therefore it can be said that this serotype can cause infection in canine host.

Typically, *Salmonella* are invasive facultative intracellular pathogens and many essential virulence traits are clustered within *Salmonella* pathogenicity islands (SPI) (Gerlach *et al.*, 2008) which allow for this characteristic. There are a number of SPI's however only a few are associated with adhesion, invasion and intracellular including, *Salmonella* Pathogenicity Island 1 and 2 (SPI1 and SPI2); these encode type III secretion systems (T3SS) with well-established roles in invasion (Patel & Galan, 2005) and intracellular survival. Effector proteins translocated by the SPI1-encoded T3SS modify signalling events of the host cell, leading to the rearrangement of the actin cytoskeleton as aforementioned in the introduction (Patel & Galan, 2005). Studies have demonstrated that SPI4 encodes a type I secretion system (T1SS) with SiiE as a secreted substrate (Gerlach *et al.*, 2007a; Morgan *et al.*, 2007); which coupled with SPI1 allow efficient entry of *Salmonella* into polarized epithelial cells. Mutant strains deficient of SPI1 and SPI4 was highly reduced in invasion and highly reduced in entering MDCK cells, respectively. Bacteria can carry varying numbers of SPI's and

are typically transferred through horizontal gene transfer events such as transfer by a plasmid, phage, or conjugative transposon (Hacker &Kaper, 2000). *Salmonella* strains with a high number of important SPI's may demonstrate advantageous adhesion and invasion abilities which may be the situation in the *S. arizonae* and *S.* Newport isolates.

The ability of different *Salmonella* serotypes to invade different host cells has been demonstrated in this chapter, suggesting that there may be a potential for a particular strain from a particular serotype to be canine host restricted. Additionally, this study demonstrated that there are serotypes that are capable of invading both cell types which suggests zoonotic capability.

6.5 Conclusion

In conclusion, statistical analysis using logistic regression and ANOVA of the adhesion and invasion assay data demonstrated that *S.* Typhimurium, *S.* Newport, *S. arizonae* and *S.* Enteritidis all adhered and invaded CaCo-2 cells and DIEC after a 20 minute incubation period. The MOI 500 data was taken forward for analysis rather than the MOI 50 as it was presumed to represent bacterial saturation for attachment sites on the cell lines. A significantly higher proportion of *S. arizonae* and *S*. Enteritidis invaded DIEC in comparison to the CaCo-2 cells and the other isolates investigated. These observations may be suggestive of canine host restricted isolates. *Salmonella* Typhimurium and *S.* Newport demonstrated no significant difference between invasions of both cell lines indicating zoonotic characteristics. Further studies using multiple representatives of candidate serotypes is required for stronger conclusions to be drawn regarding host adaptation.

Chapter 7 Final Discussion

Salmonella is the second leading cause of bacterial foodborne infection in the UK (HPA, 2011a). Regardless of education, health and safety regulation and vaccination of egg laying hens, rates of *Salmonella* infections remain high which is an increasing concern for UK public health. In an increasingly hygiene concerned society, a major barrier to pet ownership is the perceived role of companion animals in contributing to the risk of exposure to bacterial pathogens, such as *Salmonella* (Haverkos *et al.*, 2010). With over 22 million pets sharing almost half of all UK households (Murray *et al.*, 2010; PFMA, 2012), including 8 million dogs, it is important to determine the current prevalence of *Salmonella* carriage in the dog population to allow the zoonotic risks to be more accurately quantified. With many serotypes of *Salmonella* demonstrating host restricted characteristics in animal host (Bäumler *et al.*, 1998a; Pasmans *et al.*, 2003; Paulin *et al.*, 2002; Thomson *et al.*, 2008; Uzzau *et al.*, 2000), it warrants the investigation of potential host restricted strains in canine host. This thesis sought to update epidemiological data on asymptomatic carriage which are now somewhat dated (Buxton, 1957; Galton *et al.*, 1952; Gorham & Garner, 1951; McElrath *et al.*, 1952; Shimi, 1976; Timbs *et al.*, 1975) and largely non-UK related (Bagcigil *et al.*, 2007; Gorham & Garner, 1951; Lefebvre *et al.*, 2008; Leonard *et al.*, 2011; Shimi, 1976; Stucker *et al.*, 1952; Timbs *et al.*, 1975; Weber *et al.*, 1995) and investigate any potential of host restricted strains of *Salmonella* isolated from the canine host.

