MECHANISMS OF DISSEMINATION OF INTEGRON-MEDIATED MULTIPLE ANTIBIOTIC RESISTANCE IN *SALMONELLA ENTERICA*

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Abstract

Salmonella enterica is a major cause of disease in the United States and world-wide. This pathogen's increasing antibiotic resistance over the past decades has exacerbated its public health burden. To combat the rise in antibiotic resistance, it is necessary to understand how resistance spreads through the bacterial population. This requires study of the genes that determine resistance and also of the genetic relatedness of the isolates bearing those genes.

In this dissertation, three studies are presented. The aim of the first study was to examine the utility of multilocus sequence typing (MLST), which indexes sequence changes in housekeeping genes, for investigating genetic relatedness in *S. enterica*. MLST was shown to be a stable genetic typing method for *S. enterica* that is useful for long-term and global epidemiologic studies.

The aim of the second study was to examine the mechanisms of dissemination of antibiotic resistance genes mediated by a genetic structure called an integron, in a genetically diverse, global collection of *S. enterica*. Integrons are able to create variable collections of resistance genes and may be disseminated by horizontal gene transfer or by clonal expansion. Using MLST to determine genetic relatedness, the second study indicated that both of these mechanisms are important forces in the dissemination of integron mediated antibiotic resistance in *S. enterica*.

The investigation of integrons revealed three integrons not previously reported in *S. enterica*. The aim of the third study was to characterize those integrons, which contained novel aggregates of resistance genes, making them capable of conferring resistance to multiple antibiotics. One of these integrons was found in two genetically unrelated strains, reinforcing this structure's potential for contributing to horizontal gene transfer of antibiotic resistance in *S. enterica*.

Validation of MLST as a typing method for *S. enterica* has public health importance because it facilitates large scale and global studies of this organism by providing a basis for assessing genetic relatedness of diverse isolates. An understanding of the patterns of dissemination of integrons has public health significance because it enhances the ability to accomplish surveillance for changes in antibiotic resistance.

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PREFACE

I would like to thank the members of my committee, Elodie Ghedin, Lawrence Kingsley, and Gerard Nau, for their support of this research. I would like to especially thank my advisor, Lee Harrison, and Jane Marsh for the many forms of support they have given me in pursuing the work that is presented here.

1.0 INTRODUCTION

Salmonella enterica subspecies *enterica* is an important pathogen worldwide, and is responsible for a large burden of food-borne gastroenteritis. Salmonellosis is usually a self-limiting disease of limited severity but can be life-threatening in certain populations, such as the very young, the very old, and immunocompromised individuals. In 2007, the Foodborne Diseases Active Surveillance Network (FoodNet) of the Center for Disease Ccontrol and Prevention's Emerging Infections Program reported an incidence of *Salmonella* infection of 14.92 cases per 100,000 in the general population with an incidence of 62.11 cases per 100,000 in children under 5, representing approximately 38% of all food borne infection in the United States [1].

Antibiotic resistance is an increasing problem in *S. enterica*, as in many other pathogens [2]. Many of the recent *S. enterica* isolates obtained worldwide are resistant to a number of antibiotics. The multiple drug resistance (MDR) ACSSuT phenotype, with resistance to the antibiotics ampicillin, streptomycin, chloramphenicol, sulfamethoxazole, and tetracycline, is often found and is especially common in serovar Typhimurium DT 104 *S. enterica* strains [3]. Since antibiotics are the usual treatment for severe *S. enterica* associated disease, the emergence of MDR *S. enterica* displaying this phenotype is a major public health problem.

Antibiotic resistance in bacteria can develop by accumulation of point mutations or by horizontal exchange of DNA between bacteria of the same or different species. Increases in

antibiotic resistance are generally attributed to the selective pressure exerted by the use of antibiotics both in treatment of disease in humans and at sub-therapeutic levels as additives to animal feed [4, 5]. However, increases in resistance in *S. enterica* may be due at least partially to clonal dissemination of resistant strains rather than to the effects of selective pressure [6]. There are some studies that support the dissemination of specific strains of antibiotic resistant *S. enterica* in the absence of use of antibiotics that would select for those particular strains [7]. Understanding the dynamics and mechanisms of spread of antibiotic resistance is important for surveillance for increases and changes in patterns of antibiotic resistance.

1.1 GENETIC RELATEDNESS OF *S. ENTERICA*

To analyze the patterns of dissemination of antibiotic resistance in *S. enterica*, it is necessary to have a method for assessing the genetic relatedness of different isolates. In *S. enterica*, genetic relatedness is often based on serovar. Serovar is determined by the lipopolysaccharide O antigen and the two flagellar H antigens, and has been widely used historically to differentiate *S. enterica* strains. However, serovar may not be a reliable measure of evolutionary relationship [8]. Other methods that have been used to estimate genetic relatedness in groups of *S. enterica* isolates include pulsed-field gel electrophoresis (PFGE), ribosomal RNA analysis, phage typing, plasmid analysis, multilocus enzyme elecrophoresis (MLEE), and multilocus sequence typing (MLST) [9].

PFGE uses an electric field of changing orientation to move large DNA fragments, usually obtained by restriction endonuclease digestion, through a gel. While PFGE is highly

discriminatory, because it is based solely on the electrophoretic mobility of restriction endonuclease fragments, it measures only a small subset of the mutations that occur in an organism's DNA. A single point mutation in a restriction site may lead to apparent large changes in the PFGE band pattern, while more numerous changes in other locations may not be identified. Bands which appear identical by PFGE may be composed of entirely different nucleotide sequences. An additional drawback of this method is that its results do not contain any phylogenetic signal and therefore cannot be used for phylogenetic analysis. So, while PFGE is highly discriminatory and has been widely used for outbreak analysis, it is more useful for local or short term epidemiology than for long term or global epidemiology [10].

Ribosomal RNA is highly conserved in *S. enterica* and is therefore not useful for determining genetic lineage [11]. Phage typing has been used for many years to differentiate *S. enterica* strains. In this method, *S. enterica* isolates are grown on solid media and then treated with stock solutions of phages. The resulting pattern of lysis determines the phage type. Since this method relies on genetic material that is external to the *S. enterica* genome and that may be lost without significantly impacting the viability of the organism, the usefulness of phage-typing for estimating relatedness is limited [12]. Plasmid analysis suffers from similar drawbacks and does not aid in deciphering long-term or global *S. enterica* population structure [13]. MLEE looks at the similarity of the enzymes produced by an organism, and would therefore seem to be well suited to evolutionary analysis, but it relies upon protein electrophoresis which is difficult to perform reproducibly, making global comparisons a challenge [14].

MLST offers advantages over the methods described above for analysis of genetic relatedness in *S. enterica*. In this method, portions of a small number of housekeeping genes,

usually numbering between 5 and 8, are sequenced and variations in these loci are used to assign allele numbers and sequence types. Housekeeping genes are used because they accumulate selectively neutral mutations slowly [10]. Use of MLST in *S. enterica* has been limited to date. Most *S. enterica* MLST studies focus on comparison of that method with other methods for outbreak detection and do not address the utility of MLST for assessing genetic relatedness (Table 1-1). Because housekeeping genes tend to be conserved, MLST may not be discriminatory enough to be useful for outbreak detection, so this type of comparison may not be valid.

Table 1-1 MLST Studies

Study	Isolates	Methods
Fahkr [15]	85 serovar Typhimurium	PFGE, MLST
Sukhnanand [16]	25 isolates, 5 serovars	Modified sequence typing system using 3 genes
Foley [17]	128 Typhimurium	PFGE, modified sequence typing system
Kotetishvili [18]	231 isolates from Maryland	PFGE, modified sequence typing system
Torpdahl [19]	110 isolates from Denmark	PFGE, MLST, AFLP
Harbottle [20]	81 serovar Newport, 27 states, 2001 to 2003	Antibiotic resistance testing, PFGE, MLST
Kidgell [21]	26 serovar Typhi	MLST

Fahkr [15] *et al* performed MLST and PFGE on a collection of 85 *S. enterica* Typhimurium clinical isolates from cattle. Although they found 50 PFGE profiles which grouped into 3 major clusters, all 85 isolates were identical by MLST. These data demonstrate that PFGE was much more discriminatory than MLST in differentiating strains of the same serovar.

Sukhnanand *et al* [16] used a technique that is similar to MLST but utilized a combination of housekeeping and virulence genes to investigate genetic relationships in 25 *S. enterica* isolates of 5 serovars. The goal of this study was to develop a typing system that would provide adequate discrimination, reliably predict serovar, and that could be used for evolutionary analyses. This method provided adequate discriminatory power when only three gene loci (two housekeeping and one virulence) were analyzed. Their results clustered isolates by serovar in all but one case. Phylogenetic analysis using seven genes (five housekeeping and two virulence) for twenty-five isolates demonstrated that the majority of serovars represented single lineages, with the exception of serovars Newport and Kentucky, which originated from multiple lineages. The limited number of isolates and serotype used in this study make these results preliminary at best. The inclusion of virulence genes may obscure phylogenetic results, since virulence genes impart different selective pressures than housekeeping genes.

In another study using both housekeeping and virulence genes, Foley *et al* [17] developed a sequence typing scheme to study genetic relationships among *S. enterica*. They compared the results of this scheme with those of PFGE in a collection of 128 serovar Typhimurium isolates. These isolates produced 89 sequence types and 86 PFGE patterns. Although they found similar levels of discrimination in the two methods, the results of the two methods did not correlate. Their sequence typing scheme discriminated some groups of isolates that were not differentiated by PFGE, while in some cases, PFGE was more discriminatory. The use of isolates of only one serovar limits the utility of this study.

In another sequence based genotyping strategy, Kotetishvili *et al* [18] developed a typing system based on four genes (three housekeeping and one ribosomal gene) and compared this scheme to PFGE and serotyping. The ribosomal gene was found to be highly conserved and not

useful for discriminating between strains, but the authors concluded that a system using the three housekeeping genes provided more discriminatory power than either serotyping or PFGE. The authors also concluded that the genes they investigated were subject to extensive recombination. One weakness of this study was that the isolates were obtained from one geographic area (Maryland) and 75% of isolates were environmental samples, mainly from poultry. Also, the serovars Hadar (83 isolates) and Typhimurium (42 isolates) predominated in the isolate collection. For this reason, their strains may have been fairly clonal, and therefore have limited phylogentic heterogeneity. In addition, all three genes were not included for all isolates, because variable numbers of isolates failed to amplify certain loci by PCR (for example, 25% of isolates failed to amplify the *pduF* locus used in this study) resulting in different numbers of isolates being used in strain analysis for different genes.

In a study comparing the discriminatory power of a 7 gene MLST scheme, PFGE, and AFLP (amplified fragment length polymorphism) [19], 110 Danish isolates were investigated. This study found similar clustering of isolates using the three methods, but concluded that the discriminatory ability of MLST was not as great as that of AFLP or PFGE.

In a US study [20] comparing MLST and PFGE, 81 serovar Newport isolates collected from 27 states from 2001 to 2003 were examined. This study identified 43 PFGE patterns in three clusters and 12 MLST sequence types. While 61.7% of the isolates belonged to one sequence type, this may be a reflection of the lack of serovar diversity in this isolate collection. Evolutionary analysis separated the sequence types into two groups and one genetically unrelated isolate, confirming that Serovar Newport is polyphyletic, as was reported by Sukhnanand [16]. MLST was found to be a reliable predictor of antibiotic resistance in this study, with highly antibiotic resistant isolates clustering in one group and highly susceptible isolates belonging to

the other group, but this correlation may merely be coincidental due to lack of diversity of isolates in this study.

Kidgell *et al* [21] described a seven gene MLST scheme for *S. enterica*. This study included 26 isolates of serovar Typhi. The isolates included in this study defined 4 sequence types (STs) that had only minor nucleotide differences. Based on these results, the authors concluded that the Typhi isolates represent a globally distributed clone.

Although some studies on MLST in *S. enterica* have been published, these studies have not explored the diversity of this bacterium in a systematic fashion. A number of these studies include the sequencing of virulence genes, while MLST uses only housekeeping genes. This is an important distinction, since housekeeping genes are not under the same selective pressure as virulence genes. Housekeeping genes are essential and are therefore expected to provide a more stable assessment of genetic lineage than virulence genes which can display significant nucleotide diversity. This antigenic variability is due to the fact that virulence genes often encode surface proteins that are under selective pressure from the host immune system. Most of these studies were based on isolate collections that were very limited, either in numbers of isolates or in serovars included. The most serious weakness is that most of these studies compare MLST or a variant of that technique to methods such as PFGE. PFGE is very sensitive to minor genetic changes; it is designed to be used in outbreak investigation, not in phylogenetic analysis. For this reason, comparison of MLST and PFGE for discriminatory ability is inappropriate, since MLST is better suited to investigation of genetic lineage than to detection of minor genetic changes.

Most of the *S. enterica* MLST studies reviewed here have not included phylogenetic analysis of the data. *S. enterica* has been reported to be clonal in nature with limited genetic

recombination [8, 22, 23], but some recent studies have disputed this [18, 24-27]. As mentioned previously, Kotetishvili reported evidence of recombination among isolates using a 3 gene strategy [18]. In another study, statistical tests of 16 isolates of varied serovar indicated that some of the genes investigated had undergone recombination [27]. A further study including 70 *S. enterica* strains investigated DNA sequences from the *mutS* gene, which is part of a system for DNA mismatch repair, and found evidence of widespread recombination [25]. Octavia and Lan [24] found frequent recombination in 5 housekeeping genes in 15 strains of 13 serovars. Falush *et al* [26] also concluded that substantial recombination occurred among *S. enterica* serovars. These studies suggest that *S. enterica* is capable of undergoing significant recombination and may not be as clonal as previously believed.

A curated database containing MLST data for *S. enterica* is available at: http://web.mpiib-berlin.mpg.de/mlst/dbs/Senterica [28]. This database utilizes the scheme detailed by Kidgell [21] and, as of June 2008, contained information on MLST sequence types (ST), serovars, geographic location, and source for 2,237 *S. enterica* strains. A total of 192 serovars are represented in this database, with 176 serovar Kentucky, 381 serovar Newport, 343 serovar Typhimurium, 115 serovar Choleraesuis, and 163 serovar Enteritidis strains. These five serovars make up approximately half of the entries in the database. In comparison, the five most commonly reported serovars from human sources reported to the CDC in 2005 were Typhimurium, Enteritidis, Newport, Heidelberg, and Javiana, in that order of frequency [29]. Therefore, the contents of the MLST database does not exactly mirror clinical isolate frequency. There are 482 different sequence types defined and although most serovars contain more than one ST, only nine STs contain more than one serovar. Most serovars correspond to either one or a few STs. Although the contents of the database is extensive, no studies have been published

that document and describe its contents. One of the goals of this dissertation is to validate the usefulness of this method for assessing genetic relatedness of diverse strains of *S. enterica*.

1.2 INTEGRONS

A portion of the antibiotic resistance found in *S. enterica* is associated with a genetic structure called an integron [30]. An integron is a genetic unit that includes a site-specific recombination system capable of capturing and mobilizing genes contained in mobile elements called gene cassettes [31]. These cassettes are discrete units containing genes which often code for resistance to one or more antibiotics. Integrons also contain a gene encoding an integrase, responsible for insertion and excision of gene cassettes from the integron, and a promoter, which regulates expression of the gene cassettes. Integrons are often contained on plasmids or transposons [32, 33]. Plasmids are extrachrosomal, autonomously replicating DNA molecules that are capable of moving between bacterial cells. Transposons are DNA segments that are capable of moving from one location to another within the bacterial genome and between plasmids and the bacterial genome. The association of integrons with these mobile genetic elements gives integrons the ability to move between bacteria and to move within the bacterial genome. Integrons therefore provide an efficient method for transfer and acquisition of drug resistance genes in *S. enterica*. Due to their structure, integrons allow for rapid horizontal transfer of antibiotic resistance both within and between bacterial species. The general structure of an integron is shown in Figure 1.1. Integrons have been found in many bacteria of public health importance in addition to *S. enterica*, including *Pseudomonas aeruginosa, Acinetobacter baumannii*, *Klebsiella pneumoniae, Escherichia coli,* and *Serratia marcesens* [34]

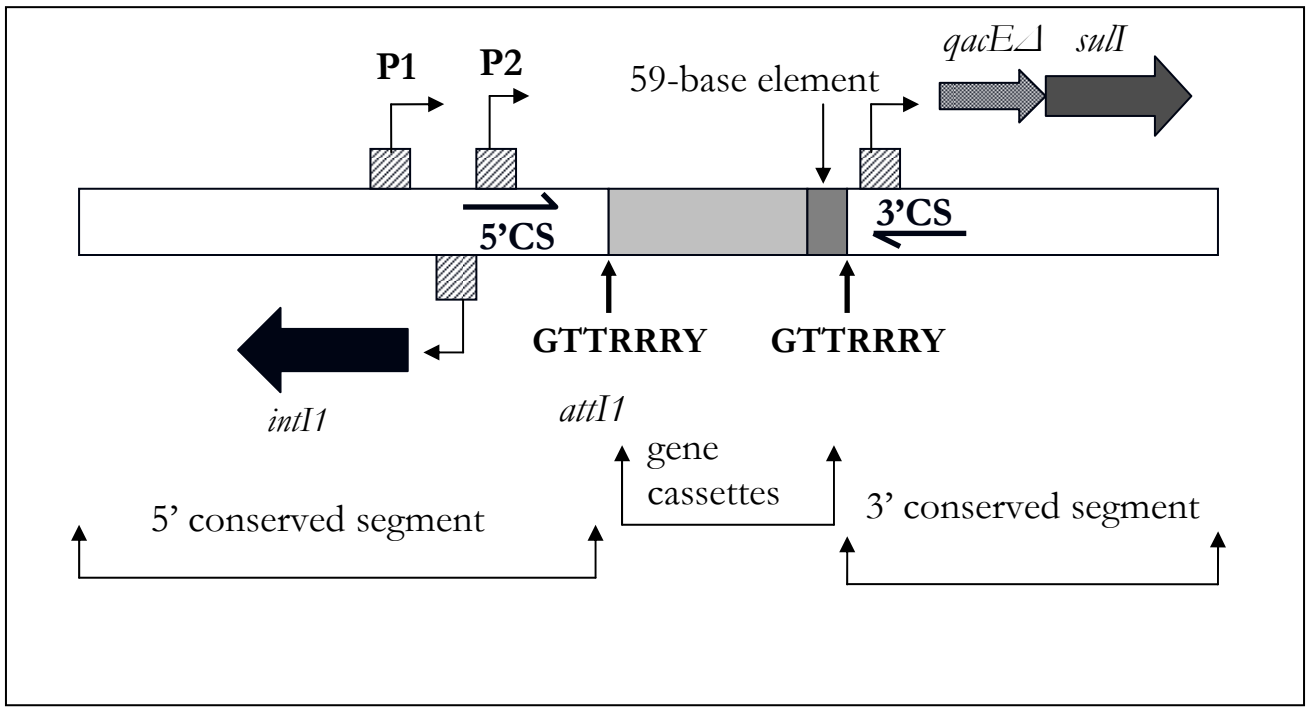

Figure 1-1 Generalized integron structure

An integron contains a variable number of gene cassettes inserted between the 5' and 3' conserved segments. The 5' conserved segment contains the gene for an integrase (*intI1*) which is responsible for insertion and excision of gene cassettes and 2 promoters (*P1* and *P2*) which regulate expression of the gene cassettes. The 3' conserved segment contains genes coding for resistance to quaternary ammonium compounds (*qacE∆1*) and sulfamethoxazole (*sulI*).

The association of the integron structure with multiple drug resistance in *S. enterica* has been investigated in a number of geographic locations, including Europe, Taiwan, China, Africa, and the United States [35-43]. However, to date there have been limited epidemiologic investigations of the association of integrons with multiple drug resistance in *S. enterica* across diverse geographic areas. In addition, many studies focus on MDR serovar Typhimurium DT104 isolates, in which resistance genes are contained in the bacterial chromosome both within

and outside of integron structures. Few investigations of multiple drug resistance in other *S. enterica* serovars have been reported.

A number of authors [41, 43-55] have characterized integrons in individual countries. Vo *et al* investigated twenty-six integron positive MDR *S. enterica* serovar Typhimurium isolates collected in the Netherlands from 1993 to 2005 [56]. They described the antibiotic resistance phenotypes, resistance genes, phage type, and PFGE patterns and determined that phage typing was not a reliable marker of relatedness among those isolates. Some isolates with different phage types exhibited the same PFGE profile and contained the same integrons, while other isolates with the same phage type had different PFGE profiles, different resistance patterns, and different integrons. Multiple drug resistance in serovar Typhimurium DT104 is often associated with chromosomally encoded integrons. Markogiannakis *et al* [47] studied MDR serovar Typhimurium DT104 strains in Greece and found five different PFGE patterns among their isolates, indicating that at least in their sample, DT104 is not always clonal. Rodriguez *et al* [52] investigated integron carriage in eight multidrug-resistant *S. enterica* strains belonging to serovars Virchow, Panama, Grumpensis, and Worthington, all isolated in Spain. In two cases, an identical integron was found in an isolate of serovar Virchow and also in one or more other *S. enterica* serovars, indicating horizontal transfer of integrons between different serovars.

A limited number of studies have examined integrons in isolates from more than one country. Murphy *et al* [57] investigated antibiotic resistance profiles and integron carriage in a group of *S. enterica* isolates. The isolates in this study consisted of two groups. Thirty-six isolates were obtained from humans or from milk filters in Ireland. The remaining isolates came from the *Salmonella* reference collection B (SARB), which was established in 1993, and which contains seventy-two isolates originating in a number of countries and having thirty-seven

different serovars [14]. The isolates in the SARB collection were obtained from a variety of sources during the 35 year period from 1953 to 1988. Antibiotic resistance testing and PCR for the integrase gene were performed on both groups of isolates. In this study, 50% of isolates amplified the integrase gene, while 25% contained integrons, indicating that some integrons did not contain any gene cassettes. DNA sequencing of gene cassettes was performed on five isolates from the SARB collection. Although the SARB collection contains isolates of 37 different serovars, the majority of Irish *S. enterica* isolates were of serovar Typhimurium. While this study does investigate *S. enterica* from different serovars and different geographic areas, the limited integron sequence data, the time interval over which isolates were collected, and the large number of Typhimuriums represented in the collection, make it difficult to make useful conclusions on the relative contributions of clonal dissemination and/or horizontal gene transfer in the spread of antibiotic resistance in *S. enterica*

In another study that compared *S. enterica* integrons from different countries, Baggesen *et al* [58] investigated a total of 136 isolates of serovar Typhimurium DT104 obtained from Europe and the United States. Ninety-three of these isolates were collected in Denmark, ten in Germany, four in Italy, five in Spain, eleven in the United Kingdom, and nine in the United States. These isolates, of both human and animal origin, were examined by antibiotic resistance testing, PCR for integrons, plasmid analysis, and PFGE. Only ten isolates did not exhibit the MDR profile (ACSSuT) that is commonly found in Typhimurium DT104. The aim of this study was to compare serovar Typhimurium DT104 isolates obtained from dairy herds in Denmark to isolates obtained in other geographic areas in order to determine if antibiotic resistance was introduced into food production animal herds by imported animals or whether the resistance emerged within Denmark as a result of selection for resistance genes. Of the 126 MDR isolates,

all but one contained two chromosomally located integrons, encoding streptomycin (*aadA1*) and β-lactams (*pse1*) resistance. These results are consistent with other published studies of MDR serovar Typhimurium DT104. When investigated by PFGE using the restriction endonuclease *Xba*I, the MDR Typhimurium DT104 isolates were remarkably similar, with the same PFGE profile shared by the majority of isolates from Denmark, Germany, Italy, Spain, the United Kingdom, and the United States. These isolates were also highly similar by PFGE using the restriction endonuclease *Bln*I. Some minor differences were noted using *Bln*I, but this study strongly suggests that serovar Typhimurium DT104 isolates have a common origin and have expanded clonally in Europe and the United States. However, this study does not provide any information concerning spread of resistance in other *S. enterica* serovars or between different genetic lineages.

Daly *et al* [36] presented evidence of transfer of integrons and other genetic elements conferring antibiotic resistance between two serovars of *S. enterica*. This study characterized only two isolates, one of serovar Typhimurium DT193, isolated in Ireland and one of serovar Enteritidis, isolated in Italy. The Typhimurium isolate harbored an integron containing the *dfrA1* and *aadA1* gene cassettes, conferring resistance to trimethoprim and streptomycin/spectinomycin, respectively. This integron was located on the bacterial chromosome. The serovar Enteritidis isolate contained an integron on a conjugative 80kb plasmid. This integron was similar to that found in the Typhimurium isolate, but was missing the 3' conserved segment and part of the *aadA1* gene cassette. The level of homology between the integrons and other antibiotic resistance genes in these two isolates supports the hypothesis that horizontal gene transfer is involved in the spread of antibiotic resistance between isolates of different serovars in different geographic areas. The authors conclude that the resistance genes

and the integron structure were originally located on the plasmid found in the serovar Enteritidis isolate and were transferred to the serovar Typhimurium isolate. This study supports the hypothesis that plasmids and horizontal gene exchange are important in the dissemination of antibiotic resistance genes among different *S. enterica* serovars. It should be noted, however, that serovar, which is not a reliable measure of genetic relatedness, was the only measure of genetic lineage employed in this study.

In a study [59] investigating integron carriage in *S. enterica* isolates from twenty different serovars obtained from Australia, Levings *et al.* searched for the presence of the Salmonella Genomic Island (SGI1), characteristic of serovar Typhimurium DT104. The SGI1 is a 43 kb region containing two integrons described above (conferring streptomycin and β-lactam resistance) as well as several other antibiotic resistance genes necessary for the expression of the MDR resistance phenotype common to serovar Typhimurium DT104. Nine strains, representing seven serovars, were positive for SGI1 or variants of that structure and were localized to the bacterial chromosome, and 2 new variants of the SGI1 were described. SGI1 and its variants were found in serovars Cerro, Derby, Dusseldorf, Infantis, Kiambu, Emek, and Paratyphi B dTartrate^{+}. The authors of this study conclude that the distribution of SGI1 in other *S. enterica* serovars supports the idea that this genomic island, although usually chromosomally located, is capable of moving between strains, and that this horizontal movement is an important factor in the dissemination of antibiotic resistance in *S. enterica*.

