Characterization of antimicrobial resistance mechanisms in multi-drug resistant *Salmonella* and *Shigella* strains from Brazil and Colombia

by

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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ABSTRACT

Multi-drug resistant (MDR) *Salmonella* spp. and *Shigella* spp. are preventing the effective and economical therapy of diarrheal diseases in Brazil and Colombia. Diarrhea continues to be a major cause of mortality in children in these two developing countries. This study was conducted in order to detect and characterize the antimicrobial resistance genes that were encoded by MDR *Salmonella* and *Shigella* strains from both Brazil and Colombia. Antimicrobial resistance gene profiles were determined for 242 MDR *Salmonella* and *Shigella* strains from both countries. To further characterize their mechanisms of resistance to β-lactams, 24 strains were screened for the presence of extended-spectrum β-lactamases (ESBLs). To determine the location of some of the resistance genes, 82 strains were screened for the presence of integrons and plasmid profiles were determined for another 21 strains with different antibiograms. These plasmids were then hybridized to bla_{TEM-1}, *tetB* and *catI* probes. The majority of the 242 *Salmonella* and *Shigella* strains encoded similar resistance mechanisms to each antimicrobial group tested and numerous putative ESBLs were observed with pI values ranging from 5.4 to approximately 9. Among the integrons sequenced, nine harboured various antimicrobial gene cassettes encoding resistance to aminoglycosides, β-lactams and trimethoprim. Twenty-three different plasmid profiles were obtained and the bla_{TEM}, *tetB* and *catI* genes were located on the 26MDa and 23MDa plasmids in several strains. Prudent use of antimicrobials in Brazil and Colombia is essential if antimicrobial therapy of serious cases of salmonellosis and shigellosis is to remain a viable route of treatment.

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1. LITERATURE REVIEW

1.1 Introduction: The problem of multi-drug resistance (MDR) in bacteria.

Since the 1940s, a wide variety of antimicrobials have been developed as a means of protection against infections caused by pathogenic bacteria. The widespread use and misuse of antimicrobial agents in medicine, agriculture and animal husbandry has provided the selective pressure responsible for the evolution of bacteria toward resistance (Levy, 2001; Tan *et al.*, 2000). As a growing number of medically important bacteria acquire antimicrobial resistance, therapeutic options for the treatment of serious bacterial infections become limited. It has become crucially important to establish effective surveillance programs worldwide in order to monitor and control the emergence and spread of antimicrobial-resistant bacteria. Despite the existence of surveillance programs founded by numerous organizations in different parts of the world including the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), novel resistance phenotypes still continue to emerge and multi-drug resistant (MDR) bacteria continue to spread worldwide (Richet, 2001).

Although a quantitative relationship has not yet been determined between the use of antimicrobials and the emergence antimicrobial resistance in bacteria, it is clear that there is a correlation between the two (Levy, 2001). For example, there is evidence that use of antimicrobials in animal husbandry causes the following disturbing sequence of events leading to transfer of resistant bacterial flora from animals to humans: a) selective pressures caused by antimicrobial use for therapy, prophylaxis and growth enhancement in animals used for food increases the resistance of their commensal and pathogenic bacterial flora; b) resistant bacteria from animals, including zoonotic

bacteria such as *Salmonella* spp., can transfer to humans via direct contact or through ingestion of contaminated meat; and finally, c) resistant bacteria from animals may colonize humans which allows for the transfer of resistance genes from animal-derived bacteria to human flora (Bogaard and Stobberingh, 2000; Levy, 1976; Nikolich *et al*, 1994; Shoemaker *et al*, 1992). It has been shown that strictly human pathogens that are present in human intestinal flora, such as *Shigella* spp., do gain resistance determinants in this manner (Hummel, 1986). The animal husbandry industry is, therefore, one potential resistance gene pool that contributes to the spread of antimicrobial resistance (Threlfall, 2002). Since the use of antimicrobials will continue in various disciplines, it has become very important to understand the types of resistance mechanisms bacteria use to survive in its presence in order to find effective methods to control the spread of antimicrobial resistance.

