

**Molecular Typing of *Salmonella* Serotypes
Isolated from Wildlife, Domesticated Animal
Species and Humans in Zimbabwe.**

By

Agness Farai Nhidza

**A Thesis submitted in Partial Fulfilment of the requirements of Masters of
Philosophy (M Phil) degree in Molecular Biology with the University of
Zimbabwe, Faculty of Science, Biochemistry Department.**

ACKNOWLEDGEMENT

My sincere gratitude goes to my husband, Robson Manjoro, for the unwavering support and encouragement. I dedicate this project to my two children, Panashe and Tapiwanashe. My heartfelt thank you goes to Central Veterinary Laboratory (CVL) in particular the Bacteriology and Molecular Biology Sections for allowing me to carry out my research at their organisation. I appreciate the effort from the supervisory team namely Dr Dexter Savadye (UZ), Professor Idah Sithole-Niang (UZ), Dr Pious Vengesai Makaya (CVL) and Mr Bamusi Saidi (CVL). I thank Mrs Kathryn Boyd, Biomedical Research and Training Institute (BRTI) for helping with the review of this document and Mr Reggie Mutetwa (BRTI) for helping with finishing up payments for my fees. Without support from all these people, it would have been difficult to come up with this document. I thank you all.

ABSTRACT

Salmonella enterica serovar *enteritidis* (*S. enteritidis*) is one of the major causative agents of diarrhoea in humans and is associated with the ingestion of contaminated animal products such as meat, poultry and poultry products. In Europe, *S. enteritidis* is involved in 80% of *Salmonella* food poisoning cases. Traditional methods of typing have been used to identify *Salmonella* up to the species level. However the advent of molecular typing techniques has made it possible to type *Salmonella* beyond the species level. This study aimed at establishing epidemiological relationships of the *Salmonella* serotypes isolated from different geographical locations and from different host species using molecular typing techniques. The study also aimed at identifying and serotyping *Salmonella* strains isolated from wildlife, domestic animals and humans using culture on selective media, biochemical tests and serotyping using the slide agglutination method.

The *Salmonella* used in this study were first typed using the slide agglutination test method using *Salmonella* antisera, before being subjected to multiplex polymerase chain reaction (Multiplex PCR), Plasmid profiling and Pulsed Field Gel Electrophoresis (PFGE) as molecular typing schemes for sub-typing beyond species level to determine their differences at molecular.

The isolate subtypes used in this study were *S. enteritidis*, Group B and Group C *Salmonella* strains. These were analysed and compared in order to pick any strain differences in relation to geographical distribution and host origin in order to determine possible epidemiological relationships. Multiplex PCR was in a position to split *S. enteritidis* strains into those with and those without *Salmonella Plasmid Virulence (spv)* genes which was not possible with plasmid profiling. *Spv* genes carry virulence genes of bacteria.

No relationship of the plasmid profiles or pulsotypes to geographical location was, however, established in this study. The present study however, managed to place the *S. enteritidis* from the Salmonellosis outbreak into a single profile based on PFGE results.

Although Group B *Salmonella* were also from an outbreak, they produced more profiles, both by plasmid and PFGE profiling though PFGE produced the highest number of profiles. According to this study, PFGE could possibly be used for typing of *Salmonella* in Zimbabwe after confirming with multiplex PCR if one wishes to show epidemiological relationships of the *Salmonella* serotypes isolated from different geographical locations and from different host species. This conclusion is drawn from the fact that in this study PFGE produced more profiles after typing compared to the other tools used. This should be complemented by the traditional typing tools.

Group C produced the largest number of plasmid profiles compared to group D and Group B possibly as a result of more species in this group compared to group D and Group B. PFGE was, however, not performed on this group as a result of lack of reagents.

In conclusion, no relationship to geographical location and host origin of isolates was established. There is need to carry out the study on a large scale to authenticate the findings.

CONTENTS

ACKNOWLEDGEMENT	I
ABSTRACT	II
TABLE OF CONTENTS.....	IV
TABLE OF FIGURES.....	VI
TABLE OF TABLES	VIII
1.0 INTRODUCTION	1
1.1 SALMONELLA	1
1.1.1 SALMONELLA ENTERITIDIS	4
1.1.2 GROUP B SALMONELLA.....	4
1.1.3 GROUP C SALMONELLA.....	5
1.2 ANTIMICROBIAL SUSCEPTIBILITY	5
1.3 PLASMIDS.....	7
1.4 TYPING TECHNIQUES.....	11
1.4.1 TRADITIONAL TYPING TECHNIQUES	12
1.4.2 MOLECULAR TYPING TECHNIQUES.....	13
1.5 OBJECTIVES	18
1.6 RESEARCH JUSTIFICATION.....	19
2.0 MATERIALS AND METHODS	20
2.1 SAMPLE COLLECTION, SALMONELLA ISOLATION AND SEROTYPING	20
2.2 SEROTYPING.....	23
2.3 ANTIMICROBIAL SUSCEPTIBILITY TESTING	23
2.4 MOLECULAR TYPING	25
2.4.1 MULTIPLEX PCR ASSAY.....	25
2.4.2 PLASMID PROFILING.....	27
2.4.3 ELECTROPHORESIS	27
2.4.4 PULSED FIELD GEL ELECTROPHORESIS (PFGE)	29
2.4.5 DISPOSAL OF THE SALMONELLA CULTURES USED IN THE STUDY.....	30
3.0 RESULTS	30

3.0 RESULTS	31
3.1 SAMPLE COLLECTION, <i>SALMONELLA</i> ISOLATION AND SEROTYPING.....	31
3.3 MULTIPLEX PCR.....	40
3.4.1 <i>S. ENTERITIDIS</i> STRAINS	43
3.4.2 GROUP B STRAINS	47
3.4.3 GROUP C STRAINS	48
3.5 PULSED FIELD GEL ELECTROPHORESIS.....	50
3.5.1 <i>S. ENTERITIDIS</i> STRAINS	50
3.5.2 GROUP B STRAINS	52
3.5.3 GROUP C STRAINS	54
4.0 DISCUSSION	55
5.0 REFERENCES	65

FIGURES

Figure 1: General Structure of <i>Salmonella</i> Bacteria.....	1
Figure 2: General Bacterial Cell Structure.....	1
Figure 3: R-Plasmid.	1
Figure 4: Pbr322 Plasmid.	1
Figure 5: Flow Chart of Sequence of Events in The Processing of Samples for <i>Salmonella</i>	1
Figure 6: Map of Zimbabwe Showing Distribution of <i>Salmonella</i> Isolates Used in this Study.....	1
Figure 7: Graph Showing Distribution of <i>Salmonella</i> Isolates in Relation to The Source.....	1
Figure 8: Graph Showing Distribution of <i>Salmonella</i> Isolates in Relation to Geographical Location.....	1
Figure 9: Percentage Sensitivity to the Selected Antibiotics for Group B <i>Salmonella</i>	1
Figure 10: Percentage Sensitivity to the Selected Antibiotics for Group C <i>Salmonella</i>	1
Figure 11: Percentage Sensitivity to the Selected Antibiotics for Group D <i>Salmonella</i>	1
Figure 12: Percentage of <i>S. Enteritidis</i> Strains Confirmed from all 282 <i>Salmonella</i> Isolates Subjected to Multiplex PCR.....	1
Figure 13: Percentage of <i>S. Enteritidis</i> Strains With <i>Spv</i> Genes relative to those Without <i>Spv</i> Genes	1

Figure 14: Multiplex PCR For Human Group D Isolates.	1
Figure 15: Plasmid Profiling of the <i>S. Enteritidis</i> Strains of Animal Origin Possessing <i>Spv</i> Gene.	1
Figure 16: Animal <i>S. Enteritidis</i> Plasmids.	1
Figure 17: Plasmid Profiles Generated From <i>S. Enteritidis</i> of Animal Origin	1
Figure 18: Human Group B <i>Salmonella</i> Plasmids.	1
Figure 19: Plasmid Profiling of Group C Isolates.	1
Figure 20: Plasmid Profiles Generated From Group C <i>Salmonella</i> Isolates	1
Figure 21: Animal <i>S. Enteritidis</i> Pulse Field Gel Electrophoresis	1
Figure 22: Pulsotypes Identified From The Pfgc Performed on the 49 <i>S. Enteritidis</i>	1
Figure 23: Group B <i>Salmonella</i> Strains Pulsed Field Gel Electrophoresis	1
Figure 24: The Pulsotypes Identified from Human Group B Isolates.....	1

TABLES

Table 1: Medically Important Representatives of <i>Salmonella</i>	1
Table 2: The Distribution of <i>Salmonella</i> Isolates Used In The Study.....	1
Table 3: Antibiotic Sensitivity Results for Group B, C and D <i>Salmonella</i> Isolates from Humans and Animals.	36

ABBREVIATIONS

AIDS	Acquired Immuno-Deficiency Syndrome
BA	Blood Agar
DNA	Deoxyribonucleic acid
CVL	Central Veterinary Laboratories
EDTA	Ethylenediaminetetraacetic Acid
ERIC	Enterobacterial Repetitive Intergenic Concensus
HIV	Human Immuno-Deficiency Virus
Kb	Kilobases
LD	Lysine Decarboxylase
MAC	MacConkey
OD	Optical Density
OIC	Opportunistic Infections Clinic
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
pSCV	<i>Salmonella choleraesuis</i> Virulence plasmid
REA	Restriction Enzyme Assay
Spv	<i>Salmonella</i> Plasmid Virulence
Ssp	Sub species
TBE	Tris Borate EDTA
TE	Tris EDTA
TSI	Triple Sugar Iron

UZ

University of Zimbabwe

1.0 INTRODUCTION

1.1 SALMONELLA

Salmonella enterica species is a gram-negative, facultative, rod shaped bacterium found in the family enterobacteriaceae (Figure 1). *Salmonella enterica* species live in the intestinal tract of warm and cold-blooded animals. Some *Salmonella* species are host adaptive for example *S. choleraesuis* for pigs, *S. gallinarum* for poultry and *S. dublin* for cattle (Boyd and Hartl, 1998). The host adapted avian pathogens (*S. pullorum* and *S. gallinarum*) are always non-motile. *Salmonella* are typically indole and urease negative but unique ones which are indole and urease positive do exist (Osborne, 2011).



Figure 1: General structure of a *Salmonella* bacterium. Available at <http://science.jrank.org/pages/5944/Salmonella-Causes-symptoms.html>.

Salmonella infections are zoonotic as they can be transmitted by humans to animals and vice versa. *Salmonella enterica* subsp. *enterica*, serovars *enteritidis* and *Typhimurium* are responsible for the vast majority of human salmonellosis (Rychlik *et al.*, 2005). Salmonellosis has been a public health concern world wide as a result of the zoonotic feature possessed by most *Salmonella spp* (Taylor *et al.*, 2001). Infection via food is also possible (Gast *et al.*, 2010). *Salmonella* infections are amongst the most prevalent causes of diarrhoeal diseases worldwide.

Salmonella is implicated in a wide range of infections ranging from life threatening typhoid to gastroenteritis and bacteraemia salmonellosis. Bacteraemia is also known as enteric fever or typhoid. Enteric fever is a result of the invasion of the bacterium into the blood stream and acute gastroenteritis is a result of food-borne intoxication (Parry and Beeching, 2009; Boyd and Hartl, 1998).

The extension of salmonellosis to various organs depends on the serotype, inoculum size and host status. When *Salmonella* is ingested in large numbers, some will survive the acidic conditions of the stomach and enter the small intestines. There, they result in diffuse mucosal inflammation, oedema and micro-abscesses. Although most non-typhoidal *Salmonella* (NTS) do not get beyond the *lamina propria* and lymphatics of the gut, *S. choleraesuis* and *S. dublin* are exceptions which result in bacteraemia with little intestinal involvement (Chartterjee *et al.*, 2000).

Salmonella also causes meningitis, which is an unusual complication of *Salmonella* sepsis. *Salmonella* sepsis occurs almost exclusively in infants and young children. Severe cases of meningitis occur in adults causing high morbidity and mortality (Karim and Islam, 2002). Due to the detrimental effects of *Salmonella* infections, there is a need to ensure food safety and ensure that accurate diagnostic assays are available. Molecular epidemiological tools can be developed for the implementation of effective disease prevention and control measures (Pan and Liu, 2002).

Medically important representatives of *Salmonella* reported in the literature are shown in Table 1 (Gast *et al.*, 2010).

Table 1: Medically important representatives of *Salmonella*

SPECIES	HOST	EFFECTS
<i>S. enterica ssp arizonae</i>	Cold blooded animals, poultry and mammals	
<i>S. choleraesuis</i>	Commensals in pigs	Hog cholera if resistance is weak
<i>S. enteritidis</i>	Intestines of cattle, rodents, ducks (and their eggs) and humans	Calf paratyphoid fever and human gastroenteritis
<i>S. paratyphi</i> i) <i>paratyphi A</i> ii) <i>paratyphi B</i>	i) Solely human pathogen ii) Humans and domesticated animals	i) Paratyphoid A ii) Paratyphoid B
<i>S. typhi</i>	Humans in temperate and subtropical Zones	
<i>S. typhimurium</i>	i) Birds and mammals ii) Humans	i) fatal, feverish intestinal infections ii) <i>Salmonella enteritis</i>
<i>S. dublin</i>	cattle	Cattle salmonellosis
<i>S. typhisuis</i>	pigs	Hog salmonellosis

In this study *S. enteritidis*, Group B *Salmonella* and Group C *Salmonella* spp based on the Kauffmann-White classification system, were used.

1.1.1 *Salmonella enteritidis*

S. enteritidis is one of the major causative agents of diarrhoea in humans and is associated with the ingestion of contaminated animal products such as meat, poultry and poultry products (Simango and Mbewe, 2000). In Europe, *S. enteritidis* is involved in 80% of *Salmonella* food poisoning cases (Nygard *et al.*, 2004).

It was noted, in a survey on infected adults, that HIV/AIDS patients are most prone to non-typhoidal salmonellosis amounting to 35% of HIV-infected adults in Africa with documented blood stream infections (Hohmann, 2001).

Diarrhoea from *S. enteritidis* does not usually require antibiotic treatment though it can be fatal in infants, HIV patients and the immuno- suppressed (Gast *et al.*, 2010).

1.1.2 Group B *Salmonella*

The common members in Zimbabwe of this group are *S. paratyphi B* and *S. typhimurium*. *S. typhimurium* is primarily a pathogen of cattle, but other organisms such as sheep, goats, pigs, birds and humans can be affected (Liebana *et al.*, 2002). Group B *Salmonella* are implicated in the vast majority of salmonellosis in humans (Tavechio *et al.*, 2009).

1.1.3 Group C *Salmonella*

This group is divided into C₁ and C₂ sub-groups. The most common in Zimbabwe are sub-group C₁, in particular *S. paratyphi C*, *S. choleraesuis* and *S. typhisuis*. These are the group O: 7 serotypes. *S. paratyphi C* is a human pathogen and the other two are animal pathogens affecting mostly pigs (Kudaka *et al.*, 2002). It has been discovered that in Zimbabwe, Group C *Salmonella* and *S typhimurium* are the primary pathogens for farmed crocodiles posing an economic threat to crocodile farmers (Foggin, 1992). Farmed crocodiles in Zimbabwe have been found to be the carriers of *Salmonella* especially *S. arizonae*, which may be considered as the normal flora of the crocodile intestines.

Obwolo and Zwart, 1993, in their research on crocodiles, found *Salmonella*, mainly *S. arizonae* in intestines of 16% of crocodiles studied (in 50, 3 year old animals) (Obwolo and Zwart, 1993). For proper treatment of pathogens, there is need to test the drugs on hand to find the best drug for the treatment against the pathogen. This can be done through performing antimicrobial susceptibility tests.

1.2 Antimicrobial Susceptibility

Disc diffusion methods, as well as Epsilon meter test (E-tests), are the most commonly used methods of testing for susceptibility of pathogens to drugs used for treatment.

An E test is similar to disc diffusion but uses a rectangular plastic strip impregnated with a stable gradient of antibiotic concentrations, sometimes up to 15 concentration gradients have been used to determine minimum inhibition concentration of pathogenic bacteria, mycobacteria or fungi (Biomérieux Product Library).