O'Mahoney *et al* [60] investigated MDR isolates from a collection of 72 isolates containing 18 serovars. They looked for integrons and plasmids in the MDR isolates. One serovar Typhimurium and one serovar Anatum isolate were found to contain an unusual integron structure that was identical in the two isolates, based on DNA sequencing. This integron

contained several open reading frames (ORFs), which matched partial sequences of gene cassettes found in other integrons, along with one complete resistance gene, which coded for ampicillin resistance. The finding of identical integrons in two different serovars supports horizontal transmission of integrons between isolates of different serovars, but this conclusion is weakened by the fact that, as mentioned previously, serovar is not a trustworthy measure of genetic relatedness, and no other assessments of genetic relatedness were attempted in this study.

Many studies have described the structure and gene cassette content of individual integrons or have compared integrons from limited geographic areas or from collections which include a limited number of serovars (Table 1-2). These studies support the hypothesis that both vertical and horizontal gene transfer are important in the dissemination of integrons. However, to date, there are few studies that compare integrons obtained from collections that originate in many locations or that include many different serovars. Current studies also lack reliable information on the genetic relatedness of the strains that have been characterized. A goal of this dissertation is to address the weaknesses of previous *S. enterica* integron studies by including isolates of varied serotypes from diverse geographic regions and by utilizing a better method for determining the genetic relatedness of those isolates.

Table 1.2 Integron Studies

Study	Isolates	Methods
Vo [56]	26 isolates from the Netherlands from 1993 to 2005	Antibiotic resistance, PCR, DNA sequencing, PFGE
Markogiannakis [47]	14 Typhimurium DT104 isolates in Greece	Antibiotic resistance, PCR, DNA sequencing, PFGE
Rodriguez [52]	8 MDR strains, 4 serovars, isolated in Spain	Antibiotic resistance, PCR, DNA sequencing, PFGE
Murphy [57]	72 from SARB (collected 1953 to 1988) 36 from Ireland	Antibiotic resistance testing, PCR, DNA sequencing
Baggesen [58]	136 Typhimurium DT104 from Europe and the United States	Antibiotic resistance testing, PCR, DNA sequencing, PFGE
Daly [36]	2 isolates, Enteriditis, from Italy and Typhimurium from Ireland	PCR, DNA sequencing
Levings [59]	20 serovars from Australia	Antibiotic resistance testing, PCR, gel electrophoresis of restriction enzyme endonuclease fragments, DNA sequencing
O'Mahoney [60]	72 isolates, 18 serovars, from Colombia	Antibiotic resistance testing, PCR, DNA sequencing, plasmid analysis
Gebreyes [38]	67 isolates, serovar Muenchen	Antibiotic resistance testing, PCR, DNA sequencing
Gebreyes [45]	484 isolates, serovar Typhimurium, USA	Antibiotic resistance testing, PCR, DNA sequencing, AFLP
Pai [51]	6 isolates, serovar Typhi, from Korea	Antibiotic resistance testing, PCR, DNA sequencing, ribotyping
Peirano [61]	135 isolates, 23 serovar, from Brazil	Antibiotic resistance testing, PCR, DNA sequencing
Zhao [55]	208 isolates from imported food in USA	Antibiotic resistance testing, PCR, DNA sequencing, PFGE
Zhao [43]	588 isolates, serovar Typhimurium, USA	Antibiotic resistance testing, PCR, DNA sequencing, PFGE
Weill [53]	47 isolates, serovar Paratyphi B	Antibiotic resistance testing, PCR mapping, PFGE
Michael [49]	2 isolates, 1 serovar from Brazil	Antibiotic resistance testing, PCR, DNA sequencing, conjugation
Miko [50]	319 isolates, 25 serovars, from Germany	Antibiotic resistance testing, PCR, DNA sequencing, hybridization
Edrington [44]	393 isolates, 23 serovar	Antibiotic resistance testing, PCR, DNA sequencing, O antigen grouping

1.3 MECHANISMS OF INTEGRON DISSEMINATION

Integrons in *S. enterica* are often found on plasmids. For this reason, plasmids have been implicated as a vehicle for the dissemination of multiple drug resistance and integrons both within *S. enterica* and between *S. enterica* and other bacteria, such as *Pseudomonas* and *Acinetobacter* [34]. Carattoli has pointed out that plasmids similar to those found in *S. enterica* have been isolated from a number of different types of gram-negative bacteria, and that the plasmids may have been transferred to *S. enterica* from other bacterial pathogens in a nosocomial setting [32]. This raises the possibility that resistance-carrying plasmids can spread worldwide among bacteria in both the hospital and community setting.

Integrons are not located on plasmids in all cases. Chromosomally located integrons have been found in a number of *S. enterica* serovars, most commonly in Typhimurium DT104, which appears to have spread globally during the 1990s. As mentioned previously, in this strain all of the genes encoding the MDR phenotype are present on a 43 kb genomic island, the SGI1, which includes two integrons and other non-integron related resistance genes and which is located in the bacterial chromosome [62]. Since this first description, variants of the SGI1 have been described in a number of other serovars, including Paratyphi B [53], Agona [63], Kiambu, Infantis, Cerro, Dusseldorf, Derby, and Emek[59]. DNA sequencing information indicates that these variant genomic islands are all inserted into the *S. enterica* genome at the same location. The SGI1 has been found to be mobilizable but not self-transmissible and has been detected as a free, circularized DNA segment [62]. These data indicate that integrons have the potential not only to accumulate and release resistance gene cassettes, but that the integron structures themselves are often capable of moving from one location to another, either to and from plasmids and the bacterial chromosome, or between different bacterial cells.

Integrons may spread geographically by clonal dissemination of the bacteria harboring them or by horizontal gene transfer. Clonal dissemination involves the vertical transmission of genetic material to offspring cells. Well-adapted clones may proliferate in one location and be introduced to new locations, where they may replace the existing populations. In this case, the bacterial population will undergo a change in its core genome reflecting the population replacement. In horizontal gene transfer, existing populations acquire genetic elements, possibly those that confer a selective advantage, from other populations. In this case the recipient population will retain its previous genetics but with the introduction of new accessory genetic elements. Depending upon the characteristics of the situation (characteristics of both the bacteria and environment) one or the other of these mechanisms may predominate, or both may play a role.

1.4 SPECIFIC AIMS

Integrons are ideally suited for accumulating and transferring antibiotic resistance and therefore make a major contribution to multiple antibiotic resistance in *S. enterica* and in other bacterial species. However, the mechanism of integron dissemination has not been well investigated. Although integrons have been studied extensively in *S. enterica*, most studies focus on isolates from one geographic area and often include one or only a few serovars or isolates. In addition, genetic relatedness of integron-carrying isolates has been addressed mainly using methods that are better suited to outbreak investigation than to phylogenetic analysis. The information obtained so far indicates that both clonal dissemination and horizontal gene transfer are important in the spread of antibiotic resistance due to integrons in *S. enterica*, but this has not

been investigated in a systematic fashion. Effective surveillance for the spread of antibiotic resistance due to integrons requires a better understanding of integron propagation in *S. enterica* populations than has been provided to date. Towards that goal the specific aims of the studies described here are (1) to validate the usefulness of MLST as a typing tool for long term and global epidemiologic analysis of *S. enterica* using a geographically and serotypically diverse collection of isolates obtained from laboratories in thirteen countries on six continents, (2). to investigate horizontal gene transfer and clonal expansion as mechanisms of dissemination of integrons in a global collection of multiply drug resistant *S. enterica* using MLST to evaluate genetic relatdeness, and (3) to characterize several unique integrons identified by these studies, which were interesting because of their size and gene cassette content, and because they were not previously reported in *S. enterica*.

2.0 MULTILOCUS SEQUENCE TYPING FOR GENETIC CHARACTERIZATION OF *SALMONELLA ENTERICA*

This study will not be published as presented here. These data will be published as part of a study that includes all of the data deposited in the global *Salmonella* MLST database, authored by a group that will include the contributors to that database.

2.1 ABSTRACT

Introduction: *Salmonella enterica* is a food borne pathogen of major importance in public health that is antigenically diverse, with over 2,500 serovars described. Despite this diversity, serovar is inadequate for studies examining the genetic relationships among isolates of this organism, due in part to the uneven frequency of the different serovars and to methodologic difficulties in serotyping. Multilocus sequence typing (MLST) is a sequence based typing method which has been employed successfully for a number of organisms. Because it relies on sequences of housekeeping genes, MLST can provide information that is useful for phylogenetic studies as well as isolate discrimination. A 7 gene MLST scheme has been developed for *S. enterica* and a global database of information is available, but as yet this method is not widely used. **Methods**: This study employed the MLST scheme used by *http://web.mpiib-berlin.mpg.de/mlst/dbs/Senterica* to investigate population structure and correlation of genetics with serovar in a collection of *S. enterica* that was highly diverse by serovar and geographic origin. MLST data was analyzed using tests for linkage disequilibrium, recombination and split decomposition. **Results:** Evidence of significant linkage disequilibrium reinforces the hypothesis that *S. enterica* is largely clonal. However, split decomposition analysis and tests of recombination indicate that there are recombinational aspects to *S. enterica* population structure. **Conclusions:** The results of this study indicate that the population structure of *S. enterica* is clonal but is also affected by recombination. Sequence types defined by MLST correlated strongly with serovar indicating that *S. enterica* is as diverse in the genetics of its housekeeping genes as it is in its serovars. MLST is a reliable method for assessing the genetic relatedness of geographically and serotypically diverse *S. enterica* isolates.

2.2 INTRODUCTION

Salmonella enterica is a widely distributed, often food-borne pathogen, capable of causing disease in humans and animals. It is a Gram-negative, rod-shaped member of the family of enterobacteriaciae and is the causative agent of typhoid fever, gastroenteritis, and occasionally systemic infection. *S. enterica* is highly antigenically diverse, comprising more than 2,500 serovars [64]. Despite this antigenic diversity, only a few serovars are responsible for the majority of sporadic human disease, with other serovars causing outbreaks and occasional sporadic cases [65]. *S. enterica* has been considered to be largely clonal in population structure [8, 22, 23], however recent studies have indicated that recombination is also important in the population dynamics of this bacterium [18, 25, 26].

The genus *Salmonella* is divided into two species, *enterica* and *bongori*. *Enterica* is further subdivided into six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). The subspecies are further divided into serovars. Determination of serovar is an agglutination test based upon the presence of O polysaccharide and H flagellar antigens. *S. enterica* isolates exhibit a large number of gene variants encoding the O and H antigens. In addition, most *S. enterica* carry 2 genes for flagellar antigens, only one of which is expressed at a time, and one or both of these flagellar genes may be missing [66].

Serovar in *S. enterica* subspecies *enterica* (SS I) is usually designated by a name, e.g., Typhimurium. Uncommon SS I serovars may be identified using antigenic formulas of the form O antigen(s):Phase1 H antigen(s):Phase 2 H antigen(s), where the absence of a flagellar H antigen is indicated by '-', e.g., 4,5,12:i:-, in which 4, 5, and 12 are O antigens, 'i' is the Phase 1 flagellar antigen, and the Phase 2 flagellar antigen is absent. Serovars that do not express O antigens are designated as 'Rough'. Serovars may be described either solely or partly by 'Group'

which refers to the dominant O antigen. The group designation is either a letter or has the form Group O:number. Examples include serovar Typhimurium which is Group O:4 or Group B and serovar Enteritidis which is group O:9 or Group D1. Subspecies other than I are referred to by antigenic formula [67].

The genetic polymorphisms in the O antigen and H flagellar antigens are responsible for the very large number of serovars exhibited by *S. enterica*. Despite its longstanding use, serovar is an imperfect and unreliable measure that often gives inconsistent results [68].

Multilocus sequence typing (MLST) is a typing method which has substantial advantages over other previously used methods. In MLST, fragments of 5 to 8 housekeeping genes are sequenced. The resulting sequences are assigned unique allele numbers which are combined to yield a sequence type (ST). The method is available to any laboratory that has access to DNA sequencing technology, and alleles and STs can be stored in global, web-enabled databases, thereby promoting sharing of typing results (www.mlst.net).

MLST has been employed for genetic characterization of many bacterial species, including *Neisseria meningitidis*, *Staphylococcus aureus*, *Bacillus cereus*, and *Candida albicans* [10, 69-72]. A global MLST database exists for *S. enterica*, but few studies have been published on the use of this method in that organism, and the majority of studies published have utilized isolate collections of relatively limited diversity [15-18, 20, 21]. In this study, we investigated the utility of MLST to study population structure and genetic relationships in a global collection of *S. enterica* isolates of diverse serovar.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial Isolates

A total of 327 *S. enterica* isolates were selected from a global collection assembled by laboratories in Argentina, Australia, Belgium, Canada, Denmark, Germany, Italy, the Philippines, South Africa, Spain, Taiwan, Uganda and the USA between September 2001 and August 2002. The collection also contained isolates obtained from the Allegheny County Department of Health, Allegheny County, Pennsylvania, the Pennsylvania Department of Health, the Ohio Department of Health, and the University of Maryland, during the years 2003 to 2007. To study MLST from a collection with the greatest diversity, one isolate representing each unique combination of serovar and geographic origin in the collection was selected for analysis (Appendix A). Serovar determination of isolates was done by the collecting laboratories, except for isolates from Taiwan, which were tested courtesy of the Pennsylvania Department of Health. No epidemiologic or clinical data is available for these isolates other than geographic origin and year of collection.

2.3.2 MLST

Genomic DNA was prepared from overnight cultures grown on Luria-Bertani agar either by boiling lysis preparation or by Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer's directions.

MLST was performed using the seven locus scheme described by the *Salmonella* MLST database (http://web.mpiib-berlin.mpg.de/mlst) (Table 2-1) [21, 73]. PCR was performed in a 50 µl volume consisting of 1x PCR Buffer II (Applied Biosystems, Foster City, CA), 1.5mM $MgCl_2$, 0.1mM dNTPs, 0.33µM forward and reverse primers, and 1.66 units Amplitaq Gold polymerase (Applied Biosystems, Foster City, CA). PCR conditions were: initial denaturation 95° C for 5 minutes, 35 cycles of 95° C for 1 minute, 58° C for 1 minute, 72° C for 1 minute, final extension 72° C for 5 minutes. PCR products were subjected to electrophoresis on 1% agarose gels, stained with ethidium bromide and visualized using UV illumination on a Gel Doc 2000 documentation system (Bio-Rad, Hercules, CA).

PCR products were purified using polyethylene glycol and were sequenced using the BigDye terminator 3.1 kit (Applied Biosystems) according to the manufacturer's instructions. Capillary sequence analysis was performed on a 3730 DNA sequence analyzer (Applied Biosystems).

Table 2.1 Multilocus sequence typing genes and primers

Locus	Gene	PCR Primer	Size (bp)	Sequencing primer	Size (bp)
aroC	chorismate synthase	F 5'-CCTGGCACCTCGCGCTATAC-3' R 5'-CCACACACGGATCGTGGCG-3'	826	sF 5'-GGCACCAGTATTGGCCTGCT-3' sR 5'-CATATGCGCCACAATGTGTTG-3'	501
dnaN	DNA polymerase III beta subunit	F 5'-ATGAAATTTACCGTTGAACGTGA-3' R 5'-AATTTCTCATTCGAGAGGATTGC-3'	833	sF 5'-CCGATTCTCGGTAACCTGCT-3' sR 5'-CCATCCACCAGCTTCGAGGT-3'	501
hemD	uropor phyrinogen III cosynthase	F 5'-ATGAGTATTCTGATCACCCG-3' R 5'-ATCAGCGACCTTAATATCTTGCCA-3'	666	sF 5'-GTGGCCTGGAGTTTTCCACT-3' sR 5'-GACCAATAGCCGACAGCGTAG-3'	432
hisD	histidinol dehydrogenase	F 5'-GAAACGTTCCATTCCGCGCAGAC-3' R 5'-CTGAACGGTCATCCGTTTCTG-3'	894	sF 5'-GTCGGTCTGTATATTCCCGG-3' sR 5'-GGTAATCGCATCCACCAAATC-3'	501
purE	phosphoribosyl aminoimidazole carboxylas	F 5'-ATGTCTTCCCGCAATAATCC-3' R 5'-TCATAGCGTCCCCCGCGGATC-3'	510	sF 5'-CGCATTATTCCGGCGCGTGT-3' sR 5'-CGCGGATCGGGATTTTCCAG-3'	399
sucA	alpha ketoglutarate dehydrogenase	F 5'-AGCACCGAAGAGAAACGCTG-3' R 5'-GGTTGTTGATAACGATACGTAC-3'	643	sF 5'-AGCACCGAAGAGAAACGCTG-3' sR 5'-GGTTGTTGATAACGATACGTAC-3'	501
thrA	aspartokinase +homoserine dehydrogenase	F 5'-GTCACGGTGATCGATCCGGT-3' R 5'-CACGATATTGATATTAGCCCG-3'	852	sF 5'-ATCCCGGCCGATCACATGAT-3' sR 5'-CTCCAGCAGCCCCTCTTTCAG-3'	501

2.3.3 Analysis and Assignment of MLST Alleles and Sequence Types

Sequences were imported into Bionumerics software V 5.10 (Applied Maths, Austin, TX). Sequences were trimmed at specific nucleotides to conform to the *Salmonella* MLST scheme. Alleles and STs were assigned by the database and all isolates and sequences have been deposited therein.

2.3.4 Statistical Analysis

DnaSP Version 4.50 was used to calculate number of polymorphic sites, number of synonymous and replacement changes, nucleotide diversity, total mutations per locus, and to determine linkage disequilibrium and estimate recombination [74]. Split decomposition analysis was accomplished using SplitsTree4 [75]. SplitsTree4 was also used for estimating recombination using the PhiTest [76]. The PhiTest (pairwise homoplasy index test), calculates a mean incompatibility score for sites. Sites are incompatible if their inferred genealogical history requires multiple mutations or convergent mutations, which implies that they have arisen due to recombination. The minimum number of recombination events in the history of the sample sequences (RM) was estimated using DnaSP by the method of Hudson and Kaplan [77]. Simpson's Index of Diversity (1-D) was calculated using the formula $D = \Sigma\, n(n-1) / N(N-1)$ where n is the frequency of each unique value (ST or allele number) and N is the total number in the sample [78].

Concatenated sequences were used to test for linkage disequilibrium, which is a result of the accumulation of mutations throughout the genome at a fairly equivalent rate. In a recombining population, a specific mutation at one site would be equally likely to be associated with any of the polymorphisms existing in the population at other sites, so mutations would tend to be in linkage equilibrium. In contrast, in a clonal population, a specific mutation at one site would tend to segregate with specific mutations at other sites existing in isolates from the same clone, resulting in linkage disequilibrium.

Linkage disequilibrium was also tested by using the MLST allele numbers to calculate the standardized Index of Association (sI_A) by the method of Haubold and Hudson using LIAN

3.0 [79]. This method uses MLST allele numbers to test for independent assortment of alleles at all loci.

Maximum likelihood (ML) phylogenetic trees were constructed using the Dnaml program of Phylip and RAxML, from sequences exported from Bionumerics [80-82]. Trees were constructed for each locus and for concatenated sequences. Trees were visualized using Phylodraw V0.82 [83, 84].

2.4 RESULTS

2.4.1 Sequence Diversity

MLST results were obtained for 324 of the original 327 isolates (Appendix A). Three isolates were removed from the study: two isolates failed to amplify any of the 7 loci, one serovar Group C from Belgium and one serovar Newport from South Africa; and one isolate of serovar O Rough from Allegheny County Health Department amplified 6 of the 7 loci but, upon repeated attempts, either failed to amplify the hisD locus or gave a smaller product than expected, which failed sequencing. The remaining 324 isolates comprised 169 unique STs (Table 2-2). The *hisD* locus provided the most discrimination, generating 94 different alleles, 101 polymorphic sites, and 109 mutations. The *hemD* locus was the least discriminatory, with 66 different alleles, 71 polymorphic sites, and 77 mutations. The average nucleotide diversity π, calculated as the average number of nucleotide differences per site between sequences, ranged from 0.0105 for *aroC* to 0.0211 for hisD. The ratio of nonsynonymous to synonymous substitutions (d_n/d_s) ranged from 0.007 for *sucA* and *thrA* to 0.209 for *hemD*, indicating that these

genes are under purifying selection, as expected for housekeeping genes (Table 2-2). Simpson's Index of Diversity was close to 1 for each locus and for the concatenated loci, indicating a relatively high level of diversity. Simpson's Index of Diversity is a measure of both the number of different types in a population and of the relative abundance of each type, and its maximum value is 1.

Table 2.2 Diversity of ST and MLST loci

	Number of alleles or STs	Number of polymorphic sites/allele or ST	Number of mutations	d_n/d_s[1]	Nucleotide diversity π[2]	Simpson's Index of Diversity	phi test[3]
ST[4]	169	594	647	0.042	0.0177	0.988	$p<0.01$[5]
aroC	81	82	84	0.024	0.0105	0.952	p=0.49
dnaN	86	72	79	0.018	0.0127	0.959	$p<0.001$[5]
hemD	66	71	77	0.209	0.0123	0.963	p=0.19
hisD	94	101	109	0.033	0.0211	0.959	$p=0.001$[5]
purE	76	90	104	0.065	0.0205	0.936	$p<0.001$[5]
sucA	67	85	94	0.007	0.0123	0.943	p=0.40
thrA	72	95	103	0.007	0.0170	0.958	p=0.14

[1]Ratio of nonsynonymous to synonymous substitutions
[2]Calculated by the method of Nei and Miller
[3]Pairwise homoplasy test
[4]Values were obtained for concatenated sequences
[5]Significant probability of recombination

There were 121 STs that were represented by single isolates. ST 19 was represented by 23 isolates: 20 were serovar Typhimurium or were closely related antigenically to Typhimurium. ST 11 contained 17 different isolates, all of which were Enteritidis or expressed variations of the Enteritidis O antigens. Two STs contained 8 isolates, 2 STs contained 7 isolates, and 3 STs contained 6 isolates and 5 isolates each. Five STs contained 4 different isolates, 15

contained 3 isolates, and 16 contained 2 isolates. There was a strong correlation between ST and serovar, regardless of geographic origin (Table 2-3). However, there were a number of exceptions to the serovar-ST association (Table 2-4).

Fifty-six serovars were represented by multiple isolates. Some serovars were consistently associated with a single ST or closely related STs. The largest group was serovar Enteritidis with all 14 isolates belonging to ST 11. Two other ST 11 isolates were serotyped as Group D, which is characterized by the same dominant O antigen as serovar Enteritidis. In addition, serovar Gallinarium, which is antigenically related to Enteriditis, was represented by a single isolate which was a double locus variant of ST 11. Serovar Typhimurium had 16 isolates of ST 19 and 4 isolates which were each a single locus variant of ST 19 (ST 34, ST 313, and 2 isolates of ST 328). Four other ST 19 isolates were closely related by serovar to Typhimurium, being monophasic or non-motile variants with the same antigenic formulas as Typhimurium or belonging to Group B, which is characterized by the same dominant O antigen as serovar Typhimurium. Forty-four serovars were represented by multiple isolates with the same ST or STs that differed at one or two loci (Table 2-3).

Some serovars appear to be polyphyletic (Table 2-5). Of the 7 serovar Newport isolates examined, 3 had identical STs (ST 118), 2 isolates differed from ST 118 at all 7 loci but had 3 alleles in common among them (ST 31 and ST 45), and 1 isolate was different from all other serovar Newport isolates at all 7 loci (ST 156). Similarly, 3 serovar Paratyphi B dT^+ isolates were identical at all 7 loci (ST 43), 2 isolates were single locus variants of ST 43 (ST 307), 2 isolates differed from ST 43 at all 7 loci but were single locus variants of each other (ST 88 and ST 127), and 1 isolate was a 7 locus variant of all Paratyphi B dT^+ isolates (ST 19). Serovar Montevideo shows a complicated pattern of relatedness. ST 138 contains 2 isolates; 2 other

isolates are single locus variants of each other that share 2 alleles with ST 318 (ST 81 and ST 316) and 1 further isolate (ST 4) has 3 loci in common with those in ST 138, 5 loci in common with ST 81 and 4 loci in common with ST 316.

In addition to the 3 polyphyletic serovars, 16 other serovars contained a total of 27 isolates in which the ST was not consistent with what would be predicted based on the serovar. Seven of these serovars were also represented by other isolates in the collection in which serovar and ST were consistently associated (for example, serovar Derby has 3 isolates of ST 40, 1 single locus variant ST 39, and 1 isolate ST 71, which is a 7 locus variant of the other 4 serovar Derby isolates). The remaining 9 serovars are present in 2 or 3 isolates each which are 6 or 7 locus variants, indicating distant genetic relatedness.