1.2 Dissemination of antimicrobial resistance genes

Extensive antimicrobial use and the presence of antimicrobial resistance genes in bacteria are the two components necessary to produce a clinical resistance problem (Levy, 2001). Whether in humans, animals or the environment, antimicrobials can alter the mix of bacteria in a population by killing susceptible bacteria and by selecting for the growth of resistant strains (Levy, 2001). In the presence of antimicrobial selective pressure, the rapid dissemination of resistance genes throughout a bacterial population is a consequence of the many different ways bacteria pass on or share genetic information. Of greatest importance is acquired resistance where horizontal gene transfer mechanisms permit the intra- and inter-species transfer of genetic information

within and between species via transformation, bacteriophage-mediated transduction, cell-cell conjugation with plasmid transfer and finally, transposable elements (Helmuth, 2000; Tan *et al*, 2000). Antibiotic resistance can also be intrinsic, resulting from an accumulation of several point mutations or from the induction of latent genes (Tan *et al*, 2000). Acquired or intrinsic resistance mechanisms can also be passed on vertically from mother to daughter cells during cell division.

1.2.1 Integrons and the spread of multi-drug resistance

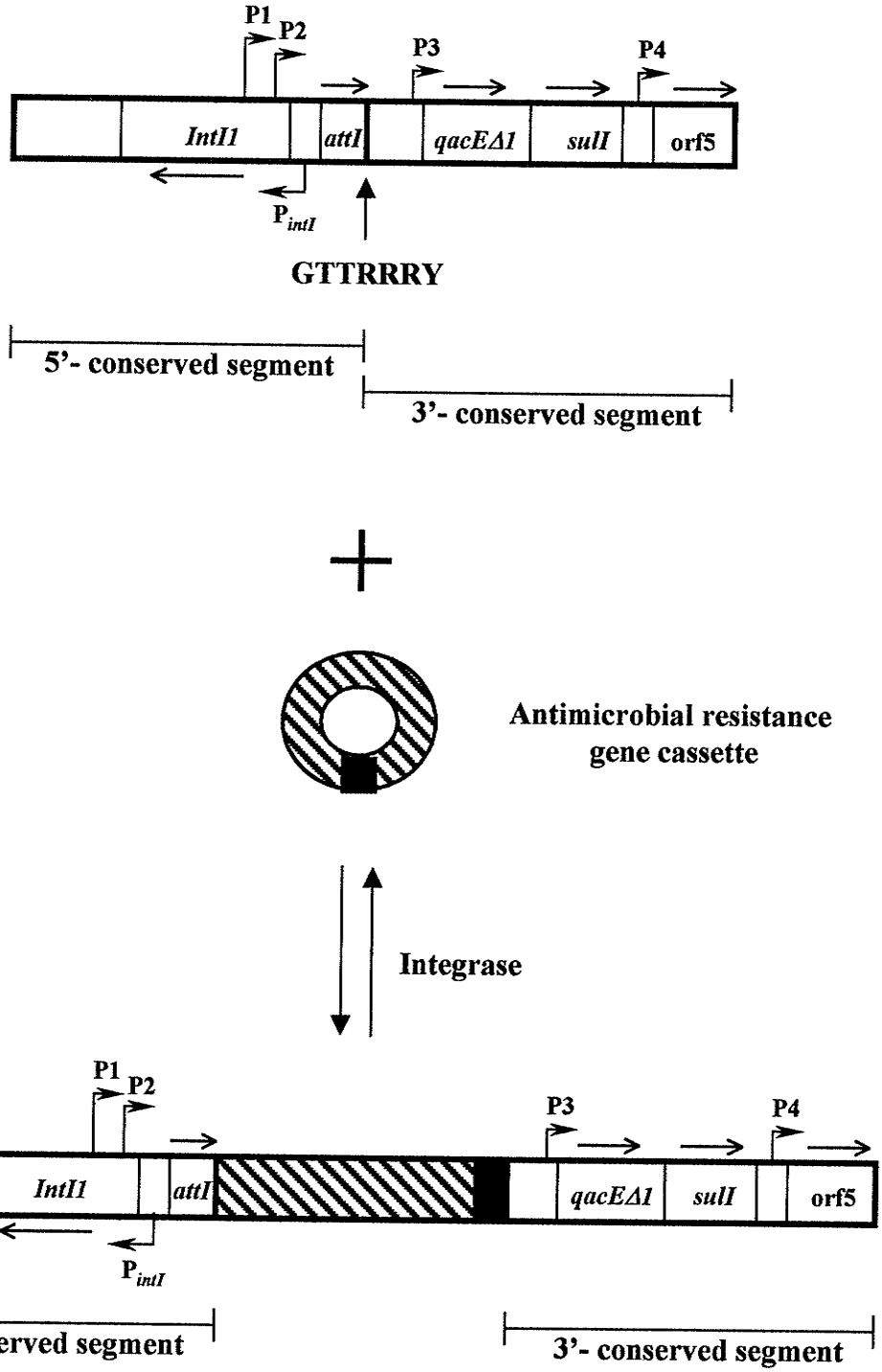
The rapid dissemination of multi-drug resistance in Gram-negative bacteria, especially in *Enterobacteriaceae*, is partly attributed to integrons (Rowe-Magnus *et al*, 1999; Brown *et al,* 2000). Integrons are gene expression elements capable of acquiring antimicrobial resistance gene cassettes by site-specific recombination (Brown *et al,* 2000; Hall, 1997). All integrons have three main components which permit the incorporation and expression of acquired gene cassettes: (a) a site-specific integrase gene (*intI*) that catalyzes the insertion or excision of gene cassettes, (b) a recombination site (*attI*) and (c) a strong promoter upstream of the insertion site for gene cassettes (Rowe-Magnus *et al*, 1999). Four different classes of integrons have been identified to date and their classification is based on the type of integrase they possess (Guerra *et al*, 2000; Naas *et al*, 2001). Class 1 integrons, the most common type found in clinical enterobacterial isolates, have a type 1 integrase encoded by the *intI1* gene and are characterized by two "conserved segments" (CS), a 5'CS and 3'CS, which flank a variable region into which various resistance gene cassettes can be inserted (Figure 1) (Guerra *et al*, 2000; Naas *et al*, 2001). Figure 1 describes the general structure of a class 1 integron before and after the insertion of an antimicrobial resistance gene cassette. Though the 3' CS of class 1