Although drugs are not recommended for treatment of uncomplicated salmonellosis, they can be useful in the treatment of extra-intestinal *Salmonella* infections such as septicaemia, endocarditis, emphysema and meningitis (Berg *et al.*, 2007). This is why it was important to include drug susceptibility testing in this study.

Some researchers have identified natural antibiotics in traditional wines in Zimbabwe. In one study, *Salmonella* group B, *S. enteritidis*, *Shigella sonnei* and *S. flexneri* were exposed to natural antibiotics from fermented mukumbi wine, a traditional Zimbabwean wine. It was observed that the natural antibiotics in the wine were comparable to artificial antibiotics on destroying the pathogens showing the importance of this wine as a form of antibiotic on complicated conditions of co-infections by these pathogens (Mugochi *et al.*, 1999).

It is also important to test the effectiveness of synthetic antibiotics to enhance further studies on combination therapy in the event of co-infections. These antimicrobial susceptibility characteristics are as a result of presence or absence of plasmids with or without the resistance genes.

1.3 Plasmids

A naturally-occurring plasmid is a circular, extrachromosomal, double stranded DNA (dsDNA) molecule that is found in bacteria and is capable of autonomous replication. Plasmids can range in size from 2 kilobases (Kb) to 1000 Kb. Furthermore they can occur in a supercoiled form, although some can occur in a linear fashion. Plasmids help bacteria to adapt to their surrounding. The adaptation is governed by a tight genetic control that involves the expression of multiple genes (Furuya *et al.*, 2006, Persing *et al.*, 2004).

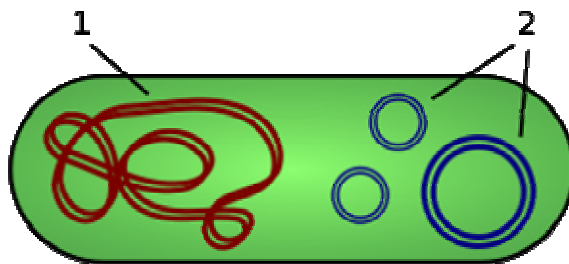


Figure 2: General bacterial cell structure showing 1-chromosomal DNA and 2-plasmids (Berg *et. al.*, 2007)

Some plasmids have a narrow host range while others have a broad host range (Kues and Stahl, 1989). Plasmids can occur naturally for example R-Plasmids which are large conjugative plasmids that carry genes which code for more than one antibiotic resistance.

R-Plasmids also code for their own replication, their own conjugal transfer and have mobile genetic elements (Sorensen *et. al.*, 2005). Figure 3 shows the general structure of an R- plasmid. This is just an example amongst hundreds of plasmids existing in nature.

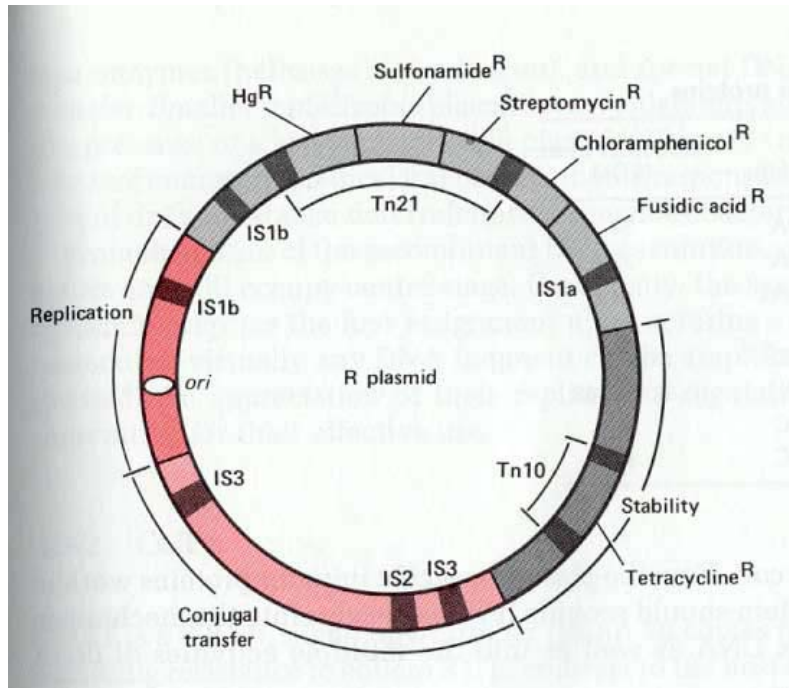


Figure 3: R-plasmid (Berg *et. al.*, 2007).

Another naturally occurring plasmid is the tumour inducing (*Ti*) plasmid found in *Agrobacterium tumefaciens* which infect dicotyledonous plants. These plasmids have genes which code for a number of plant growth hormones such as auxins and cytokinins, as well as derivatives of the amino acid arginine, known as opines. These substances lead to tumour formation as a result of unregulated cell division.

Other notable examples in addition to the ones mentioned above encode various functions such as colicin resistance, symbiosis and nitrogen fixation (Berg *et. al.*, 2007). Cloning plasmids, however, are much smaller units with three basic elements, namely, a cloning site, also known as a polylinker or multiple cloning site, an origin of replication (*ori*) and a selectable marker.

The multiple cloning site is where unique restriction enzymes cleave. The origin of replication is where replication initiates while the selectable marker is commonly a gene for antibiotic resistance e.g. the ampicillin resistance gene). Typical examples include pBR322 plasmids and the more recently identified the pUC series of plasmids. Figure 4 shows the structure of the pBR322 plasmid.

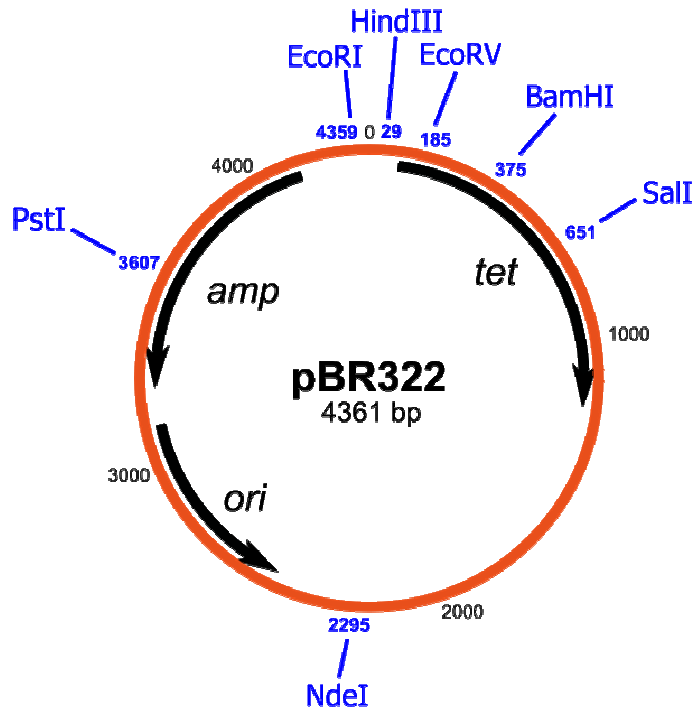


Figure 4: pBR322 plasmid (Berg *et. al.*, 2007).

In nature some plasmids can be transferred from one bacterium to the next through conjugation. They have to encode all of the protein functions that are necessary for mobilisation and transfer from a host to a recipient cell. If this characteristic is lacking in a plasmid co-existing with another plasmid possessing these characteristics and present in the same cell, then the former plasmid can also be mobilized for conjugation.

The ability to be mobilised is dependent on the presence of specific nucleotide sequences that are recognised by the proteins encoded by the self-mobilising plasmid (Carattoli, 2003).

The ability of plasmids to be transferred from one bacterium to another contributes to the spread of drug resistance in bacterial species (Rychlik *et al.*, 2005). This is why an entire colony of bacteria may become resistant to a particular drug for example kanamycin. This characteristic has been exploited in genetic engineering in order to identify recombinant factors (Chen and Dubnau, 2004)

Similarly the plasmid copy number can be manipulated to over-express the gene of interest (Sorensen *et al.*, 2005). For example the copy number in the pUC series of plasmids is 500 per cell.

1.3.1 *spv* Genes

Infections resulting in Salmonellosis are usually due to the presence of *Salmonella* virulence genes that can either be on a plasmid or chromosome (Ridley and Threlfall, 1998). Those occurring in the chromosome can be found on pathogenicity islands.

Salmonella virulence plasmids are heterogeneous in size and can range from 50-125 kb and all share a 7.8 kb region required for bacterial multiplication in the reticuloendothelial system.

Other loci of the plasmid such as the fimbrial operon, *pef*, the conjugal transfer gene, *traT* and the *rck* and *rsk* loci may play a role in other stages of the infection process (Boyd and Hartl, 1998; Rotger and Casadesus, 1999).

Virulence plasmids have been identified in some invasive serovars such as *S. typhimurium*, *S. enteritidis*, *S. dublin*, *S. cholerasuis*, *S. gallinarium*, *S. pullorum* and *S. abortusovis*. Some of these virulence plasmids are species- specific, for example, a 50 kb pSCV plasmid of *S. cholerasuis*, a 94 kb pSLT plasmid of *S. typhimurium* and a 55 kb plasmid of *S. enteritidis* (Rychlik *et al.*, 2008).

The sizes of virulence plasmids of *S. cholerasuis*, (pSCV), have been seen to vary from 50 kb to more than 125 kb. Plasmids larger than 50 kb result from the recombination of a 50 kb pSCV plasmid with non-pSCVs (Chu and Chiu, 2006; Chishih *et al.*, 2001). Plasmids can be used as a typing tool for bacteria besides other typing tools described below.

1.4 Typing techniques

In this study, serotyping, biochemical testing and molecular typing techniques were used to characterise *Salmonella* species isolated from humans and animals to determine their molecular epidemiological relationship and determine possible routes of transmission cycles among humans, domestic animals and wildlife. Antimicrobial susceptibility testing was also performed.

1.4.1 Traditional typing techniques

The traditional typing techniques involve the use of selective culture media, biochemical and serological tests (Boyd and Hartl, 1998, Prager *et al.*, 2003). These traditional methods do not highlight differences of organisms beyond species or serotype levels. The advent of molecular techniques has made it possible to identify differences within the same serogroup. Consequently traditional typing methods should always be complemented by molecular typing techniques to enhance their utility (Namoos *et al.*, 1994). Some of the traditional typing methods are briefly mentioned below.

1.4.1.1 Biochemical testing

Identification of bacteria using biochemical tests relies on their ability to utilise a particular carbohydrate in the media. The test makes use of enzymatic activities to differentiate bacteria (Norman, 2005).

Products of biochemical reactions will result in a colour change to the medium where bacteria have been inoculated. For example, the reactions can result in change in the pH of the inoculated medium. The pH indicator in the medium shows a colour change indicating the availability of an exo-enzyme released by the bacteria causing the formation of a product with the colour change effect. Examples of biochemical tests rely on chemical reactions such as oxidation, fermentation, hydrolysis and degradation (Norman, 2005; Hendriksen, 2003).

Bacteria in the same group do not necessarily utilise the same carbohydrate, even when they appear to be similar morphologically. This is why serotyping and other typing methods like molecular typing need to be employed to complement the biochemical tests. It is important to have a proper identification in order to treat infections timeously.

1.4.1.2 Serotyping

This is a way of classifying microorganisms by means of surface antigens.

Salmonella are classified according to the Kauffman-White scheme. The scheme first determines the O antigen based on the polysaccharide associated with the lipopolysaccharide (somatic antigens). Secondly the H antigen is determined based on the flagellar proteins. The principle behind this test is antibody-antigen agglutination (Grimont and Weill, 2007).

1.4.2 Molecular typing techniques

In Zimbabwe very little has been done on the molecular epidemiology of *Salmonella*, hence the need to carry out this study to add value to the little information available at present. Molecular epidemiology is the application of molecular biology in the study of the causes, distribution and control of diseases in a population (<http://dictionary.reference.com/browse/epidemiology>).

Molecular epidemiology can be important in public health in order to find the most effective way to deploy public health nurses or personnel in the local health departments and point of care sites.

The following are some of the techniques that have been used to type *Salmonella* species and other pathogens at the molecular level: Multiplex polymerase chain reaction (multiplex PCR), ribotyping, pulsed- field gel electrophoresis (PFGE), plasmid profiling, IS200 typing (Foley, Zhao and Walker, 2007) and enterobacterial repetitive intergenic consensus (ERIC) PCR (Bennasar *et al.*, 2000).

The evolution of drug resistant strains of *Salmonella* has posed a big challenge to epidemiologists and clinicians (Novak, 2011; Rowe, Ward and Threlfall, 1997).

One of the most effective approaches to monitoring the spread of salmonellosis is the use of molecular typing techniques. This present study has been limited to multiplex PCR, Plasmid profiling and PFGE as molecular typing tools due to limited funding.

Restriction enzyme analysis (REA) methods such as ribotyping and PFGE are able to discriminate between *Salmonella* involved in human and *Salmonella* involved in animal infections even to the strain level within the same species providing information on the epidemiology and genetic relationships that might occur among serotypes.

The molecular typing techniques used in this study are described below.

1.4.2.1 Multiplex PCR

Multiplex PCR is a variant of the ordinary PCR which deals with the use of more than one primer per reaction to simultaneously amplify many target regions. It is usually applied in the analysis of multiple markers, detection of pathogens or genetically modified organisms (GMOs) or for microsatellite analyses. The setting up of multiplex PCR requires intensive optimisation of the procedures.

Multiplex PCR has the advantage that each amplicon provides an internal control for the other amplified fragment. This is because failure of one fragment to amplify while the others amplify will be an indicator that the reaction has not failed (Foley, Zhao and Walker, 2007).

1.4.2.2 Plasmid Profiling

This study used plasmid profiling as one of the typing tools. Plasmid profiling of an organism involves the isolation of plasmid DNA from an organism of interest, followed by the separation of these molecules based on their size by agarose gel electrophoresis.

Although plasmids can be transferred between organisms, their presence or absence can be an important epidemiological marker (Ridley and Threlfall, 1998). In general plasmids of 10 kb or less occur in 10% of *Salmonella* field strains (Ridley and Threlfall, 1998).

These low molecular weight plasmids do not encode distinguishing traits. Low molecular weight plasmids were mostly used in molecular typing as opposed to the high molecular weight ones that were known to encode distinguishing traits. It has however been discovered that these low molecular weight plasmids code for retron reverse transcriptase and tend to influence phage resistance (Rychlik *et al.*, 2001).

1.4.2.3 PFGE

Pulsed Field Gel Electrophoresis (PFGE) is essentially the comparison of large genomic DNA fragments after digestion with a restriction enzyme. PFGE was once considered the golden method of molecular typing but is now being superseded by more versatile variable number tandem repeats (VNTRs) and multi locus sequence typing (MLST). From their studies on the stability of multi-locus variable number of tandem repeats in *S. typhimurium*, Hopkins *et al.*, (2007) discovered that VNTR was more stable and provided better discrimination compared to PFGE (Namoos *et al.*, 1994; Hopkins *et al.*, 2007). It has been shown to be highly effective in epidemiological studies involving a variety of bacteria including *Salmonella* serovars *enteritidis* and *typhimurium* (Karim and Islam, 2002).

Since the bacterial chromosome is typically a circular molecule, digestion with a rare-cutting restriction enzyme such as *Not I* yields several linear molecules of DNA. The rationale is that when comparing two strains that are clonal the sites at which the restriction enzymes act on the DNA and the length between these sites would be identical.

Therefore, after digestion of the DNA and electrophoresis through an agarose gel, if the DNA banding patterns between any two isolates is identical, then these isolates are considered the same strain, and referred to as being monomorphic.

On the other hand, if two isolates are not the same strain, then the sites at which the restriction enzymes act on the DNA and the length between these sites would be different; thus their DNA banding patterns on gels will be different (Basim and Basim, 2001) and are, therefore, referred to as being polymorphic.