Table 2.3 Serovars associated with one ST or closely related STs

Serovar	Main ST (# isolates)	Variant STs (# isolates, # loci difference)
Abony	442 (2)	-
Agona	13 (6)	-
Albany	292 (2)	-
Anatum	64 (6)	-
Berta	435 (3)	-
Bovismorbificans	142 (3)	-
Branderup	22 (4)	-
Brandenburg	65 (3)	334 (1, SLV)
Chandans	465 (2)	-
Chester	411 (1)	343 (1, DLV) , 436 (1, DLV)
Choleraesuis	145 (2)	66 (1, SLV)
Derby	40 (3)	39 (1, SLV)
Dublin	10 (3)	-
Eastbourne	24 (1)	410 (1, DLV)
Enteritidis	11 (14)	-
Goldcoast	358 (3)	-
Hadar	33 (6)	-
Heidelberg	15 (9)	-
Hvittingfoss	446 (2)	-
Group C (8,20:r:-)	292 (2)	-
Infantis	32 (7)	-
Isangi	216 (1)	335 (1, SLV), 336 (1, DLV), 337 (1, SLV)
Javiana	24 (3)	432 (1, SLV), 589 (1, SLV)
Kentucky	152 (4)	-
Kiambu	309 (2)	-
Litchfield	449 (1)	453 (1, SLV)
London	155 (3)	-
Mbandaka	413 (4)	-
Muenchen	112 (3)	82 (2, SLV)
Napoli	474 (1)	542 (1, DLV)
Ohio	329 (2)	-
Panama	48 (3)	-
Paratyphi A	129 (3)	85 (2, SLV)
Rissen	469 (3)	-
Saintpaul	27 (2)	49 (1, SLV), 50 (3, SLV)
Sandiego	20 (1)	126 (1, DLV)
SS I 6,8:r:-	142 (2)	-
SS IV Group V O:44	433 (2)	-
Stanley	29 (4)	-
Schwarzengrund	96 (4)	-
Thompson	26 (1)	459 (1, SLV)
Typhimurium	19 (16)	34 (1, SLV), 313 (1, SLV), 328 (2, SLV)
Virchow	16 (4)	-
Weltevreden	365 (3)	-

SLV, single locus variant
DLV, double locus variant

Table 2-4 Inconsistent sequence type and serovar correlation

Serovar	Number of Isolates	STs
Bareilly	2	203, 464 (7 locus variants)
Derby*	1	71 (7 locus variant of others)
Eastbourne*	1	414 (7 locus variant of others)
Group B (4,5,12:i:-)	2	19, 142 (7 locus variants)
Havana	2	578, 588 (7 locus variants)
Heidelberg*	1	32 (6 locus variant)
Hvittingfoss*	2	434, 438 (7 locus variants of others)
Kottbus	2	30, 582 (7 locus variants)
Livingstone	3	457, 543, 586 (7 locus variants)
Mississippi	2	425, 448 (7 locus variants)
Oranienburg	2	23, 407 (7 locus variants)
Poona	2	308, 447 (6 locus variants)
Saintpaul*	1	95 (7 locus variant of others)
Sandiego*	1	95 (7 locus variant of others)
Stanleyville	2	444, 591 (7 locus variants)
Thompson*	1	52 (6 locus variant of others)

*Variants of these serovars are consistent in ST-serovar correlation

Table 2-5 Polyphyletic serovars

Serovar	ST	aroC	dnaN	hemD	hisD	purE	sucA	thrA	# of isolates
Montevideo	138	11	41	55	42	34	58	4	2
Montevideo	4	43	41	16	13	34	13	4	1
Montevideo	81	43	41	16	42	35	13	4	1
Montevideo	316	43	41	16	42	35	13	111	1
Newport	31	2	2	15	14	15	20	12	1
Newport	45	10	7	21	14	15	12	12	1
Newport	118	16	2	45	43	36	39	42	3
Newport	156	63	14	6	12	5	14	58	1
Newport	559	84	7	25	197	167	71	3	1
Paratyphi B dT^+	43	2	14	24	14	2	19	8	3
Paratyphi B dT^+	307	2	14	24	14	2	19	107	2
Paratyphi B dT^+	19	10	7	12	9	5	9	2	1
Paratyphi B dT^+	88	46	44	46	46	38	18	34	1
Paratyphi B dT^+	127	46	54	46	46	38	18	34	1

2.4.2 Evidence for Clonality

MLST sequences were tested for evidence of clonality and recombination. The results indicate that the population structure of S. enterica has both clonal and recombinational aspects

Concatenated sequences were tested for linkage disequilibrium. The sequence was 3,336 nucleotides long with 546 polymorphic sites resulting in 148,785 pairwise comparisons. The number of significant pairwise comparisons by the chi-square test was 29,395 and was 14,503 after applying the Bonferroni correction for multiple comparisons. Linkage disequilibrium is a non-random association of mutations which occurs in a clonal population because recombination does not act to break up the linkage of mutations arising at different loci within a clonal group of organisms. Significant linkage disequilibrium supports the supposition that *S. enterica* is predominantly clonal in population structure.

However, while the six subspecies of *S. enterica* are considered to belong to the same species, genetic distances may effectively prevent recombination between them [24]. Therefore the test for linkage disequilibrium was repeated after removing isolates clustering with Subspecies II, III, and IV (M10, M17, M23, M27, M31, M32, M33, M54, M83, and M98). This resulted in a lower value for linkage disequilibrium (408 polymorphic sites resulting in 83,028 pairwise comparisons, number of significant pairwise comparisons by chi-square test 10,049, using the Bonferroni procedure, 4,985). This result indicates that much of the linkage disequilibrium was due to inclusion of isolates not from Subspecies I, and therefore the evidence for exclusive clonality in Subspecies I is weaker.

Linkage disequilibrium was also investigated using the standardized Index of Association (sI_A) for the multilocus data set. The value of sI_A is 0 for a population in linkage equilibrium and is 1 for complete linkage disequilibrium [79]. The value of sI_A, as calculated by LIAN for this

data set, was 0.2299, so this value indicates a degree of disequilibrium. LIAN tests the null hypothesis that the data set is in linkage equilibrium. For this data set, the null hypothesis was not supported, at a $p<0.01$, indicating statistically significant linkage disequilibrium.

2.4.3 Evidence for Recombination

Although the results of linkage disequilibrium support a clonal population structure in *S. enterica*, tests for recombination were also undertaken and their results indicate that evidence of recombination is also present in this data set. Split decomposition, which does not assume that the data fit a tree-like structure, allows the conflicting phylogenetic signals in the data that are characteristic of recombination, to be visualized [85, 86]. SplitsTree4 was used to create a network representation of the relationships contained in the concatenated sequence. Neighborhood net graphs were created for all 7 loci and for the concatenated sequences (Figure 2-1). These graphs all demonstrated some degree of network structure, indicative of recombination. In addition, a splits network analysis of concatenated sequence showed a star shaped structure indicative of recombination (star pylogeny) (Figure 2-2) [85].

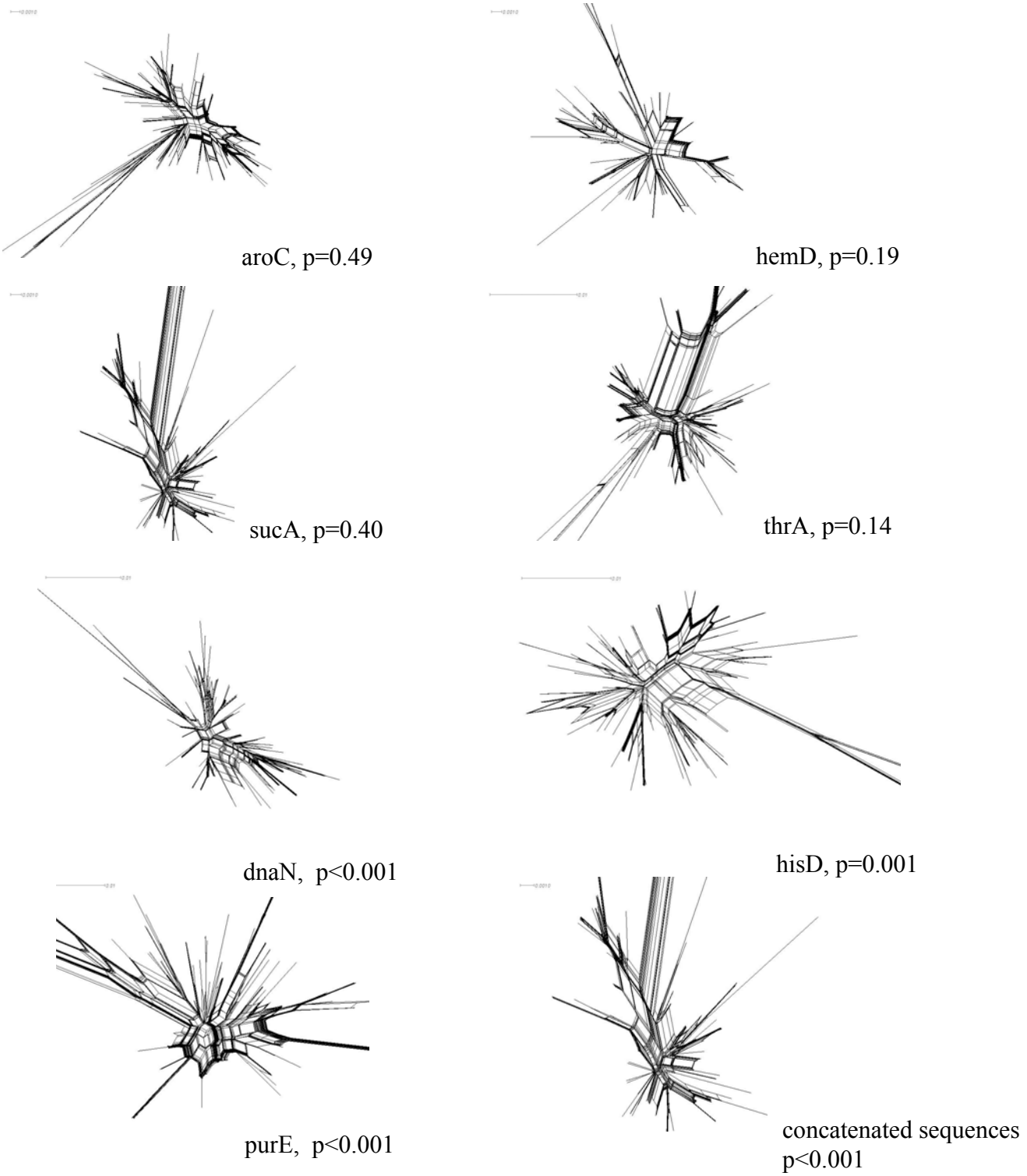

Figure 2-1 Neighborhood net graphs

Neighborhood net graphs of sequences of 7 loci and concatenated sequences, showing network structure characteristic of recombination. Loci aroC, hemD, sucA, and thrA did not show statistical evidence for recombination by the pairwise homoplasy test PhiTest (p>0.05) while loci dnaN, hisD, and purE, and concatenated sequences did show statistically significant recombination (p <=0.001).

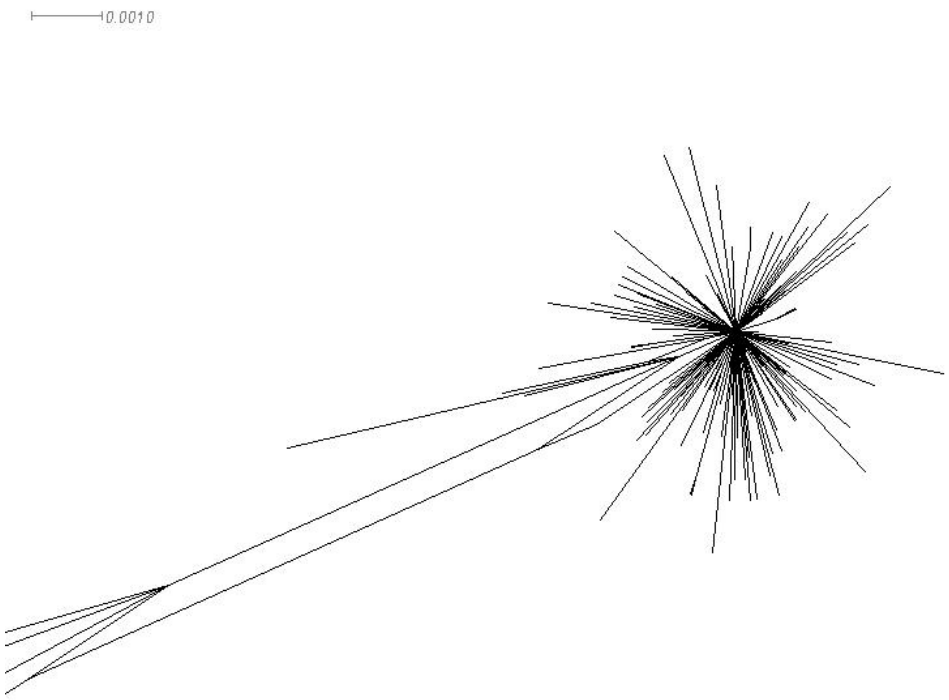

Figure 2-2 Split decomposition graph

Split decomposition graph of concatenated sequences of 7 loci, showing star shaped pattern characteristic of data sets in which phylogenetic signal is obscured by recombination.

Recombination in the concatenated sequences was estimated using the pairwise homoplasy test PhiTest in SplitsTree4. Statistically significant evidence for recombination was found in the concatenated sequence at $p<0.01$. Not all individual loci showed significant recombination (Table 2-2). Sequences from the loci dnaN, hisD, and purE showed highly significant recombination, as did the concatenated sequences, while the other 4 loci did not (p ranged from 0.14 to 0.49) (Figure 2-1). The minimum number of recombination events in the history of the concatenated sequences, RM, was estimated at 94 for the total collection and 67 for STs from Subspecies I. For comparison, *Neisseria meningitidis* is considered to be highly recombinant. Using the same test, RM was estimated for sequences obtained from the *Neisseria*

meningitidis MLST database (http://pubmlst.org/neisseria/). RM for the 7 loci was 374. This result indicates that *S. enterica* is not exclusively clonal, but is also not as recombinant as *N. meningitidis*.

2.4.4 Maximum Likelihood Grouping of STs

Concatenated sequences of the 7 loci were used for creating a ML tree to explore genetic relatedness of STs (Appendix B). Some STs cluster distantly from the main group. The majority of these STs belong to isolates that were typed as subspecies II, III, IIIb and IV which would be expected to be genetically different from subspecies I. The majority of STs associated with Subspecies I isolates show very little segregation into well defined groups. Rather, many minor clusters of related isolates are evident in the data.

A radial tree depiction of the ML tree obtained from the unique STs of isolates in this study presents a similar picture, with the isolates being divided into five main groups (Figure 2-3). Most serotypes were present in only one group, which is not surprising, considering that a large number of serotypes were represented by only one isolate. STs of Subspecies II, III, IIIb, and IV were closely related to each other and less closely related to the majority of Subspecies I. Polyphyletic serovars such as Newport appear in more than one group. In this data, the correlation of ST with serovars in Subspecies I shows an extent of diversity in housekeeping gene sequence that is comparable to the diversity in genes responsible for determining serovar.

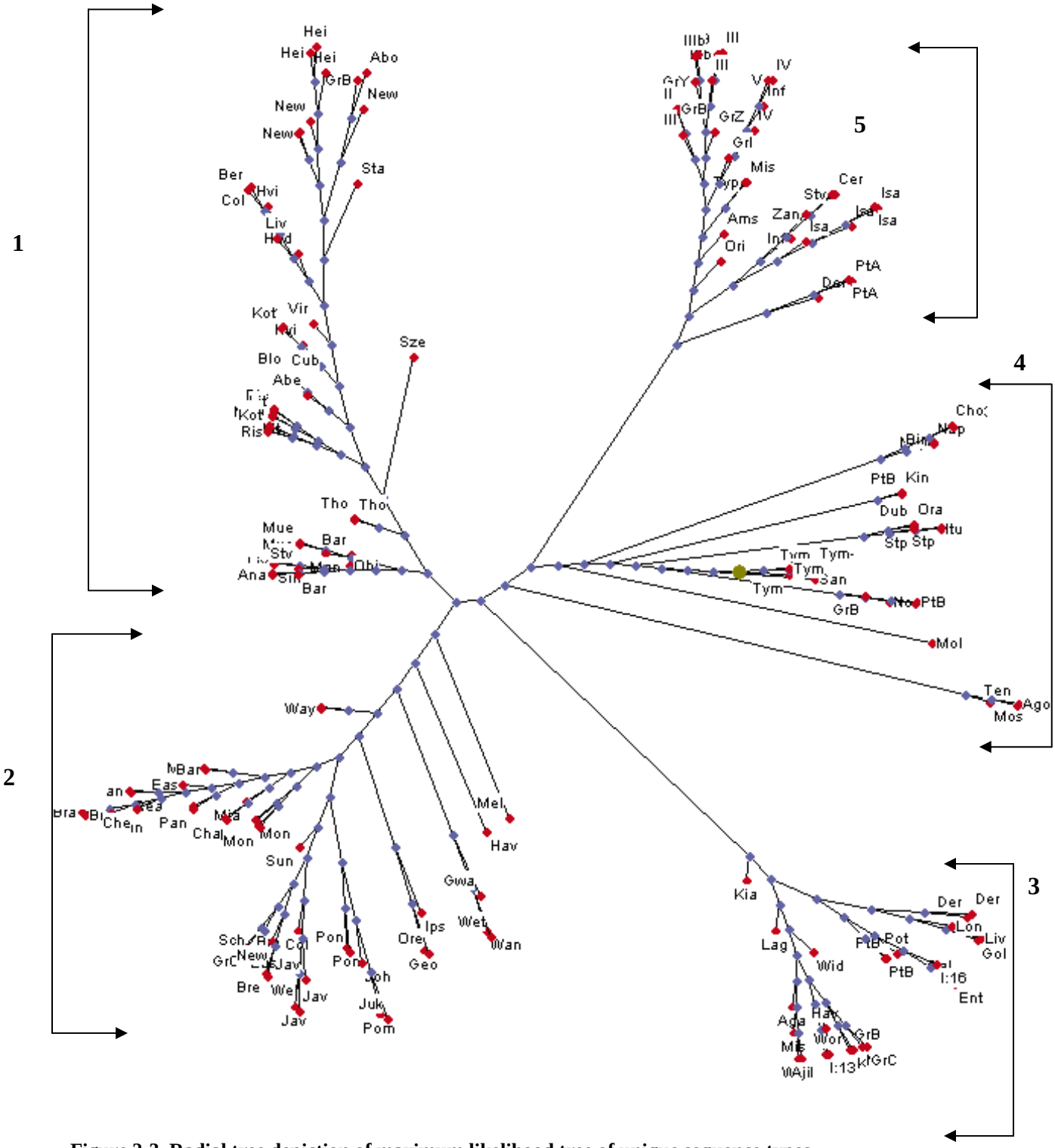

Figure 2-3 Radial tree depiction of maximum likelihood tree of unique sequence types
Tree of unique sequence types (STs) from 169 *S. enterica* isolates, labeled by serovar (key in Appendix C).

2.5 DISCUSSION

In *S. enterica,* a concordance of serovar and whole genome has been previously noted [87] but was not supported by multilocus enzyme electrophoresis studies [14]. The results of this study indicate that serovar is closely related to ST in *S. enterica*. Serovars Enteritidis (ST 11) and Typimurium (ST 19) are quite consistent in their association with a single ST and that association holds in isolates obtained globally. A similar consistency appears in serovars represented by fewer numbers of isolates in this study. This is a particularly interesting finding in the case of *S. enterica*, which manifests a very large number of different serotypes. Since housekeeping genes are not under antigenic selective pressure, they might be expected to be less diverse than the genes encoding serovar, but this is not the case. The large number of STs and the strong correlation of serovar and ST argue that the forces creating serovar, whether mutation or recombination, are acting at a similar level on the whole genome.

Although in our results, isolates of the same serovar usually belong to the same or a few related STs, in some cases, STs of isolates with the same reported serovar are vastly different, both by allele number and also by differences in concatentated sequences, from the most common ST for that serovar. Some serovars, notably Newport and Paratyphi B, appear to be polyphyletic. Paratyphi B contained 2 unrelated lineages. Serovar Newport contained 4 unrelated STs but 2 of these contained only 1 isolate, so these may not reflect established lineages. Serovar Montevideo demonstrates an interesting and complicated pattern of relatedness. ST 4 potentially diverged by mutations into 2 different lineages represented by ST 138 and the single locus variants ST 81 ands ST 316.

In a number of cases, serovar and ST seem to have no correlation; some serovars represented by two or three isolates have one or no loci in common and a number of serovars

show a consistent relationship with ST that is broken by one divergent isolate. These mismatches in ST-serovar correlation may reflect adaptations to escape immunity, either by mutation or recombination or they may represent stable lineages that are underrepresented in the isolate collection used in this study.

Although the surface antigens that determine a particular serovar may be the same, organisms belonging to these serovars may be quite different in their core genome. This may be due to either horizontal transfer of serovar determinants or to mutation due to intense selective pressure. In these instances, serovar information can be misleading in depicting relatedness in genetically distinct populations and ST would be correspondingly misleading in predicting serovar.

Moreover, serotyping results are often unreliable. In a 2002 study in which serovars were determined for eight isolates by a number of different laboratories, the results were only 73% accurate [68]. Although the isolates in that study had common and easily identifiable serovars, only 9 (30%) of the 30 participating laboratories typed all 8 isolates correctly. For these reasons, serovar is a less than ideal measure of relatedness in *S. enterica* isolates, and ST may provide a more reliable and reproducible measure.

S. enterica was originally believed to have a clonal population structure [8, 22, 23], although recent evidence has modified this view [18, 24-26]. Studies of *Escherichia coli*, which is evolutionarily and ecologically closely related to the *Salmonellae*, indicate that, although the *E. coli* population structure is predominantly clonal, recombination does occur [88, 89]. Our study uses MLST data to confirm a similar situation in *S. enterica*. Clonality is supported by linkage disequilibrium in mutations in the concatenated sequence of the 7 MLST loci by the chi-square test with the Bonferroni correction for multiple comparisons. In this case, where

comparisons are not independent, the use of the Bonferroni correction should result in a conservative estimate of significance. However, split decomposition and recombination tests indicate that recombination is also evident in the isolates included in this collection.

The results of this study reinforce the diverse genetic character of *S. enterica* and help validate the usefulness of MLST in exploring phylogeny and accomplishing surveillance for that organism. Because the isolates included in this study were extremely varied in genetic background, as evidenced by the number of serovars and STs, they present a more complete picture of the diversity of *S. enterica* population structure than previous studies using more genetically limited collections. The concordance of ST with serovars suggests the possibility that whatever forces have caused the great diversity in genes encoding serovar have also acted to cause similar diversity in housekeeping genes. This diversity may be a result of recombination, mutation, or both. It has been suggested that isolates in which DNA mismatch repair systems are damaged may contribute significantly to the extent of recombination and therefore to diversity in S. enterica [26]. This intriguing hypothesis deserves further investigation. Although recombination appears to occur, the long-term existence of stable clones, such as ST 19 (usually serovar Typhimurium) and ST 11 (usually serovar Enteritidis) indicates that fit clones can persist over long periods of time. The consistency in serovar-ST concordance in this study supports the hypothesis that *S. enterica* exists as a large number of stable lineages, in which events such as mutation, recombination, and selection act to create new clonal lineages that differ from their ancestors in both antigenic and housekeeping genes. Our results validate the use of MLST for typing large and varied collections of *S. enterica* for which other existing typing schemes are inappropriate.

3.0 HORIZONTAL GENE TRANSFER AND CLONAL EXPANSION OF INTEGRON MEDIATED MULTIPLE DRUG RESISTANCE IN A GLOBAL COLLECTION OF NON-TYPHOIDAL *SALMONELLA ENTERICA*

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3.1 ABSTRACT

Salmonella enterica has become increasingly resistant to antibiotics, partly as a result of genes carried on integrons. Both clonal expansion and horizontal gene transfer may contribute to the spread of antibiotic resistance integrons in this organism. We investigated antibiotic resistance and integron carriage among 90 isolates with the ACSSuT phenotype (resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) in a global collection of *S. enterica* isolates. Four integrons, *dfrA12/orfF/aadA2*, *dfrA1/aadA1*, *dfrA7*, and *arr2/blaOXA30/cmlA5/aadA2*, were found in genetically unrelated isolates from 8 countries on 4 continents, supporting a role for horizontal gene transfer in the global dissemination of *S. enterica* multiple drug resistance. Typhimurium isolates containing identical integrons with the gene cassettes *blaPSE1* and *aadA2* were found in 4 countries on 3 continents, supporting the role of clonal expansion. This study demonstrates that both clonal expansion and horizontal gene transfer contribute to the global dissemination of antibiotic resistance in *S. enterica*.

3.2 INTRODUCTION

Salmonella enterica is a leading cause of food borne disease world-wide [90, 91]. In the United States, the Centers for Disease Control and Prevention estimates that there are as many as 1.4 million cases of *S. enterica* associated disease annually [92, 93]. While usually self-limiting, salmonellosis may require antimicrobial treatment in the very young, very old or those who are immunocompromised. However, antibiotic resistance has become increasingly common in *S. enterica*, which can complicate therapy. The National Antimicrobial Resistance Monitoring System (NARMS) reported that in 2004, 15.0% of non-Typhi isolates were resistant to 2 or more classes of antibiotics and 8.1% were resistant to 5 or more classes. The most common *S. enterica* multiple drug resistance pattern in 2004 was ACSSuT (resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) [65, 94].

Antibiotic resistance can occur by point mutations in the bacterial genome or through through the horizontal transfer of genetic elements carrying resistance genes. Resistance may be disseminated through clonal expansion of resistant strains or through horizontal transfer of genetic elements coding for resistance determinants. *S. enterica* populations change through the introduction of strains that expand and displace existing populations [6, 95]. Such population dynamics enable antibiotic resistance in *S. enterica* to spread as a result of clonal expansion. The global dissemination of the multidrug resistant (MDR) *S. enterica* serotype Typhimurim phage type DT104 clone is an example of the role of clonal expansion in the spread of antibiotic resistance determinants across multiple countries and continents [3]. Clonal expansion is also

probably responsible for the dissemination of nalidixic acid resistance in *S. enterica* ser. Typhi isolates obtained in southern Asia and Africa [96].

Horizontal transfer of genetic material among *S. enterica* or from other bacterial species also plays an important role in the dissemination of drug resistance in this pathogen [34]. Evidence indicates that horizontal gene transfer plays a major role in the dissemination of antibiotic resistance in other bacterial species, such as *Escherichia coli* [97] and *Stenotrophomonas maltophilia* [98]. The location of antibiotic resistance genes on mobile genetic elements, such as plasmids, transposons, and integrons, facilitates the mobilization of antibiotic resistance from one organism to another [32].

Integrons are genetic structures capable of capturing and excising gene cassettes, which usually encode antibiotic resistance determinants. While integrons are not self-mobilizable, they are usually found associated with transposons and are often located on plasmids, facilitating their mobility [32]. Integrons are thus ideally suited for the dissemination and recombination of antibiotic resistance genes. Integrons are common in *S. enterica*, and make an important contribution to the extent of antibiotic resistance in this species [32-34]. Because of their plasmid and transposon association, integrons are assumed to be mobilized predominantly through horizontal gene transfer [34]. However, the clonal nature of *S. enterica* suggests that clonal expansion may also play a role in dissemination of drug resistance. An example of clonal expansion of integron bearing *S. enterica* is the global distribution of the serovar Typhimurium DT104 clone, which harbors a genetic resistance island known as the Salmonella Genomic Island 1 (SGI1). This region contains a number of resistance elements including 2 integrons with the gene cassettes *blaPSE1* and *aadA2* and genes for tetracycline and chloramphenicol resistance which are not integron associated [62].