Epidemiological investigation revealed that asymptomatic carriage of *Salmonella* in dogs in the UK was *ca.* 0.20% which suggests a low exposure risk and low potential for zoonotic transmission between canines and humans. The low levels of carriage revealed in this study remains within the lower end of the spectrum of incidence rates demonstrated in previous studies, 0-43% (McElrath *et al.*, 1952) and 1-35% (Finley *et al.*, 2007; Galton *et al.*, 1969; Greene *et al.*, 1998). On par with this low incidence rate the longitudinal study investigating *Salmonella* shedding in a cohort of ten dogs over a 9 month period revealed an absence of asymptomatic *Salmonella* carriage. It is possible that a longer period of sampling might provide additional information regarding seasonality shedding (Foley *et al.*, 2007; Pires *et al.*, 2009; Tanaka *et al.*, 1976). Additionally, in regards to the low (*ca* 0.20%) asymptomatic carriage of *Salmonella* in canines revealed in the epidemiological study, a larger population size of canines may be sampled for investigations into seasonality shedding in future studies. Whilst *Salmonella* was not recovered from canine faeces investigated in this study there were several zoonotic pathogens isolated and are regarded as typical gut associated bacteria, including *Escherichia*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Proteus*, *Serratia* which have been observed by similar studies undertaken into isolation of bacterial species in faeces (Crowther, 1971; Guentzel, 1996; Rice *et al.*, 2003).

Investigations into the survival of *Salmonella* in canine faeces presented in chapter 2 at room temperature and outdoor environment revealed positive survival of *Salmonella* after 48 hours and 6 month period, respectively. Temperature investigations into *Salmonella* survival are carried out in the food industry to determine an effective storage temperature that will destroy bacteria or keep them at a minimal level. Observations of survival at room temperature in this thesis have also been suggested by

a similar study (Zhuang *et al.*, 1995). This leads to concerns of potential cross contamination to individuals and surrounding environments therefore caution should be taken when dealing with canine faeces and hygiene is of utter most importance for the reduction of potential contamination risk (Rice *et al.*, 2003; Rusin *et al.*, 1998).

The absence of *Salmonella* suggests concern regarding zoonotic transmission of this organism from canines to humans ought not to be a barrier to dog ownership however improper hygiene practices could potentially lead to cross contamination to individuals and their environments. The low incidence of *Salmonella* demonstrated in this thesis aids in the promotion of companion dog ownership as the benefits of Human-Animal Interactions (HAI). These interactions are associated with improvement in health and reduction of minor illnesses after acquisition of a pet; pet owners visit the doctor less often and spend less money on medication; a strong attachment to a pet is associated with significantly less depression after the loss of a spouse; and adults and children are more likely to meet the guidelines for daily activity if they own a dog (Esposito *et al.*, 2011; McCune & Serpell, 2012; McNicholas, 2004; Melson, 2003; Serpell, 1991 & 2001). To evaluate the risk of zoonotic diseases from pet to human and vice versa, studies into other pathogenic microorganisms from different pet host are needed.

Salmonella isolation and stability in faecal specimens was thoroughly evaluated, which adds confidence to the true absence of *Salmonella* in canine faeces sampled in this thesis; if it had of been present the comprehensive methodological approach would have detected it. The coupling of well-established media for *Salmonella* isolation were successful in positive isolation of artificially inoculating faecal samples that were used

for a positive control during each isolation experiment. The use of BPW and RV broth has been extensively used in previous studies and the use of the selective agars XLD, HE and BS have also demonstrated positive results in the isolation of *Salmonella* in other studies (Bager & Petersen, 1991; De Boer, 1998, Cantor *et al.*, 1996; Dusch & Altwegg, 1995; Edel & Kampelmacher, 1974; Finley *et al.*, 2007; Schotte *et al.*, 2007; Taylor, 1965; Vassiliadis, 1981 & 1983).