integrons may vary, most class 1 integrons encode the 3'CS that is shown in Figure 1 (Naas *et al*, 2001). Gene cassettes are small mobile genetic elements that consist of one gene, most often without its own promoter, and a "59-base element" (or *attC*) at the 3' end that varies in sequence and size except for two inverted repeats with 7bp core regions corresponding to the GTTRRRY insertion site on the integron (Recchia and Hall, 1997). As is illustrated in Figure 1, gene cassettes that are not integrated into an integron exist as non-replicating circular molecules (Recchia and Hall, 1997). However, if one or more cassettes become inserted into an integron, they are co-transcribed from one or both of the promoters located in the 5'CS (Naas *et al*, 2001). Of the approximately 60 known gene cassettes, the majority are antimicrobial resistance genes that confer resistance to aminoglycosides, chloramphenicol, ß-lactams and trimethoprim (Guerra *et al*, 2000; Recchia and Hall, 1997).

Though integrons are incapable of self-transposition, they are often found on conjugative plasmids and transposons which permit the intra- and inter-species transfer of the antibiotic resistance genes they carry (Hall, 1997). The ability for integrons to capture and express many antibiotic resistance gene cassettes is one of the most important reasons why the misuse and overuse of antimicrobials must be prevented. As integrons are often found on plasmids and transposons already encoding antimicrobial resistance genes, the selection pressure caused by antimicrobial use not only selects for one resistance determinant but selects for many (Fluit and Schmitz, 1999).

Figure 1:

Schematic representation of a class 1 integron. The general structure of a class 1 integron consists of a 5'-conserved segment (5'CS) that encodes: an integrase (*intI*) that is preceeded by its promoter P_{int}; one (P1) or two (P2) strong promoters that direct the transcription of inserted antimicrobial resistance gene cassettes; and, finally, an *attI* site downstream of the strong promoters where integrase catalyzes the recombination of circularized gene cassettes. The GTTRRRY consensus sequence (G = guanine, T = thymine, R = purine, Y = pyrimidine) is the target of recombination and the exact point of insertion of gene cassettes occurs between the G and first T. The 3'CS encodes a promoter (P3) that enables the transcription of both *qacEΔ1* and *sulI* genes that encode resistance to quaternary ammonium compounds and to sulfonamides, respectively. The P3 promoter controls the expression of open-reading frame 5 (orf5) of unknown function. Integrase catalyzes the insertion or excision of gene cassettes at the GTTRRRY site either at the *attI* of the 5'CS or at the 59'base element of an existing integrated gene cassette though the former is preferred (Fluit and Schmitz, 1999). The black box at the end of the antimicrobial resistance gene cassette represents the 59-base element.