Clonal expansion of integron-bearing *S. enterica* would account for the occurrence of a particular genetic lineage with a specific integron in a variety of geographic regions. Horizontal gene transfer would account for the existence of identical integrons in isolates of different genetic lineages. In order to explore the roles of clonal expansion and horizontal gene transfer in the dissemination of antibiotic resistance due to integrons, we investigated the integron structure and genetic lineage of 90 MDR *S. enterica* isolates from a global collection comprised of over 1,900 isolates from 13 countries and 6 continents. A goal of this study was to improve our understanding of the contributions of clonal expansion and horizontal gene transfer to the dissemination of integrons carrying antibiotic resistance genes in *S. enterica* to enable the development of improved strategies for the control of antibiotic resistance in this organism as well as other emerging pathogens of public health importance.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial Isolates

A total of 1,920 *S. enterica* isolates were investigated. 1,743 consecutive isolates were collected by laboratories in Argentina, Australia, Belgium, Canada, Denmark, Germany, Italy, the Philippines, South Africa, Spain, Taiwan, Uganda and the USA, between September 2001 and August 2002 (Table 3-1). These isolates were collected as part of a separate study that aimed

to identify a genetically and geographically diverse group of *Salmonella enterica* isolates with reduced susceptibilities to the fluoroquinolones. The isolates were not selected but rather were collected consecutively without regard to antibiotic susceptibility. In addition, 179 isolates were collected by the Allegheny County Department of Health in 2002 and 2003 as part of routine public health surveillance. Serotyping was performed by the collecting laboratories, except for isolates from Taiwan, which were serotyped by the Pennsylvania Department of Health.

Table 3.1 Laboratories that provided *S. enterica* isolates

Country	Institution	Contact Person
USA	CDC Foodborne and Diarrheal Diseases Laboratory Section	Timothy J. Barrett
Canada	Ontario Public Health Laboratory	Frances Jamieson
Canada	Laboratory for Foodborne Zoonoses, Population and Public Health Branch	Cornelius Poppe
Argentina	Centro de Estudios en Antimicrobianos	Jose Maria Casellas
Australia	Queensland Health Scientific Services	John Bates
Belgium	Antwerp University Hospital	Herman Goossens
Germany	BGVV, Berlin	Andreas Schroeter
South Africa	South African Institute for Medical Research	Karen Keddy
Spain	Institute of Health Carlos III, Enteric Bacteria Laboratory	Miguel Usera
Italy	Istituto Superiore di Sanita, Rome	Alessandra Carattoli
Denmark	Hvidovre Hospital, Copenhagen	Dennis Hansen
Taiwan	National Cheng Kung University	Wen-Chien Ko

The ACSSuT resistance phenotype has become increasingly prevalent in *S. enterica* and that phenotype has been commonly associated with integron carriage in this species. For these reasons, we selected a subset of isolates from the collection that exhibited the ACSSuT resistance phenotype for further investigation. Isolates selected for integron investigation were confirmed to be *S. enterica* by PCR with primers specific for the *invA* region of the *inv* locus [99] (Table 3-2).

3.3.2 Antibiotic Resistance Testing

Antibiotic resistance was determined using the disc diffusion method on Mueller–Hinton agar according to the manufacturer's directions (Becton, Dickinson and Co, Sparks, MD). Susceptibility to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline was determined using the manufacturer's breakpoints.

3.3.3 Integron Detection and Characterization

Genomic DNA from *S. enterica* isolates was prepared using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer's directions. Integron carriage was determined by PCR using primers specific to the *intI* region of the integrase gene. Isolates positive for integrase were further characterized by PCR using primers specific for the 5' and 3' conserved segments (CS) of the integron structure. PCR was performed in a 50 µl volume consisting of 1x PCR Buffer II (Applied Biosystems, Foster City, CA), 1.5mM $MgCl_2$, 0.1mM dNTPs, 0.33µM forward and reverse primers, and 1.66 units Amplitaq Gold polymerase

(Applied Biosystems, Foster City, CA). PCR conditions were: initial denaturation 95° C for 5 minutes, 35 cycles of 95° C for 1 minute, 58° C for 1 minute, 72° C for 5 minutes plus 5 seconds each cycle, final extension 72° C for 7 minutes. Long range PCR was performed to detect integrons larger than 2.0 kb, using the Gene Amp HiFidelity Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's directions. Long range PCR conditions were: initial denaturation for 2 minutes at 94° C, 10 cycles of 94° C for 15 seconds, 58° C for 30 seconds, 68° C for 4 minutes, followed by 20 cycles of 94° C for 15 seconds, 58° C for 30 seconds, 68° C for 4 minutes plus 5 seconds each cycle, with a final extension of 72° C for 7 minutes. PCR products were subjected to electrophoresis on 1% agarose gels, stained with ethidium bromide and visualized using UV illumination on a Gel Doc 2000 documentation system (Bio-Rad, Hercules, CA). For isolates that amplified multiple integrons, PCR products were separated by gel electrophoresis and purified using either the Qiaquick Gel Extraction Kit (Qiagen) or the Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Column Kit (BioRad). Some PCR products were cloned prior to sequencing using the Topo TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) following the manufacturer's directions.

Isolates in this study were investigated for the presence of the Salmonella Genomic Island 1 (SGI1) and variant SGI1s by PCR using published primers specific for the right (104-RJ, 104-D) and left (U7-L12, LJ-R1) junctions of the chromosomal insertion site [100]. Isolates were considered to be positive for the left or right junction of the SGI1 if they generated PCR product of the appropriate size with primers specific for that junction. Using this method, isolates with the SGI1 would be positive for the left junction but not for the right junction due to the presence of a retronphage between the 104-RJ and 104-D.

3.3.4 Gene Cassette Identification

Class 1 integron gene cassettes were identified by PCR and sequence analysis using the 5' and 3' CS primers. Sequencing was performed with the BigDye terminator 3.1 kit (Applied Biosystems) according to the manufacturer's instructions. Capillary sequence analysis was performed on a 3730 DNA sequence analyzer (Applied Biosystems). Sequences were analyzed and additional primers were designed using the Lasergene 7.0.0 software package (DNAStar, Madison, WI). Gene cassette homology searches were performed using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) [101].

Ninety-one of the 121 integrons found in this study were sequenced in their entirety. In some cases, when integrons were identified that were of the same size as ones previously sequenced, their gene cassettes were identified by PCR using primer pairs designed in this study. Primers used in this study are listed in Table 3-2.

Table 3.2 Primers

Primer	Sequence(5'-3')	Target	Reference
5'CS	GGCATCCAAGCAGCAAGC	5' conserved segment	Levesque[102]
3'CS	AAGCAGACTTGACCTGAT	3' conserved segment	Levesque[102]
int_F	CGATGCGTGGAGACCGAAACCTT	intI1	Maguire[9]
int_R	GTAACGCGCTTGCTGCTTGGATGC	intI1	Maguire[9]
invA_F	ACACAGCTCGTTTACGACCTGAAT	invA	Rahn[99]
invA_R	AGACGACTGGTACTGATCGATATT	invA	Rahn[99]
sul1_F	GCGCGGCGTGGGCTACC	sul1	This study
sul1_R	CCGCAAGGCTCGCTGGAC	sul1	This study
aadA1_R	CGATGACGCCAACTACCTCTGATA	aadA1internal primer	This study
arr2_F	ATTGTTGGCGTTGTTGAAGACTGG	arr2 internal primer	This study
cmlA5_F	GAATGGGAATGGGATGCCTGATAG	cmlA5 intrnal primer	This study
oxa10_R	TTTACAAAGCACGAAGACACCATT	blaOXA10 intrnal primer	This study
cmlA_F	GCAGGTCGCGAGGAAAGTAATG	cmlA 5' forward primer	This study
cmlA_R	ACACCGCCCAAGCAGAAGTAGA	cmlA 3' reverse primer	This study
blaOXA30_F	TCGCAAGAAATAACCCAAAAA	blaOXA30 internal primer	This study
aacA4_F	AAGCGGGGTTTGAGAGG	aacA4 forward primer	This study
aacA4_R	CGCGTACTCCTGGATCGGTTTCTT	aacA4 reverse primer	This study
dfr_1_F	TTTAGGCCAGTTTTTACCCAAGAC	dfr1 internal primer	This study
ere_est_R	GCGCCAGCAGAATTATCCTTACAT	ereA2 internal primer	This study
aac(6')IIC_F	CCGCGGGATTGACCAGT	aac(6')IIC internal primer	This study
dfrA12_F	GCTGCGCATTTTGGTTCC	dhrA12 internal primer	This study
aadA2_R	TGTCATTGCGCTGCCATTCTCC	aadA2 internal primer	This study
qacH_F	GCGTCGCCGTTCTAAATCTGCTAT	qacH internal primer	This study
aac_R	GGGCGCCGGGTGTCTGGAG	aacA4 internal primer	This study
IS_F	GTCACGCCCCGACCATCACCTTCC	IS1247 internal primer	This study
TNP_F	CCGCGCTGGCCGACCTGAAC	transposase A internal primer	This study
ere_F	CCTAACCGGGCGATTCAA	erythromycin esterase internal primer	This study
cmlA_R_internal	ATCACACGCCCCATAAAACGAG	cmlA internal primer	This study
arr_R2	GCGGGATCCAGAACCAGGCGACAT	arr-2 internal primer	This study
arr_accA_R	AGAGCGGCTTTGGTTCC	internal primer arr-2—accA junction	This study
ere_F2	CGCTGATTTCGCTGTCCTGA	ereA internal primer	This study
dfrA17_F	AAAAAGGCTAACAAGTCGT	dfrA17 internal primer	This study
cml_R2	GCTGAATTGTGCTCGCTGTCGTA	cml internal primer	This study
aadA_con_F	CGACATCATYCCGTGGCGTTAT	aadA forward consensus primer	This study
aadA_con_R	CGGCAGCCACATCCTTC	aadA reverse consensus primer	This study
aacA4_F	ATGACCTTGCGATGCTCT	aacA4 internal primer	This study
aacA4_R	CTCGATGGAAGGGTTAGG	aacA4 internal primer	This study
blaOXA30_F	ACACAATACATATCAACTTCGC	blaOXA30-aadA internal primer	This study
aadA1_R_S	GGATAACGCCACGGAATGATGTC	aadA1 internal primer	This study
albany_PSE1a_F	CCTTTGGGGCCACCTACAG	blaPSE1 primer	This study
albany_PSE1b_F	ATCAAAATTATGGGGTTACTTACA	blaPSE1 primer	This study
albany_dfr1_F	ATGGTAGCTATATCGAAGAATGGA	dfr primer	This study
albany_dfr2_F	AAGTACTGGCTATTGCCTTAGGAG	dfr primer	This study
U7-L12	ACACCTTGAGCAGGGCAAAG	SGI1 left junction	Mulvey[62]
LJ-R1	AGTTCTAAAGGTTCGTAGTCG	SGI1 left junction	Mulvey[62]
104-RJ	TGACGAGCTGAAGCGAATTG	SGI1 right junction	Mulvey[62]
C9-L2	AGCAAGTGTGCGTAATTTGG	SGI1 right junction	Mulvey[62]
104-D	ACCAGGGCAAAACTACACAG	SGI1 right junction	Mulvey[62]

3.3.5 Multilocus Sequence Typing

Genetic relatedness of isolates was assessed using multilocus sequence typing (MLST). MLST uses sequences of approximately 500 bp in length from seven housekeeping genes to define a sequence type (ST). Isolates with the same alleles at all seven loci are considered to be genetically indistinguishable by MLST and therefore define an ST. Isolates with the same alleles at six loci are considered to be closely related genetically.

MLST was performed using the seven locus scheme described by the Salmonella MLST database (http://web.mpiib-berlin.mpg.de/mlst) [19-21, 26]. Visualization of PCR products and sequencing of gene fragments was accomplished as described above for integron gene cassettes. Sequences were analyzed using Bionumerics software V 5.10 (Applied Maths, Austin, TX). Alleles and STs were assigned by the *Salmonella* MLST database. All isolates in this study and their associated sequence types have been deposited in the *Salmonella* MLST database.

3.3.6 Definitions of Horizontal Gene Transfer and Clonal Expansion

Horizontal gene transfer was defined as *S. enterica* isolates belonging to different STs (STs that differ at more than one locus) but bearing the same integron. Clonal expansion was defined as *S. enterica* isolates bearing the same integron and belonging to the same ST or STs differing at only one locus, but occurring in more than one geographic location. Because isolates in this study were collected consecutively over a limited period of time in each location, and because geographic source was the only epidemiologic information available, it was not possible to determine if genetically related isolates bearing the same integron in a given location were part of an outbreak or if they reflected clonal expansion beyond an outbreak. Therefore, *S.*

enterica isolates collected from a single geographic location that belonged to the same ST and harbored identical integron structures were considered to be one isolate for classification as either horizontal gene transfer or clonal expansion.

3.4 RESULTS

3.4.1 ACSSuT Resistance

Of the 1,920 isolates initially screened by antibiotic susceptibility testing, 104 (4.9%) exhibited the ACSSuT resistance phenotype. The proportion of ACSSuT-resistant isolates ranged from zero in Australia, Argentina, Belgium, and Canada to 19% in Taiwan and South Africa (Table 3-3).

Table 3.3 Geographic source and ACSSuT resistance in the isolate collection

Geographic Source	No. Isolates	No. (%) ACSSuT resistant
Argentina	148	0 (0.0)
Australia	146	0 (0.0)
Belgium	66	0 (0.0)
Canada	144	0 (0.0)
Denmark	153	8 (5.2)
Germany	150	1 (0.7)
Italy	156	3 (1.9)
Philippines	67	6 (8.9)
Spain	151	8 (5.3)
South Africa	160	30 (18.8)
Taiwan	150	29 (19.3)
USA/ACHD	179	8 (4.5)
USA/CDC	150	1 (0.7)
Uganda	100	10 (10.0)
TOTAL	1920	104 (5.4)

3.4.2 Integron Detection and Characterization

Of the 104 *S. enterica* isolates with the ACSSuT resistance phenotype, 90 (86.5%) were positive for the integrase gene and amplified gene cassette regions by PCR with primers for integron 5'CS and 3'CS regions. The majority of cassette positive isolates contained either one (n=61, 68%) or two (n=26, 29%) integrons (Table 3-4). Three isolates contained three or more integrons. While a total of 16 different integrons were found in this collection, 19 distinct integron profiles could be identified due to the occurrence of multiple integrons in some isolates (Table 3-4). Six integrons contained only one gene cassette, *aacA4*, *aadA2*, *aadB*, *blaPSE1*, *dfrA7*, or *dfrA15*. Seven integrons contained two or three gene cassettes, *aadB/catB3, aac3A-Id/aadA7*, *blaOXA30/aadA1*, *dfrA12/orfF/aadA2*, *dfrA1/orfC*, *dfrA1/aadA1*, and *tnpA/dfrA7.* A

4.0 kb integron containing the cassettes *arr2/cmlA5/blaOXA10/aadA1*was found alone or in combination with an integron containing the single gene cassette *aacA4*. Two other unique large integrons were found in this collection, a 5.8 kb integron with the gene cassettes *qacH/dfrA17/ereA/aadA2/cmlA/aadA1* and a 6.0 kb integron with cassettes *aac(6')-IIc/ereA2/IS1247/aac3/arr/ereA2*.

A total of 121 class 1 integrons were identified. Of these, 91 were fully sequenced (Table 3-4). With 2 exceptions, point mutations in gene cassette sequences were not observed. One integron containing the single cassette *aadA2* exhibited a single base difference from other *aadA2* gene cassettes found in this study (a T to C transition at position 39 of the gene cassette, resulting in a synonymous change). One *blaOXA30* gene cassette exhibited a point mutation (A to G) at position 31, also a synonymous change. All other gene cassettes within the study showed 100% nucleotide identity to GenBank reported cassettes, except for the *dfrA17* gene cassette identified in the unique 5.8kb integron in the Stanley isolates from Taiwan (Table 3-4). This cassette showed 91% sequence identity to a gene cassette found in uncultured bacteria, GenBank Accession no. FM179325 and in *E. coli* GenBank Accession no. EU687490.The majority of gene cassettes found in this study confer resistance to the aminoglycosides (*aadA1*, *aadA2*, *aadA7*, *aadB*, *aacA4*, *aac*, *aac3A-Id*, *aac(6')-IIc*), trimethoprim (*dfrA1*, *dfrA7*, *dfrA12*, *dfrA15*, *dfrA17*) and ß-lactams (*blaPSE1*, *blaOXA10*, *blaOXA30*). Other resistance cassettes included those coding for chloramphenicol resistance (*cmlA, cmlA5, catB3*), erythromycin resistance (*ereA2*), rifampin resistance (*arr2*) and resistance to quaternary ammonium compounds (*qacH*). Since resistance to the aminoglycoside streptomycin and the ß-lactam ampicillin were selection criteria for isolates in this study, the predominance of cassettes encoding resistance to those antibiotics is not unexpected. Phenotypic resistance to chloramphenicol was also a selection

criterion, but only 3 integrons contained gene cassettes for this resistance. None of the integrons identified in this study carried genes coding for tetracycline resistance, although phenotypic resistance to this antibiotic was also an inclusion criterion. The SGI1 contains genes for chloramphenicol and tetracycline resistance which are not located within integrons, so tetracycline and chloramphenicol resistant isolates that are SGI1 positive most likely contain these genes. Alternatively, resistance to chloramphenicol and tetracycline may be encoded elsewhere on the chromosome or on structures other than integrons in isolates which are not positive for the SGI1.

The SGI1 was identified in 17 isolates (19%). Twelve of the SGI1 positive isolates belonged to ST 19 and exhibited the integron pattern (*blaPSE1*, 1.0 kb and *aadA2*, 1.2 kb) characteristic of serotype Typhimurium phage type DT 104. An additional Typhimurium ST 19 isolate containing four integrons, including *blaPSE1* and *aadA2*, was also positive for SGI1. Four serotype Albany isolates ST 292 were also positive for a variant SGI1 which includes the integrons *dfrA1/orfC* and *blaPSE1* [62]. This result indicates chromosomal location of these integrons in these isolates.

3.4.3 Genetic relatedness of integron bearing *S. enterica*

The 90 integron containing *S. enterica* isolates represented 17 different STs. Thirty-three (37%) Typhimurium isolates belonged to ST19 or to STs that differ from 19 at only 1 locus. These isolates contained 11 different integrons which combined to create 12 different integron profiles (Table 3-4). Eleven (12%) isolates belonged to ST 216 or to STs that differ from 216 at

1 or 2 loci. These isolates contained 3 integrons and 4 integron profiles. The remaining isolates represented diverse STs, which differed from each other at 6 or 7 of the MLST loci.

Table 3.4 Description of integrons and associated serotypes, MLST results, and countries of origin of 90 isolates with the ACSSuT phenotype in this study

Integron Profile	No. Isolates	Serotype	MLST Results							ST (No.)	Country
			aroC	dnaN	hemD	hisD	sucA	purE	thrA		
aacA4	1	Isangi	117	73	41	79	71	65	72	335(1)	South Africa
	1	Isangi	117	73	41	79	71	65	72	335(1)	Uganda
	1	Typhimurium	10	7	12	9	5	9	2	19(1)	South Africa
aac(6')-IIc/ereA2/IS1247/ aac/arr/ereA2	3	Heidelberg	2	7	9	9	5	9	12	15(3)	Philippines
aadA2	1	Isangi	2	73	41	79	71	65	72	216(1)	South Africa
arr2/cmlA5/blaOXA10/aadA1	8	Isangi	2	73	41	79	71	65	72	216(5)	South Africa
			117	73	41	79	71	65	72	335(1)	
			10	73	41	79	71	9	72	336(1)	
			2	73	41	79	71	65	8	337(1)	
	3	Typhimurium	10	7	12	9	5	9	2	19(3)	
blaOXA30/aadA1	1	Typhimurium	116	7	12	9	5	9	2	328(1)	Taiwan
dfrA1/aadA1	1	Goettingen	11	10	13	32	10	13	112	334(1)	Spain
	2	Brandenburg	11	10	13	32	10	13	112	334(2)	
	1	Typhimurium	10	19	12	9	5	9	2	34(1)	Germany
dfrA12/orfF/aadA2	5	Cholerasuis	34	31	35	14	26	6	8	66(4)	Taiwan
			16	16	20	18	8	12	18	29(1)	
	1	Stanley	16	16	20	18	8	12	18	29(1)	
	1	Schwarzengrund	43	47	49	49	41	15	3	96(1)	
	1	Schwarzengrund	43	47	49	49	41	15	3	96(1)	Denmark
	1	Typhimurium	10	7	12	9	5	9	2	19(1)	USA/CDC
	1	Heidelberg	2	7	9	9	5	9	12	15(1)	Philippines
	2	Typhimurium	10	7	12	9	5	9	2	19(2)	South Africa
dfrA7	6	Enteritidis	5	2	3	7	6	6	11	11(6)	Uganda
	2	Enteritidis	5	2	3	7	6	6	11	11(2)	South Africa
	4	Paratyphi A	45	4	8	44	27	9	8	85(4)	Denmark
qacH/dfrA17/ereA/aadA2/ cmlA/aadA1	13	Stanley	16	16	20	18	8	12	18	29(13)	Taiwan
tnpA/dfrA7	1	Enteritidis	5	2	3	7	6	6	11	11(1)	Uganda
aacA4 arr2/cmlA5/blaOXA10/aadA1	6	Typhimurium	10	7	12	9	5	9	2	19(6)	South Africa
aadA2 blaOXA30/aadA2	1	Typhimurium	10	7	12	9	112	9	2	313(1)	South Africa
aadB blaOXA30/aadA1	1	Typhimurium	10	7	12	9	112	9	2	313(1)	South Africa
aadB/catB3 blaOXA30/aadA1	1	Typhimurium	10	7	12	9	5	9	2	19(1)	Taiwan
	1	Typhimurium	116	7	12	9	5	9	2	328(1)	Philippines
blaPSE1 aadA2	4	Typhimurium	10	7	12	9	5	9	2	19(4)	USA/ACHD
	2	Typhimurium	10	7	12	9	5	9	2	19(2)	Spain
	3	Typhimurium	10	7	12	9	5	9	2	19(3)	Italy
	3	Typhimurium	10	7	12	9	5	9	2	19(3)	South Africa
dfrA1/orfC blaPSE1	4	Albany	104	100	54	78	104	9	48	292(4)	Taiwan
dfrA15 blaPSE1 aac3A-Id/aadA7	1	Newport	63	14	6	12	5	14	58	156(1)	Denmark
aadB aadA2 blaOXA30/aadA1	1	Typhimurium	10	7	12	9	112	9	2	313(1)	South Africa
dfrA15 blaPSE1 aadA2 dfrA12/orfF/aadA2	1	Typhimurium	10	7	12	9	5	9	2	19(1)	USA/ACHD

[a]Order of gene cassettes is as listed
[b]Number of integrons sequenced
[c]Positive for SGI1 or variant SGI1
[d]3 *aacA4* cassettes were fully sequenced, *arr2/cmlA5/blaOXA10/aadA1* gene cassettes were identified by partial sequencing and PCR with multiple primer pairs internal to gene casettes

3.4.4 Integron distribution across *S. enterica* genetic lineages

This study identified 5 integrons that were distributed across different genetic lineages, supporting the role of horizontal gene transfer in the dissemination of antibiotic resistance in *S. enterica*.

The integron *dfrA12/orfF/aadA2*, which confers resistance to trimethoprim and streptomycin/spectinomycin, was identified in *S. enterica* from 5 different serotypes belonging to 5 STs (Figure 3-1, Panel A). This integron was geographically widespread, being found in isolates from Europe, the United States, Taiwan, the Philippines, and South Africa. The integron containing the single trimethoprim-encoding *dfrA7* cassette was present in isolates from 2 serotypes, 2 STs and 3 different geographic areas (Figure 3-1, Panel B). The integron *dfrA1/aadA1* was found in isolates from 3 serotypes, 2 STs and 2 different geographic areas (Figure 3-1, Panel C).

Isolates of ST 216 (serovar Isangi) and ST 19 (serovar Typhimirium) from South Africa contained an identical 4.0 kb integron not previously reported in *S. enterica*, with the cassettes *arr2/cmlA5/blaOXA10/aadA1* (Figure 3-1 Panel D). In some ST 19 isolates, this integron was found in combination with the *aacA4* integron. The presence of a unique integron in genetically unrelated isolates from the same geographic area indicates that this integron may have been horizontally transferred.