Investigations into host restricted or adapted strains were of interest in this thesis and a panel of isolates from symptomatic canines and humans were metabolically profiled and analysed using established microbiological tests. The phenotypic properties of clinical isolates of *Salmonella* from both host populations were investigated using API 20E test, Biolog Inc. Microbial Identification Systems GEN III MicroPlate™, motility test and antibiotic profiling (MIC and disc diffusion). The results from these phenotypic tests were used to form a profile of each isolate depending on the resulting reaction. Therefore each isolate gave a unique profile based on their phenotypic characteristics which were analysed to investigate associations between host origin and serotype of the isolate. The API 20E tests are well-established and are cost effective (Nucera *et al.*, 2006; O'Hara *et al.*, 1992; Peele *et al.*, 1997). The API test results identified profiles that were generally homogenous, only demonstrating differences in one or two reactions either inositol, VP and TDA reactions that were provided on the test strip. Reactions from each isolate were manipulated to form binary profiles and these were analysed by hierarchical clustering. There were a number of groups formed by Dice coefficients which were represented by UPGMA clustering from the dendrogram produced using GelCompar. Group 1 were isolates that were negative for inositol fermentation and VP production. Group 2 demonstrated a negative reaction to indole

however a positive reaction to VP. Group 3 demonstrated positive reactions to both indole and VP. Group 4 demonstrated positive reaction to indole however a negative reaction to VP. However, there was no significant relationship with serotypes and profiles, neither was there any association with host origin of isolates and profiles produced. This method was simple and allowed for rapid profiling however demonstrated a lack of depth in discrimination of each isolate. A correspondence in *Clinical Infectious Diseases* highlighted problems concerning the potential misidentification of bacteria when commercial identification systems are used (Frederiksen, 2001). For this thesis the fact that only 20 reactions could be investigated resulted in a limited number of characteristics therefore the Biolog Inc. Microbial Identification Systems GEN III MicroPlate™, a much more comprehensive test containing 96 reactions was carried out to investigate differences between each isolate. Each isolate produced a profile consisting of absorbance readings of each metabolic test and these data when interrogated with PCA statistics revealed two isolates that were phenotypically distinct from the majority of the *Salmonella* population. These two isolates were from a canine origin, and of serotypes *S.* Typhimurium and *S.* Newport, which were selected for further analysis. Previous studies have exploited the phenotypic system for bacterial identification (Wilson & Jackson 1996) rapid comparison methods of microbes (Truu *et al.*, 1999), and metabolic capabilities of microorganisms and even compromised host cell lines (Bochner *et al.*, 2011). As has been demonstrated here, these phenotypic methods appear to work well when considering time, cost and simplicity of interpretation of the resulting data; testing in this manner leads to profiles which can be analysed for differences or similarities.

Further phenotypic profiling was carried out on the *Salmonella* isolates based on their antibiotic sensitivity patterns, using the well-established methods of MIC and antibiotic

disc diffusion (Andrews, 2001a & 2001b; Brown & Kothari 1975). Sensitivity patterns were used to reveal any association between the resistance pattern of an isolate to antibiotics and where it was isolated from, either canine or human host. The tests revealed that canine isolates were significantly more resistant to the panel of antibiotics including ampicillin, amoxicillin chloramphenicol, gentamicin, tetracycline, and trimethoprim. This result is not unexpected as the prescribing practice of antibiotics in the veterinary setting (Hughes *et al.*, 2012), especially in animals reared for human consumption (Breuil *et al.*, 2000; Collignon, 2012; Ebner & Mathew, 2000) is much more relaxed than the human clinical settings. This implies that bacterial infections in companion canine animals may be treated improperly which contributes to the increase in antibiotic resistance as demonstrated in the canine isolates from this study (Prescott *et al.*, 2002). This is also concerning from a clinical point of view as zoonotic infections originating from dogs may demonstrate limited antibiotic therapy options. Overall the phenotypic profiles revealed that in general isolates from both canine and clinical *Salmonella* share similar characteristics. Canine isolates demonstrated a higher level of resistance to antibiotics however there was no clear association of isolates from the same serotype sharing the same phenotypic profiles whether it was antibiotic sensitivity or biochemical reactions. No association with isolates from the same serotype was demonstrated and no discrimination with isolates from different host groups was demonstrated using the panel of isolates investigated in this thesis; however these phenotypic tools are useful for the identification of minor differences between certain isolates.