P1
P2
P3
P4
IntI1
attI
qacEΔ1
sulI
orf5
P intI
GTTRRRY
5'- conserved segment
3'- conserved segment
Antimicrobial resistance gene cassette
Integrase
P1
P2
P3
P4
IntI1
attI
qacEΔ1
sulI
orf5
P intI
5'- conserved segment
3'- conserved segment

1.3 *Salmonella* spp., *Shigella* spp. and multi-drug resistance

1.3.1 Description of *Salmonella* spp.

Salmonellosis is one of the leading causes of bacterial food-borne illness worldwide and has a significant impact on society regarding human suffering and financial burden. In 2002, the total yearly cost associated with salmonellosis in the United States alone is estimated at $2.4 billion (http://www.ers.usda.gov) whereas only 2 years earlier, the estimate was $1.4 billion (The World Health Organization, 2000). Salmonellosis is generally divided into four syndromes: gastroenteritis, enteric or typhoid fever, septicemia (with or without gastroenteritis) and an asymptomatic carrier state (Galan and Sansonetti, 1996; Janda and Abbott, 1998). The type of infection that will develop depends on several factors including the serotype of the invading *Salmonella,* the host being invaded and the state of the host's immune system (Galan and Sansonetti, 1996). The natural reservoir of non-typhoidal *Salmonella* are animals and therefore humans most often become infected by ingesting contaminated red meat, poultry, eggs and dairy products (Buck and Werker, 1998).

Salmonella spp. are Gram-negative, facultatively anaerobic bacilli belonging to the family *Enterobacteriaceae* (Janda and Abbott, 1998). The genus *Salmonella* has two species- *Salmonella enterica* and *S. bongori* (Popoff and Le Minor, 1997). *Salmonella enterica* is further subdivided into 6 subspecies wherein over 2000 different serotypes have been identified. The majority of *Salmonella* strains isolated from humans are *Salmonella enterica* subsp. *enterica* (Agasan *et al*, 2002). The serotype of a *Salmonella* isolate is determined by the somatic (O), flagellar (H) and Vi antigens that are found on the surface of the outer membrane (Agasan *et al*, 2002). All *Salmonella* serotypes

mentioned in this thesis are *Salmonella enterica* subspecies *enterica* (Popoff and Le Minor, 1997). Some *Salmonella* serotypes only infect a specific host, such as *S.* Typhi and *S.* Paratyphi that only infect humans, while others have a broad host range such as *S.* Typhimurium, *S.* Agona, *S.* Enteritidis and *S.* Infantis that are capable of invading both human and animal hosts (Galan and Sansonetti, 1996; Janda and Abbott, 1998).

1.3.2 Description of *Shigella* spp

Shigellosis is endemic throughout the world and is especially a problem in developing countries where unsanitary conditions and malnutrition are common (Galan and Sansonetti, 1996). *Shigella* spp.are transmitted via the oral-fecal route, often through the consumption of *Shigella*-contaminated food and water. Unlike *Salmonella* spp., *Shigella* spp. are host-adapted to humans only and cause bacillary dysentery which is characterized by abdominal pain, fever and watery diarrhea which often contains blood and mucus (Janda and Abbott, 1998). Although shigellosis is usually self-limiting, infants and young children are at greatest risk of developing life-threatening symptoms including severe sepsis, dehydration, intestinal occlusion and/or perforation and subsequent chronic malnutrition (Galan and Sansonetti, 1996). The infectious dose of *Shigella* is as low as 10-100 organisms and thus, it is extremely contagious (Janda and Abbott, 1998). For these reasons, antimicrobial therapy has often been used for patients with shigellosis as it minimizes both the duration of clinical symptoms and the period of fecal excretion of *Shigella* organisms .

Shigella spp. are Gram-negative, facultatively anaerobic bacilli that are non-motile. The serotype of *Shigella* isolates is determined by the cell wall's somatic O-antigens. *Shigella* spp. are divided into 4 serogroups, three of which have multiple serotypes: Group A: *Shigella dysenteriae* (15 serotypes); Group B: *Shigella flexneri* (6 serotypes); Group C: *Shigella boydii* (18 serotypes); and Group D: *Shigella sonnei* (1 serotype) (Janda and Abbott, 1998). In addition, the Group D *Shigella sonnei* produce colonies that are either smooth (form I) or rough (form II) (Janda and Abbott, 1998). *S. flexneri* is the most prevalent species in developing countries whereas *S. dysenteriae*, a highly virulent serogroup which is known to cause deadly epidemics, is rare (Galan and Sansonetti, 1996).