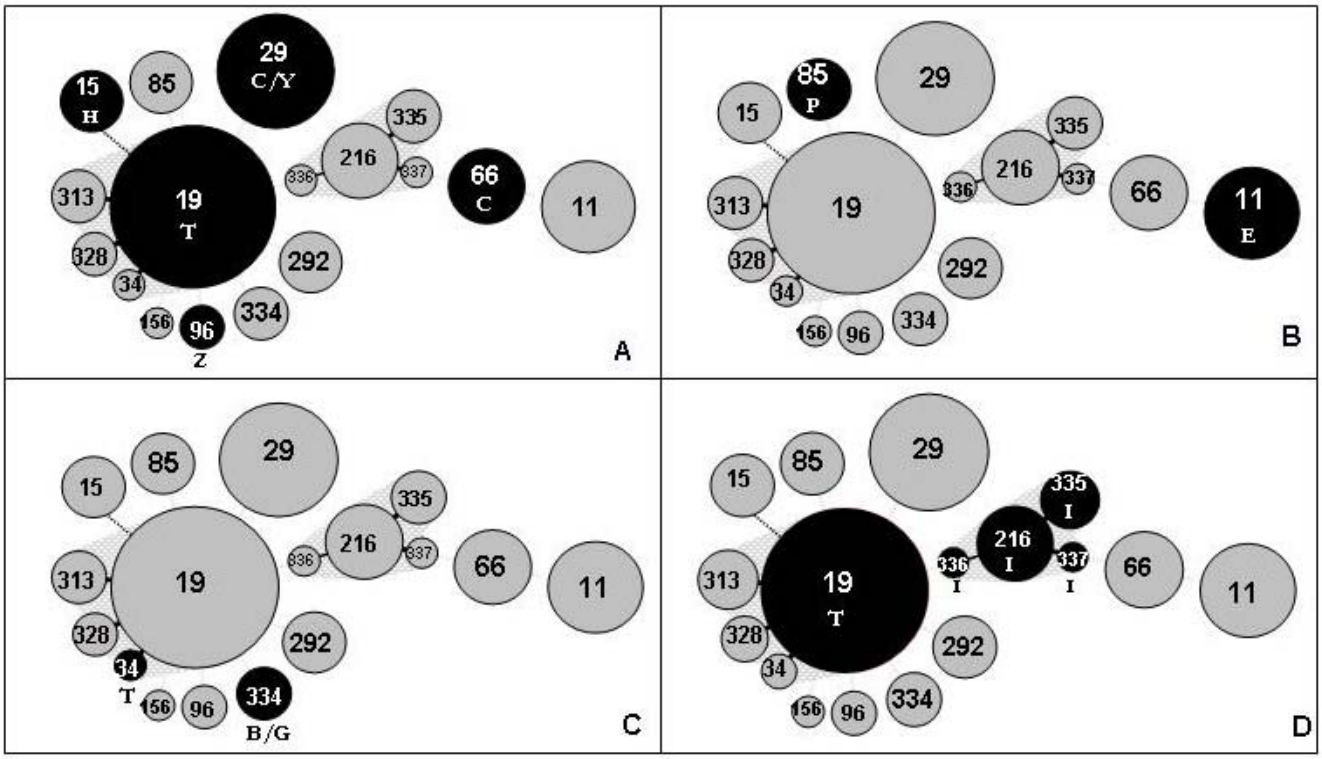

Figure 3-1 Minimum spanning trees depicting integron distribution across *S. enterica* genetic lineages

Panel A: *dfrA12/orfF/aadA2*; Panel B: *dfrA7*; Panel C: *dfrA1/aadA1*; Panel D: *arr2/blaOXA30/cmlA5/aadA2*
Circles represent unique STs. Red circles represent the STs which carried the integron involved in horizontal gene transfer. Numbers in circles represent the ST. Circle size reflects number of isolates in each ST. Pink and green shading indicate closely related groups of isolates.
Letters refer to serotypes; B, Brandenburg; C, Cholerasuis; E, Enteriditis; H, Heidelberg; G, Goettingen; I, Isangi; P, Paratyphi A; Z, Schwarzengrund; Y, Stanley; T, Typhimurium.
Geographic source of isolates is as follows:
Panel A: ST 66, serotype C, Taiwan; ST 29, serotype C, Taiwan; ST 29 serotype Y, Taiwan; ST 96, serotype Z, Denmark; ST 96, serotype Z, Taiwan; ST 19, serotype T, USA/CDC and South Africa; ST 15, serotype H, Philippines
Panel B: ST 11, serotype E, Uganda and South Africa; ST 85, serotype P, Denmark
Panel C: ST 334, serotype G, Spain; ST 334, serotype B, Spain; ST 34, serotype T, Germany
Panel D: ST 19, serotype T, South Africa; ST 216, 335, 336, 337, serotype I, South Africa.

An integron containing the single gene cassette *aacA4* was found alone in isolates in isolates of serovar Isangi with ST 335 from Uganda and South Africa. This integron was also found in isolates with ST 19 both alone and in combination with the integron *arr2/cmlA5/blaOXA10/aadA1*. These isolates belong to different genetic lineages, in that they

differ at all 7 MLST loci. The occurrence of the same integron in these genetically unrelated isolates further supports a role of horizontal gene transfer in the dissemination of antibiotic resistance in *S. enterica*.

3.4.5 Evidence for Clonal Expansion of integron mediated MDR in *S. enterica*

The serotype Typhimurium phage type DT104 integron pattern, with two integrons of sizes 1.0 and 1.2 kb and bearing the gene cassettes *blaPSE1* and *aadA2* was found in 12 (13%) isolates of ST 19 (Table 3-5) and in one further serotype Typhimurium ST 19 isolate which also contained 2 other integrons. This result is consistent with the hypothesis that a common ancestor has undergone clonal expansion in a number of geographic areas.

Table 3-5 Evidence of clonal expansion

Integron Profile	No. of Isolates	Serotype	ST	Country
blaPSE1 aadA2	4	Typhimurium	19	USA/ACHD
	2	Typhimurium	19	Spain
	3	Typhimurium	19	Italy
	3	Typhimurium	19	South Africa
blaOXA30/aadA1	1	Typhimurium	328	Philippines
	2	Typhimurium	19, 328	Taiwan
	1	Typhimurium	313	South Africa
aadB/catB3	1	Typhimurium	19	Taiwan
	1	Typhimurium	328	Philippines
dfrA12/orfF/aadA2	1	Schwarzengrund	96	Taiwan
	1	Schwarzengrund	96	Denmark
dfrA12/orfF/aadA2	1	Typhimurium	19	USA/CDC
	2	Typhimurium	19	South Africa
aacA4	1	Isangi	335	South Africa
	1	Isangi	335	Uganda
dfrA7	6	Enteritidis	11	Uganda
	2	Enteritidis	11	South Africa

Serovar Typhimurium isolates of ST 19 from Taiwan and ST 328 from the Philippines contained two integrons with the cassettes *aadB/catB3* and *blaOXA30/aadA1*. The integron *blaOXA30/aadA1* was also found alone in an isolate of serovar Typhimurium ST 328 from Taiwan and in combination with an integron containing the cassette *aadB* in an isolate of serovar Typhimurium ST 313 from South Africa. ST 313 and ST 328 are closely related to ST 19, differing from it at only one locus (ST 313 differs from ST 19 at the MLST locus sucA, ST 328 and ST 19 differ at MLST locus aroC, see Table 3-4). Therefore, these isolates are all closely related and their integrons may represent an instance of clonal expansion.

While the class 1 integron, *dfrA12/orfF/aadA2*, appears to have been circulated through horizontal gene transfer, this integron has also spread through clonal expansion of non-typhoidal *S. enterica*. This integron appeared in isolates of serovar Schwarzengrund ST 96 in Taiwan and

Denmark and in serovar Typhimurium isolates of ST 19 in the United States and South Africa. Similarly, integrons with the single gene cassettes *aacA4* and *dfrA7* also exhibited both horizontal gene transfer and clonal expansion. The *aacA4* integron was found in serovar Isangi isolates of ST 335 in Uganda and South Africa. The *dfrA7* integron was found in serovar Enteritidis ST 11, also in Uganda and South Africa.

3.5 DISCUSSION

To better understand the dissemination of integron mediated antibiotic resistance, this study characterized the class 1 integrons and genetic lineages associated with 90 multiply drug resistant isolates obtained from a global collection of non-typhoidal *S. enterica*. Integrons found in this collection were diverse in size, gene cassette combination and distribution and presented evidence for roles of both clonal expansion and horizontal gene transfer.

Horizontal gene transfer is known to be an important factor in the dissemination of antibiotic resistance genes, particularly when those genes are associated with mobile elements such as plasmids, transposons and integrons [33, 34, 97]. In this study, the widespread distribution of the *dfrA12/orfF/aadA2* integron among several STs and across several distinct geographic regions is an example of horizontal gene transfer as is the presence of the integrons *dfrA7*, *dfrA1/aadA1*, and *arr2/cmlA5/blaOXA10/aadA1* in different genetic backbones (when assessed by MLST). These integrons are potentially capable of transmitting resistance to other *S. enterica* isolates or to other bacteria.

Integrons are widely distributed among bacterial species. The integron found in the greatest number of different genetic lineages in this study, *dfrA12/orfF/aadA2*, has been previously reported in a number of species, including *E. coli* (GenBank accession number AF335108, unpublished), *Serratia marcesens* (Genbank accession number AF284063, unpublished), and *Salmonella* [103]. The integron *dfrA1/aadA1* has been documented in *E. coli* from Turkey [104], and cited in *E. coli* in GenBank entries from India (GenBank accession number EF417897, unpublished) and Kenya (GenBank accession number EF417897, unpublished), in *Klebsiella pneumonia* from Poland (GenBank accession number AY007807, unpublished) as well as in *S. enterica* (GenBank accession number AM746675, unpublished). Transfer of integrons between different bacterial species has been documented in the clinical setting, which poses a serious threat to containment of nosocomial infections [105]. The existence of identical integrons in different types of bacteria and the ability of these integrons to be transferred *in vivo* indicates that many bacteria draw integrons from a common pool. This is an important consideration in efforts to halt the development and spread of antibiotic resistance.

In this study, clonal expansion of *S. enterica* appears to be responsible for a significant fraction of the dissemination of drug resistance integrons. Although several integrons demonstrated evidence of clonal expansion, including *aacA4*, *aadB/catB3*, *blaOXA30/aadA1*, *dfrA7*, and *dfrA12/orfF/aadA2,* the strongest evidence is presented by the prevalence and geographic ubiquity of the serotype Typhimurium DT104 pattern clone, in which the resistance-encoding integrons (*blaPSE1* and *aadA2*) are chromosomally integrated. These integrons are still mobilizable [106] but their chromosomal location may make them more likely to be disseminated through clonal expansion than through horizontal gene transfer, particularly in the absence of antibiotic selective pressure.

Our study assessed mechanisms of dissemination of integrons in a collection that is more genetically and geographically diverse than is typical for studies of integrons in *S. enterica*. MLST, used for assessment of genetic relatedness of isolates in this study, is better suited to analysis of global populations than other commonly-used methods, such as pulsed field gel electrophoresis. In addition, this study focused on the relative contributions of clonal expansion and horizontal gene transfer to the dissemination of class 1 integron borne genes coding for antibiotic resistance, which has not previously been well explored.

Antibiotic resistance is a serious and increasing problem in *S. enterica* and in other Gram-negative pathogens, such as *Pseudomonas aeruginosa, Acinetobacter baumannii*, *K. pneumoniae* and *E. coli* [60]. The genes that code for antibiotic resistance in these pathogens have proven to be remarkably mobile and widely distributed within and between species. The dissemination of integrons bearing antibiotic resistance gene cassettes in *Salmonella* and other bacteria is a complex process that involves both the horizontal transfer of mobile genetic elements and the expansion of particularly fit clones. The combined effect of these mechanisms is that, if integrons confer an adaptive benefit due to the presence of antibiotic selective pressure or if clones harboring these integrons have increased fitness due to other factors, then the integrons may disseminate rapidly both geographically and among diverse species. It is important to understand these mechanisms of transmission to develop methods for surveillance and control of antibiotic resistance.

4.0 NOVEL INTEGRONS IN A GLOBAL COLLECTION OF MULTI-DRUG RESISTANT NON-TYPHOIDAL *SALMONELLA ENTERICA*

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4.1 ABSTRACT

Investigation of integron carriage in a global collection of multi-drug resistant *Salmonella enterica* identified 3 unique class 1 integrons not previously reported in this species. This study used PCR and DNA sequence analysis to characterize the structure of these integrons. A ~4.0 kb integron containing the gene cassettes *arr2/cmlA5/blaOXA10/aadA*1 was found in isolates belonging to serovars Isangi and Typhimurium from South Africa. This integron was shown to be located on a plasmid and therefore may have been acquired by horizontal gene transfer. A ~6.0 kb integron containing the gene cassettes *aac(6')IIc/ereA2/IS1247/aac/arr/ereA2* was found in isolates belonging to serovar Heidelberg from the Philippines. This integron contains the insertion sequence *IS1247*, and two putative resistance gene cassettes which disrupt the erythromycin resistance gene cassette. Finally, a ~6.0 kb integron containing the gene cassettes *qacH/dfrA17/ereA/aadA2/cmlA/aadA1* was found in serovar Stanley isolates from Taiwan. This integron, which has not been previously reported in any bacterial species, contains a dihydrofolate reductase gene cassette (*dfrA17*) with only 91% sequence similarity to previously reported *dfrA* cassettes. The *S. enterica* integrons described in this study are complex and represent novel collections of resistance genes which confer multi-drug resistance and have the potential to be widely disseminated among *S. enterica* as well as other bacterial species.

4.2 INTRODUCTION

Salmonella enterica is a leading cause of bacterial gastroenteritis in humans worldwide [90]. While most *S. enterica* infections are self-limiting, serious cases often require treatment with antibiotics. For several decades, an increase in antibiotic resistance has been noted in *S. enterica*, as in other Gram-negative bacteria [65]. Class 1 integrons contribute significantly to antibiotic resistance in Gram-negative organisms [107]. Integrons are genetic structures capable of capturing and integrating gene cassettes that typically encode antibiotic resistance determinants. Integrons therefore have the ability to confer novel combinations of drug resistance to the bacteria in which they reside. Integrons are frequently associated with plasmids and are therefore easily transferable among and between different bacterial species [32]. Due to their mobility and ability to quickly acquire diverse resistance determinants, integrons are uniquely adapted to transfer and disseminate antibiotic resistance.

Commonly reported integrons in *S. enterica* are small, usually containing 1 to 3 antibiotic resistance cassettes [52, 57, 108]. However, integrons containing a larger numbers of cassettes have been reported in other bacteria [109, 110]. In a previous study, we found that the majority of integrons in a global collection of *S. enterica* contained 1 to 3 gene cassettes [73]. The present study describes the gene cassette contents and plasmid localization of 3 large, novel integrons identified from the same global collection of multi-drug resistant *S. enterica*.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial Isolates

S. enterica isolates were obtained from laboratories in South Africa, Taiwan, and the Philippines between 2001 and 2002 as part of a large global collection investigating multi-drug resistant *S. enterica*. Serovars for isolates from South Africa and the Philippines were determined by the collecting laboratories. The Pennsylvania Department of Health determined serovars for isolates from Taiwan. Multilocus sequence typing (MLST) was performed as described previously [73] to confirm the genetic lineage of isolates.

Isolates from this global collection were screened for resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (ACSSuT). Those isolates exhibiting resistance to these 5 antibiotics were investigated for integron carriage and gene cassette content as previously described [73]. Representative isolates of serovar Isangi and Typhimurium from South Africa, Heidelberg from the Philippines and Stanley from Taiwan were selected for integron characterization (Table 4-1).

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Table 4-1 Description of *S. enterica* strains

Strain	Serovar	Geographic Origin	Integron	Resistance Pattern
SAL01259	Isangi	South Africa	*arr2/cmlaA5/blaOXA10/aadA1*	AmpChlStrTetSamCazCefNalGenAtmSxtRif
SAL01261	Isangi	South Africa	*arr2/cmlaA5/blaOXA10/aadA1*	AmpChlStrTetSamCazCefNalGenAtmSxtRif
SAL01274	Isangi	South Africa	*arr2/cmlaA5/blaOXA10/aadA1*	AmpChlStrTetSamCazCefNalGenAtmSxtRif
SAL02433	Typhimurium	South Africa	*arr2/cmlaA5/blaOXA10/aadA1*	AmpChlStrTetSamCazCefGenAtmSxtRif
SAL02519	Heidelberg	Philippines	*aac(6')IIc/ereA2/IS1247/aac/arr2/ereA2*	AmpChlStrTetSamCazNalGenAtmSxtRif
SAL02520	Heidelberg	Philippines	*aac(6')IIc/ereA2/IS1247/aac/arr2/ereA2*	AmpChlStrTetSamCazNalGenAtmSxtRif
SAL02521	Heidelberg	Philippines	*aac(6')IIc/ereA2/IS1247/aac/arr2/ereA2*	AmpChlStrTetSamCazNalGenAtmSxtRif
SAL02530	Stanley	Taiwan	*qacH/dfrA17/ereA/aadA2/cmlA/aadA1*	AmpChlStrTetSxtSamEry
SAL02533	Stanley	Taiwan	*qacH/dfrA17/ereA/aadA2/cmlA/aadA1*	AmpChlStrTetSxtSamEry
SAL02536	Stanley	Taiwan	*qacH/dfrA17/ereA/aadA2/cmlA/aadA1*	AmpChlStrTetSamEry

Amp, ampicillin; Chl, chloramphenicol, Str, streptomycin; Tet, tetracycline; Sam, ampicillin-sulbactam; Caz, ceftazidime; Cef, cephalothin; Nal, nalidixic acid; Gen, gentamycin; Atm, azteonam; Sxt, trimethoprim-sulfamethoxzole; Rif, rifampin; Ery, erythromycin

4.3.2 Antibiotic Resistance Testing

Antibiotic resistance was determined using the disc diffusion method (Becton, Dickinson and Co, Sparks, MD) on Mueller–Hinton agar according to the guidelines of the Clinical Laboratory Standards Institute. Antibiotics tested were: ampicillin (10µg), tetracycline (30µg), chloramphenicol (30µg), streptomycin (10µg), sulfamethoxazole (250µg), ampicillin/sulbactam (10µg /10µg), cephalothin (30µg), cefoxitin (30µg), cefotaxime (30µg), cefotaxime/clavulanic acid (30µg /10µg), ceftazidine (30µg), ceftazidine/clavulanic acid (30/10µg), ceftiofur (30µg), piperacillin/tazobactam (100µg /10µg), cefipime (30µg), imipenem (10µg), meropenem (10µg), nalidixic acid (30µg), ciprofloxacin (5µg), levofloxacin (5µg), gatifloxacin (5µg), gentamycin

(10µg), aztreonam (30µg), trimethoprim-sulfamethoxazole(1.25µg/23.75µg), rifampin (5µg), and erythromycin (15µg). *Escherichia coli* ATCC25922 was included as a control in all antibiotic susceptibility testing.

4.3.3 Integron Characterization

The Qiagen DNeasy Tissue Kit (Qiagen, Maryland) was used to prepare genomic DNA from *S. enterica* isolates, following the manufacturer's directions. Integrons were amplified by PCR using primers specific for the 5' and 3' conserved segments (CS) (Table 4-2). Integron gene cassette regions were amplified by long range PCR using the Gene Amp HiFidelity Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's directions. Long range PCR conditions were: initial denaturation for 2 minutes at 94° C, followed by 10 cycles of 94° C for 15 seconds, 58° C for 30 seconds, 68° C for 4 minutes, followed by 20 cycles of 94° C for 15 seconds, 58° C for 30 seconds, 68° C for 4 minutes plus 5 seconds per cycle, with a final extension of 72° C for 7 minutes. Electrophoresis on 1% agarose gels was performed to resolve integron PCR products. Gels were stained with ethidium bromide and visualized using UV illumination with a gel documentation system (Gel Doc 2000; Bio-Rad, Hercules, CA). For subsequent sequence analysis, PCR products were treated with ExoSap-It (USB, Cleveland, OH) according to the manufacturer's directions.

Table 4-2 Primers

Primer	Sequence(5'-3')	Target	Reference
5'CS	GGCATCCAAGCAGCAAGC	5' conserved segment	Levesque[102]
3'CS	AAGCAGACTTGACCTGAT	3' conserved segment	Levesque[102]
aadA1_R	CGATGACGCCAACTACCTCTGATA	aadA1internal primer	This study
arr2_F	ATTGTTGGCGTTGTTGAAGACTGG	arr2 internal primer	This study
cmlA5_F	GAATGGGAATGGGATGCCTGATAG	cmlA5 intrnal primer	This study
oxa10_R	TTTACAAAGCACGAAGACACCATT	blaOXA10 intrnal primer	This study
cmlA_F	GCAGGTCGCGAGGAAAGTAATG	cmlA5 5' forward primer	This study
cmlA_R	ACACCGCCCAAGCAGAAGTAGA	cmlA5 3' reverse primer	This study
ere_est_R	GCGCCAGCAGAATTATCCTTACAT	erythromycin esterase internal primer	This study
aac(6')IIC_F	CCGCGGGATTGACCAGT	aac(6')IIC internal primer	This study
aadA2_R	TGTCATTGCGCTGCCATTCTCC	aadA2 internal primer	This study
qacH_F	GCGTCGCCGTTCTAAATCTGCTAT	qacH internal primer	This study
aac_R	GGGCGCCGGGTGTCTGGAG	aacA4 internal primer	This study
IS_F	GTCACGCCCCGACCATCACCTTCC	IS1247 internal primer	This study
TNP_F	CCGCGCTGGCCGACCTGAAC	transposase A internal primer	This study
ere_F	CCTAACCGGGCGATTCAA	erythromycin esterase internal primer	This study
cmlA_R_internal	ATCACACGCCCCATAAAACGAG	cmlA internal primer	This study
arr_R2	GCGGGATCCAGAACCAGGCGACAT	arr-2 internal primer	This study
arr_accA_R	AGAGCGGCTTTGGTTCC	internal primer arr-2—accA junction	This study
ere_F2	CGCTGATTTCGCTGTCCTGA	erythromycin esterase internal primer	This study
dfr17_F	AAAAAGGCTAACAAGTCGT	dfrA17 internal primer	This study
cml_R2	GCTGAATTGTGCTCGCTGTCGTA	cml internal primer	This study

4.3.4 Gene Cassette Identification

Initial sequencing of integron PCR products was performed with the 5' and 3' CS primers using the BigDye terminator 3.1 kit (Applied Biosystems) according to the manufacturer's instructions. Capillary sequence analysis of sequencing products was performed on a 3730 DNA sequence analyzer (Applied Biosystems). Sequence analysis and design of additional primers utilized the Lasergene 7.0.0 software package (DNAStar, Madison, WI). BLAST analysis was

performed on resulting sequences to determine gene cassette homologies (http://www.ncbi.nlm.nih.gov/BLAST) [101].

4.3.5 Plasmid Analysis

Plasmids were isolated from *S. enterica* strains using the Qiagen Midi Kit (Qiagen, Maryland) according to the manufacturer's directions. Electroporation of plasmids into electrocompetent *E. coli* Top 10 cells (Invitrogen, Carlsbad, CA) was performed using a MicroPulser (Bio-Rad, Hercules, CA). *E. coli* transformants were selected on Luria Bertani agar supplemented with the appropriate antibiotic (150 µg/ml erythromycin, 25 µg/ml chloramphenicol, or 50 µg/ml rifampin). Antibiotic susceptibility testing and plasmid isolation was performed on *E. coli* transformants as described above. The resulting plasmid preparations were PCR amplified with 5' and 3' CS primers to identify integrons.

For isolates which did not generate transformants, plasmid carriage was further investigated by performing restriction endonuclease digestion on the purified plasmid preparations with 20 units of *Hind*III in a 10 µl volume containing 1x NEB2 buffer (New England Biolabs, Ipswich, MA) and 0.1 µg BSA. The digestion mixture was incubated at 37° C for 2 hours and subjected to electrophoresis on a 0.7% gel and stained with ethidium bromide to visualize plasmid bands.

4.4 RESULTS

4.4.1 Antibiotic resistance

Based on the selection criteria, all *S. enterica* isolates tested exhibited resistance to ampicillin, tetracycline, chloramphenicol, streptomycin, and sulfamethoxazole and were additionally resistant to ampicillin/sulbactam. Isolates closely related by serovar displayed similar resistance patterns. The Isangi isolates, Sal01259, Sal01261, and Sal01274 were resistant to aztreonam, ceftazidime, gentamycin, and nalidixic acid, as well as rifampin. Serovar Heidelberg isolates, Sal02519, Sal02520, and Sal02521 were resistant to aztreonam, gentamicin, and cephalothin and had intermediate sensitivity to nalidixic acid while serovar Stanley isolates Sal02530, Sal02533, and Sal02536 were also resistant to erythromycin (Table 4-1). The Typhimurium isolate, Sal02433, was resistant to aztreonam, ceftazidime, gentamicin, and cephalothin, as well as rifampin (Table 4-1). None of the *S. enterica* isolates were resistant to ciprofloxacin, levofloxacin, gatifloxacin, imipenim or meropenem

4.4.2 Integron Characterization

The Isangi isolates and the Typhimurium isolate from South Africa contained a ~4kb integron comprised of the gene cassettes *arr2/cmlA5/blaOXA10/aadA1* (Figure 4-1, A). The *arr2* gene cassette, which was originally described in *P. aeruginosa*, encodes an ADP-ribosyl

transferase and confers resistance to rifampin [111]. The *cmlA5* cassette encodes a chloramphenicol efflux protein and contains its own promoter, which is unusual for a gene cassette [110]. The *blaOXA10* gene cassette is contiguous with the *aadA*1 cassette due to the lack of a majority of the 59 base pair element which usually terminates an integron gene cassette [110]. The *blaOXA10* cassette encodes resistance to the ß-lactam class of antibiotics while *aadA*1 confers resistance to aminoglycoside antibiotics, including streptomycin. All of the isolates carrying the *arr2/cmlA5/blaOXA10/aadA1* integron were resistant to rifampin, chloramphenicol, ampicillin, and streptomycin, as expected if all cassettes in this integron were expressed.

To determine the location of the *arr2/cmlA5/blaOXA10/aadA1* integron, plasmids were prepared from the serovar Isangi isolate Sal01259 and the serovar Typhimurium isolate Sal02433. PCR amplification of the resulting plasmid DNA with integron specific 5'and 3' CS primers generated products of the exact same size as the original *S. enterica* genomic DNA (data not shown) suggesting that the *arr2/cmlA5/blaOXA10/aadA1* integron was plasmid located. To confirm these results, plasmids isolated from Isangi Sal1259 and Typhimurium Sal2433 were transferred by electroporation to *E. coli*. The resulting transformants exhibited resistance to tetracycline, sulfamethoxazole, chloramphenicol, ampicillin, ampicillin-sulbactam ceftazidime, gentamicin, trimethoprim-sulfamethoxazole and rifampin (Table 4-3). PCR amplification of plasmid transformed *E. coli* cell lysates with 5' and 3' CS primers generated a product identical in size to the integron from the original isolates and from the plasmid preparation (data not shown). Plasmids isolated from Isangi Sal01261 and Sal01274 gave similar transformation results (Table 4-3).