Further into the thesis it was of interest to investigate the incidence of serotypes associated with canine salmonellosis and commonly associated serotypes within the human population by comparison to the HPA PFGE database of human salmonellosis; a form of genotypic characterisation. Isolates from canine and human host were characterised using a harmonised PFGE method. The well-established PFGE method is used world-wide and has demonstrated to be a reliable reproducible method for comparison of profiles from different cases (Avery *et al.*, 2002; Lawson *et al.*, 2001; Lomonaco *et al.*, 2008; Sandt *et al.*, 2006; Soyer *et al.*, 2010; Swaminathan *et al.*, 2001 & 2006; Thong *et al.*, 1995; Threlfall *et al.*, 1999). Profiles produced were much more diverse then the phenotypic profiles for different *Salmonella* serotypes. However dendrographic representation of the relationship between isolates revealed that isolates from the same serotypes whether they were from human or canine host, tended to cluster closely together differing in only a few bands (Zou *et al.*, 2010). No major clustering was observed representing isolates from a single host; canine and human isolates tended to be interdispersed amongst the clusters suggesting zoonotic transmission rather than infections predominantly within a restricted host. On-going investigations should be carried out on circulating isolates from symptomatic canines to investigate any potential for an evolving host restricted strain.

The resulting PFGE profiles coupled with analysis did not reveal any isolates that were clustered differently depending on their host origin. However phenotypic characterisation using Biolog Inc. Microbial Identification Systems GEN III MicroPlate™ did reveal two canine isolates, *S.* Typhimurium (VLA 39) and *S.* Newport (VLA 31) that were markedly different from the population of other canine and human

Salmonella isolates. Therefore due to the differences in metabolic reaction, involving stachyose and NAG, it was of interest to investigate if these differences were involved with the degree of pathogenicity. Pathogenicity is heavily associated with adhesion and invasion of host cells as demonstrated by numerous investigation (Cossart & Sansonetti, 2004; Dibb-Fuller *et al.*, 1999; Galan *et al.*, 1992; Letourneau *et al.*, 2011; Kusters *et al.*, 1993; Shi & Casanova, 2006; Suarez & Russmann, 1998; Mroczenski-Wildey *et al.*, 1986) additionally if a strain shows a high affinity to invade a particular host cell then this demonstrates host restriction characteristics. An invasion assay may be utilised for the identification of canine host restricted strains of *Salmonella* by demonstrating significant invasion into intestinal cells isolated from dogs (DIEC) but not those from a human source (CaCo-2). Bacterial suspensions of the two clinical canine strains (*S.* Typhimurium 39 and *S.* Newport 31), one human clinical isolate (*S.* Enteritidis ID 18), one canine strain isolated from an asymptomatic dog (*S. arizonae*) and two control isolates, positive (*S.* Typhimurium, NCTC 74) and negative (*E. coli* ATCC 10536) were applied to a monolayer of CaCo-2 cells and DIECs. Following incubation of these cells lines with the bacteria the cells were lysed and bacterial cell counts were enumerated to reveal the number of adhering and invading *Salmonella*. Gentamicin antibiotic was used for the invasion assays to eliminate all *Salmonella* cells that were not invaded. The application of these assays and ANOVA statistics enabled the significant interactions of each *Salmonella* isolate with each cell line, CaCo-2 representing human host and DIEC representing canine host, to be investigated. The analysis revealed that *S. arizonae* and *S.* Enteritidis invaded the DIEC significantly more than the other strains and significantly more than in the CaCo-2. The two isolates *S. arizonae* and *S.* Enteritidis could be potential candidates for host restricted isolates of *Salmonella* in canine host. The association of *S. arizonae* isolation from canine hosts is