The preparation of genomic DNA suitable for PFGE begins by lysing bacteria that are embedded in agarose blocks (Karim and Islam, 2002). After multiple washes, the DNA within the agarose is digested with restriction enzymes and electrophoresed using PFGE.

PFGE differs from conventional agarose gel electrophoresis in that the orientation of the electric field across the gel is periodically changed in contrast to being unidirectional and constant in standard electrophoresis. The variability in the electric field allows PFGE to resolve the very large fragments (>600 kb) associated with this analysis. The banding patterns obtained are the pulsotypes used in typing to identify the different strains of organisms (Basim and Basim, 2001)

1.5 OBJECTIVES

1.5.1 Overall objective

To subject *Salmonella* isolates from particular sites in Zimbabwe, isolated from wildlife, domestic animals and humans, to selected molecular typing schemes, analyse and compare the subtypes in relation to strain, geographical distribution and host relationships.

1.5.2 Specific objectives

1. To identify and serotype *Salmonella* isolates using traditionally recognised laboratory methods.
2. To subject the isolates to selected molecular typing schemes for sub-typing beyond species level and determine their differences at molecular level.
3. To analyse and compare the subtypes and document differences of strains associated with geographical distribution and host origin to determine possible epidemiological relationships.

1.6 RESEARCH JUSTIFICATION

This study will provide more information on *Salmonella* epidemiology to scientists in research on zoonotic diseases especially those involved in disease control and drug research. The different *Salmonella* serotypes affecting wildlife in Zimbabwe are at present not well known and this study was meant to help in identifying some of the different *Salmonella* serotypes of wildlife origin. The study will also help to establish possible transmission routes of salmonellosis from wildlife to domesticated animals since these animals usually share grazing lands. This knowledge will help in disease control.

The knowledge of the effect of geographical locations on the strains will help to locate sources of disease outbreaks and to try and prevent further spread of the disease through targeting the source of an outbreak. Animals and animal products are the routes for transmission of *Salmonella* to humans, and hence, the inclusion of humans in this study to provide information that can be used in the control of zoonotic disease outbreaks in humans.

2.0 MATERIALS AND METHODS

2.1 Sample collection, *Salmonella* isolation and serotyping

The sampling sites were Mutare, Bulawayo, Kariba, and Harare. Animal *Salmonella* strains were isolated from cattle, birds and crocodiles. Swabs and organs from animals were taken from various farms to the Central Veterinary Laboratory (CVL) in Harare. Once received, the swabs and organs were immediately put in selenite broth, an enrichment medium for *Salmonella*. These samples were stored at 4°C and cultured within a week.

One hundred and seventeen (117) outbreak *Salmonella* strains of human origin were collected as isolates from Harare hospital, a referral hospital in Zimbabwe, and 18 from Central Veterinary Laboratory (CVL), bacteriology section. The CVL human Isolates were isolated from anal swabs taken from employees of Colcom pig and Kariba crocodile abattoirs, which are meat-processing companies in Zimbabwe.

The samples in selenite broth were incubated at 37°C overnight before culturing on blood agar (BA) and MacConkey (MAC) agar and then incubated overnight at 37°C. Suspected *Salmonella* colonies were further cultured on Xylose Lysine Deoxycholate (XLD) agar. Triple sugar iron (TSI), Lysine-Decarboxylase (LD) and Urease were the biochemical tests used to confirm XLD *Salmonella* suspected colonies.

The samples in selenite broth were incubated at 37°C overnight before culturing on blood agar (BA) and MacConkey (MAC) agar and then incubated overnight at 37°C. Suspected *Salmonella* colonies were further cultured on Xylose Lysine Deoxycholate (XLD) agar.

XLD is a selective agar used for the selection of *Salmonella* and *Shigella* species. The phenol red indicator, which is bright pink or red, in the media will turn yellow if pH is reduced below its normal of 7.4 as a result of sugar fermentation. Most gut bacteria including *Salmonella* will ferment the sugar xylose. After exhausting xylose, the *Salmonella* will decarboxylate lysine increasing pH again and turning red. Some *Salmonella* metabolise thiosulphate in the medium producing hydrogen sulphide giving the colonies a black centre with red outskirts which differentiates them from *Shigella*. In addition to XLD, Triple sugar iron (TSI), Lysine-Decarboxylase (LD) and Urease were the biochemical tests used to confirm XLD *Salmonella* suspected colonies (Nye et al., April 2002)

The *Salmonella* strains were serotyped in accordance with the Kauffmann-White scheme based on one serotype –one species concept on the basis of somatic (O) and flagella (H) antigens (Brennar *et al.*, 2000) to identify the serovars, which were now, typed using molecular typing techniques. Figure 5 summarises the sequence of events in the processing of the samples and isolates.

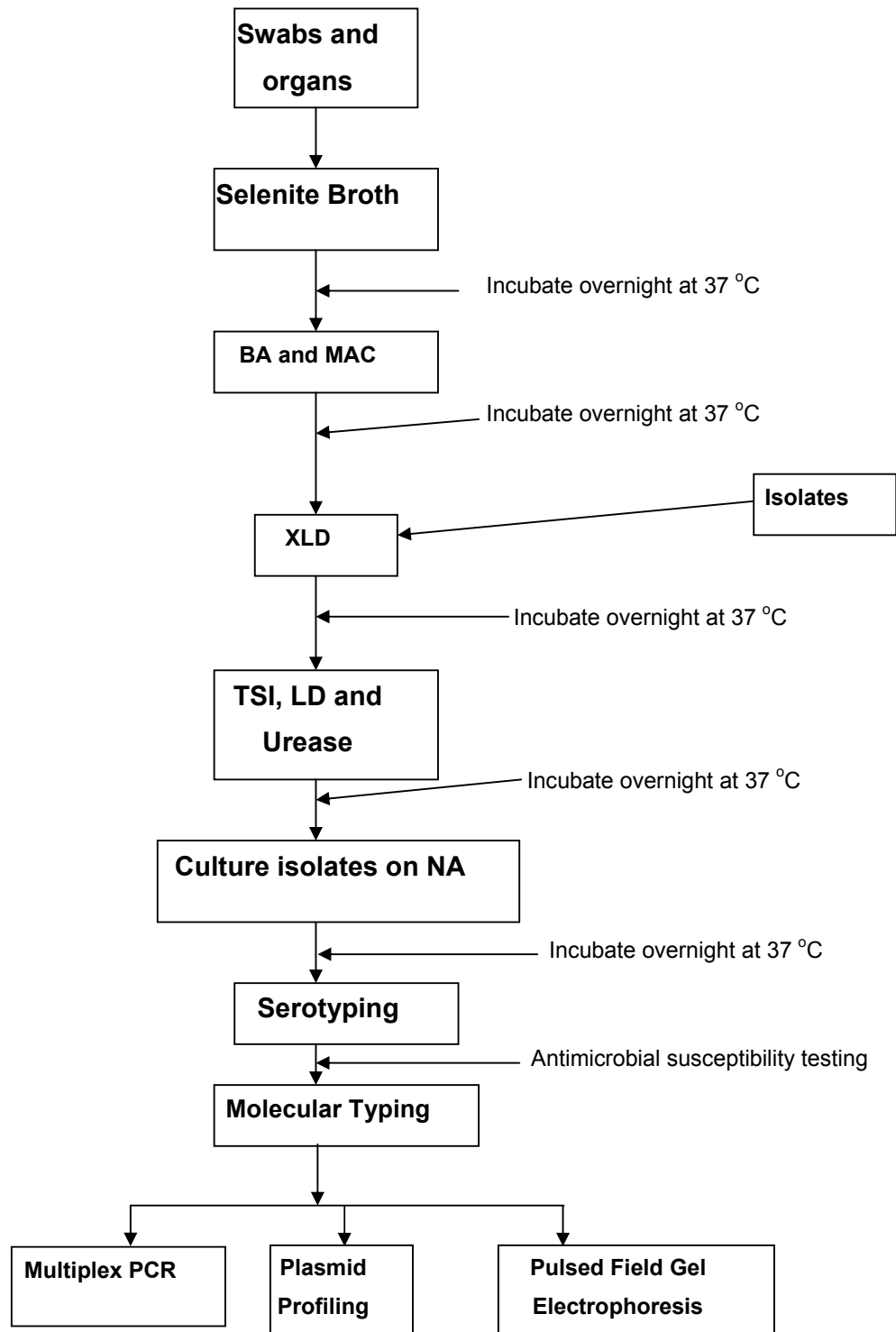


Figure 5: Flow chart summarizing sequence of events in the processing of samples for *Salmonella*

2.2 Serotyping

The isolates were serotyped for the different *Salmonella* groups using Kauffmann-White scheme based on one serotype –one species concept on the basis of somatic (O) and flagella (H) antigens (Brennar *et al.*, 2000). Slide agglutination was used. A drop of sterile physiological saline (0.9% saline) was put on a clean slide and a colony of bacteria suspended in this solution. A drop of antisera was then added and gently mixed for up to 1 min before checking for any agglutination which indicates a positive reaction.

The positive control was *S. typhimurium* and negative control was sterile saline. These were subjected to the same treatment as the sample and results observed and recorded. The results of the samples were only accepted if the Quality Control (QC) results came out as expected, namely agglutination with *S. typhimurium* and no agglutination with sterile physiological saline (results not shown).

2.3 Antimicrobial Susceptibility Testing

The sensitivities of the identified serotypes to antibiotics was tested using a panel of 19 antimicrobial discs supplied by the University of Sassari, that included furazolidone (FR 50) and nitrofurantoin (Ni 50mcg) which are nitrofurans antibiotics, ampicillin (Amp or AP 10 mcg), cloxacillin (OB or OT 5 mcg), ticarcillin (Tc 75mcg) and penicillin (P 10 mcg) which are the penicillin antibiotics, erythromycin (E 15 mcg)

which is a macrolide antibiotic, streptomycin (S 30 mcg), neomycin (Ne 10 mcg), gentamycin (Gm or CN 30 mcg) and kanamycin (K) which are aminoglycosides.

Included also were chloramphenicol (C 5 mcg) which is a phenocol antibiotic, tetracycline (T 30 mcg) and oxytetracyclin (OT or OB 30 mcg) which are the tetracycline antibiotics, sulphamethaxazole trimethoprim (Sxt 25 mcg) which is a sulphonamide antibiotic, lincomycin (My or L) which is a lyncosamide antibiotic, ciprofloxacin (Ci), nalidixic acid which are quinolone antibiotics and vancomycin (Va) which is a glycopeptide antibiotic.

The isolates used in the test were standardized using optical density (OD) between 0.5 and 0.55 at 600 nm wavelength so that approximately the same number of bacteria was used in each sensitivity plate to avoid false sensitive or resistance results.

The standardised culture was used to inoculate Mueller Hinton agar (MHA) plates or nutrient agar plates and the different antimicrobial discs used. The plates were incubated over night at 37°C and the degree of clearance read to determine if the strain was resistant or sensitive to a particular antibiotic.

2.4 Molecular Typing

2.4.1 Multiplex PCR assay

Multiplex PCR was performed on isolates identified as *Salmonella* both of human and animal origin.

The reason for this assay was two-fold: a) to confirm the identity of both the genus *Salmonella*, and species *Salmonella enteritidis* isolates, as well as, b) to identify pathogenic strains with *spv* genes and those without *spv* genes which could be used as another characterisation tool.

The DNA was extracted using hexadecyltrimethyl ammonium bromide (CTAB) DNA extraction method (Maldonado, 1994). For the PCR the following reagents were used: 1.5 mM magnesium chloride; 1X PCR buffer; 0.2 mM deoxynucleoside triphosphates (dNTPs which are dGTP, dCTP, dTTP and dATP); 0.2 μ M primer as mentioned below; 0.125U *Taq* polymerase enzyme; two micro-litres (2 μ l) (approximately 5ng/ μ l) sample DNA estimated using DNA strips from Invitrogen, (San Diego, USA) according to the manufacturer's instructions. The reaction mixture was made up to required volume using sterile double distilled water.

Multiplex PCR was carried out using the following primer pairs: ST11 (GCCAACCATTGCTAAATTGGCGCA) / ST 14 (GGTAGAAATTCCCAGCGGGTACTGG) which amplifies a randomly cloned sequence of approximately 429 bp specific for the genus *Salmonella*, SEFA 2 (GCAGCGGTTACTATTGCAGC) / SEFA 4 (TGTGACAGGGACATTTAGCG) which amplifies the *Salmonella enteritidis* fimbril antigen gene of approximately 310 bp and S1 (GCCGTACACGAGCTTATAGA) / S4 (ACCTACAGGGGCACAATAAC) which amplifies the *Salmonella* plasmid virulence gene (*spv*) of approximately 250 bp and is specific to *S. enteritidis* (Pan and Liu, 2002). *S. enteritidis* can have the first two bands but lacking the third *spv* gene band or it can have all the three bands occurring.

The PCR was run using the following programme: 1 cycle of pre-denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94°C for 30 sec; annealing at 56°C for 90 sec and extension at 72°C for 30 sec, and a final cycle of final extension at 72°C for 7 min was performed before storage at 4°C.

The amplicons were loaded on 1% agarose gel containing ethidium bromide (final concentration of 0.5 µg/ml) and electrophoresed at 90 V in 0.5 X Tris-borate EDTA (TBE) electrophoresis buffer. A 100 bp ladder was used as molecular weight marker.

The gel was viewed under a UV transilluminator and photographed using the Kodak Gel Logic 100 imaging system, supplied by Eastman Kodak Company, (Rochester, New York, USA). All isolates identified as *Salmonella* were genotyped by the plasmid profiling technique.

2.4.2 Plasmid profiling.

Plasmid extraction was performed using the modified Kado and Liu alkaline lysis method (Kado and Liu, 1981) on *S. enteritidis* strains and group B *Salmonella* isolates. The number and size of the plasmids was used as a differential tool.

The extracts were run on 0.7% agarose gel using V517 and 39R861 *E. coli* plasmids as markers. The gel was stained using 10 mg/ml stock solution of ethidium bromide to a final concentration of 0.5 µg/ml.

After electrophoresis, the gel was viewed under a UV transilluminator and a photograph taken using the Kodak Gel Logic 100 imaging system, (Eastman Kodak Company, Rochester, New York, USA). The gel photographs were analysed to identify the different profiles shown and relate the profiles to host and geographical location.

2.4.3 Electrophoresis

One gram of molecular grade agarose powder was added to 100 ml of 0.5X TBE Buffer. The mixture was melted in a microwave for 2-5 min and shaken gently at intervals until all the agarose grains were molten.

The gel casting apparatus was set up with a comb inside. The gel was left to cool down to approximately 55 °C while setting up the casting apparatus. A spirit level was used to ensure that the apparatus was on a level surface.

To the cooled gel, ethidium bromide was added to a final concentration of 0.5 µg/ml and mixed thoroughly then gently poured into the casting apparatus while ensuring that bubbles did not form during the process. If bubbles formed, they were gently removed with a pipette tip before the gel began to set within 15-20 min. Once the gel had set, the blockers and the comb were gently removed avoiding cracking the gel. The gel was lowered into the electrophoresis tank and sufficient 0.5 XTBE buffer was poured into the electrophoresis tank to cover the gel.

Six times loading dye was mixed with a specific volume of sample that was enough to dilute the dye to a final concentration of 1X. For example if 2 µl of the 6X loading dye was used, then 12 µl of sample would be used. The first and last wells were loaded with known markers, while the samples with dye were gently loaded into the wells avoiding punching the gel and floating the loads. The gel was electrophoresed at 90 V for 90 min.

The electrophoresis was left to run for approximately 1 ½ hr checking the progressions in between at 30 min intervals to avoid overrunning the gel. The gel was stopped at approximately 2/3 run down the gel. The run gel was viewed under a UV transilluminator using the Gel Logic 100 imaging system and gel photographs taken.

2.4.4 Pulsed Field Gel Electrophoresis (PFGE)

All isolates on which plasmid fingerprinting was performed were further analysed using PFGE. PFGE was performed according to the modified Threlfall protocol (Ridley and Threlfall, 1998) on the 49 *S. enteritidis* strains and 32 Group B *Salmonella* isolate strains. Plugs were prepared by mixing 20 µl of proteinase K (20 mg/ml), 500 µl of sample suspension of 0.5-0.55 O.D at 600 nm wavelength and 500 µl of 2% low melting point (LMP) agarose.