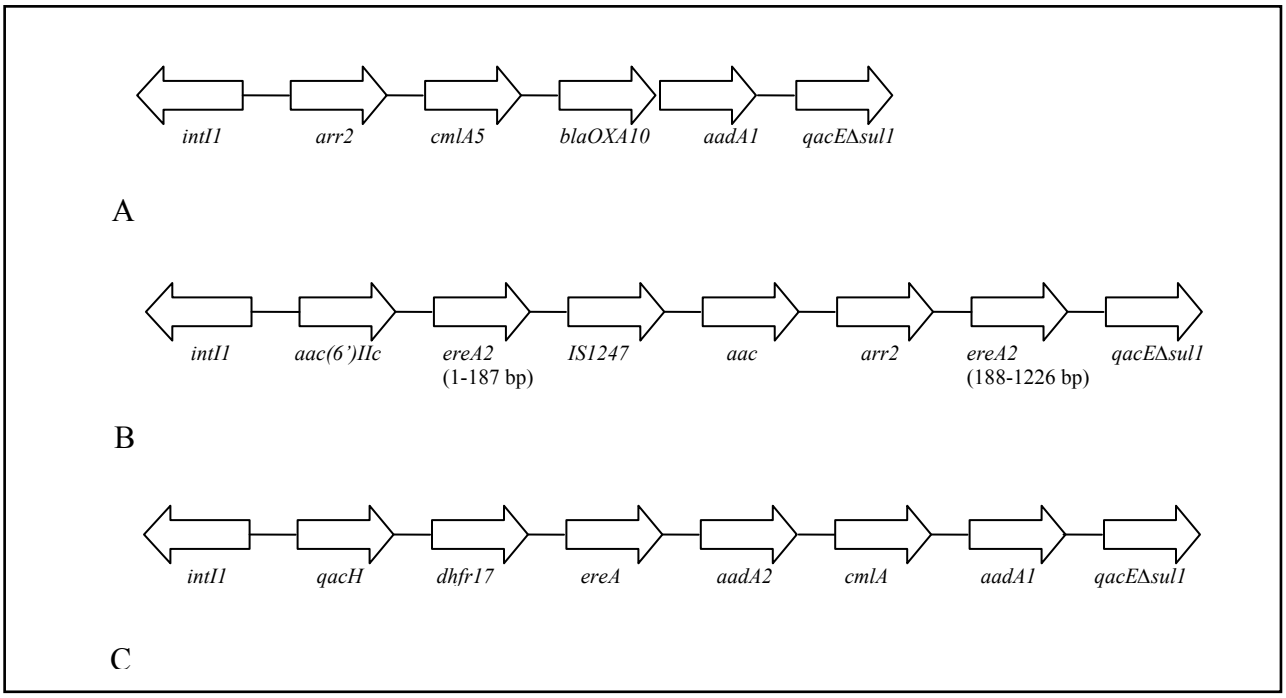


Figure 4-1 Integron structures

Gene cassettes are shown as arrows indicating coding direction. Gene names appear below cassettes.
(A) Integron obtained from isolates Sal01259 and Sal01261 and Sal01274 (serovar Isangi) and Sal02433 (serovar Typhimurium) from South Africa, in which the *blaOXA*10 cassettes is fused to the following *aadA1* cassette.
(B) Integron obtained from isolates Sal02519, Sal02520 and Sal02521 (serovar Heidelberg) from the Philippines.
(C) Integron obtained from isolates Sal02530, Sal02533 and Sal02536 (serovar Stanley) from Taiwan.

Table 4-3 Transfer of resistance to *E. coli* by plasmid electroporation

Plasmid	Donor resistance phenotype	Electorporate resistance	Integron	Resistance not transferred
pSAL01259	AmpChlStrTetSamCazCefNal GenAtmSxtRif	AmpChlStrTetSamCaz CefGenAtmSxtRif	*arr2/cmlaA5/blaOXA10/ aadA1*	Nal
pSAL01261	AmpChlStrTetSamCazCefNal GenAtmSxtRif	AmpChlStrTetSamCaz CefGenAtmSxtRif	*arr2/cmlaA5/blaOXA10/ aadA1*	Nal
pSAL01274	AmpChlStrTetSamCazCefNal GenAtmSxtRif	AmpChlStrTetSamCaz CefGenAtmSxtRif	*arr2/cmlaA5/blaOXA10/ aadA1*	Nal
pSAL02433	AmpChlStrTetSamCazCefGen AtmSxtRif	AmpChlStrTetSamCaz CefGenAtmSxtRif	*arr2/cmlaA5/blaOXA10/ aadA1*	none

Amp, ampicillin; Chl, chloramphenicol, Str, streptomycin; Tet, tetracycline; Sam, ampicillin-sulbactam; Caz, ceftazidime; Cef, cephalothin; Nal, nalidixic acid; Gen, gentamycin; Atm, azteonam; Sxt, trimethoprim-sulfamethoxzole; Rif, rifampin; Ery, erythromycin
E. coli used for electroporation was resistant to streptomycin.

These data indicate that the rifampin, sulfamethoxazole, chloramphenicol and ampicillin resistances were transferred to *E. coli* on a plasmid bearing the integron *arr2/cmlA5/blaOXA10/aadA1*. The finding of aztreonam, ceftazidime, gentamicin, and trimethoprim resistance in *E. coli* transformants indicates that other resistance determinants may be located on the *arr2/cmlA5/blaOXA10/aadA1* containing plasmid or on a separate plasmid that was also transferred to *E. coli* by electroporation. Nalidixic acid resistance was not transferred from serovar Isangi isolates to *E. coli*; this resistance is most likely due to mutation in chromosomally encoded topoisomerase. Streptomycin resistance due to the transfer of *aadA*1 could not be evaluated due to the inherent resistance of the *E. coli* cells used for transformation in these studies.

MLST indicated that the serovar Isangi isolates Sal01259, Sal01274 and Sal01261 all belong to the same genetic lineage. ST216 and ST337 are single locus variants of each other and therefore are genetically closely related (differ at thrA, Table 4-4). On the other hand, the serovar Typhimurium isolate Sal02433 belongs to ST19 which differs from the Isangi STs at all 7 MLST loci (Table 4-4). The identification of an identical plasmid-localized integron in isolates from

distinct genetic lineages defined by MLST indicates that the *arr2/cmlA5/blaOXA10/aadA1* integron likely was either transferred horizontally between the 2 different genetic lineages or transferred to the Isangi and Typhimurium serovars from another common source.

Table 4-4 Genetic relatedness of Serovar Isangi and Typhimurium strains by MLST

Strain	Serovar	ST	Allele Number						
			aroC	dnaN	hemD	hisD	purE	sucA	thrA
SAL01259	Isangi	216	2	73	41	79	71	65	72
SAL01261	Isangi	216	2	73	41	79	71	65	72
SAL01274	Isangi	337	2	73	41	79	71	65	8
SAL02433	Typhimurium	19	10	7	12	9	5	9	2

The Heidelberg isolates contained a ~6 kb integron with the gene cassettes *aac(6')IIc/ereA2/IS1247/aac3/arr/ereA2* (Figure 4-1, B). The *aac(6')IIc* gene cassette encodes a 6'-N-aminoglycoside acetyltransferase which confers resistance to gentamicin [112]. The *ereA2* gene cassette, which confers resistance to erythromycin [113, 114], is interrupted at position 188 by *IS1247* [115] and the gene cassettes *aac3* and *arr*. The *aac3* gene cassette shows 79.8% sequence similarity to the N-acetyltransferase, *aac3-Vb* from *Serratia marcescen*s, which confers resistance to gentamicin [116, 117]. All 3 Heidelberg isolates were resistant to gentamicin suggesting that the *aac3* gene cassette may encode N-acetyltransferase activity. The *arr* gene cassette displays limited sequence similarity to a rifampin ADP-ribosyl transferase from a *Rhodopseudomonas palustris* gene [117, 118]. Thus, the rifampin resistance in the Heidelberg strains could be explained by the presence of this unique *arr* cassette. The 3' end of the *ere2* cassette resides downstream of the *arr* gene cassette. Although the *ereA2* cassette is interrupted, the Heidelberg strains were resistant to erythromycin, and it is possible that a truncated gene product retains some or all of the antibiotic resistance activity of the intact protein. It is also

possible that other resistance genes were present in these strains and were responsible for the observed resistance phenotypes.

Repeated attempts to purify plasmids from Heidelberg isolates Sal02519, Sal02520 and Sal02521 failed to yield *E. coli* transformants on appropriate selection. Plasmids were not evident in these isolates when assessed by restriction endonuclease digestion with *Hind*III (data not shown). These data suggest that the *aac(6')IIc/ereA2/IS1247/aac/arr/ereA2* integron may be chromosomally located in the Heidelberg isolates.

Serovar Stanley isolates contained an integron that has not been previously described. This novel ~6 kb integron contains the gene cassettes *qacH/dfrA17/ereA/aadA2/cmlA/aadA1* (Figure 4-1, C). All of the Stanley isolates were resistant to erythromycin, streptomycin, chloramphenicol, and trimethoprim/sulfamethoxazole, consistent with the resistance cassettes carried on the integron. The *qacH* cassette encodes an efflux pump that confers resistance to quaternary ammonium compounds and ethidium bromide [119].

The second gene cassette in this integron exhibited 90.7% sequence identity to *dfrA17*, a dihydrofolate reductase integron gene cassette, which typically confers resistance to trimethoprim (found in uncultured bacteria, GenBank Accession no. FM179325 and in *E. coli* GenBank Accession no. EU687490). The *dfrA17* gene cassette identified in the Stanley integron encodes a 157 amino acid protein variant which differs from FM179325 at 11 amino acid residues (Figure 4-2). These amino acid changes are conservative; therefore the trimethoprim resistance observed in all of the Stanley strains is predicted to be due to expression of this variant *dfrA17* cassette.

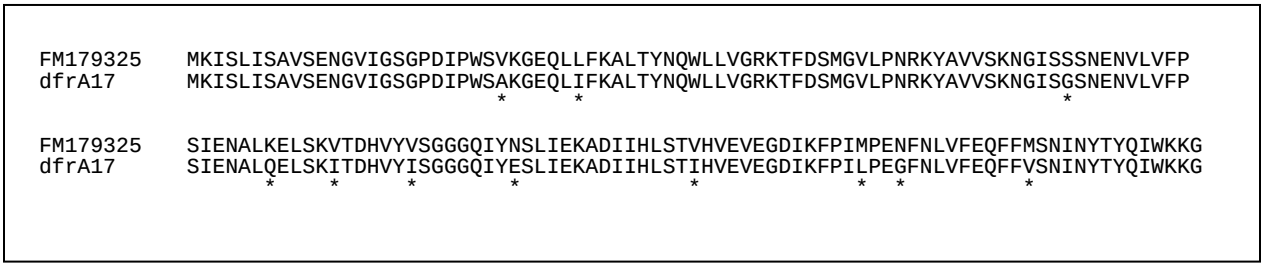


Figure 4-2 Amino acid sequence of *dfrA17* gene cassette

Comparison of amino acid sequence of *dfrA17* gene cassette obtained from Stanley integron Sal02530, Sal02533, and Sal02536 to amino acid sequence of closest match, *dfrA1*7 from GenBank Accession no. FM179325. Asterisks indicate amino acid substitutions.

The *ereA* gene cassette encodes a type 1 erythromycin esterase, which has been reported in *E. coli* (GenBank Accession no. DQ157752) and *P. aeruginosa* (GenBank Accession no. EF207719). Expression of this integron cassette would explain the erythromycin resistance phenotype observed in the *S. enterica* Stanley strains. The gene cassette series *aadA2/cmlA/aadA1* has been reported in *E. coli* (GenBank Accession no EF113389) and *S. enterica* serovar Typhimurium [120]. Both *aadA2* and *aadA1* confer resistance to aminoglycosides while *cmlA* confers chloramphenicol resistance, which may explain these resistances observed in the Stanley isolates.

While a plasmid band was detected in the serovar Stanley isolates by restriction endonuclease digestion, plasmid preparations from these strains did not amplify a 6kb integron product nor were plasmid transformations of *E. coli* successful (data not shown).

4.4.3 Nucleotide sequence accession numbers

The nucleotide sequences of integrons *arr2/cmlaA5/blaOXA10/aadA1, aac(6')IIc/ereA2Δ/IS1247/aac3/arr/ereA2*, and *qacH/dfrA17/ereA/aadA2/cmlA/aadA1* have been deposited in the GenBank/EMBL/DDBJ.

4.5 DISCUSSION

This study describes several previously unidentified integrons in *S. enterica*. These integrons are interesting not only for their size and content but also for their possible origins and their implications for spread of multiple antibiotic resistance.

The integron *arr2/cmlA5/blaOXA10/aadA1* identified in Isangi and Typhimurium *S. enterica* isolates contains a series of gene cassettes identical to a segment of a previously reported larger, complex class 1 integron in *Acinetobacter baumannii* strain AYE from France [109]. This sequence of gene cassettes has also been reported in *P. aeruginosa* clinical isolates collected in Thailand in 1999 [121], but has not been reported as a complete integron and has not been reported in *S. enterica.*

The *arr2/cmlA5/blaOXA10/aadA1* integron reported in this study is located on a plasmid in *S. enterica* ST 216 and 337 serovar Isangi and ST 19 serovar Typhimurium strains. The existence of identical integrons on plasmids from *S. enterica* isolates of different sequence types and serovars supports the role of horizontal gene transfer in the dissemination of drug resistance

in *S. enterica*. Movement of integron-bearing plasmids may occur among *S. enterica* serovars and/or plasmids may be transferred to *S. enterica* via other drug resistant bacterial species. The gene cassette *blaOXA10* in the *A. baumannii* and *P. aeruginosa* integrons and in the integrons reported in this study has lost the majority of its 59 base element, suggesting that these cassettes would mobilize as a unit in the event of excision, rather than as separate entities. The existence of this unique fused two cassette structure in integrons in *A. baumannii* and *P. aeruginosa* as well as in an integron found in two serovars of *S. enterica* in this study increases the likelihood that these integrons have a common origin and participated in horizontal gene exchange.

Interestingly, the series of gene cassettes *arr2/cmlA5/blaOXA10/aadA1* has been found in *A. baumannii* as part of an 86 kb chromosomally located resistance island which contains a number of additional resistance determinants. In the study of *P. aeruginosa* isolates from Thailand, this series of 4 gene cassettes was found as part of several different integrons, and was localized to a plasmid in one case [121]. It is possible that a plasmid borne *arr2/cmlaA5/blaOXA10/aadA1* integron from *S. enterica* acquired additional elements, such as the insertion sequence *IS1999* and the additional gene cassettes *bla*VEB-1 and *aadB* which are present in the *A. baumannii* integron, and was subsequently transferred to other bacterial species. Conversely, the *arr2/cmlaA5/blaOXA10/aadA1* integron identified in isolates in this study may have been generated by gene cassette excision events from a larger integron. It has been suggested that chromosomally located bacterial super integrons, such as that found in *A. baumannii*, are the source of gene cassettes found in smaller mobile integrons [122], and this offers a plausible source for the integron found in this study.

The *aac(6')IIc/ereA2/IS1247/aac/arr/ereA2* integron is unusual in that it contains an insertion sequence interrupting the gene cassette *ereA2,* which normally confers erythromycin

resistance. A potential ancestor of this integron is the integron *aac(6')IIc/ere*A2, found on a plasmid in the newly emerged *S. enterica* serovar Keurmassar in Senegal in 2000 [123]. The *ereA2* gene cassette contains a 4 bp repeat sequence which may have provided an insertion target for *IS1247* [117]. *IS1247* carrying the *aac3* and *arr* cassettes may have excised from a plasmid or chromosomal location and inserted into the 4 bp repeat sequence in the *ereA2* cassette, thereby resulting in generation of the *aac(6')IIc/ereA2/IS1247/aac/arr/ereA2* integron. The *ereA*2 cassette may retain its functionality, since the isolates containing the interrupted gene cassette in this study displayed an erythromycin resistant phenotype. The *aac(6')IIc/ereA2/IS1247/aac/arr/ereA2* integron has previously been reported in a *Klebsiella oxytoca* isolate from a single patient in Paris [117]. Although plasmid localization was not demonstrated for the *K. oxytoca* integron, the existence of this identical and unusual integron in both *K. oxytoca* and *S. enterica* suggests that this unique structure can be mobilized across bacterial species.

To our knowledge, the *qacH/dfrA17/ereA/aadA2/cmlA/aadA1* integron found in serovar Stanley isolates has not been reported in any bacterial species. The 3' end of this *S. enterica* integron resembles a plasmid-borne 3.2 kb *aadA2/cmlA/aadA1* integron reported in *E. coli* [110]. Since integrons are believed to preferentially add cassettes at the first position [30], this *E. coli* integron could be a progenitor of the *S. enterica* serovar Stanley integron described here. The *dfrA17* cassette in this integron is novel in that it demonstrates only 90.7% sequence similarity to any reported dihydrofolate reductase genes. The source of this newly described *dfrA17* variant is not known, but it contains numerous nucleotide changes from its nearest match. Interestingly, the gene cassette series *aadA2/cmlA/aadA1* has also been found inverted following an insertion sequence on a plasmid in *S. enterica* serovar Cholerasuis [124] This series of gene

cassettes has also been found preceded by the cassette *dfrA12* and an unidentified ORF and followed by a *qacH* cassette and transposase in an *S. enterica* serovar Typhimurim integron [120]. This suggests the intriguing hypothesis that *qacH/dfrA17/ereA/aadA2/cmlA/aadA1* could have arisen due to insertion sequence and transposase activity, as well as by the usual integrase-catalyzed integron modification events. It has recently been suggested that insertion sequences play a role in the evolution of integron gene cassettes [125] and this may be true for the integron described here.

The gene cassette diversity and plasmid localization of the integrons reported here demonstrate the capacity of this structure for creating and disseminating complex collections of resistance elements. The existence of multiple drug resistance determinants in one genetic structure exacerbates the problem of antibiotic resistance as selection for any gene cassette within an integron selects for all of the cassettes. In addition, the existence of genes coding for resistance to disinfectants, such as the *qacH* gene cassette, may allow common disinfection methods to select for multidrug resistant pathogens.

The integrons reported here also underscore the importance of horizontal gene transfer in the expansion of antibiotic resistance. Integrons and the gene cassettes they contain exist as a common pool which has the capacity to be exchanged by many bacterial species. Gene cassettes from integrons in one species may soon appear in another species. This study further emphasizes the vital role integrons play in the spread of antibiotic resistance throughout the microbial community.

5.0 DISCUSSION

S. enterica is one of the leading causes of food borne illness in the United States and around the world and is responsible for a number of diseases of varying severity, including enterocolitis, bacteremia, and typhoid fever. In healthy adults, symptoms of salmonellosis typically resolve within a week, but in vulnerable populations, treatment with antibiotics may be required for recovery. Alarming increases in antibiotic resistance over the past several decades have made that treatment more challenging [126]. The studies presented here focus on mechanisms of dissemination of antibiotic resistance mediated by integrons, which are capable of rapidly spreading resistance to multiple drugs simultaneously, and so contribute extensively to the increased burden of antibiotic resistance in *S. enterica* and other organisms.

Integrons may be spread by horizontal gene transfer or by clonal expansion. Differentiating between the two mechanisms requires that genetic relatedness of isolates be determined using a method that provides adequate discrimination while not obscuring relatedness by being overly discriminatory. MLST has been investigated in a number of types of bacteria and has been found to be a reproducible and reliable method for indexing genetic relatedness, while being suitable for large scale studies of isolates of varied genetic backgrounds. However, this technique has not been used to characterize a large and varied collection of *S. enterica* strains prior to the studies presented here.

To determine if MLST would be a suitable method for determining genetic relatedness in *S. enterica*, our first study used this technique to investigate a large and highly diverse collection of isolates of this species. Our results showed that the degree of diversity in housekeeping genes in *S. enterica* is comparable to the degree of diversity reflected by serovar, which is quite high in this organism. ST was found to correlate closely with serovar in a majority of isolates. Although, because of limited discriminatory power within the most commonly encountered serovars, MLST is likely not useful for outbreak detection, this work demonstrated that MLST is a valid method for determining global genetic relatedness in *S. enterica* and provides a basis for the use of MLST in studies of large and varied collections of that species, in which methods such as PFGE would be overly discriminatory.

In addition to its use in determining relatedness of isolates in epidemiologic studies, MLST may also be important in determining genetic relatedness of isolates in other situations. In some cases, serovars are polyphyletic and so isolates of the same serovar may be genetically quite different. MLST provides information which could be used to discriminate isolates which appear to be related based on serovar, but which actually are not. Change of serovar may occur in respose to increased immunity. MLST would also be useful in detecting possible cases in which isolates switched serovar.

The first study also confirms that *S. enterica* populations are clonal, although not exclusively so. The MLST data provide genetic lineage information that is necessary to our investigation of mechanisms of dissemination of integron mediated antibiotic resistance. Research indicates that in many cases, distinct clones are responsible for the majority of cases of bacterial infection [127] and this appears to be the case for *Salmonella*. The *S. enterica* clonality observed in this study indicates that surveillance for antibiotic resistant clones which may have

increased virulence and/or infectious potential may be required to limit the public health burden of severe disease.

The main focus of the studies presented here was the investigation of mechanisms of transfer of multiple antibiotic resistance encoded by genes contained on integrons. Integrons can be disseminated vertically, through clonal expansion, or horizontally, through lateral gene transfer. Because of their frequent association with plasmids and transposons, integrons can be expected to be spread by horizontal gene transfer. However, due to the clonality of *S. enterica*, clonal expansion may also contribute to the expansion of integron mediated antibiotic resistance. In the second study reported here, we found identical integrons in isolates of the same ST in a variety of geographic locations, indicating the importance of clonal expansion in integron dissemination. We also found identical integrons in genetically unrelated isolates, confirming the importance of horizontal gene transfer. Our results indicate that both vertical and horizontal transmission contribute to the dissemination of integrons in *S. enterica.* Clonal expansion was found to be particularly important when integrons are located on the bacterial chromosome, as in the serovar Typhimurium DT104. Therefore, our study indicates that surveillance for chromosomal location of integrons can be useful in predicting changes in *S. enterica* antibioitic resistance patterns.

The second study identified several integrons not previously reported in *S. enterica*. These novel *S. enterica* integrons were characterized in the third study of this dissertation and are interesting for several reasons. These integrons contain a larger number of gene cassettes than are usually reported in *S. enterica*. This finding is important because the presence of multiple cassettes increases both the extent of horizontal transfer, since all cassettes in an integron will move together, and the burden of resistance, because all cassettes present in a particular integron

will be selected for together, allowing for selection by one drug to maintainresistance to a number of drugs. The plasmid localization of these integrons and transformation data indicate that at least one of these novel integrons has likely been transferred between different *S. enterica* genetic lineages. In addition, two of the integrons described in the third study have been identified in other bacterial genera, one as part of a larger chromosomal integron, supporting the hypothesis that integrons are mobile between species. This mobility emphasizes the importance of integron surveillance, since the appearance of an integron in any one of several bacterial species, such as *P. aeruginosa, A. baumannii*, *K. pneumoniae* and *E. coli*, predicts the appearance of the same integron in the other species.

One of the novel integrons described in the third study has an insertion sequence interrupting a gene cassette. Insertion sequences have recently been implicated in the evolution of integrons [107] and have been found next to *qnrA*, which provides some resistance to quinolone activity. The *qnrA* gene has been found on plasmids [128-130] and has been described as a part of an integron [131]. This suggests the possibility that insertion sequences and transposition events may be involved in the creation of new integron gene cassettes from chromosomally integrated non-integron genes. One of the most interesting aspects of integrons concerns the origin of gene cassettes, and the possibility that new gene cassettes will be added to those that currently exist, in response to the introduction of new antibiotics. Our finding of an insertion sequence with 2 putative resistance cassettes internal to an integron supports the possibility that insertion sequences are the vehicles by which new gene cassettes are created.