becoming a common phenomenon as observations of isolation in canines have been made from researchers at Liverpool Veterinary School and The Royal Veterinary College (Personal communications with Professor Martin Woodward and Liverpool Veterinary School). However the trend demonstrated in *S.* Enteritidis is somewhat unusual as this serotype is the leading infection causing serotype of *Salmonella* in human infection (Angulo & Swerdlow, 1999; Chai *et al.*, 2012; Janmohamed *et al.*, 2011; HPA 2011c; Woodward *et al.*, 2002). The two selected isolates from the Biolog Inc. Microbial Identification Systems GEN III MicroPlate™, *S.* Typhimurium (VLA 39) and *S.* Newport (VLA 31), demonstrated no significant difference with the DIEC in comparison to the other isolates in the study. *Salmonella* Typhimurium and *S.* Newport demonstrated equal invasion into both cell lines indicating zoonotic potential, supported by published data (Hughes *et al.*, 2008; Lawson *et al.*, 2011; Nakamura *et al.*, 1986; Ojo & Adetosoye, 2009; Pitout *et al.*, 2003; Poppe *et al.*, 2006; Zhao *et al.*, 2003). The differences observed in this study between the isolates and the invasion ability into CaCo-2 cells and DIEC demonstrates the potential of zoonotic and host restricted strains of *Salmonella.* The use of these models alone to characterise host restriction has provided insight into the microorganism ability to invade different cell lines; these observations demonstrate the need for further investigations into the relationship between *Salmonella* serotype and its ability to adhere and invade different host cells.

In conclusion this thesis has demonstrated that the prevalence of *Salmonella* within UK dogs is extremely low at approximately 0.2% representing a significant reduction compared to historical figures. The reduction is likely as a result of changes in animal feeding habits towards commercially prepared pet food and further integration of dogs

into the home in recent years. Detailed phenotypic characterisation of a panel of *Salmonella* isolates obtained from symptomatic canines and humans revealed no significant differences between the metabolic profiles of *Salmonella* originating from the two host groups. Two isolates, a *S.* Typhimurium and *S.* Newport, did demonstrate marked heterogeneity in their utilisation of stachyose and NAG; these minor metabolic differences amongst thousands of homogeneous reactions revealed by PCA analysis and demonstrating the power of this data mining technique. The significance of these metabolic differences warrants further investigation and may be associated with the ability of these strains to cause infection in the canine host as they were both isolated from a symptomatic canines. Genotypic characterisation of canine and human isolates by PFGE revealed both indistinguishable and highly related profiles in phylogenetic clusters composed of strains from human and canine origin. The presence of indistinguishable strains distributed throughout the human and canine clusters supports the zoonotic potential of *Salmonella*. Invasion assays in DIEC's and CaCo-2 cells confirmed the ability of *Salmonella* to invade both of these cells lines, however *Salmonella arizonae* and *S.* Enteritidis demonstrated greater invasion in DIEC's. The enhanced capacity for invasion observed in these two isolates is worthy of further exploration and may be suggestive of emerging host adaptation, although invasion assays with additional and multiple representation of these serotypes is required.

In an increasingly hygiene concerned society, a major barrier to pet ownership is the perceived role of companion animals as a potential reservoir of zoonotic pathogens. Dogs represent the most frequently owned pet with over 8 million homed in the UK. For these reasons it is important to determine the prevalence of pathogens in the pet

population to enable a quantitative risk assessment to be made, and this thesis has made a significant contribution to informing such an assessment specifically in relation to the infection risk of *Salmonella*. Whilst the risk of zoonotic infection of *Salmonella* is reassuringly low it is likely that dogs may harbour other zoonotic pathogens and therefore it is recommended that good hygiene should always be implemented alongside pet ownership.