1.3.3 Multi-drug resistance in *Salmonella* spp. and *Shigella* spp.: the need for surveillance

Multi-drug resistance strains of *Salmonella* spp. first emerged in the 1960s and have since spread throughout the world (Helmuth, 2000; Janda and Abbott, 1998; Threlfall, 2002). Since the 1990s, an MDR *Salmonella* strain that has become a global health problem is *Salmonella* Typhimurium definitive phage type 104 (PT104, also known as DT104 in the United Kingdom) that is commonly known to be a penta-resistant strain with chromosomally-encoded resistance to ampicillin (A), chloramphenicol (C), streptomycin (S), sulphonamides (Su) and tetracycline (Threlfall, 2002). Within the last few years, *S.* Typhimurium PT104 strains with additional resistance or to trimethoprim, gentamicin, extended-spectrum cephalosporins and /or fluoroquinolones have been reported (Glynn *et. al.*, 1998; Low *et.al.*,1997; Molbak *et. al.*, 1999; Threlfall *et.al.*,

1997). Most humans become infected with multi-drug resistant PT104 (MDR PT104) through contaminated meat or contact with infected animals (Poppe *et. al.*, 1998). Since it was first isolated from cattle in the United Kingdom (Threlfall *et.al.*, 1994), MDR PT104 has been isolated from various animals including porcine and avian hosts (Besser *et. al.*, 1997; Low *et.al.*,1997; Ridley and Threlfall, 1998) and has been observed in different parts of the world including other European countries (Baggesen et al, 2000), Canada (Poppe *et. al.*,1996) and the United States (Besser *et al.*, 1997). In addition to MDR PT104, there are a growing number of other MDR *S.* Typhimurium phage types as well as other MDR *Salmonella* serotypes that have been reported (Gebreyes and Altier, 2002; Nastasi *et. al.*, 2000; Threlfall, 2002; Winokur *et. al.*, 2000). Unfortunately, not all countries consider *Salmonella* infection as a notifiable disease and thus, without proper surveillance, it is difficult to ascertain the true magnitude of the MDR problem in *Salmonella* spp worldwide (Baggesen *et al.*, 2000).

Since the emergence of sulfonamide-resistant *Shigella* spp. in the 1950s, *Shigella* spp. have acquired resistance to commonly used antimicrobials such as ampicillin, tetracycline, sulfamethoxazole-trimethoprim (SXT), streptomycin, chloramphenicol and nalidixic acid in both industrialized and developing countries (McIver *et. al.*, 2002; Navia *et. al.*, 1999). The treatment of shigellosis caused by MDR *Shigella* strains is often limited to second- or third-generation cephalosporins, quinolones or fluoroquinolones (Aysev and Guriz, 1998; Jamal *et. al.*, 1998; Laureillard *et. al.*, 1998; Replogle *et. al.*, 2000). It is only a matter of time before MDR *Shigella* strains acquire resistance to all known antimicrobials since fluoroquinolone-resistant *Shigella flexneri* and *Shigella*

dysenteriae have already been isolated in India (Ballal *et. al.*, 1998; Thirunarayanan *et. al.*, 1993).

Since 1996, the Pan American Health Organization (P.A.H.O), a branch of the World Health Organization, has been sponsoring a project aimed at improving the surveillance of antimicrobial resistance in *Salmonella* spp., *Shigella* spp.and *Vibrio cholerae* spp. in the Americas. With the collaboration of Health Canada's National Microbiology Laboratory (N.M.L.) (formerly known as the Laboratory Center for Disease Control), participating laboratories from Central and South America are tested on their performance of laboratory techniques required for the surveillance of antimicrobial resistance in *Salmonella* spp., *Shigella* spp.and *Vibrio cholerae* spp. The main objective of performance testing is to ensure the proper use of standardized techniques for antimicrobial susceptibility testing and serotyping in each country by training lab personnel as well as providing the necessary test materials. Quarterly surveillance reports were submitted to the N.M.L. and P.A.H.O. by each participating country which included the total number of *Salmonella*, *Shigella* and *Vibrio cholerae* strains isolated from patients, their serotypes, corresponding epidemiological data (if available) and antimicrobial susceptibility profiles. A summary of results for all participating countries from 1996-1999 is published on P.A.H.O.'s website http://www.paho.org.