Disease outbreaks caused by *Salmonella* are common, as exemplified by the 2008 serovar Saintpaul outbreak and the 2007 serovar Tennessee outbreak, both of which occurred in the US. Outbreaks due to antibiotic resistance bacteria are more serious than those caused by pan

susceptible species, because the treatment of resistant strains is more difficult. Because integrons are intimately associated with increases in antibiotic resistance and with emergence of resistance to new antibiotics, surveillance for integrons and their associated gene cassettes is an important component of the effort to limit the problem caused by that resistance. *S. enterica* has also been used for bioterrorism, and integrons have the potential to increase that threat, since minor genetic alterations may generate a novel integron which can be spread through the population via food contamination. Our findings support a role for both horizontal gene transfer and clonal expansion of integron mediated multiple drug resistance in *S. enterica*. The studies described in this dissertation demonstrate that MLST can be used effectively to investigate *S. enterica* population structure and the modes of antibiotic resistance spread in this orgamism. Continued investigations can improve surveillance strategies to limit the public health burden caused by *S. enterica.*

APPENDIX A

CHAPTER 2: MLST ISOLATES

ID	ST	Country of Origin	Serotype	aroC	dnaN	hemD	hisD	purE	sucA	thrA	Group
M1	307	ACHD/USA	Paratyphi B dT^{+}	2	14	24	14	2	19	107	4
M2	96	ACHD/USA	Schwarzengrund	43	47	49	49	41	15	3	2
M3	316	ACHD/USA	Montevideo	43	41	16	42	35	13	111	2
M4	11	ACHD/USA	Enteriditis	5	2	3	7	6	6	11	3
M5	447	ACHD/USA	Poona	13	127	92	113	114	107	4	2
M6	19	ACHD/USA	Typhimurium	10	7	12	9	5	9	2	4
M7	152	ACHD/USA	Kentucky	62	53	54	60	5	53	54	3
M8	15	ACHD/USA	Heidelberg	2	7	9	9	5	9	12	1
M9	33	ACHD/USA	Hadar	2	5	6	7	5	7	12	1
M10	433	ACHD/USA	SS IV Group V O:44	30	28	31	27	22	29	30	5
M11	118	ACHD/USA	Newport	16	2	45	43	36	39	42	1
M12	162	ACHD/USA	SS IV 6,7:Z4, Z24:-	30	28	31	57	57	69	30	5
M13	407	ACHD/USA	Oranienburg	45	14	82	9	128	6	34	4
M14	80	ACHD/USA	Miami	42	40	17	41	33	36	41	2
M15	24	ACHD/USA	Javiana	13	12	17	16	13	16	4	2
M16	112	ACHD/USA	Muenchen	41	42	43	58	9	12	2	1
M17	573	ACHD/USA	SS III Group J O:17	184	25	29	211	129	178	131	5
M18	26	ACHD/USA	Thompson	14	13	18	12	14	18	1	1
M19	22	ACHD/USA	Norwich	12	2	15	14	11	14	16	4
M20	413	ACHD/USA	Mbandaka	15	70	93	78	113	6	68	3
M21	435	ACHD/USA	Berta	2	2	3	124	2	2	6	1
M22	13	ACHD/USA	Agona	3	3	7	4	3	3	7	4
M23	574	ACHD/USA	SS III Group Y O:48	29	173	30	212	21	145	132	5
M24	404	ACHD/USA	Paratyphi B dT+	46	122	3	18	6	138	133	4

ID	ST	Country of Origin	Serotype	aroC	dnaN	hemD	hisD	purE	sucA	thrA	Group
M25	405	ACHD/USA	Hartford	2	15	21	138	130	139	12	1
M26	464	ACHD/USA	Bareilly	10	129	18	153	8	150	22	1
M27	575	ACHD/USA	Group I O:16	31	28	136	28	23	29	81	5
M28	49	ACHD/USA	Ituri	5	14	21	9	6	12	17	4
M29	112	ACHD/USA	Muenchen	41	42	43	58	9	12	2	1
M30	22	ACHD/USA	Braenderup	12	2	15	14	11	14	16	4
M31	433	ACHD/USA	SS IV Group V O:44	30	28	31	27	22	29	30	5
M32	430	ACHD/USA	SS IV Group Z O:50	145	26	30	144	21	145	28	5
M33	445	ACHD/USA	SS IIIb 48:k:z53	33	26	30	147	131	145	134	5
M34	40	ACHD/USA	Derby	19	20	3	20	5	22	22	3
M35	22	ACHD/USA	Braenderup	12	2	15	14	11	14	16	4
M36	20	ACHD/USA	San Diego	11	10	13	13	10	13	4	2
M37	449	ACHD/USA	Litchfield	14	72	114	12	6	19	15	1
M38	27	ACHD/USA	Saintpaul	5	14	18	9	6	12	17	4
M39	129	ACHD/USA	Paratyphi A	45	4	8	44	27	56	8	5
M40	1	ACHD/USA	Typhi	1	1	1	1	1	1	5	5
M41	75	ACHD/USA	Salinatis	14	37	39	33	30	19	37	1
M42	406	ACHD/USA	Richmond	14	37	108	139	105	19	135	1
M43	532	ACHD/USA	Baranquilla	180	161	132	21	12	13	4	2
M44	64	ACHD/USA	Anatum	10	14	15	31	25	20	33	1
M46	307	ACHD/USA	Paratyphi B dT^+	2	14	24	14	2	19	107	4
M47	537	ACHD/USA	Georgia	14	117	133	192	27	132	136	2
M48	11	ACHD/USA	Enteritidis	5	2	3	7	6	6	11	3
M49	43	ACHD/USA	Paratyphi B dT^+	2	14	24	14	2	19	8	4
M50	48	ACHD/USA	Panama	22	11	25	21	10	23	68	2
M51	4	ACHD/USA	Montevideo	43	41	16	13	34	13	4	2
M52	448	ACHD/USA	Mississippi	48	128	96	119	116	119	118	5
M53	88	ACHD/USA	Paratyphi B dT^+	46	44	46	46	38	18	34	3
M54	576	ACHD/USA	SS III Gr O:58	33	152	30	213	131	87	134	5
M55	410	ACHD/USA	Eastbourne	13	11	17	16	13	15	4	2
M56	443	ACHD/USA	Monschaui	5	75	8	96	39	146	97	4
M57	450	ACHD/USA	Telelkebir	83	46	25	148	33	23	63	2
M58	557	ACHD/USA	Sundsvall	175	11	125	199	9	9	160	2
M59	465	ACHD/USA	Chandans	83	63	49	154	33	58	137	2
M60	538	ACHD/USA	Jukestown	84	109	25	193	12	23	23	2
M61	11	ACHD/USA	Gp D Rough	5	2	3	7	6	6	11	3
M62	411	ACHD/USA	Chester	11	10	25	42	4	13	4	2
M63	329	ACHD/USA	Ohio	82	38	26	12	115	78	70	1

ID	ST	Country of Origin	Serotype	aroC	dnaN	hemD	hisD	purE	sucA	thrA	Group
M64	577	ACHD/USA	Gwale	185	37	98	204	172	179	167	2
M65	152	ACHD/USA	Kentucky	62	53	54	60	5	53	54	3
M66	446	ACHD/USA	Hvittingfoss	15	126	101	88	8	19	18	1
M67	451	ACHD/USA	Pomona	111	109	17	149	41	13	23	2
M69	96	South Africa	Schwarzengrund	43	47	49	49	41	15	3	2
M70	358	Spain	Goldcoast	5	110	35	122	2	19	22	3
M71	82	South Africa	Muenchen	41	42	43	12	9	12	2	1
M72	32	South Africa	Infantis	17	18	22	17	5	21	19	5
M73	64	South Africa	Anatum	10	14	15	31	25	20	33	1
M74	544	South Africa	Molade	4	164	134	195	132	46	158	4
M75	142	South Africa	Bovismorbificans	2	59	23	64	38	61	12	1
M76	31	South Africa	Newport	2	2	15	14	15	20	12	1
M77	33	South Africa	Hadar	2	5	6	7	5	7	12	1
M78	10	South Africa	Dublin	5	2	3	6	5	5	10	4
M79	11	South Africa	Enteritidis	5	2	3	7	6	6	11	3
M80	19	South Africa	Typhimurium	10	7	12	9	5	9	2	4
M81	96	South Africa	Schwarzengrund	43	47	49	49	41	15	3	2
M82	329	Spain	Ohio	82	38	26	12	115	78	70	1
M83	53	Spain	4,12:b:-	24	22	27	22	18	24	24	5
M84	469	Spain	Rissen	92	107	79	156	64	151	87	1
M85	33	Spain	Hadar	2	5	6	7	5	7	12	1
M86	11	Spain	Enteritidis	5	2	3	7	6	6	11	3
M87	457	Spain	Livingstone	137	2	36	152	2	7	6	1
M88	32	Spain	Infantis	17	18	22	17	5	21	19	5
M89	71	Spain	Derby	39	35	8	36	29	9	36	5
M90	52	Spain	Blockley	23	9	15	12	17	20	12	1
M91	409	Spain	Ajiobo	138	41	109	94	39	132	3	3
M92	306	Spain	Bredeney	111	47	49	16	41	15	4	2
M93	142	Spain	Bovismorbificans	2	59	23	64	38	61	12	1
M94	142	Spain	4,5,12,:i:-	2	59	23	64	38	61	12	1
M95	11	Spain	9,12:-:-	5	2	3	7	6	6	11	3
M96	358	Spain	Goldcoast	5	110	35	122	2	19	22	3
M97	19	Spain	4,5,12:-I:-	10	7	12	9	5	9	2	4
M98	439	Spain	48:k:1,5	88	26	30	84	21	68	80	5
M99	13	Spain	Agona	3	3	7	4	3	3	7	4
M100	48	Spain	Panama	22	11	25	21	10	23	23	2
M101	466	Australia	Zanzibar	147	13	15	123	140	7	17	5
M102	424	Australia	Birkenhead	143	31	18	143	5	142	22	4

ID	ST	Country of Origin	Serotype	aroC	dnaN	hemD	hisD	purE	sucA	thrA	Group
M103	426	Australia	Aberdeen	46	124	112	12	36	19	18	1
M104	539	Australia	Mgulani	168	14	15	146	5	90	12	1
M105	343	Australia	Chester	11	10	25	118	10	13	4	2
M106	442	Australia	Abony	17	8	3	146	97	19	14	1
M107	434	Australia	Hvittingfoss	18	2	18	12	25	7	12	1
M108	82	Australia	Muenchen	41	42	43	12	9	12	2	1
M109	50	Australia	Saintpaul	5	21	18	9	6	12	17	4
M110	558	Australia	Orientalis	110	167	135	202	166	45	102	2
M111	32	Australia	Infantis	17	18	22	17	5	21	19	5
M112	24	Australia	Javiana	13	12	17	16	13	16	4	2
M113	413	Australia	Mbandaka	15	70	93	78	113	6	68	3
M114	309	Australia	Kiambu	112	105	3	98	111	115	108	3
M115	578	Australia	Havana	139	174	68	132	133	9	168	3
M116	16	Australia	Virchow	6	7	10	10	8	10	14	1
M117	540	Australia	Waycross	61	158	25	194	136	45	126	2
M118	43	Australia	Paratyphi B dT^+	2	14	24	14	2	19	8	4
M119	19	Australia	Typhimurium	10	7	12	9	5	9	2	4
M120	453	Australia	Litchfield	14	72	115	12	6	19	15	1
M121	138	Australia	Montevideo	11	41	55	42	34	58	4	2
M122	408	Australia	Potsdam	2	2	9	7	134	19	86	3
M123	467	Australia	Agama	136	130	40	94	2	9	95	3
M124	579	Australia	Welikade	111	46	137	16	40	58	63	2
M125	468	Australia	SS IIIb	33	86	30	155	135	87	134	5
M126	365	Australia	Weltevreden	130	97	25	125	84	9	101	2
M128	523	Australia	Wangata	101	64	25	163	84	6	101	2
M129	24	Australia	Eastbourne	13	12	17	16	13	16	4	2
M130	442	Australia	Abony	17	8	3	146	97	19	14	1
M131	580	Australia	Orion	99	175	46	11	111	180	1	5
M132	13	Australia	Agona	3	3	7	4	3	3	7	4
M133	64	Australia	Anatum	10	14	15	31	25	20	33	1
M134	462	Australia	Singapore	117	14	23	127	6	7	22	1
M135	469	Germany	Rissen	92	107	79	156	64	151	87	1
M136	27	Germany	Saintpaul	5	14	18	9	6	12	17	4
M137	142	Germany	SS I 6,8:r:-	2	59	23	64	38	61	12	1
M138	11	Germany	Enteritidis	5	2	3	7	6	6	11	3
M139	470	Germany	Gallinarum	4	2	3	7	31	6	11	3
M140	19	Germany	SS I Rough Form	10	7	12	9	5	9	2	4
M141	15	Germany	Heidelberg	2	7	9	9	5	9	12	1

ID	ST	Country of Origin	Serotype	aroC	dnaN	hemD	hisD	purE	sucA	thrA	Group
M142	142	Germany	Bovismorbificans	2	59	23	64	38	61	12	1
M143	34	Germany	SS I 4,5,12:i:-	10	19	12	9	5	9	2	4
M144	32	Germany	Infantis	17	18	22	17	5	21	19	5
M145	64	Germany	Anatum	10	14	15	31	25	20	33	1
M146	155	Germany	London	10	60	58	66	6	65	16	3
M147	581	Germany	SS II 30:-:z6	25	176	27	214	18	181	169	5
M148	412	Germany	Reading	11	10	25	13	10	58	4	2
M149	582	Germany	Kottbus	2	5	116	88	5	19	12	1
M150	583	Germany	Woodinville	138	152	135	215	136	9	3	3
M151	584	Germany	Colindale	6	18	46	124	2	175	12	1
M152	465	Germany	Chandans	83	63	49	154	33	58	137	2
M153	432	Germany	SS IIIb 61:-:1,5,7	88	26	30	55	21	68	80	5
M154	430	Germany	SS IIIb 50:k:-	145	26	30	144	21	145	28	5
M155	34	Germany	SS I 4,12:i:-	10	19	12	9	5	9	2	4
M156	203	Germany	Bareilly	81	69	36	12	68	12	17	1
M157	585	Germany	SS I 16:e,h:l,w	5	64	40	216	173	103	170	3
M158	10	Germany	Dublin	5	2	3	6	5	5	10	4
M159	452	Germany	SS IV 51:z4,z23:-	30	28	31	150	22	29	30	5
M160	454	Germany	SS IV 48:g,z51:-	30	28	31	151	22	148	30	5
M161	16	Germany	Virchow	6	7	10	10	8	10	14	1
M162	142	Germany	6,8:r:-	2	59	23	64	38	61	12	3
M163	279	Germany	SS I 4,12:d:-	62	95	54	96	100	9	100	3
M164	33	Germany	Hadar	2	5	6	7	5	7	12	1
M165	13	Germany	Agona	3	3	7	4	3	3	7	4
M166	365	Germany	Weltevreden	130	97	25	125	84	9	101	2
M167	541	Germany	Cerro	14	162	43	123	118	115	120	5
M168	586	Germany	Livingstone	47	98	36	152	174	61	171	3
M169	138	Germany	Montevideo	11	41	55	42	34	58	4	2
M170	587	Germany	SS I 13,23:z:-	117	70	68	132	175	9	172	3
M171	588	Germany	Havana	186	35	78	75	39	182	97	2
M172	413	Germany	Mbandaka	15	70	93	78	113	6	68	3
M173	18	Denmark	Manhattan	9	9	6	12	9	12	2	1
M174	427	Denmark	Szentes	144	2	108	12	115	19	1	1
M175	414	Denmark	Eastbourne	50	10	25	72	10	13	4	2
M176	455	Denmark	Lagos	146	89	8	33	139	149	4	3
M177	413	Denmark	Mbandalea	15	70	93	78	113	6	68	3
M178	469	Denmark	Rissen	92	107	79	156	64	151	87	1
M179	542	Denmark	Napoli	161	163	96	178	155	12	33	4

ID	ST	Country of Origin	Serotype	aroC	dnaN	hemD	hisD	purE	sucA	thrA	Group
M180	64	Denmark	Anatum	10	14	15	31	25	20	33	1
M181	11	Belguim	Enteritidis	5	2	3	7	6	6	11	3
M182	543	Belguim	Livingstone	117	135	18	12	162	162	38	1
M183	19	Belguim	Typhimurium	10	7	12	9	5	9	2	4
M184	19	Belguim	Typhimurium	10	7	12	9	5	9	2	4
M185	428	Belguim	SS I 3,10:eh: -	13	80	113	49	10	107	23	2
M186	16	Belguim	Virchow	6	7	10	10	8	10	14	1
M187	11	Belguim	Group D	5	2	3	7	6	6	11	3
M188	129	Belguim	Paratyphi A	45	4	8	44	27	56	8	5
M189	39	Belguim	Derby	19	20	3	20	5	2	22	3
M191	358	Belguim	Goldcoast	5	110	35	122	2	19	22	3
M192	11	Philippines	Enteritidis	5	2	3	7	6	6	11	3
M193	145	Philippines	Choleraesuis var. kunzendorf	36	31	35	14	26	6	8	4
M194	40	Philippines	Derby	19	20	3	20	5	22	22	3
M195	155	Philippines	London	10	60	58	66	6	65	16	3
M196	365	Philippines	Weltevreden	130	97	25	125	84	9	101	2
M197	434	Philippines	Rhydyfelin	18	2	18	12	25	7	12	1
M198	444	Philippines	Stanleyville	14	112	43	123	118	147	120	5
M199	29	Philippines	Stanley	16	16	20	18	8	12	18	1
M200	589	Philippines	Javiana	187	12	17	16	13	16	4	2
M201	590	Philippines	Amersterdam	188	112	121	14	176	183	1	5
M202	152	Philippines	Kentucky	62	53	54	60	5	53	54	3
M203	50	Philippines	Saintpaul	5	21	18	9	6	12	17	4
M204	13	CDC/USA	Agona	3	3	7	4	3	3	7	4
M205	112	CDC/USA	Muenchen	41	42	43	58	9	12	2	1
M206	33	CDC/USA	Hadar	2	5	6	7	5	7	12	1
M207	118	CDC/USA	Newport	16	2	45	43	36	39	42	1
M208	49	CDC/USA	Saintpaul	5	14	21	9	6	12	17	4
M209	329	CDC/USA	SS I 9,12: undetermined	82	38	26	12	115	78	70	1
M210	11	CDC/USA	Enteritidis	5	2	3	7	6	6	11	3
M211	446	CDC/USA	Hvittingfoss	15	126	101	88	8	19	18	1
M212	19	CDC/USA	Typhimurium Var Copenhagen	10	7	12	9	5	9	2	4
M213	19	CDC/USA	Typhimurium	10	7	12	9	5	9	2	4
M214	591	CDC/USA	Stanleyville	51	48	138	88	177	2	22	1
M215	52	CDC/USA	Thompson	23	9	15	12	17	20	12	1
M216	19	CDC/USA	4,5,i:-	10	7	12	9	5	9	2	4
M217	96	CDC/USA	Schwarzengrund	43	47	49	49	41	15	3	2

ID	ST	Country of Origin	Serotype	aroC	dnaN	hemD	hisD	purE	sucA	thrA	Group
M218	10	CDC/USA	Dublin	5	2	3	6	5	5	10	4
M219	19	CDC/USA	Group B	10	7	12	9	5	9	2	4
M220	15	CDC/USA	Heidelberg	2	7	9	9	5	7	12	1
M221	95	CDC/USA	Sandiego	3	36	43	38	16	42	38	4
M222	152	CDC/USA	Kentucky	62	53	54	60	5	53	54	3
M223	129	CDC/USA	Paratyphi A	45	4	8	44	27	56	8	5
M224	24	CDC/USA	Seremban	13	12	17	16	13	16	4	2
M225	308	CDC/USA	Poona	22	104	25	110	12	83	4	2
M226	32	CDC/USA	Infantis	17	18	22	17	5	21	19	5
M227	22	CDC/USA	Braenderup	12	2	15	14	11	14	16	4
M228	309	CDC/USA	Kiambu	112	105	3	98	110	115	108	3
M229	11	Canada	Enteritidis	5	2	3	7	6	6	11	3
M230	15	Canada	Heidelberg	2	7	9	9	5	9	12	1
M231	36	Canada	Group B (4,5,12:-:1,2)	18	14	12	9	5	18	21	4
M232	19	Canada	Typhimurium	10	7	12	9	5	9	2	4
M233	33	Canada	Hadar	2	5	6	7	5	7	12	1
M234	13	Canada	Agona	3	3	7	4	3	3	7	4
M235	459	Canada	Thompson	14	13	18	12	14	18	7	1
M236	592	Canada	Worthington	189	70	68	132	175	9	172	3
M237	15	Canada	Heidelberg	2	7	9	9	5	9	12	1
M238	26	Canada	Thompson	14	13	18	12	14	18	1	1
M239	45	Canada	Newport	10	7	21	14	15	12	12	1
M240	112	Canada	Muenchen	41	42	43	58	9	12	2	1
M241	463	Canada	Meleagridis	92	125	78	128	138	9	141	2
M242	33	Canada	Group C (6,8:-:enx)	2	5	6	7	5	7	12	1
M243	423	Canada	Group B (4,5,12:b:-)	20	4	23	14	16	19	140	1
M244	435	Canada	Berta	2	2	3	124	2	2	6	1
M245	32	Canada	Infantis	17	18	22	17	5	21	19	5
M246	15	Canada	Heidelberg	2	7	9	9	5	9	12	1
M247	126	Canada	Sandiego	11	10	25	13	10	35	4	2
M248	425	Canada	Mississippi	127	90	111	33	122	143	95	3
M249	593	Canada	Ipswich	61	177	25	183	136	9	126	2
M250	438	Canada	Hvittingfoss	2	14	15	145	5	20	22	1
M251	371	Canada	Group D (9, 12:-:15)	13	12	17	16	117	16	4	2
M252	43	Canada	Paratyphi B dT^+	2	14	24	14	2	19	8	4
M253	440	Canada	Adelaide	140	105	85	93	14	10	3	4
M254	29	Canada	Stanley	16	16	20	18	8	12	18	1
M255	64	Canada	Anatum	10	14	15	31	25	20	33	1

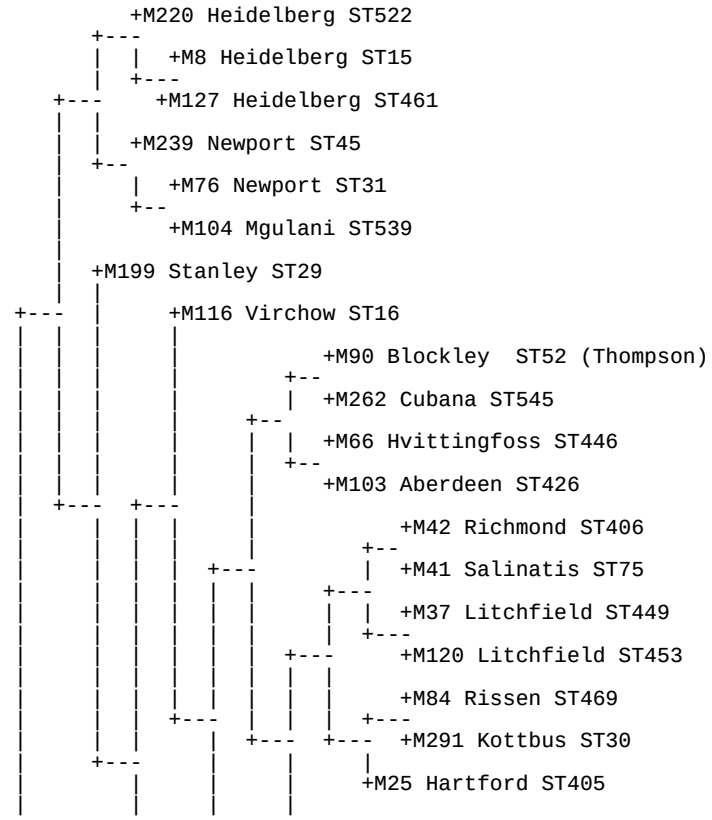
ID	ST	Country of Origin	Serotype	aroC	dnaN	hemD	hisD	purE	sucA	thrA	Group
M256	292	Canada	Group C (8,20:r:-)	104	100	54	78	104	9	48	3
M257	22	Canada	Braenderup	12	2	15	14	11	14	16	4
M258	22	Canada	Group C (6,7:eh:-)	12	2	15	14	11	14	16	4
M259	292	Canada	Group C (8,20:r:-)	104	100	54	78	104	9	48	2
M260	546	Canada	Kingabwa	14	14	122	172	6	7	2	4
M261	436	Canada	Chester	14	10	25	42	40	13	4	2
M262	545	Canada	Cubana	45	15	15	12	130	172	18	1
M263	437	Canada	Javiana	13	12	63	16	13	16	4	2
M264	64	Canada	Group E (3,10:e,h:-)	10	14	15	31	25	20	33	1
M265	19	Canada	Group B (4,5,12:i:-)	10	7	12	9	5	9	2	4
M266	292	Canada	Albany	104	100	54	78	104	9	48	3
M267	319	Canada	Tennessee	118	107	8	51	2	117	16	4
M268	65	CDC/USA	Brandenburg	11	10	13	32	10	13	4	2
M270	24	CDC/USA	Javiana	13	12	17	16	13	16	4	2
M271	16	CDC/USA	Virchow	6	7	10	10	8	10	14	1
M272	521	CDC/USA	Nima	52	63	48	186	36	165	3	2
M273	145	CDC/USA	Choleraesuis	36	31	35	14	26	6	8	4
M274	505	CDC/USA	Bredeney	157	142	49	16	40	35	3	2
M275	547	CDC/USA	Widemarsh	182	36	3	192	163	173	159	3
M276	85	CDC/USA	Paratyphi A	45	4	8	44	27	9	8	5
M277	19	CDC/USA	Lindenberg	10	7	12	9	5	9	2	4
M278	29	CDC/USA	Stanley	16	16	20	18	8	12	18	1
M280	413	CDC/USA	Mbandaka	15	70	93	78	113	6	68	3
M281	26	CDC/USA	16,7:-:1,5	14	13	18	12	14	18	1	1
M282	19	CDC/USA	Typhimurium	10	7	12	9	5	9	2	4
M283	65	CDC/USA	Brandenburg	11	10	13	32	10	13	4	2
M284	471	CDC/USA	Johannesburg	13	131	49	157	12	13	4	2
M285	435	CDC/USA	Berta	2	2	3	124	2	2	6	1
M286	85	CDC/USA	Group B	45	4	8	44	27	9	8	5
M287	19	CDC/USA	Paratyphi B dT^{+}	10	7	12	9	5	9	2	4
M288	48	Italy	Panama	22	11	25	21	10	23	23	2
M289	155	Italy	London	10	60	58	66	6	65	16	3
M290	15	Italy	Hiedelberg	2	7	9	9	5	9	12	1
M291	30	Italy	Kottbus	10	17	21	12	15	20	18	1
M292	474	Italy	Napoli	161	147	96	178	155	12	142	4
M293	32	Italy	Infantis	17	18	22	17	5	21	19	5
M294	32	Maryland/USA	Heidelberg	17	18	22	17	5	21	19	5
M295	19	Maryland/USA	Typhimurium	10	7	12	9	5	9	19	4