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9.0 Appendix

9.1 Pet Consent form

Pet Consent Form

Aston University, Life and Health Sciences, is analysing faecal samples from dogs.

We would like you to collect 3 consecutive faecal samples from your dog each month for 12 months. This will be used to analyse the bacteria present in the faeces. Once analysed, the collective results from a number of dogs will be expected to add important knowledge and understanding of gastrointestinal health.

By signing this form you agree to provide faecal samples and give permission for your pet to be entered into the study.

We would also like you to complete a short questionnaire about your pet.

Thank you for your help in this study.

I agree to the above and to providing 3 consecutive faecal samples from my pet. I understand that the samples will become the property of Aston University Life and Health Sciences Department and will be used for research purposes.

Name of pet: _____

Name of owner: _____

Signature: _____ Date: _____

9.2 Owner survey

Owner Survey

We would appreciate it if you could answer the following questions about your pet. The content of the survey will remain private and confidential.

1. Your name

2. Name of pet: _____

3. Breed of pet: _____

4. Date of birth of pet (or age in years): _____

5. Sex: _____

6. Is your pet neutered or entire? _____

7. Current weight (if known): _____

8. Typical main diet your pet regularly consumes: (e.g. canned meat, dry complete, home prepared etc.)
   _____

9. Snacks and treats: (please give details of any chews/treats/extras you feed to your pet including non-pet food)
   _____

10. Is your dog on any medication? _____
    If so, what? _____

11. Has your dog had any previous gastrointestinal problems? _____
    If so, please describe_____

12. Is your dog copraphagic (eats faeces)?_____

13. Does your dog eat anything else unusual? _____

Pet Consent Form

The Waltham Centre for Pet Nutrition, Leicestershire, UK is analysing faecal samples from dogs.

We would like you to collect a fresh faecal sample from your dog. This will be used to analyse the bacteria present in the faeces. Once analysed, the collective results from a number of dogs will be expected to add important knowledge and understanding of gastrointestinal health.

By signing this form you agree to provide a faecal sample(s) and give permission for your pet to be entered into the study.

We would also like you to complete a short questionnaire about your pet.

Thank you for your help in this study.

I agree to the above and to providing a faecal sample(s) from my pet. I understand that the samples will become the property of the Waltham Centre for Pet Nutrition and will be used for research purposes.

Name of pet: _____

Name of owner:_____

Signature _____ Date:_____

Thank you for completing this survey and consent form.

9.3 Epidemiological *Salmonella* data of reported cases of infection throughout the year from 2000-2010.

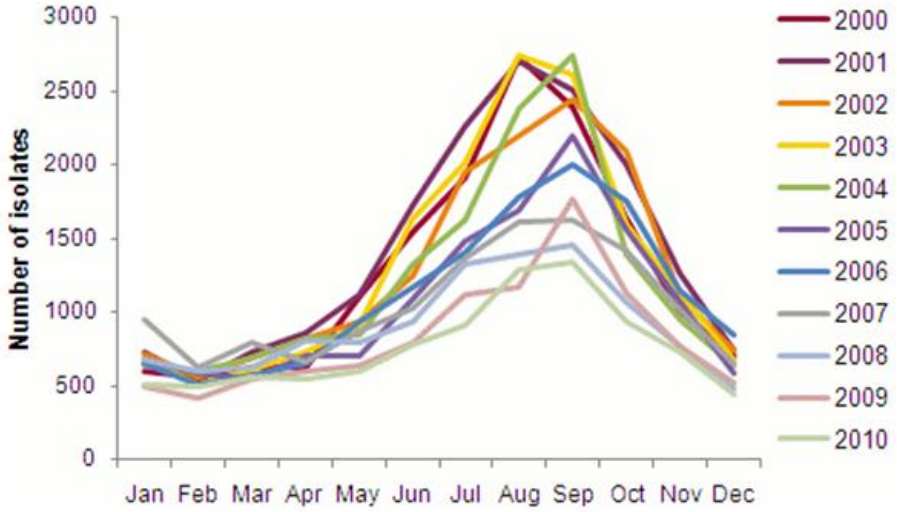

Figure 9.3 Seasonal incidences of salmonellosis in the UK over 10 years. During the winter months there is steady level of cases however incidences peak during the summer months (HPA, 2012).