The mixture was set in plug moulds for 10 min at 4°C for the agarose gel to set. The plugs were removed from moulds and left in TE buffer for at least a week before restriction digestion was performed. Restriction digestion was performed overnight using 20 units of *Xba*I restriction enzyme.

Two thirds of each of the digested plugs was loaded on a 1 % ordinary agarose gel. On the gel, the sample plugs were sandwiched by a commercial PFGE marker. The PFGE programme was run for 22 hrs at 6V/cm (200V) and 14 °C with switch time of 2-64 sec and a 120° linear corner using the BioRad PFGE apparatus.

A volume of 2.5 L of 0.5X TBE buffer was used during electrophoresis as the electrolyte. The gel was stained in 0.5 µg/ml ethidium bromide solution for 20 min and then de-stained in 1 L of distilled water for 30 min. The gel was viewed under a UV-transilluminator and photographs taken using the Gel Logic 100 imaging system.

The different pulsotypes were identified and used for analyses to determine isolate differences.

2.4.5 Disposal of the *Salmonella* cultures used in the study

All the *Salmonella* cultures used in the study were autoclaved first before being sent for incineration at Central Veterinary Laboratory to avoid contamination of working areas, laboratory staff and the environment with the pathogenic bacteria.

3.0 RESULTS

3.1 Sample collection, *Salmonella* isolation and serotyping

A total of 214 samples were collected from animals for isolation of *Salmonella*. Of the 214 samples collected, 35 (16.35 %) were positive for *Salmonella* after serotyping. Two other isolates were dropped after Multiplex PCR failed to confirm them as *Salmonella*, hence remaining with 33.

In total, 148 *Salmonella* isolates were prepared from animal species (35 from the 214 samples collected, 57 isolates supplied by CVL and 58 isolates from Mutare Veterinary Laboratory) as shown in Table 2. The 57 isolates from CVL were from Victoria Falls (crocodiles n=4), Kariba (crocodile n=12), Binga (crocodile n=10), Harare (chickens n=23, caprine n=1), Chinhoyi (bovine n=6) and Norton (porcine n=1).

For human isolates, a total of 135 *Salmonella* isolates were collected of which 117 were from Harare Hospital and 18 were from CVL. See Table 2 and Figure 6 for the distribution of isolates used in this study.

Table 2: The distribution of *Salmonella* isolates used in the study

	Location	Source	Number of isolates
From animals	Victoria Falls	Crocodile	4
	Kariba	Crocodile	12
	Binga	Crocodile	10
	Harare	Chickens	23
		Caprine	1
	Chinhoyi	Bovine	6
	Norton	Porcine	1
	Mutare	Bovine	58
	Bulawayo North	Ostriches	33
From humans	From CVL and Harare Hospital		135 total <i>Salmonella</i> isolates from humans. 117 isolates from Harare Hospital
	From Harare Hospital		
			40 group C
	-Harare		27
	-Norton		1
	-Beatrice		2
	-Gokwe		2
	-Zaka		1
	-unknown		7
	-Harare		33 Group B
	-unknown		18
			15
	-Harare		8 Group D
	-Kariba		4
			4
	Harare		35 <i>Salmonella</i> species
	-Beatrice		29
	-Chinhoyi		1
	-Kariba		1
			4
	Unknown		1 Group G
			1
	From CVL		18 <i>Salmonella</i> Species
	-Harare		7
	-Chinhoyi		1
	-Kariba		10

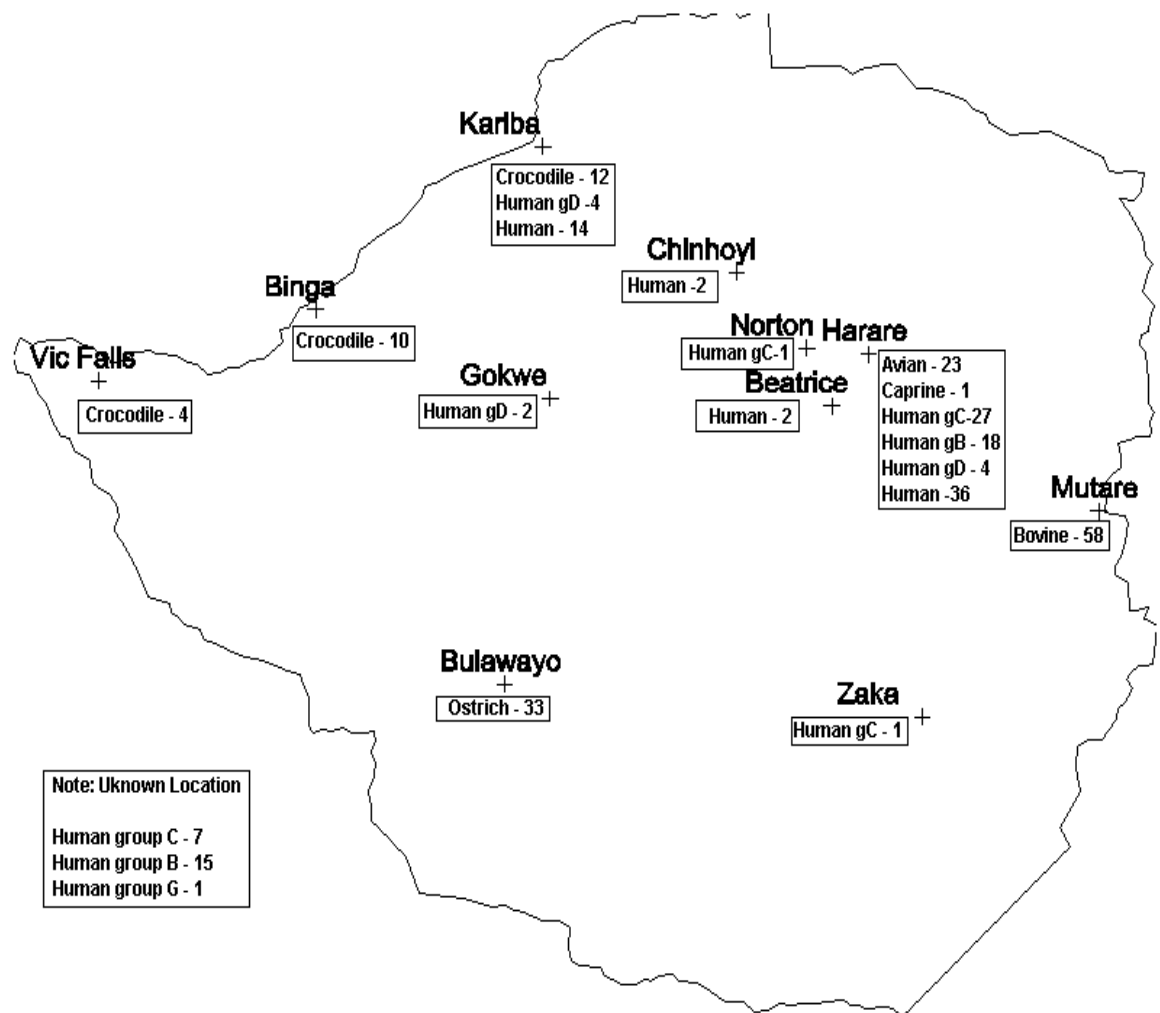


Figure 6: Map of Zimbabwe showing distribution of *Salmonella* isolates used

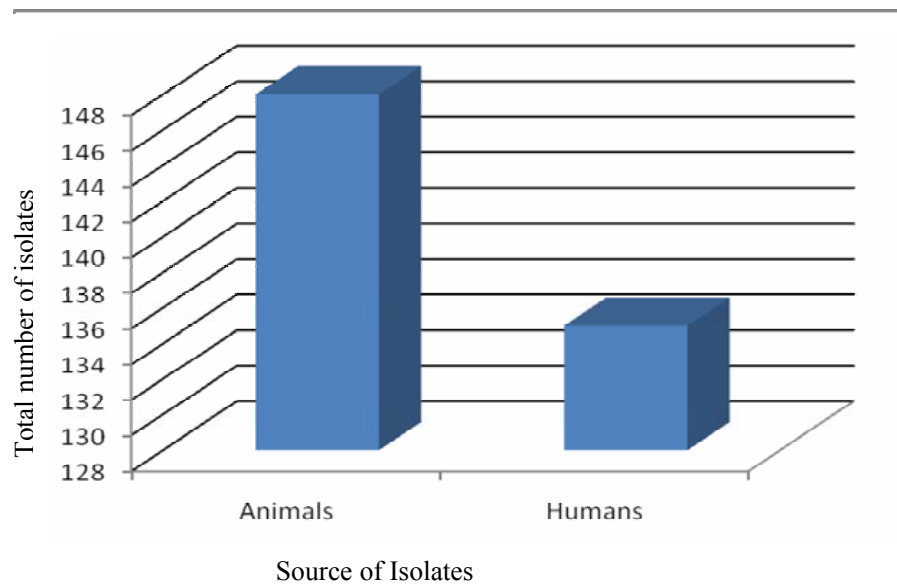


Figure 7: Graph showing distribution of *Salmonella* isolates in relation to the source

Figure 7 indicates that the 52% of isolates (148/283) was from animals and 48% (135/283) from humans.

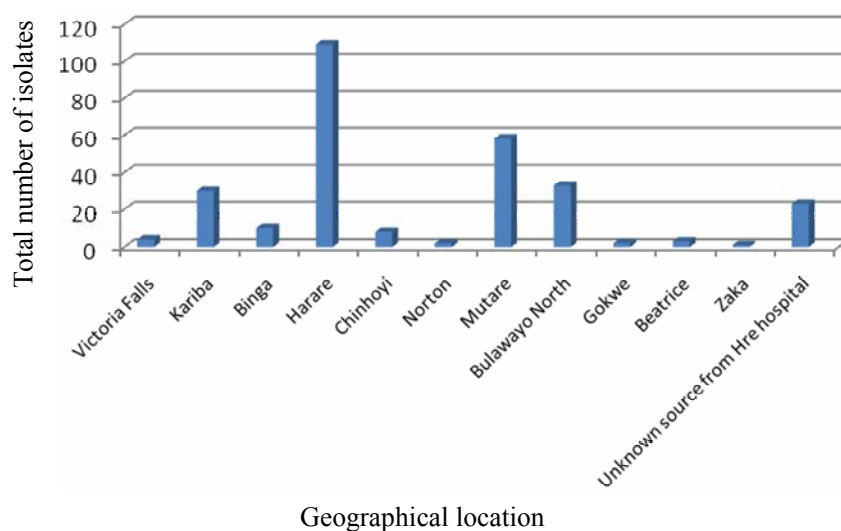


Figure 8: Graph showing distribution of *Salmonella* isolates in relation to geographical location

Figure 8 indicates that Harare had the largest number of isolates used in the study.

3.2 Antibiotic susceptibility testing

The *Salmonella* isolates were at least 95% sensitive to the following antibiotics, Gentamycin, Kanamycin (Group B, 100%), Ciprofloxacin (Groups B and D, 100%: Group C not tested), and Furazolidone (Group C, 100%) and resistant to the rest of the antibiotics used. The results are summarised in Table 3.

Table 3: Antibiotic sensitivity results for group B, C and D *Salmonella* isolates from humans and animals.

Antibiotic type	Total number of isolates								
	sensitive			% sensitive			% resistant		
	Grp B	Grp C	Grp D	Grp B	Grp C	Grp D	Grp B	Grp C	Grp D
Ni, 50µg	0/11	nd	0/7	0	nd	0	100	nd	100
Gm/CN,30µg	10/11	5/40	3/7	91	12.5	43	9	81.5	57
Cx, 5µg	0/11	nd	0/7	0	nd	0	100	nd	100
E, 15µg	6/11	10/40	1/7	55	25	14	45	75	86
S, 10µg	8/11	2/40	2/7	73	5	29	27	95	71
T, 25µg	0/11	1/40	5/7	0	2.5	71	100	97.5	29
Amp/AP,10µg	4/11	3/40	2/7	36	7.5	29	64	92.5	71
K, 30µg	11/11	10/40	6/7	100	25	86	0	75	14
Cip, 5µg	11/11	nd	7/7	100	nd	100	0	nd	0
Tc, 75µg	4/11	nd	3/7	36	nd	43	64	nd	57
My/L, 2µg	1/11	0/40	0/7	9	0	0	91	100	100
Ne, 10µg	7/11	11/40	1/7	64	27.5	14	36	72.5	86
Na, 30µg	10/11	nd	6/7	91	nd	86	9	nd	14
P, 10 units	9/11	1/40	6/7	82	2.5	86	18	97.5	14
C, 5µg	4/11	nd	0/7	36	nd	0	64	nd	100
Fr	nd	40/40	nd	nd	100	nd	nd	0	nd
OB/OT	nd	0/40	nd	nd	0	nd	nd	100	nd
Va	nd	0/40	nd	nd	0	nd	nd	100	nd
Sxt	nd	0/40	nd	nd	0	nd	nd	100	nd

Key: nd= not done, Fr =furazolidone, Amp/AP= ampicillin, Cx = cloxacillin, P = penicillin, E =erythromycin, S = streptomycin, Ne =neomycin, K= kanamycin, Gm/CN = gentamycin, T =tetracycline, OB/OT =oxytetracyclin, Sxt = sulphamethaxazole trimethoprim, My/L=Lincomycin, Cip = ciprofloxacin, Va = vancomycin, Tc=Ticarcillin, C= chloramphenicol, Na=Nalidixic acid and Ni=Nitrofurantoin

The following graphs have been developed using information indicated in Table 3.

Group B

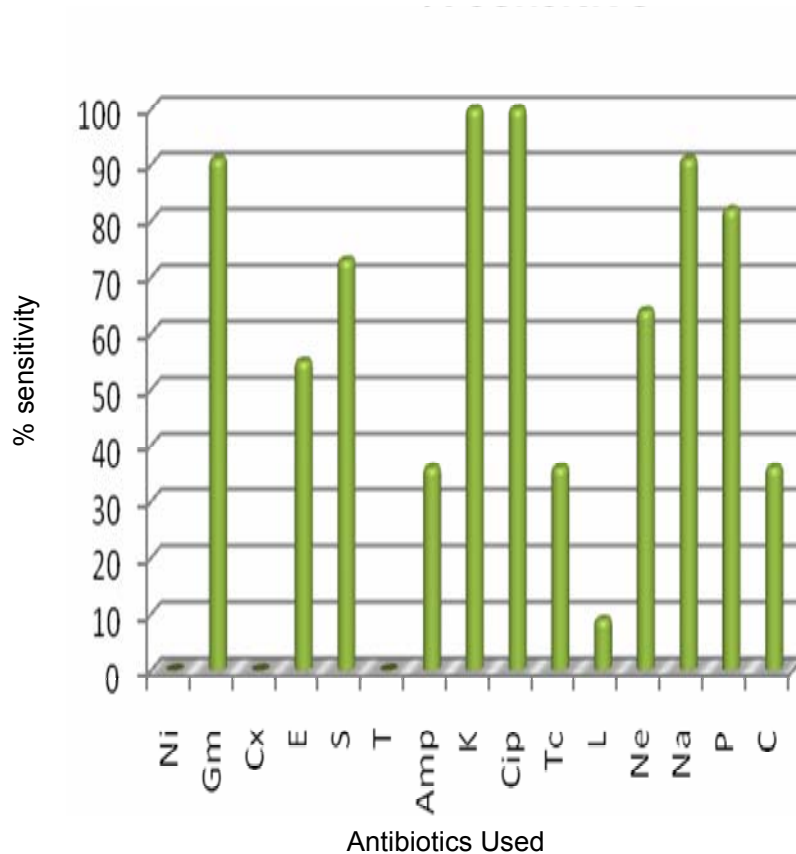


Figure 9: Percentage sensitivity to the selected antibiotics for the 11 group B *Salmonella* tested

Key: Ni=nitrofurantoin, Gm=gentamycin, Cx= cloxacillin, E=erythromycin, S=streptomycin, T=tetracycline, Amp= ampicillin, K=kanamycin, Cip=ciprofloxacin, Tc= ticarcillin, L=lincomycin, Ne=neomycin, Na=nalidixic acid, P=penicillin and C=chloramphenicol

Figure 9 indicates that the antibiotics to which group B isolates were most sensitive with at least 95% sensitivity were Kanamycin (100%) and Ciprofloxacin (100%) and the rest were below 95% indicating partial sensitivity of the group B isolates to the antibiotics used.