1.4 Antimicrobials and resistance mechanisms in bacteria

Antimicrobials have varied spectra of action due to different mechanisms used to target bacteria. Broad spectrum antimicrobials are those that inhibit a variety of

pathogens (both Gram-positive and Gram-negative bacteria) whereas narrow spectrum antimicrobials act on a single group of organisms (Brock *et al.*, 1994). In general, antimicrobial agents have mechanisms that target one of the following bacterial structures or pathways: (a) the bacterial cell wall; (b) the cytoplasmic membrane; (c) protein synthesis; (d) nucleic acid synthesis; and, finally, (e) metabolic pathways (Neu, 1992).

A wide variety of antimicrobials have been developed or synthesized as a means of protection against infections caused by pathogenic bacteria. The classification of antimicrobials is base primarily on their chemical structure and the addition or subtraction of chemical functional groups from the core structure leads to the various members of the group. Up to date, there are six main groups of antimicrobials that are used to treat Gram-negative infections: ß-Lactams, Aminoglycosides, Chloramphenicol, Tetracyclines, Sulfonamides/Trimethoprim and Quinolones. The specific details of each antimicrobial group are outlined below as well as the resistance mechanisms that bacteria have developed against them.

1.4.1 ß-Lactams

1.4.1.1 Structure and classification of ß-lactams.

Eleven years after Fleming isolated penicillin from *Penicillium* mold in 1928, H. W. Florey and E. B. Chain realized its therapeutic potential and developed it as a chemotherapeutic agent for the treatment of bacterial infections in humans (Livermore and Williams, 1996). Since its first clinical use in 1940, benzylpenicillin (penicillin G) gave rise to the modern antibiotic era that is marked by the widespread use and development of chemotherapeutic agents.

Due to their broad-spectrum activity and generally low toxicity, the ß-lactam family is the most widely used in clinical medicine and has grown to include many natural and semi-synthetic members. The chemical structures of all members of the ß-lactam family are based on the active structure- the four-membered ß-lactam ring. ß-lactams consist of penicillins (penams), cephalosporins (cephems), carbapenems, monobactams and the ß-lactam /ß-lactamase inhibitors combination drugs. As is shown in Figure 2, the penicillins and cephalosporins are the two classical ß-lactam groups and are based on 6-aminopenicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA), respectively (Yao and Moellering, 1995). Table 1 lists the various classes of penicillins that are grouped according to a combination of factors: chemical structure, spectrum of activity and susceptibility to ß-lactamases (see section 1.4.1.3 for details on ß-lactamases) (Yao and Moellering, 1995).

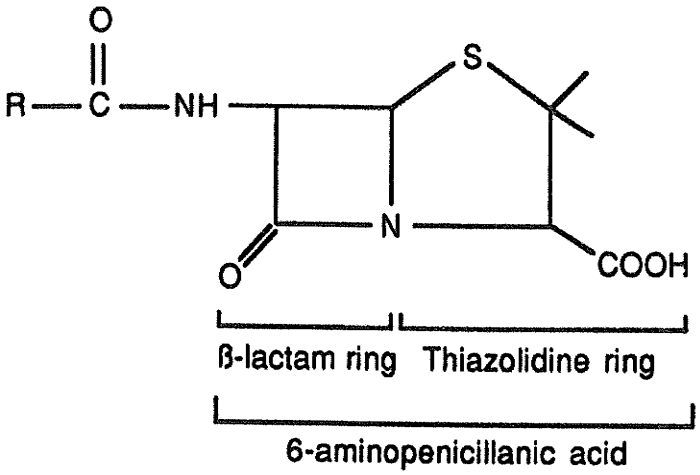
Table 1: Spectra of activity of various penicillin classes.

Penicillin Class	Example	Spectrum of Activity
Natural Penicillins Benzylpenicillin Phenoxymethyl penicillin	 Penicillin G Penicillin V	Susceptible: Penicillinase- Gram+ bacteria, most Gram- bacteria; Resistant: *Bacteroides fragilis*, *Enterobacteriaceae*, *Pseudomonas aeruginosa*
Semi-synthetic Penicillinase-resistant Isoxazolyl penicillins	 Methicillin Oxacillin	Susceptible: Penicillinase+ staphylococci Resistant: MRSA, enterococci, *B. fragilis*, *Enterobacteriaceae*, *Pseudomonas* spp.
Extended Spectrum Aminopenicillins Carboxypenicillins Ureidopenicillins	 Ampicillin Carbenicillin Piperacillin	Susceptible: Penicillinase- Gram+ and ß-lactamase- Gram- bacteria (Carboxy- and ureidopenicillins are more active against some ß-lactamase+ *Enterobacteriaceae* and *P. aeruginosa*) Resistant: penicillinase+ staphylococci
Penicillin + ß-lactamase inhibitors	Ampicillin-sulbactam Ticarcillin-clavulanate Piperacillin-tazobactam	Susceptible: ß-lactamase+ Gram- bacteria (class A), penicillinase+ staphylococci Resistant: class C ß-lactamase+ bacteria