1.0 Conferences attended and other professional activities

Publications in preparation

Preena Mistry, Nicola Williams, Malcolm Bennett, Sian Wilson, Corrin Wallis, Zoe Marshall-Jones, Sandra McCune and Anthony Hilton. Current perspective on asymptomatic carriage of *Salmonella* in canines. *American Journal of Public Health.*

Preena Mistry, Corrin Wallis and Anthony Hilton. Characteristics of clinical human and veterinary *Salmonella* isolates by phenotypic micro-arrays and antibiotic sensitivity patterns. *Applied and Environmental Microbiology.*

Preena Mistry, Corrin Wallis and Anthony Hilton. Potential for zoonoses/anthroponosis investigated by comparison of PFGE profiles derived from human and veterinary isolates of *Salmonella*. *American Journal of Public Health.*

Electronic Publications

McCune, S. Mistry, P. Hilton, A.C. Wallis, C. (2012) Reducing barriers to pet ownership- WALTHAM website www.waltham.com.

Poster presentations

Mistry, P., Hilton, A. C., Wallis, C., Marshall-Jone, Z (2009). *Salmonella* in companion animals. Aston University Postgraduate poster day, 3rd July, Aston University, Birmingham, UK.

Mistry, P., Hendry, E. R., Davies, M. and Chaudhry, R. (2011). Microbiology: Infectious disease, investigation and prevention. Aston University Postgraduate poster day, 16th March, Aston University, Birmingham, U.K.

Mistry, P (2011). Current perspective on asymptomatic carriage of *Salmonella* in canines. Aston University Postgraduate Poster Day, 29th June, Aston University, Birmingham, UK.

Mistry, P., Hilton, A.C., Wallis, C., Marshall-Jone, Z., McCune, S. (2011). Current perspective on asymptomatic carriage of *Salmonella* in canines. ISAZ Conference 4-7th August. Human-Animal Interactons: Challenges and Rewards Indianapolis, Indiana.

Mistry, P., Hilton, A.C., Wallis, C. (2012). *Salmonella* in companion dogs. Human-Animal Interaction Research Consortium Meeting. ISAZ Pre-Conference Meeting at, 9th-10th July, WCPN, Waltham.

Oral Presentations

Mistry, P. (2009). *Salmonella* in companion animals. First year Research in Progress presentation, 20th May. Aston University, Birmingham, UK.

Mistry, P. (2009). *Salmonella* in companion animals- Waltham epidemiological study, 29th October. WCPN, Waltham, UK.

Mistry, P. (2010). Epidemiological investigations into *Salmonella* carriage in companion canines. Second year Research in Progress presentation, 17th March. Aston University, Birmingham, UK.

Mistry, P. (2011). *Salmonella* in companion dogs. Final year Research in Progress presentation, 1st June. Aston University, Birmingham, UK.

Professional activities completed

53rd International Basic Training Workshop on Bionumerics® and Gelcompar ll® software. Organised by Applied Maths NV, April 20th-21st 2009, Sint-Martens-Latem, Belgium

Essential Statistics and Experimental Design training course, Mars University R&D College. April 2009, WCPN, Waltham, UK.

Infection 2009. E-Bug education and activity stand volunteer, 11th-13th November 2009. International Conventional Centre, Birmingham, UK.

4th Broadening microbiology horizons meeting. SFAM Spring meeting, 16 April 2010, Stratford upon Avon, UK.

Microbiology teaching assistant and demonstrator for microbiology undergraduate modules including food microbiology, biotechnology and microbiological pathogeneses. 2009-2012.