Group C Isolates

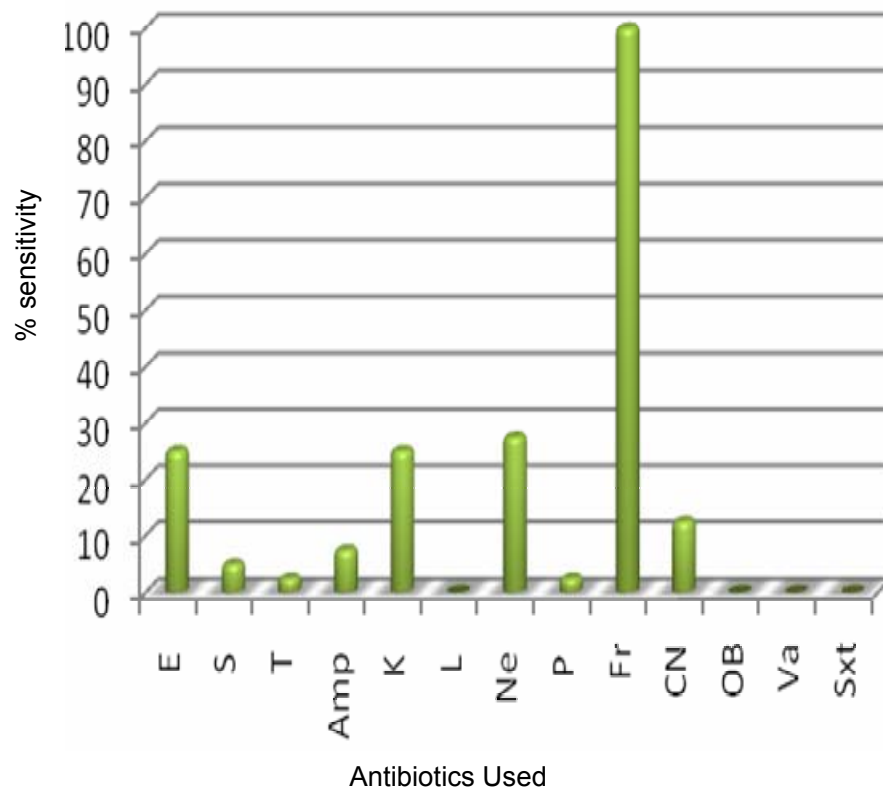


Figure 10: Percentage sensitivity to the selected antibiotics for the 4 group C *Salmonella* tested

Key: E=erythromycin, S=streptomycin, T=tetracycline, Amp= ampicillin, K=kanamycin, L=lincomycin, Ne=neomycin, P=penicillin, Fr=Furazolidone, CN=gentamycin, OB=oxytetracycline, Va=vancomycin and SXT= sulphamethaxazole trimethoprim.

Figure 10 indicates that all group C isolates (100%) were sensitive to Furazolidone but 21% sensitive to erythromycin, 5% sensitive to ampicillin, 21% sensitive to kanamycin, 25% sensitive to neomycin and 10% sensitive to gentamycin.

Group D Isolates

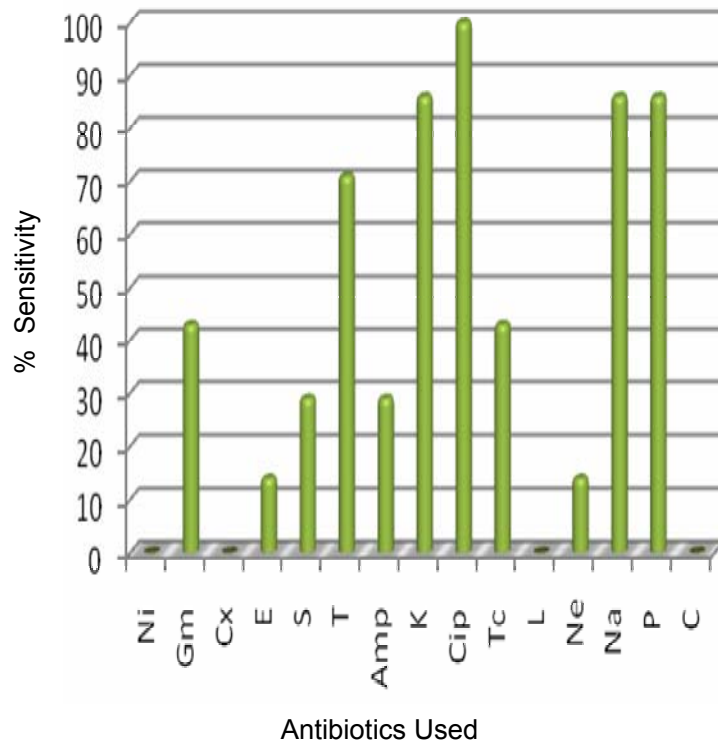


Figure 11: Percentage sensitivity to the selected antibiotics for the 7 group D *Salmonella* tested

Key: Ni=nitrofurantoin, Gm=gentamycin, Cx= cloxacillin, E=erythromycin, S=streptomycin, T=tetracycline, Amp= ampicillin, K=kanamycin, Cip=ciprofloxacin, Tc= ticarcillin, L=lincomycin, Ne=neomycin, Na=nalidixic acid, P=penicillin and C=chloramphenicol

Figure 11 indicates that the group D isolates were most sensitive to Ciprofloxacin (100%) and 10% sensitive to the antibiotics E and Ne, 84% sensitive to K, Na and P then 70% sensitive to T, then 40% sensitive to Gm and Tc and 25% sensitive to S and Amp. The isolates were 100% resistant to Ni, Cx, C and L antibiotics.

3.3 Multiplex PCR

Two of the 35 isolates that had been shown to be *Salmonella* positive by serotyping were dropped after Multiplex PCR failed to confirm their status as *Salmonella* bringing the number to 33, which is 15.42% of the 214 samples analysed. All the 282 *Salmonella* isolates from animals and humans were subjected to Multiplex PCR assay.

Multiplex PCR confirmed 14 bovine *Salmonella* isolates collected from Mutare Veterinary Laboratory to be *S. enteritidis*. In addition, 15 /44 *Salmonella* isolates, (34.09%), collected from CVL, all isolates from avian sources, were confirmed to be *S. enteritidis* strains. For human isolates 12 (9.52%) from CVL and 8 (6.35%) from Harare hospital, were confirmed to be *S. enteritidis*. All in all, 49 out of 282 isolates, which is 17.38%, were confirmed *S. enteritidis*. This is illustrated in Figure 12.

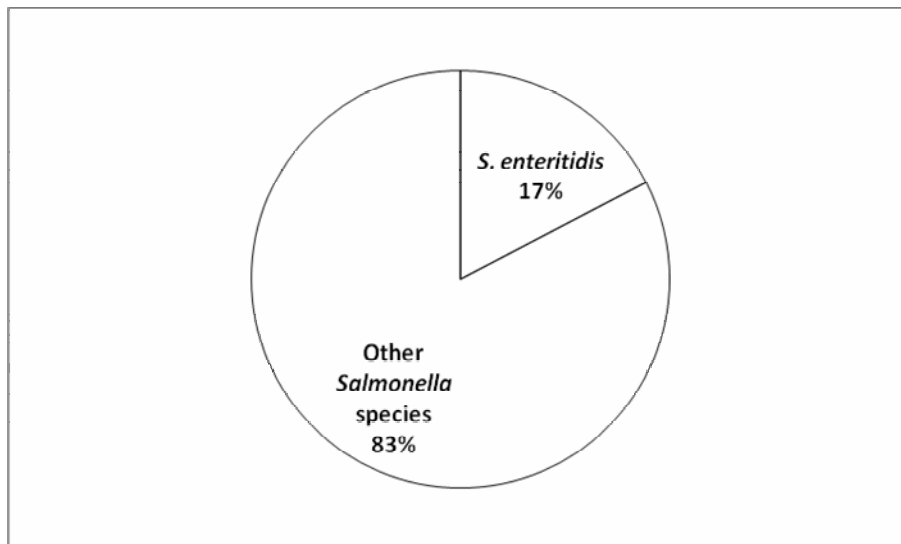


Figure 12: Percentage of *S. enteritidis* strains confirmed from the 282 *Salmonella* isolates subjected to multiplex PCR.

Multiplex PCR was also in a position to group *S. enteritidis* strains into 2 groups, those with *spv* genes and those without *spv* genes. Of the 49 *S. enteritidis* isolates confirmed, 39 (79.59%) had the *spv* gene as shown by the presence of a band of approximately 250 bp of which 31 (79.49%) were of animal origin and 8 (20.51%) were of human origin (all from outbreak), and 10 (20.41%) did not have *spv* gene of which 5 (50%) were of animal origin (all from Harare) and 5 (50%) were of human origin (4 from Kariba and the other one with an unknown source). See figure 14. Figure 13 shows the percentage distribution of *S. enteritidis* strains with and without *spv* genes.

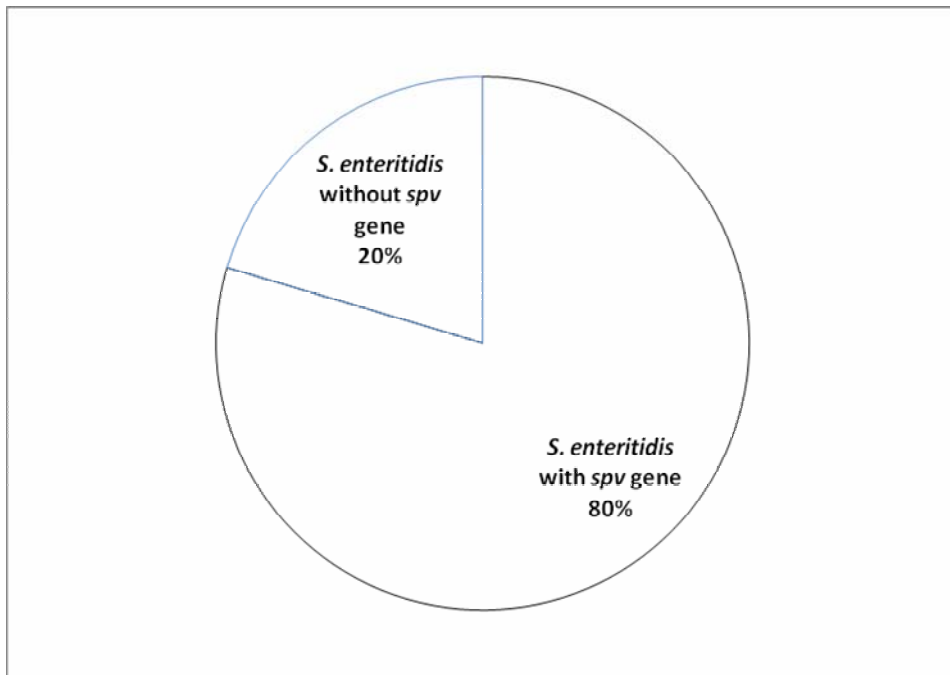


Figure 13: Percentage of *S. enteritidis* strains with *spv* genes compared to those without *spv* genes

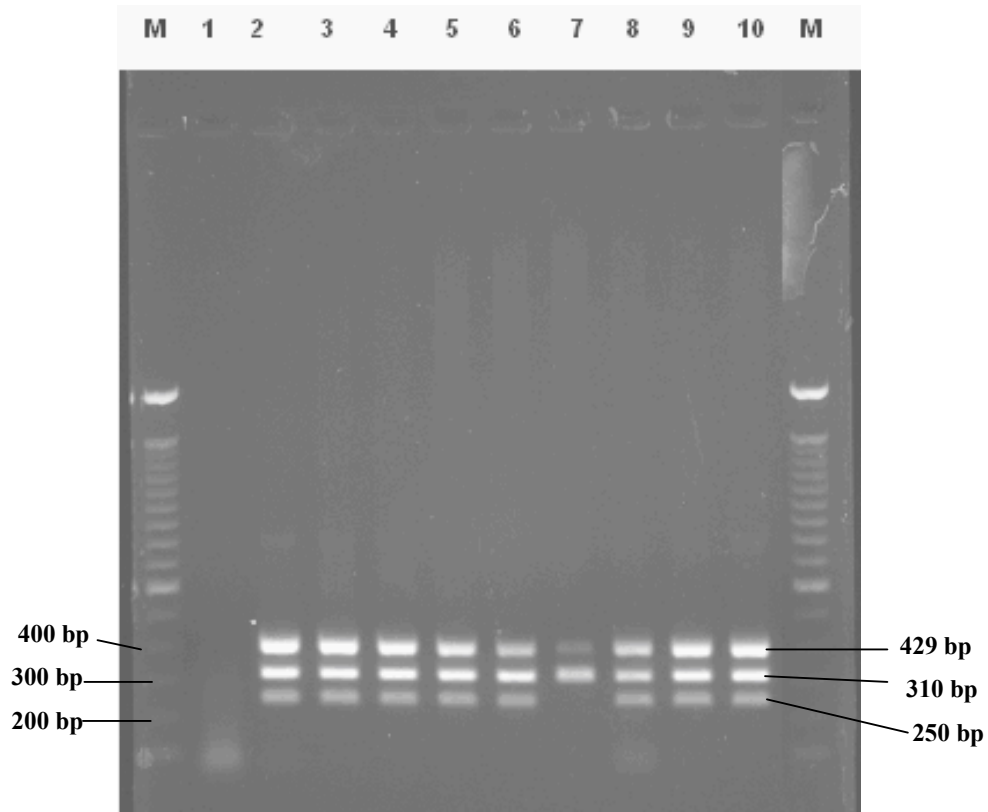


Figure 14: Multiplex PCR for human group D isolates. M is the 100 bp ladder. Lane 1 is negative control, lane 2-10 are group D isolates

In Figure 14, the band corresponding to 429 bp indicates the genus *Salmonella*, the band of 310 bp indicates the species *S. enteritidis* and the band of 250 bp indicates the presence of *spv* genes. Lanes 2 to 6 and 8 to 10 show presence of *S. enteritidis* strains with the *spv* gene loci and lane 7 indicates *S. enteritidis* strain without the *spv* gene locus

3.4 Plasmid extraction and profiling

3.4.1 *S. enteritidis* strains

All the 8 *S. enteritidis* outbreak strains of human origin, with and without the *spv* gene, shared a common heavy plasmid of approximate size of 54 Kb. Of the 32 strains from animals, 12 (37.5%) lacked the heavy plasmid despite their carrying the *spv* gene (Figure 14).

A total of 11 plasmid profiles have been identified from strains with *spv* gene and 3 from the strains without *spv* gene (Figures 15 and 16). Five out of seven (71.4%) *S. enteritidis* isolates from ostriches shared the same profile. Of the 5 isolates, 3 (60%) were from Bulawayo province and 2 (40%) from Harare province. Sharing the same profile with ostriches were 2 chicken isolates from Harare province. Five plasmid profiles have been identified (Figure 15).