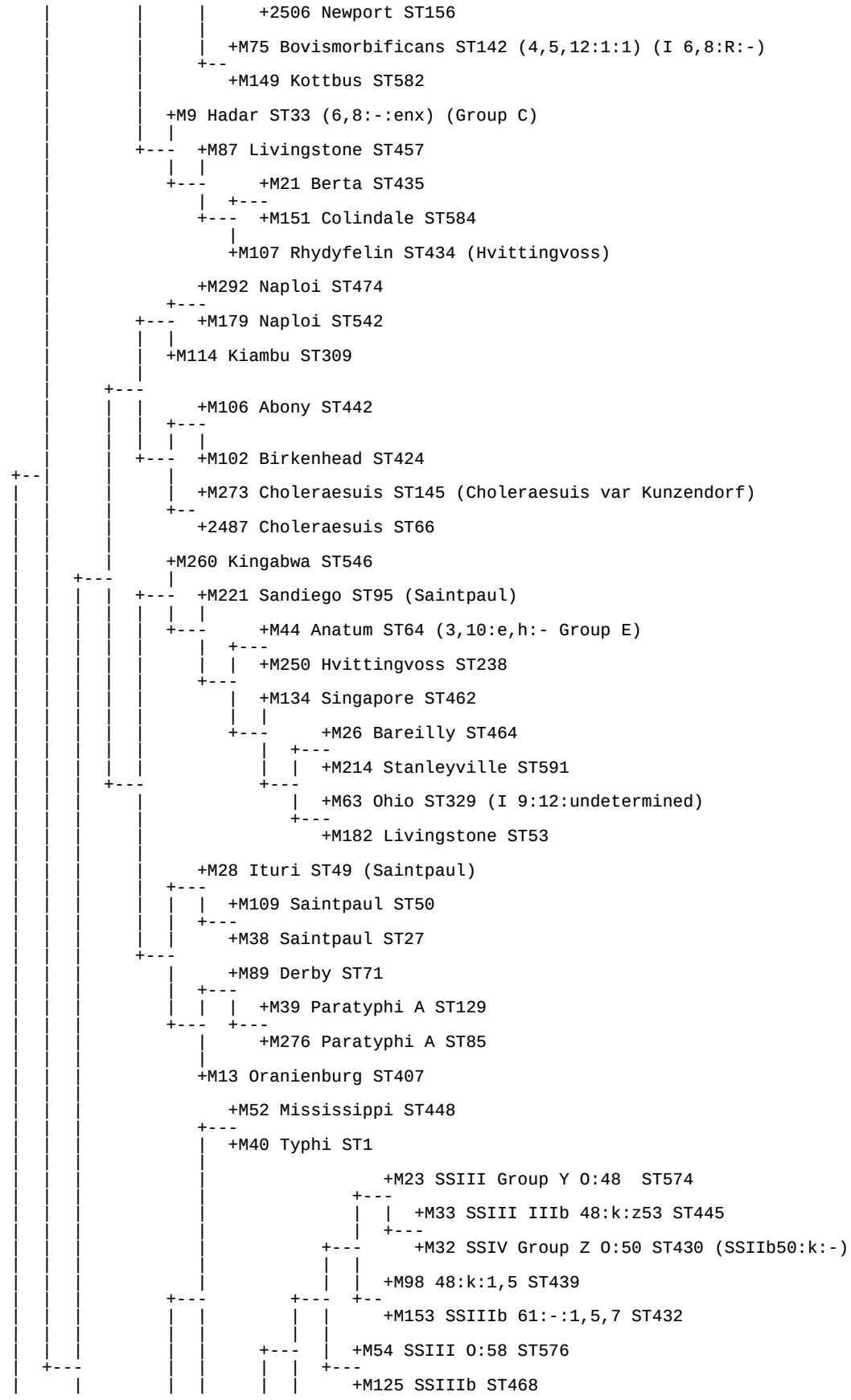
ID	ST	Country of Origin	Serotype	aroC	dnaN	hemD	hisD	purE	sucA	thrA	Group
M296	11	Maryland/USA	Enteritidis	5	2	3	7	6	6	11	3
M297	118	Maryland/USA	Newport	16	2	45	43	36	39	42	1
M298	11	Ohio/USA	Enteritidis	5	2	3	7	6	6	11	3
M299	50	Ohio/USA	Saintpaul	5	21	18	9	6	12	17	4
M300	559	PA/USA	SS I Rough:eh:1,5	84	7	25	197	167	71	3	2
M301	127	PA/USA	Paratyphi B dT^{+}	46	54	46	46	38	18	34	3
M302	81	PA/USA	Montevideo	43	41	16	42	35	13	4	2
M303	95	PA/USA	Saintpaul	3	36	43	38	16	42	38	4
M304	23	CDC/USA	Oranienburg	13	11	16	15	12	15	4	5
M305	40	CDC/USA	Derby	19	20	3	20	5	22	22	3
M306	11	Italy	Enteriditis	5	2	3	7	6	6	11	3
1259	216	SouthAfrica	Isangi	2	73	41	79	71	65	72	5
1261	337	SouthAfrica	Isangi	2	73	41	79	71	65	8	5
1284	335	SouthAfrica	Isangi	117	73	41	79	71	65	72	5
2348	336	SouthAfrica	Isangi	10	73	41	79	71	9	72	5
2427	19	SouthAfrica	Typhimurium	10	7	12	9	5	9	2	4
2443	334	Spain	Brandenburg	11	10	13	32	10	13	112	2
2444	19	Spain	Typhimurium	10	7	12	9	5	9	2	4
2446	19	Italy	Typhimurium	10	7	12	9	5	9	2	4
2448	11	Uganda	Enteriditis	5	2	3	7	6	6	11	3
2487	66	Taiwan	Cholerasuis	34	31	35	14	26	6	8	4
2488	29	Taiwan	Stanley	16	16	20	18	8	12	18	1
2491	292	Taiwan	Albany	104	100	54	78	104	9	48	3
2492	19	Taiwan	Typhimurium	10	7	12	9	5	9	2	4
2499	19	CDC/USA	Typhimurium	10	7	12	9	5	9	2	4
2500	96	Denmark	Schwarzengrund	43	47	49	49	41	15	3	2
2501	85	Denmark	Paratyphi A	45	4	8	44	27	9	8	5
2506	156	Denmark	Newport	63	14	6	12	5	14	58	1
2508	34	Germany	Typhimurium	10	19	12	9	5	9	2	4
2509	19	ACHD/USA	Typhimurium	10	7	12	9	5	9	2	4
2519	15	Philippines	Heidelberg	2	7	9	9	5	9	12	1
2522	328	Philippines	Typhimurium	116	7	12	9	5	9	2	4
2526	328	Taiwan	Typhimurium	116	7	12	9	5	9	2	4
2541	11	SouthAfrica	Enteritidis	5	2	3	7	6	6	11	3
2542	313	SouthAfrica	Typhimurium	10	7	12	9	112	9	2	4

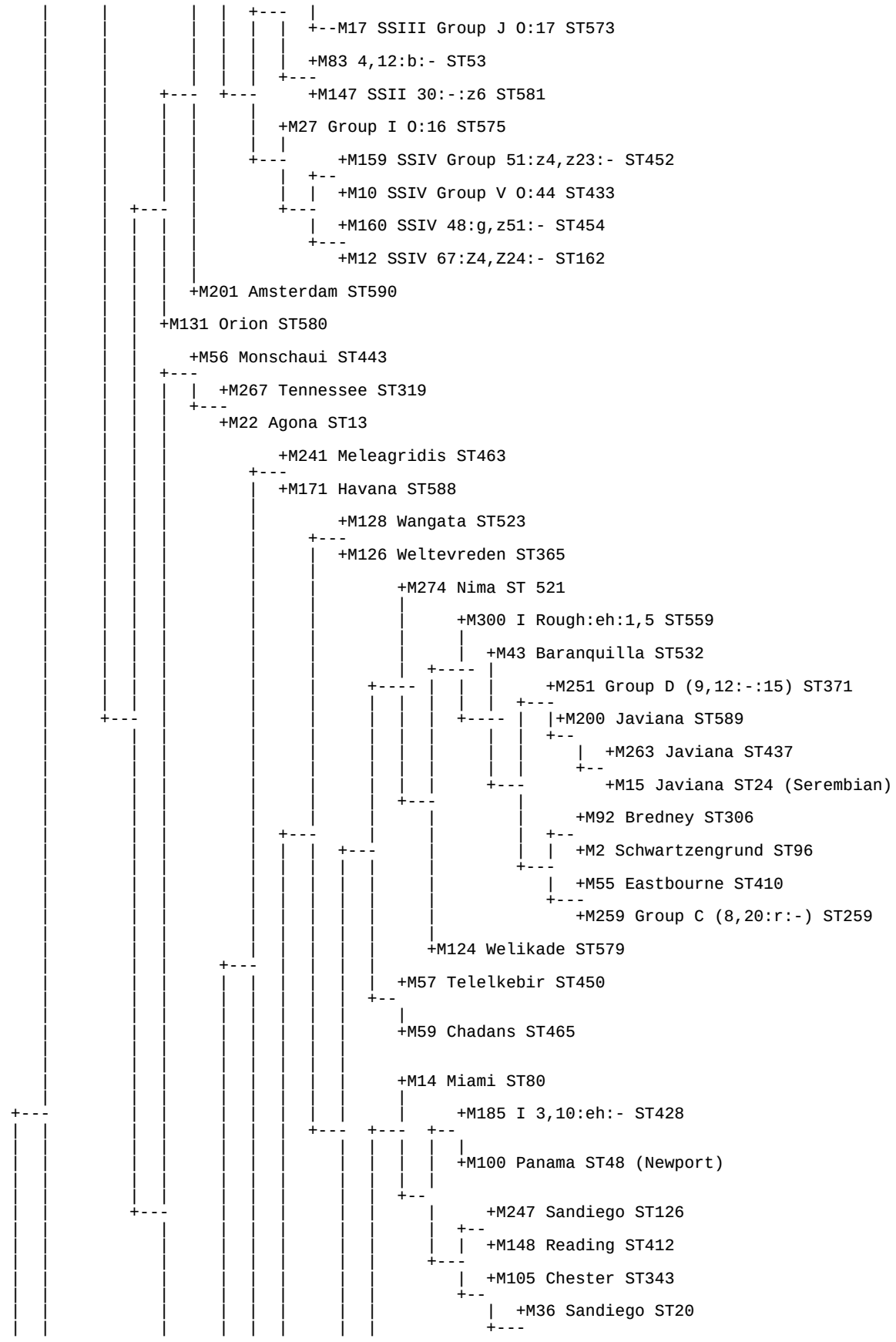
APPENDIX B

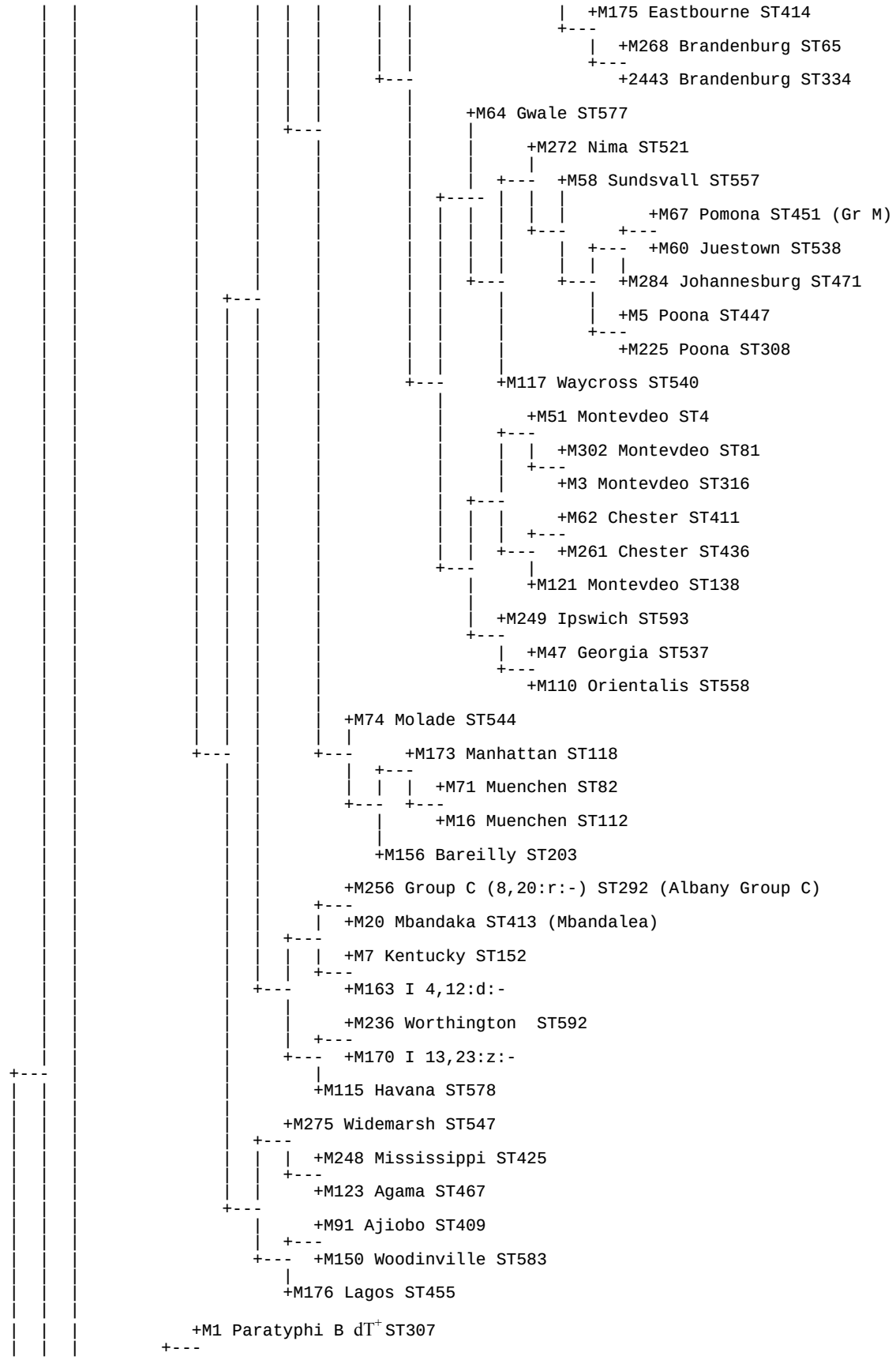
CHAPTER 2: MAXIMUM LIKELIHOOD TREE OF MLST STS AND ISOLATES

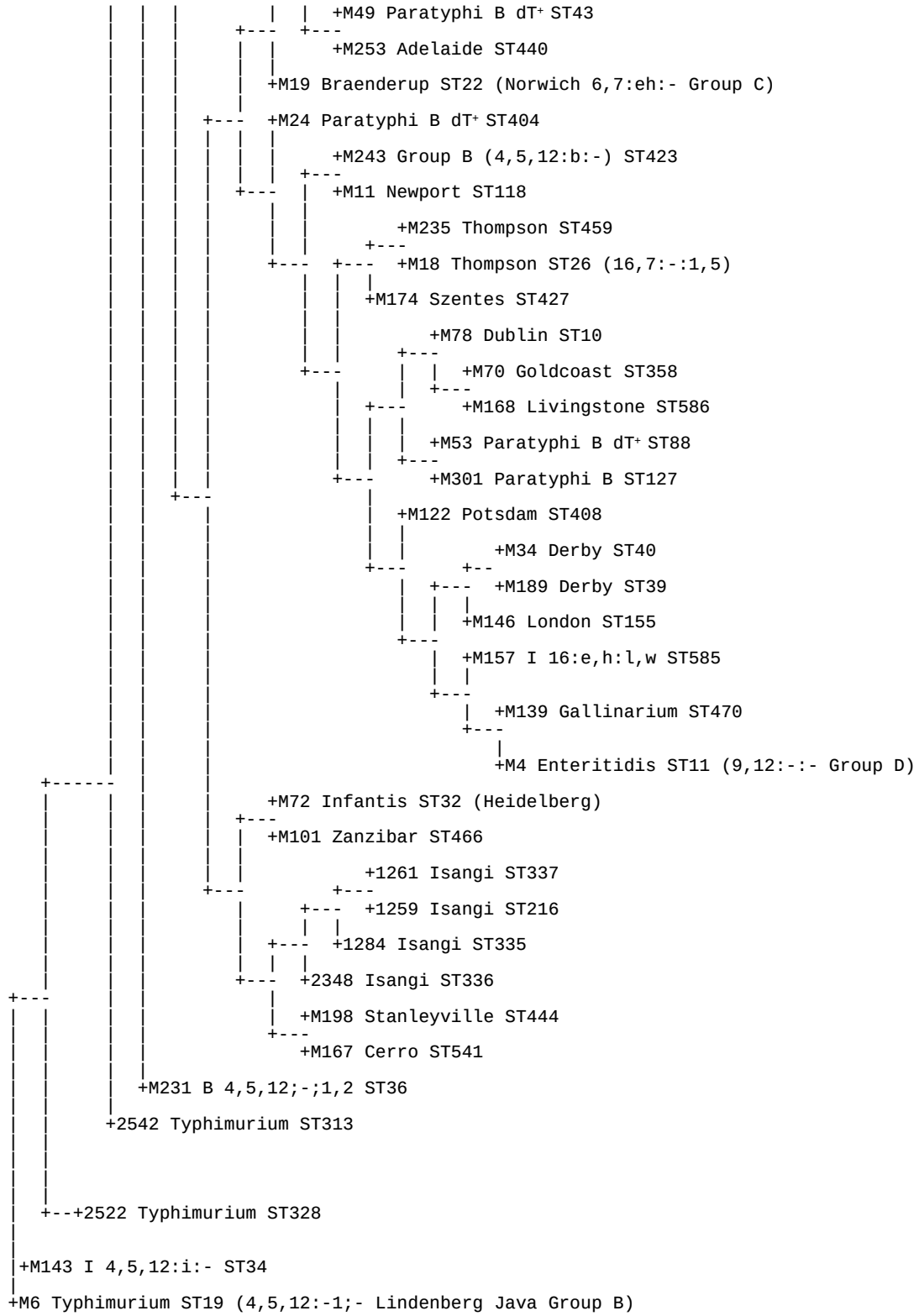
Maximum likelihood tree of unique sequence types (STs) from 169 *S. enterica* isolates, labeled by isolate ID and serovar. When more than one isolate had the same ST and serovar, only one ID and serovar is shown. When an ST contained more than one serovar, alternate serovars are listed after the ST, in parentheses.




+2506 Newport ST156
+M75 Bovismorbificans ST142 (4,5,12:1:1) (I 6,8:R:-)
+M149 Kottbus ST582
+M9 Hadar ST33 (6,8:-:enx) (Group C)
+M87 Livingstone ST457
+M21 Berta ST435
+M151 Colindale ST584
+M107 Rhydyfelin ST434 (Hvittingvoss)
+M292 Naploi ST474
+M179 Naploi ST542
+M114 Kiambu ST309
+M106 Abony ST442
+M102 Birkenhead ST424
+M273 Choleraesuis ST145 (Choleraesuis var Kunzendorf)
+2487 Choleraesuis ST66
+M260 Kingabwa ST546
+M221 Sandiego ST95 (Saintpaul)
+M44 Anatum ST64 (3,10:e,h:- Group E)
+M250 Hvittingvoss ST238
+M134 Singapore ST462
+M26 Bareilly ST464
+M214 Stanleyville ST591
+M63 Ohio ST329 (I 9:12:undetermined)
+M182 Livingstone ST53
+M28 Ituri ST49 (Saintpaul)
+M109 Saintpaul ST50
+M38 Saintpaul ST27
+M89 Derby ST71
+M39 Paratyphi A ST129
+M276 Paratyphi A ST85
+M13 Oranienburg ST407
+M52 Mississippi ST448
+M40 Typhi ST1
+M23 SSIII Group Y O:48 ST574
+M33 SSIII IIIb 48:k:z53 ST445
+M32 SSIV Group Z O:50 ST430 (SSIIb50:k:-)
+M98 48:k:1,5 ST439
+M153 SSIIIb 61:-:1,5,7 ST432
+M54 SSIII O:58 ST576
+M125 SSIIIb ST468


+M17 SSIII Group J O:17 ST573
+M83 4,12:b:- ST53
+M147 SSII 30:-:z6 ST581
+M27 Group I O:16 ST575
+M159 SSIV Group 51:z4,z23:- ST452
+M10 SSIV Group V O:44 ST433
+M160 SSIV 48:g,z51:- ST454
+M12 SSIV 67:Z4,Z24:- ST162
+M201 Amsterdam ST590
+M131 Orion ST580
+M56 Monschaui ST443
+M267 Tennessee ST319
+M22 Agona ST13
+M241 Meleagridis ST463
+M171 Havana ST588
+M128 Wangata ST523
+M126 Weltevreden ST365
+M274 Nima ST 521
+M300 I Rough:eh:1,5 ST559
+M43 Baranquilla ST532
+M251 Group D (9,12:-:15) ST371
+M200 Javiana ST589
+M263 Javiana ST437
+M15 Javiana ST24 (Serembian)
+M92 Bredney ST306
+M2 Schwartzengrund ST96
+M55 Eastbourne ST410
+M259 Group C (8,20:r:-) ST259
+M124 Welikade ST579
+M57 Telelkebir ST450
+M59 Chadans ST465
+M14 Miami ST80
+M185 I 3,10:eh:- ST428
+M100 Panama ST48 (Newport)
+M247 Sandiego ST126
+M148 Reading ST412
+M105 Chester ST343
+M36 Sandiego ST20


+M175 Eastbourne ST414
+M268 Brandenburg ST65
+2443 Brandenburg ST334
+M64 Gwale ST577
+M272 Nima ST521
+M58 Sundsvall ST557
+M67 Pomona ST451 (Gr M)
+M60 Juestown ST538
+M284 Johannesburg ST471
+M5 Poona ST447
+M225 Poona ST308
+M117 Waycross ST540
+M51 Montevdeo ST4
+M302 Montevdeo ST81
+M3 Montevdeo ST316
+M62 Chester ST411
+M261 Chester ST436
+M121 Montevdeo ST138
+M249 Ipswich ST593
+M47 Georgia ST537
+M110 Orientalis ST558
+M74 Molade ST544
+M173 Manhattan ST118
+M71 Muenchen ST82
+M16 Muenchen ST112
+M156 Bareilly ST203
+M256 Group C (8,20:r:-) ST292 (Albany Group C)
+M20 Mbandaka ST413 (Mbandalea)
+M7 Kentucky ST152
+M163 I 4,12:d:-
+M236 Worthington ST592
+M170 I 13,23:z:-
+M115 Havana ST578
+M275 Widemarsh ST547
+M248 Mississippi ST425
+M123 Agama ST467
+M91 Ajiobo ST409
+M150 Woodinville ST583
+M176 Lagos ST455
+M1 Paratyphi B dT+ ST307


+M49 Paratyphi B dT+ ST43
+M253 Adelaide ST440
+M19 Braenderup ST22 (Norwich 6,7:eh:- Group C)
+M24 Paratyphi B dT+ ST404
+M243 Group B (4,5,12:b:-) ST423
+M11 Newport ST118
+M235 Thompson ST459
+M18 Thompson ST26 (16,7:-:1,5)
+M174 Szentes ST427
+M78 Dublin ST10
+M70 Goldcoast ST358
+M168 Livingstone ST586
+M53 Paratyphi B dT+ ST88
+M301 Paratyphi B ST127
+M122 Potsdam ST408
+M34 Derby ST40
+M189 Derby ST39
+M146 London ST155
+M157 I 16:e,h:l,w ST585
+M139 Gallinarium ST470
+M4 Enteritidis ST11 (9,12:-:- Group D)
+M72 Infantis ST32 (Heidelberg)
+M101 Zanzibar ST466
+1261 Isangi ST337
+1259 Isangi ST216
+1284 Isangi ST335
+2348 Isangi ST336
+M198 Stanleyville ST444
+M167 Cerro ST541
+M231 B 4,5,12;-;1,2 ST36
+2542 Typhimurium ST313
+2522 Typhimurium ST328
+M143 I 4,5,12:i:- ST34
+M6 Typhimurium ST19 (4,5,12:-1;- Lindenberg Java Group B)

APPENDIX C

CHAPTER 2: SEROTYPE ABBREVIATIONS

Serotype	Abbreviation	Serotype	Abbreviation	Serotype	Abbreviation
Aberdeen	Abe	Hvittingfoss	Hvi	Orion	Ori
Abony	Abo	Infantis	Inf	Panama	Pan
Adelaide	Ade	Ipswich	Ips	Paratyphi A	PtA
Agama	Aga	Isangi	Isa	Paratyphi B dT+	PtB
Agona	Ago	Ituri	Itu	Pomona	Pom
Ajiobo	Aji	Javiana	Jav	Poona	Poo
Albany	Alb	Johannesburg	Joh	Potsdam	Pot
Amersterdam	Ams	Jukestown	Juk	Reading	Rea
Anatum	Ana	Kentucky	Ken	Rhydyfelin	Rhy
Baranquilla	Bar	Kiambu	Kia	Richmond	Ric
Bareilly	Bae	Kingabwa	Kin	Rissen	Ris
Berta	Ber	Kottbus	Kot	Saintpaul	Stp
Birkenhead	Bir	Lagos	Lago	Salinatis	Sal
Blockley	Blo	Lindenberg	Lin	Sandiego	San
Bovismorbificans	Bov	Litchfield	Lit	Schwarzengrund	Sch
Braenderup	Brn	Livingstone	Live	Seremban	Ser
Brandenburg	Bra	London	Lon	Singapore	Sin
Bredeney	Bre	Manhattan	Man	SS I 4,5,12:i:-	Tym-
Cerro	Cer	Mbandaka	Mbk	SS I Rough Form	I:R
Chandans	Cha	Mbandalea	Mbl	SS I 48:k:1,5	I:48
Chester	Che	Meleagridis	Mel	SS II	II
Choleraesuis	Cho	Mgulani	Mgu	SS III	III
Choleraesuis var kunzendorf	ChK	Miami	Mia	SS IIIb	IIIb
Colindale	Col	Mississippi	Mis	SS IV	IV
Cubana	Cub	Molade	Mol	Stanley	Sta
Derby	Der	Monschaui	Mos	Stanleyville	Stv

Dublin	Dub	Montevideo	Mot	Sundsvall	Sun
Eastbourne	Eas	Muenchen	Mue	Szentes	Sze
Enteriditis	Ent	Napoli	Nap	Telelkebir	Tel
Gallinarum	Gal	Newport	New	Tennessee	Ten
Georgia	Geo	Nima	Nim	Thompson	Tho
Goldcoast	Gol	Norwich	Nor	Typhi	Typ
Group B	Gp B	Ohio	Ohi	Typhimurium	Tym
Group C	Gp C	Oranienburg	Ora	Virchow	Vir
Group D	Gp D	Orientalis	Ore	Wangata	Wan
Group E	Gp E	Orion	Ori	Waycross	Way
Group I O:16	Gp I	Newport	New	Welikade	Wel
Gwale	Gwa	Nima	Nim	Weltevreden	Wet
Hadar	Had	Norwich	Nor	Widemarsh	Wid
Hartford	Har	Ohio	Ohi	Woodinville	Wod
Hartford	Har	Oranienburg	Ora	Worthington	Wor
Havana	Hav	Orientalis	Ore	Zanzibar	Zan
Heidelberg	Hei				

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