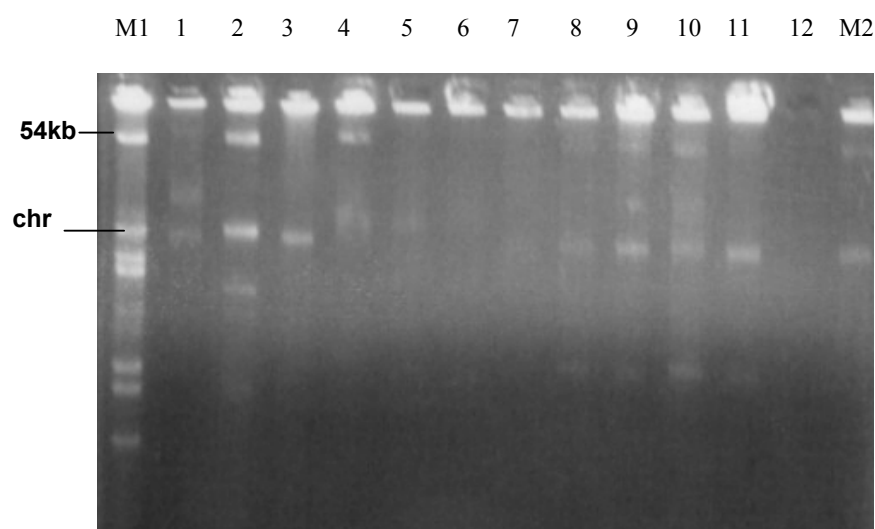


Figure 15: Plasmid profiling of some of the *S. enteritidis* strains of animal origin found to be possessing *spv* gene. M1 is V517 marker, M2 is 39R861 marker and lanes 1-12 are *S. enteritidis* strains possessing *spv* gene and chr is the chromosomal DNA

Lanes 1, 2, 4, 8, 9 and 10, in Figure 15, indicate isolates with the 54 kb heavy plasmids typical of *S. enteritidis* and associated with the *spv* gene loci. The rest of the samples in lanes 3, 5, 6, 7, 11 and 12, do not show presence of this heavy plasmid.

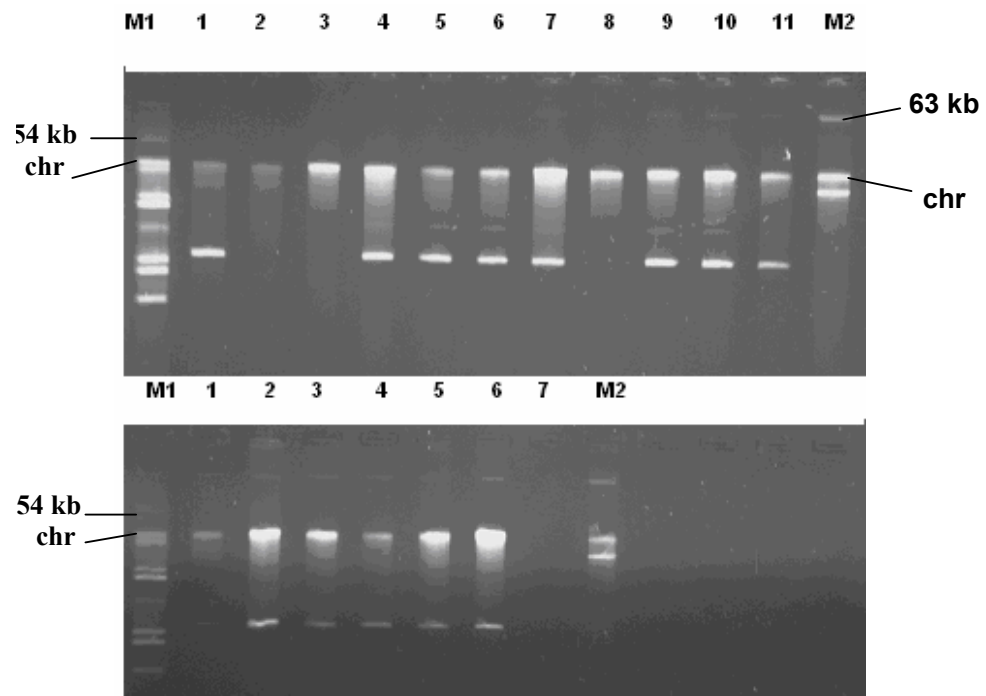


Figure 16: Animal *S. enteritidis* plasmids. M1 is V517 marker, M2 is 39R861 marker, 1-11 upper lanes and 1-7 bottom lanes are animal *S. enteritidis* strains. chr is the chromosomal DNA band

All the isolates shown in Figure 16 do not show the 54 kb heavy plasmid associated with *spv* gene loci of *S. enteritidis*.

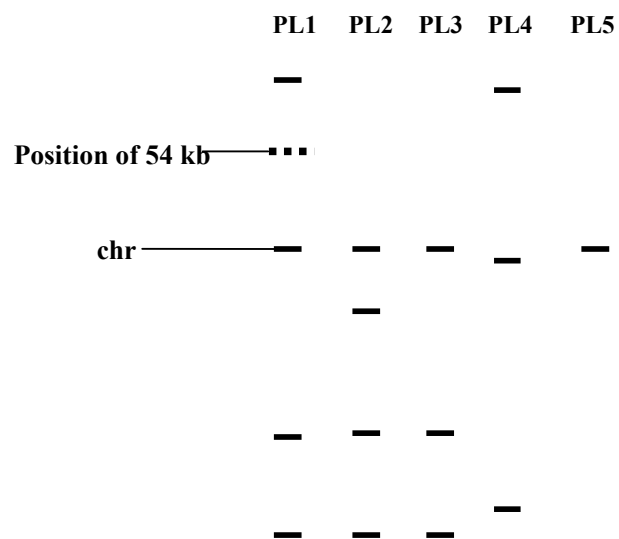


Figure 17: Plasmid profiles generated from *S. enteritidis* of animal origin. PL1 to PL5 are the different profiles identified.

3.4.2 Group B strains

A total of 11 profiles were obtained from all the 32 outbreak strains of human origin (Figure 24). Two strains from the opportunistic Infections Clinic (OIC) were included in the same groups as other isolates obtained from other sources.

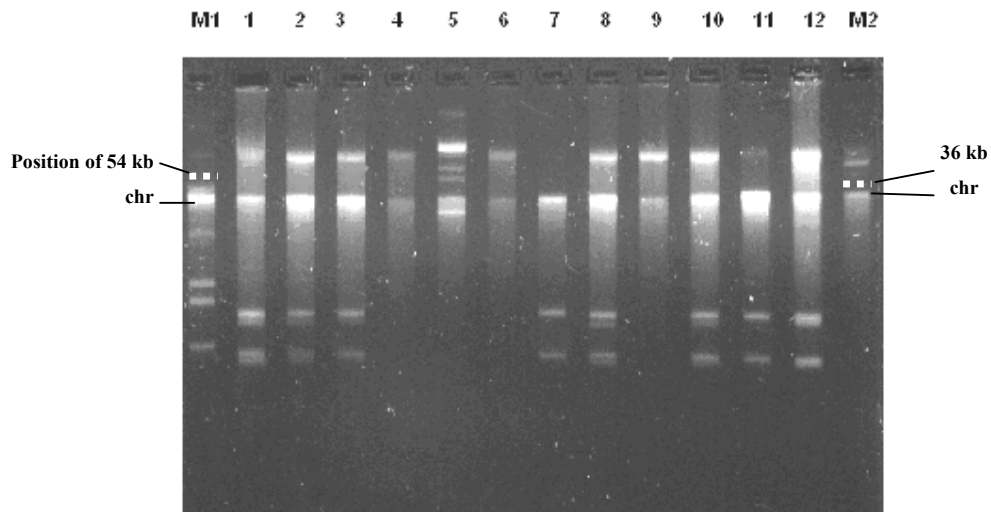


Figure 18: Human group B *Salmonella* plasmids. M1 is V517 marker, M2 is 39R861 marker. chr is chromosomal DNA

Lanes 1, 2, 3, 8 and 10, in Figure 18, have similar banding pattern (profile). The same applies for lanes 4, 6 and 9 and also 7 and 11. Lane 5 has a unique profile as well as lane 12.

3.4.3 Group C strains

From the 40 Group C strain isolates analysed, 17 plasmid profiles were obtained.

Three isolates, one from Gokwe, one from Mbare and one from an unknown source, obtained from Harare Hospital, were placed in the same profile (PL7). Also 2 isolates, one from Gokwe and the other from Zaka were placed in the same profile (PL8). The two isolates from Beatrice were placed in the same profile (PL5). In another profile, 2 isolates from Highfields, 1 from Glenview, 1 from Waterfalls and 1 from Budiriro were grouped in the same profile (PL9), (Figure 19).

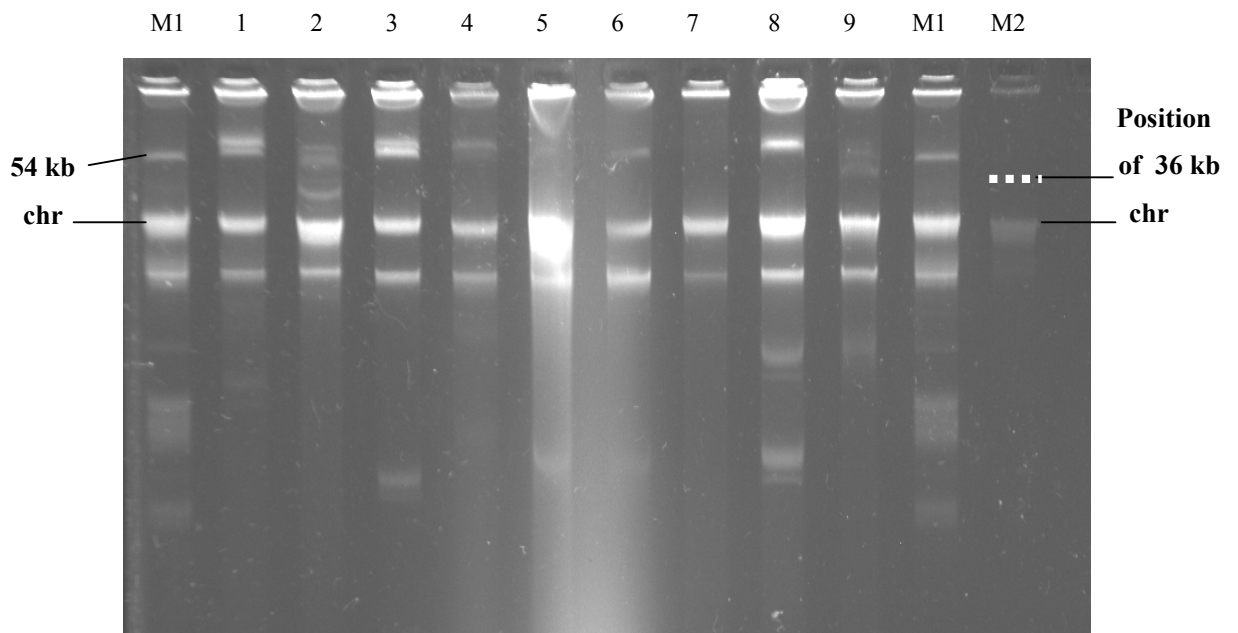


Figure 19: Plasmid profiling of Group C isolates. M1 is V517 marker, M2 is 39R861 marker, 1-9 are human group C *Salmonella* plasmids. Cr represents chromosomal DNA band.

Lanes 4 and 6, in Figure 19, have a similar plasmid profile and the rest of the lanes each has a unique pattern.

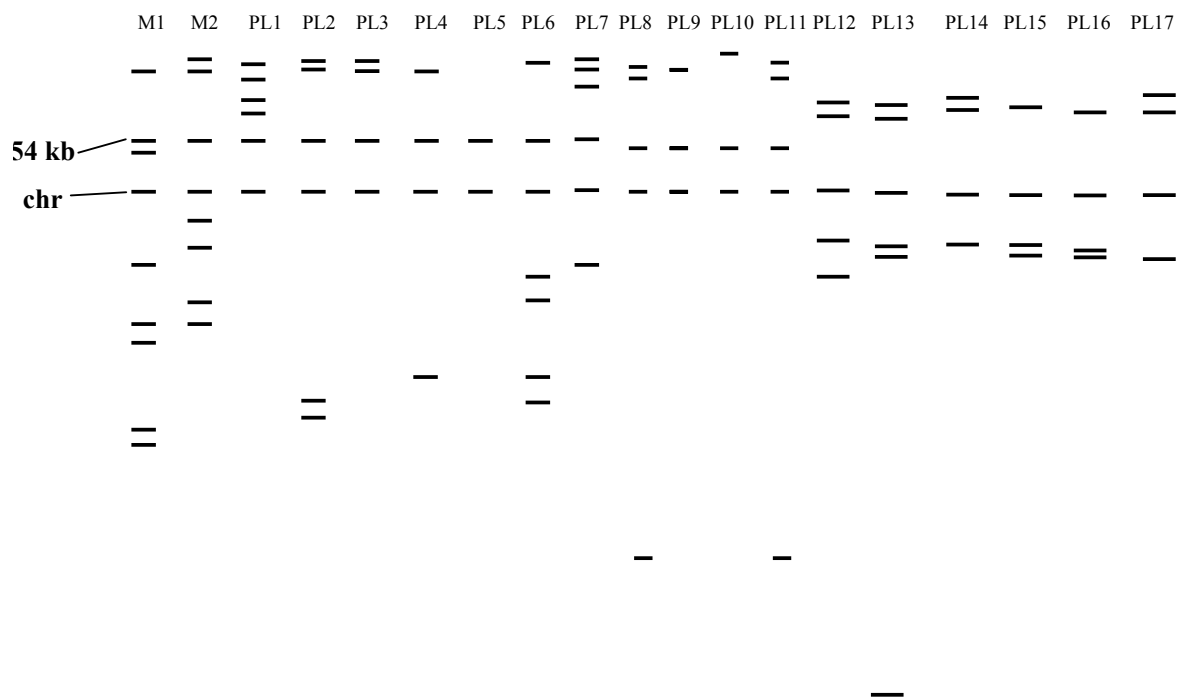


Figure 20: Plasmid profiles generated from the 40 group C *Salmonella* isolates obtained from outbreak of Salmonellosis in humans from Harare Hospital, Zimbabwe. M1 and M2 are the plasmid markers (V517 marker, M2 is 39R861 respectively). PL1-PL17 are the plasmid profiles generated from the 40 group C *Salmonella* isolates analysed.

3.5 Pulsed field gel electrophoresis

3.5.1 *S. enteritidis* strains

PFGE identified 10 pulsotypes of which pulsotype 1 was from clinical human isolates from 2005 salmonellosis outbreak and the other 9 were for animal isolates (Figures 21 and 22). Of the 13 bovine isolates from Mutare, 8 (61.5%) were identified under one profile using PFGE and 5 (38%) of the same isolates from Mutare had the same profile. Generally, *S. enteritidis* strains obtained from animals produced more profiles compared to those isolated from humans.

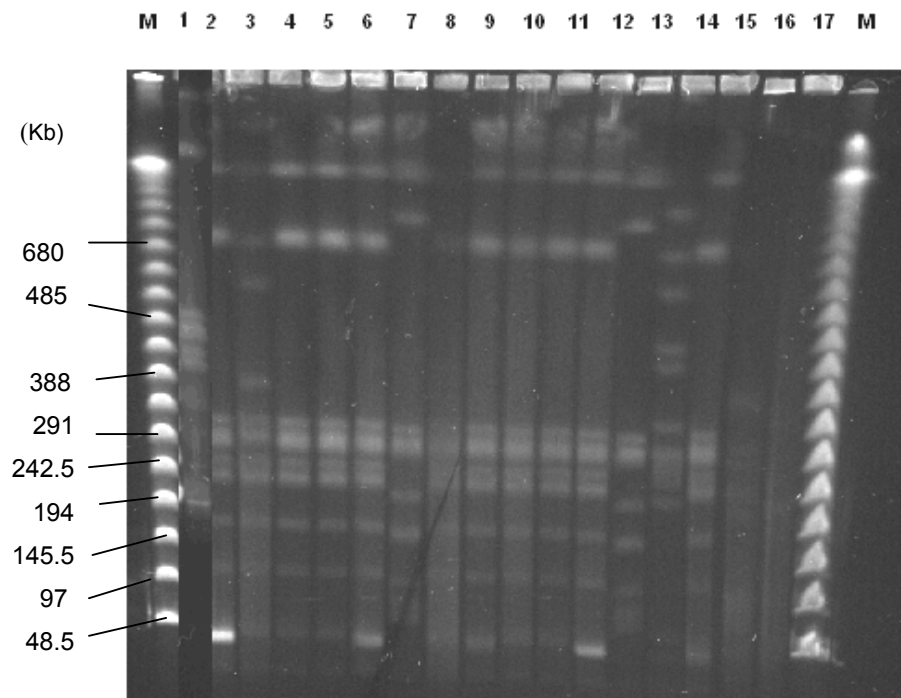


Figure 21: Animal *S. enteritidis* pulse field gel electrophoresis of chromosomal DNA digested with *Xba*I. M is the pulse field marker, 1-17 are Animal *S. enteritidis* strains

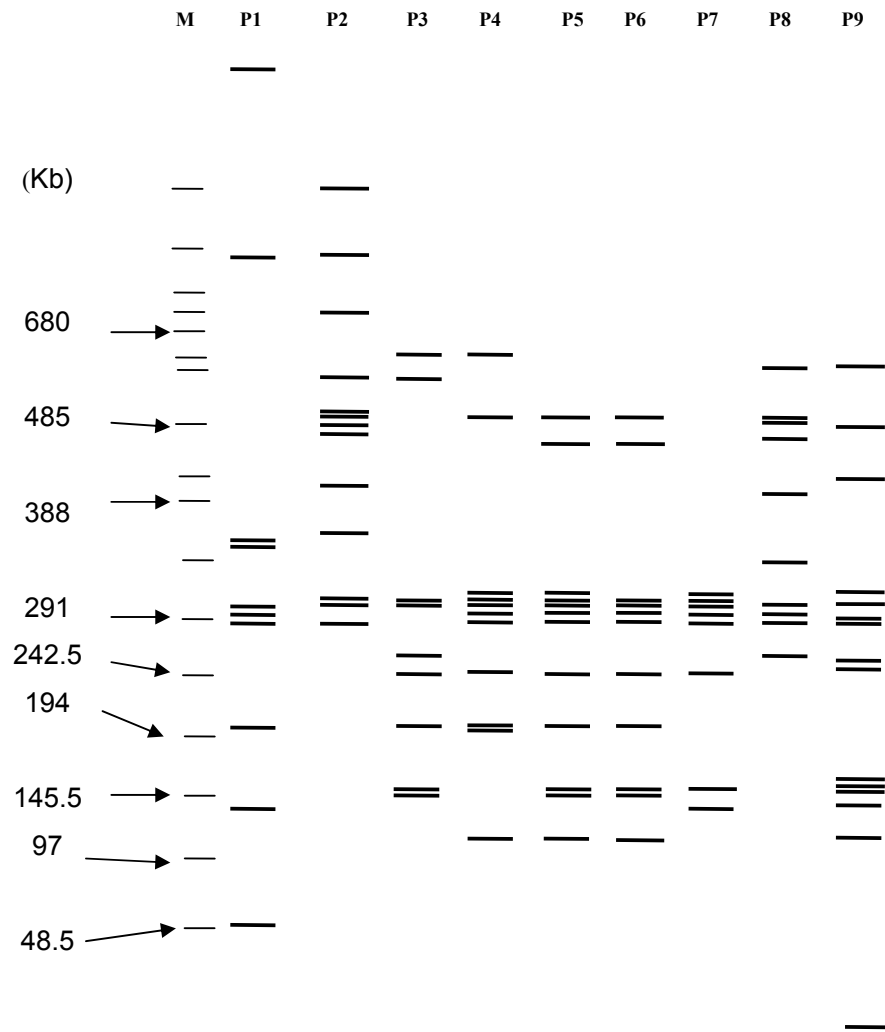


Figure 22: Pulsotypes identified from the PFGE performed on the 49 *S. enteritidis*. P1 to P9 are the identified pulsotypes and M is the PFGE marker.

In Figure 21, lanes 4 and 5 show a similar banding pattern (pulsotypes). The rest of the lanes each indicates a unique pulsotype for the samples electrophoresed in these lanes. The pulsotypes are denoted by P, in Figure 22, which shows all the identified pulsotypes for the 49 *S. enteritidis* strains.

3.5.2 Group B strains

The 32 group B isolates from plasmid profiling, which were further analysed using PFGE, were further split into 19 pulsotypes from the 11 plasmid profiles obtained (Figure 24). For example, 3 isolates, 2 from Harare and 1 from an unknown source and collected from Harare Hospital, which were previously put in one plasmid profile were further split into 3 different pulsotypes. Figures 23 and 24 show the pulsotypes obtained.

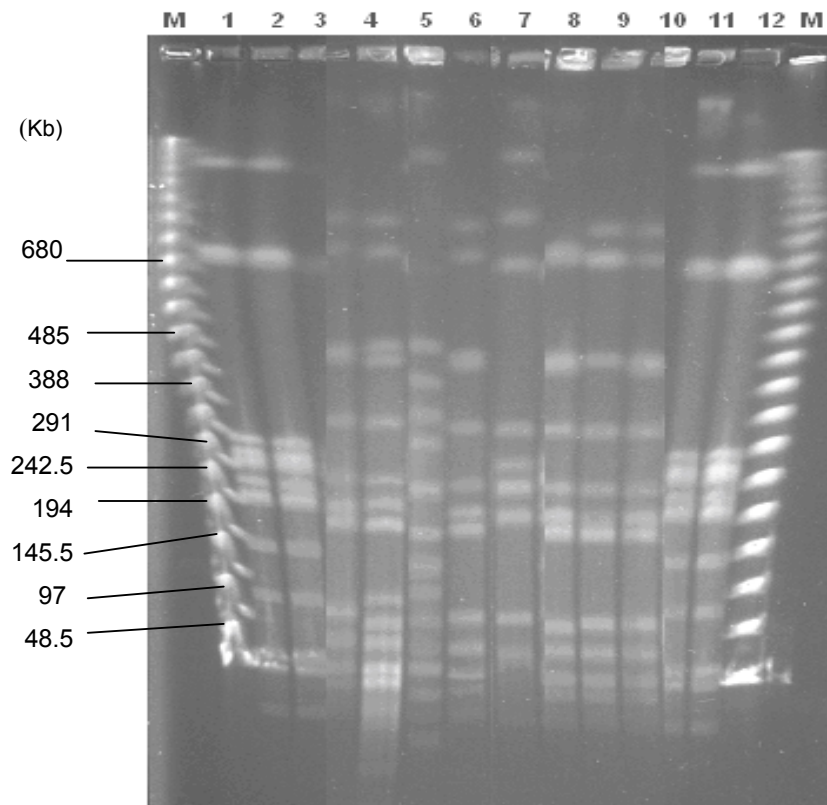


Figure 23: Group B *Salmonella* strains pulsed field gel electrophoresis of chromosomal DNA digested with *Xba*I. M is the pulsed field marker, lanes 1-12 are the group B *Salmonella* strains.

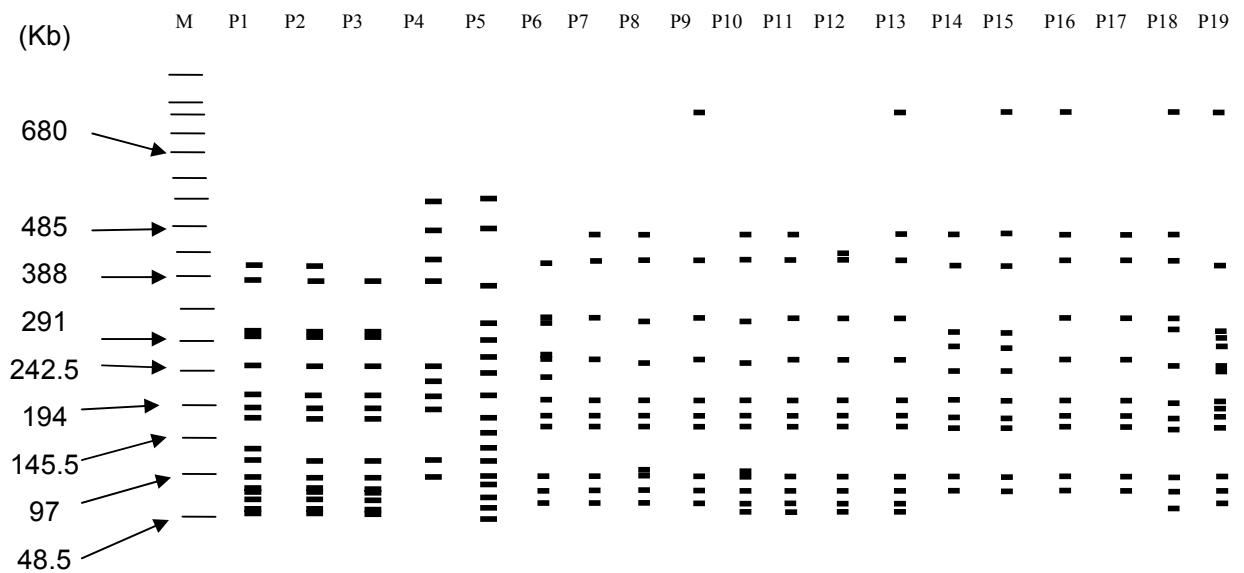


Figure 24: The Pulsotypes identified from human group B isolates. P1-P19 are the pulsotypes obtained as a result of PFGE on the 32 human group B isolates. M is the PFGE marker.

In Figure 23, lanes 1, 2 and 12 show a similar banding pattern (pulsotypes). Also lanes 9 and 10 have a similar pulsotype. The rest of the lanes indicate each a unique pulsotype for the samples electrophoresed in these lanes. The pulsotypes are denoted by P in Figure 24 which shows all the identified pulsotypes for the 32 Group B *Salmonella* strains.

3.5.3 Group C Strains

This group was not analysed by PFGE due to a lack of reagents when funding ceased.

4.0 DISCUSSION

The 2005 *Salmonella* outbreak in Zimbabwe might have been aggravated by the economic challenges that Zimbabwe was facing during that period. It was difficult for Zimbabweans to put food on the table. The literature states that one is more prone to salmonellosis if one is elderly, has other medical conditions like a weakened immune system as a result of cancer or HIV or if one is malnourished (WHO Global Foodborne Infections Network, 2010).

The results from antimicrobial susceptibility testing indicate that isolates were at least 95-100% resistant to the following antibiotics: Chloramphenicol (Group D, 100%), Lincomycin (Group C and Group D, 100%), Tetracycline (Group B, 100%, Group C, 97.5%), Cloxacillin (Group B and Group D, 100%, Group C not done) and Nitrofurantoin (Group B and Group D, 100%, Group C not done). This 95-100% resistance may be due to the fact that these drugs are the most commonly used antibiotics in the clinical treatment of salmonellosis and that patients might be abusing the drugs by buying them without the doctor's prescription. As a result, the pathogenic bacteria might have developed resistance to the antibiotics.

Tetracycline and Chloramphenicol antibiotics are commonly used for treatment of campylobacteriosis and salmonellosis in animals which will be used as food by humans.

In some circumstances, a small quantity of antibiotics will be added to animal feed as health promoters in order to destroy pathogens in the feed hence promoting animal health and growth. As a result, there will be carryover of antibiotic residue in the meat or animal products used for human consumption. The same drugs for example chloramphenicol, are used for treatment of *Salmonella* infections such as typhoid fever which is as a result of infections due to *S. typhi*. Although the use of antibiotics was banned in 2006 by EU, one will still find use of these antibiotics illegally to boost growth of his/her animals. This results in resistance to treatment of salmonellosis when these antibiotics are used in disease treatment or control (NOAH, 2010).

Antibiotic sensitivity tests performed on *Salmonella* group C indicated partial sensitivity and resistance of this group to all antibiotics tested, except furazolidone. This finding suggests that furazolidone is still effective in the treatment of diseases due to Group C *Salmonella*. Since these isolates are from an outbreak of salmonellosis, the resistance pattern is hardly surprising given that the same antibiotics might have been used previously to treat the outbreak, and hence the development of resistance.

Generally, there was no link established between antibiotic patterns and possession of *spv* gene loci in *S. enteritidis* strains. This is possibly due to the presence of antibiotic resistance genes either on the plasmid or on the chromosome. Also, no correlation could be ascertained between the antibiotic resistance patterns with geographical locations of the host. This can be as a result of migration of both

animals and humans which makes it difficult to assign a fixed geographical location to the host animals.

Another pattern observed from group C *Salmonella* was that the group C *Salmonella* from the outbreak mainly affected children below 10 years of age. This might have been due to the fact that the immune system of this group will not have been fully developed. The literature states that although *Salmonella* can affect all ages, in most cases it affects children, young adults and the very old (Brent *et al.*, 2006; Hoefer, 2009). The severity of this disease, however, depends on the number of bacteria ingested, age and general health condition of the patient (Hoefer, 2009; Green and Cheesebrough, 1993).

Plasmid profiling of group C produced more profiles compared to group B *Salmonella*, possibly due to more species in group C that are common pathogens compared to group B. Literature states that, *S. typhimurium*, which is a group B *Salmonella* is the most common pathogen presenting in humans suffering from salmonellosis (WHO Global Foodborne Infections Network, 2010). It might be possible that most of those strains from group B were *S. typhimurium*, hence, the fewer profiles compared to group C *Salmonella* plasmid profiles.

According to literature, molecular typing has a higher differential power as a typing tool compared to the traditional methods of typing (Kudaka *et al.*, 2002). Molecular typing is able to split organisms from species level to the different strains within the same species.

The different molecular typing tools also have different discriminatory powers hence the comparison in this study. The isolates used in this study were subjected to different molecular typing tools, which were in a position to further split the *Salmonella* beyond species level.

The isolates from wildlife, domestic animals and humans, which were identified and serotyped, using traditional methods, were confirmed using multiplex PCR. The fact that 2 of the 35 isolates serotyped as *Salmonella* were negative after multiplex PCR, indicates the importance of complementing traditional typing methods with the molecular typing methods. It also reflects the higher discriminatory power of molecular typing methods compared to the traditional typing methods.

In the present study, Multiplex PCR was able to split *S. enteritidis* strains into those with and those without *spv* genes. According to this study, no link was however established between the presence or absence of *spv* gene with geographical distribution of isolates. For example, the 8 *S. enteritidis* outbreak strains were grouped into a single profile by both plasmid and PFGE fingerprinting though 4 were from Kariba and the other 4 were from Harare region.

The absence of the link is most probably due to migration of animals and humans from one region to another, which results in the spreading of the strains peculiar to a region to other regions. This poses difficulties to assign strains to a geographical location.

This study attempted to establish a link between the distribution of *spv* genes in *S. enteritidis* and the relationship of these genes, plasmid profiles and PFGE profiles to geographical distribution and host organism of the *S. enteritidis* isolates studied.

It was not possible to split the isolates into those with *spv* genes and those without the genes using plasmid profiling. For example some of the isolates, which were observed to contain the *spv* gene after multiplex PCR analysis (Figure 14), lacked the heavy plasmid associated with virulence, after plasmid profiling (Figure 19). This could imply that the virulence gene for some isolates is located on the chromosome.

However, with PFGE analysis, it was possible to split the isolates into two, those with *spv* genes and those without *spv* genes. This indicates the high discriminatory power of PFGE compared to plasmid analysis technique. This was also indicated in the group B isolates, where 11 plasmid profiles were further split into 19 pulsotypes by PFGE. Furthermore, this observation could imply that the isolates had these virulence genes on the chromosomal DNA and not on plasmids.

Although group B was also from an outbreak, it produced more profiles, both by plasmid and PFGE profiling. This might be because the isolates were many (32) compared to the *S. enteritidis* ones (8). This increased chances of obtaining a different strain from a group.

The results indicate a relationship between *S. enteritidis* isolate and its host. This might be an indicator of the strains that are host specific. As an example, ostriches from Bulawayo and chickens from Harare (all avian) shared the same plasmid profile. The same strains shared the same plasmid profile with clinical outbreak human isolates from Harare and Kariba. This might be as a result of humans eating undercooked, infected meat. This could also indicate the root cause of the salmonellosis outbreak. It can be explained by the fact that during the period under study, people were eating more of the chicken meat compared to beef since chicken was cheaper during that period. The findings also confirm that food needs to be cooked thoroughly.

The 2 strains obtained from the Opportunistic Infectious Clinic (OIC) were grouped together with other isolates from different sources. This suggests that the presence or absence of the HIV in an individual has no effect on the *Salmonella* isolate. This, however, cannot be stated with total certainty since the sample size was very small. There is need for further research to include a larger sample size of *Salmonella* from people living with HIV and AIDS (PLWHA) and compare the results with those from the HIV negative group.

In plasmid profiling, 11 profiles were obtained from the 49 strains analysed. Makaya (unpublished data) obtained 5 profiles from 179 *S. enteritidis* isolates obtained from poultry. The difference in the number of plasmid profiles might be as a result of mutations.

It can also be as a result of the fact that the strains used by Makaya (unpublished data) were all from Zimbabwean poultry, while those used in this study were from a wider range of sources including humans, poultry, bovine and crocodile, resulting in a broader range of fingerprints.

The outbreak strains from humans produced the same fingerprint after PFGE analysis, which might indicate that the isolates were clonal. The fingerprint can be considered as the outbreak fingerprint, which can be used as a reference fingerprint if the outbreaks recur. PFGE was able to split the *Salmonella* isolates into more groups compared with other methods. This means PFGE has a higher differential power compared to the other methods used in this study.

Generally, strains obtained from animals produced more profiles compared to those isolated from humans after typing with PFGE. This might be because most of the isolates from humans were from an outbreak, hence, were most probably the same strain that was spread from one individual to the other.

It appears the strain type of *S. enteritidis* is not affected by the location within the host. This is indicated by the fact that the *S. enteritidis* from humans produced the same pulsotype regardless of whether it was isolated from stool or blood. There is a high possibility that the isolates were clonal since *Salmonella*, under severe cases, can migrate to the lymph tracts, which carry water and protein to the blood (Worden, 2009), and the blood itself and sometimes infects other organs (Brennan, 2010; Brenner *et al.*, 2000).

Most field strains of *S. enteritidis* collected from farms were from healthy animals. This might be an indicator that animals are reservoirs of *Salmonella*. There is, therefore, a possibility of avian strains (and other strains) being transmitted to humans through the food chain. The results confirm the need to thoroughly cook meat and animal products before consumption.

From this study, we recommend the use of PFGE for typing of *Salmonella* after confirming with multiplex PCR, if one wants to show epidemiological relationships of the *Salmonella* serotypes isolated from different geographical locations and from different host species. This is because the present study shows that PFGE produced more profiles after typing compared to the other tools used. Nevertheless, this should be complemented by the traditional typing tools (Namoos *et al.*, 1994). However, there is still need to compare PFGE with other tools such as IS200 typing, Ribotyping and ERIC PCR, which were supposed to be done in this study but could not be performed due to limited resources.

A greater sample size of isolates also needs to be considered in future to ascertain the relationships between an isolate strain and its geographical location. At the moment there is limited documented and published literature on *Salmonella* outbreak statistics in Zimbabwe.

In conclusion, no relationship to geographical location and host origin of isolates was established. There is need to carry out the study on a large scale to authenticate the findings. There is also a need to put surveillance in place to monitor *Salmonella* outbreaks in Zimbabwe since *Salmonella* has been recorded as one of the deadliest pathogens worldwide (Nygard *et al.*, 2004). Literature states that the two disease agents of greatest concern to immune compromised individuals are *Salmonella spp* and *Toxoplasma gondii* (Nygard *et al.*, 2004). Statistical records and relevant findings in Zimbabwe should be documented and published for easy access by researchers, epidemiologists, farmers and human health workers.

Study Limitations

There is need to carry out the study with a larger sample size to authenticate the findings from the current study. It would have been more informative if Southern blot analysis and DNA sequencing had been used to complement the PCR methods.

5.0 REFERENCES

1. Basim, E and Basim, H. 2001. Pulsed-Field Gel Electrophoresis (PFGE) Techniques and its use in Molecular typing. **Turkey Journal of Biology: 25.** 405-418.
2. Bennasar, A, De Luna, G, Cabrer, B and Lalucat, J. 1999. Rapid identification of *Salmonella typhimurium*, *S. enteritidis* and *S. virchow* isolates by Polymerase Chain Reaction based fingerprinting methods. **International Microbiology: 3.** 31-38.
3. Berg, J. M, Tymoczko, J. L., and Stryer, L. 2007. "Biochemistry". 6th ed. W.H. Freeman and Company, NY
4. Biomerieux Product library. Available at http://www.biomerieux-diagnostics.com/servlet/srt/bio/clinical-diagnostics/dynPage?doc=CNL_CLN_PRD_G_PRD_CLN_22. Accessed on 03 October 2012.
5. Boyd, F and Hartl, D. L. 1998. *Salmonella* virulence plasmid: Modular Acquisition of the *spv* virulence region by an F-plasmid in *Salmonella enterica* sub-species I and insertion into the chromosome of sub-species II, IIIa, IV and VII isolates. **Genetics: 149.** 1183-1190.
6. Brent, A. J, Oundo, J. O, Mwangi, I, Ochola L, Lowe, B and Berkley, J. A. 2006. *Salmonella* Bacteraemia in Kenyan Children. **Paediatric Infectious Disease Journal: 25 (3).** 230-236.

7. Brennan, J. *Salmonella* Group B Defined. Available at
http://www.ehow.com/about_6640815_Salmonella-group-defined.html.
 Accessed on 29 November 2010.
8. Brenner, F. W, Villar, R. G, Angulo, F. J, Tauxe, R and Swaminathan, B. 2000.
Salmonella nomenclature. **Journal of Clinical Microbiology**: **38**. 2465-2467.
9. Carattoli, A . 2003. Plasmid-Mediated Antimicrobial Resistance
 in *Salmonella enterica*. **Current Issues Molecular Biology**: **5**. 113-122.
10. Chartterjee, A, O'Keefe, K, and Varman, M. 2010. *Salmonella* Infection.
 Emedicine. webMD
11. Chen, I and Dubnau, D. 2004. DNA uptake during bacterial transformation.
Nature Reviews Microbiology: **2 (3)**: 241–9.
12. Chi-shih Chu, Cheng-Itsun Chiu, Wan-Yuwu, Chi- Hong Chu, Tsui-Ping Liu
 and Jonathan, T. Ou. 2001. Large drug resistance virulence plasmids of
 clinical isolates of *Salmonella enterica* serovar *cholerasuis*. **Antimicrobial
 Agents and Chemotherapy**: **45**. 2299-2303.
13. Chu, C, and Chiu, C. H. 2006. Evolution of the virulence plasmids of non-
 typhoid *Salmonella* and its association with antimicrobial resistance. **Microbes
 and Infection**. Jun; **8(7)**:1931-6.
14. Epidemiology. Available at
<http://dictionary.reference.com/browse/epidemiology>. Accessed on 06/07/10
15. Foggin, C, M. 1992. Disease trends on crocodile farms in Zimbabwe. In
 crocodile proceedings of 11th working meeting of the Crocodile Specialist
 Group. October 2007

16. Foley, S. L, Zhao, S and Walker, R. D. 2007. Comparison of molecular typing methods for the differentiation of *Salmonella* food borne pathogens. **Fall: 4(3):253-76.**
17. Furuya, E. Y and Lowy, F. D. (2006). Antimicrobial-resistant bacteria in the community setting. **Nature. Microbiology: 4.** 36-45.
18. Gast, R. K, Jones, D. R, Anderson K. E, Guraya R, Guard J and Holt P. S. 2010. In vitro penetration of *Salmonella* Enteritidis through yolk membranes of eggs from 6 genetically distinct commercial lines of laying hens. **Poultry Science: 89** (8): 1732–1736.
19. Green, S. D and Cheesebrough, J. S. 1993. *Salmonella* Bacteremia among Children at a Rural Hospital in Western Zaire. **Annals of Tropical Paediatrics: 13 (1).** 45-53.
20. Grimont P.A.D and Weill F.X.2007. Antigenic Formulae of the *Salmonella* Serovars 9th edition. WHO Collaborating Centre for Reference and Research on *Salmonella*, Institut Pasteur, Paris.
21. Hendriksen, R. 2003. A global surveillance and laboratory support project of the World Health Organization. **Laboratory Protocols. Level 1 Training Course. Identification of *Salmonella*.** 4th Edition.
22. Hohmann, E. L. 2001. Nontyphoidal Salmonellosis. **Clinical Infectious Diseases: 32.** 263-269. Hoefer. H. *Salmonella* and Pet Reptiles. Available at http://www.heidihoefer.com/pages/reptiles/Salmonella_reptiles.pdf. Accessed on 17 July 2009.
23. Kado, C. I and Liu, S. T. 1981. Rapid procedure for detection and isolation of large and small plasmids. **Journal of Bacteriology: 145.** 1365-1373.

24. Karim, M and Islam, N. 2002. *Salmonella* meningitis: report of 3 cases of adults and literature review. **Infections: 30.** 104-108.
25. Kudaka, J, Itokazu, K, Taira, K, Iwai, A, Kondo, M, Susa, T and Iwanaga, M. 2006. Characterisation of *Salmonella* isolated in Okinawa, Japan. **Journal of Infectious Diseases: 59.** 15-19.
26. Kues, U and Stahl, U. December 1989. Replication of plasmids in gram-negative bacteria. **Microbiological Review: 53(4):** 491–516
27. Liebana, E, Migura, L. G, Clouting, C, Clifton-Hadley, F. A, Lindsay, E, Threlfall, E. J, McDowell, S. W. J and Davies, R. H. 2002. Multiple Genetic typing of *Salmonella enterica* Serotype *Typhimurium* Isolates of Different Phage Types (DT104, U302, DT204b and DT49) from Animals and Humans in England, Wales and Northern Ireland. **Journal of Clinical Microbiology. 40:** 4450-453.
28. Maldonado, R. CTAB Help. Available at [Rafael at genetics.med.utah.edu](mailto:Rafael@genetics.med.utah.edu). Accessed on 06 February 2007
29. Mugochi, T, Parawira, W, Mpofu, A, Simango, C and Zvauya, R. 1999. Survival of some species of *Salmonella* and *Shigella* in Mukumbi, a traditional Zimbabwean wine. **International Journal of Food Sciences and Nutrition. 50; 6.** 451-455
30. Namoos , B, Threlfall, E. J, Rowe, B and Stanely, J.1994. Comparative evaluation of molecular typing of strains from a national epidemic due to *Salmonella brandenburg* by rRNA gene, IS200 probes and pulse field gel electrophoresis. **Journal of Clinical Microbiology: 32.**1876.

31. **National Office of Animal Health (NOAH).** Growth promoters – some Questions & Answers. Available at <http://www.noah.co.uk/issues/briefingdoc/16-digestiveenhancers.htm>. Accessed on 18 October 2012.
32. Norman, L, 2005. Biochemical Tests for Identifying unknowns. Available at <http://web.fccj.org/~lnorman/unknowns.htm?index=2>. Accessed on 29 November 2010
33. Novak, S. Aug 2011. Drug Resistant Salmonella Prompts Recall of 36 Million Pounds of Turkey. Food & health. Available at <http://www.treehugger.com/feeds/authors/snovak.xml>. Accessed on 10 August 2011.
34. Nye, K. J, Fallon, D, Frodsham, D, Gee, B, Graham, C, Howe, C, Messer, S, Turner, T and Warren, R. E. April 2002. An evaluation of the performance of XLD, DCA, MLCB, and ABC agars as direct plating media for the isolation of *Salmonella enterica* from faeces. *Journal of Clinical Pathology*. **55** (4): 286–8
35. Nygård, K, De Jong, B, Guerin, P, Andersson, Y, Olsson, A and Giesecke, J. 2004. Emergency of new *S. enteritidis* phage types in Europe? Surveillance of infections in returning travellers. **BMC Medicine**: **2**. 32
36. Obwolo, M, J. and Zwart, P. 1993. Prevalence of *Salmonella* in the intestinal tract of farm reared crocodiles (*Crocodylus niloticus*) in Zimbabwe. **Journal of Zoo and Wildlife Medicine**: **24**. 175-176
37. Osborne, C. 2011. Zoonotic Diseases in Immune-compromised Persons. Available at <http://www.carolonpets.com/blog/avoiding-disease/zoonotic-diseases-in-immune-compromised-persons/>. Accessed on 29 November 2010.

38. Pan, T. M and Liu, Y. J. 2002. Identification of *Salmonella enteritidis* isolates by polymerase chain reaction and multiplex polymerase chain reaction. **Journal of Microbiology, Immunology and Infections: 35.** 147-151.
39. Parry, C.M, and Beeching, N. J. 2009. Treatment of enteric fever. **BMJ 338:** b1159–b1159.
40. Persing, D. H, Tenover, F. C, Versalovic, J, Tang, Y-W, Unger, E.R, Relman, D. A and White T. J. 2004. **Molecular Microbiology; Diagnostic Principles and Practice.** Washington, D.C: American Society for Microbiology.
41. Prager, R, Kabsch, W, Streckel, W, Voigt, W, Tietze, E and Tschape, H. 2003. Molecular Properties of *Salmonella enterica* Serotype Paratyphi B Distinguish between Its Systemic and Its Enteric Pathovars. **Journal of Clinical Microbiology: 41.** 4270-4278.
42. Ridley, A and Threlfall, E. J. 1998. Molecular Epidemiology of Antibiotic Resistance Genes in Multiresistant Epidemic *Salmonella Typhimurium* DT104. **Microbial Drug Resistance. 4:** 113-118
43. Rotger, R. and Casadesus, J. 1999. The virulence plasmids of *Salmonella*. **International Microbiology: 2.** 177-184
44. Rowe, B, Ward, L. R and Threlfall, E. J. 1997. Multidrug-resistant *Salmonella typhi*: a worldwide epidemic. **Clinical Infectious Diseases.** Jan; 24 Suppl 1:S106-9.
45. Rychlik, I, Gregorova, D and Hradecka, H. 2005. *Distribution and function of plasmids in Salmonella enterica.* **Veterinary Microbiology: 112.** 1-10.

46. Rychlik, I, Hradecka, H. and Malcova, M. 2008. *Salmonella enterica* serovar *Typhimurium* typing by prophage-specific PCR. **Microbiology: 154**. 1384-1389.
47. Rychlik, I, Sebkova, A, Gregorova, D and Karpiskova, R. 2001. Low Molecular Weight Plasmid of *Salmonella enterica* Serovar Enteritidis Codes for Retron Reverse Transcriptase and Influences Phage resistance. **Journal of Bacteriology: 183 (9)**. 2852-2858.
48. *Salmonella*-Causes and symptoms. Available at <http://science.irank.org/pages/5944/Salmonella-Causes-symptoms.html>. Accessed on 17 July 2009
49. Simango, C and Mbewe, C. 2000. *Salmonella enteritidis* diarrhoea in Harare, Zimbabwe. **Tropical Medicine and International Health: 5**. 503–506
50. Sorensen, S, J, Bailey, M, Hansen, L. H, Kroer, N and Wuertz, S. September 2005. Studying Plasmid Horizontal Transfer *in situ*: A critical review. **Nature Reviews: Microbiology: 3**. 701
51. Tavechio, A. T, Fernandes, S. A, Ghilardi, A. C, Soule, G, Ahmed, R and Melles, C. E. 2009. Tracing Lineage by phenotypic and genotypic markers in *Salmonella enterica* subsp. Enteric serovar 1, 4, [5], 12: i - and *Salmonella Typhimurium* isolated in state of Sao Paulo, Brazil. Enteric Disease Program, National Microbiology Laboratory, Health Canada, Winnipeg, Manitoba, Canada.
52. Taylor, L. H, Latham, S. M and Woolhouse, M. E. 2001. Risk factors for human disease emergence. **Philosophical Transactions of the Royal Society B 356(1411)**:983-9

53. WHO Global Foodborne Infections Network. Laboratory Protocol. Isolation of *Salmonella* species from food and Animal Faeces. 5th Edition. June 2010.
54. Worden, J. *Salmonella* and Food Poisoning. Available at http://www.netdoctor.co.uk/health_advice/facts/Salmonella.htm. Accessed on 17 July 2009.