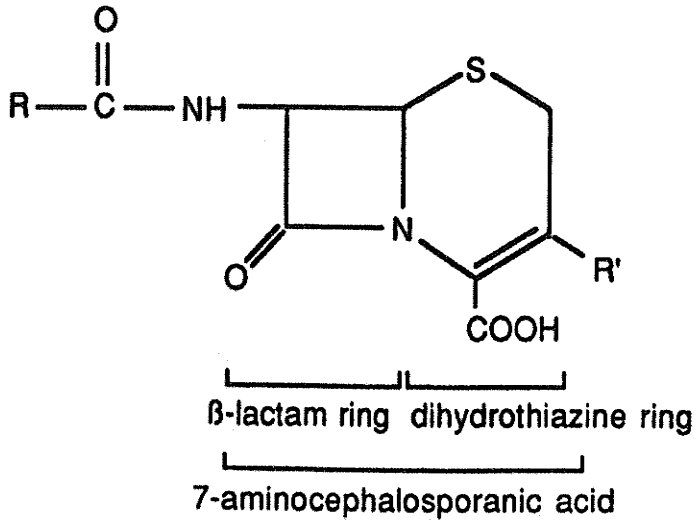
Figure 2:

Chemical structures of penicillins and cephalosporins. The 6-APA structure of penicillins consist of a ß-lactam ring fused to a thiazolidine ring and various side-chain modifications at position 6 differentiate one penicillin from another due to the resulting differences in antibacterial and pharmacokinetic properties. In contrast to penicillins, the 7-ACA of cephalosporins has a dihydrothiazine ring fused to the ß-lactam ring and various members of this ß-lactam group are derived by substituting the functional groups at positions 3 and 7 (Yao and Moellering, 1995).

(A) **Penicillins**



(B) **Cephalosporins**



Cephalosporins, derived from *Cephalosporium acremonium*, were developed in the 1950s due to the appearance of penicillin-resistant staphylococci (Livermore and Williams, 1996). Though several classification schemes have been suggested for cephalosporins, there is no universally accepted scheme to date. The most common classification of cephalosporins is the "generational" scheme that is divided into four generations based on increasing activity against Gram-negative bacteria and decreased susceptibility to ß-lactamases (see Table 2) (Livermore and Williams, 1996).

Table 2: Cephalosporins: generational classification and spectra of activity.

Cephalosporin class	Examples	Spectrum of activity
1st generation: Narrow spectrum	Cefazolin Cephalothin	Gram+ bacteria, penicillin-susceptible and -resistant *S. aureus* and *Streptococcus pyogenes,* some Gram- bacteria
2nd generation: Extended spectrum	Cefoxitin Cefuroxime Cefotetan	ß-lactamase+ Gram-negative bacteria, anaerobic bacteria including *Bacteroides fragilis* (cephamycins only: eg. cefotetan, cefoxitin, cefmetazole), some Gram+ cocci
3rd generation: Broad spectrum	Ceftazidime Cefixime Cefpodoxime	ß-lactamase+ Gram-negative bacteria, *Enterobacteriaceae*, *Pseudomonas* spp., some Gram+ cocci
4th generation:	Cefepime Cefpirome	Aerobic Gram-negative bacteria including many of those resistant to ceftriaxone, cefotaxime and/or ceftazidime, *Pseudomonas* spp. and penicillin-susceptible *S. aureus, S. pyogenes* and *S. pneumoniae*

The non-classical ß-lactams, namely the monobactams, carbapenems and the ß-lactamase inhibitors, have different structures and antibacterial properties in comparison to those of classical ß-lactams. The monobactams, for example, are structurally unique

since they consist of a single ß-lactam ring structure whereas all other ß-lactams have a fused double-ring structure. Aztreonam is the only monobactam in clinical use and is only effective against aerobic Gram-negative bacteria. In contrast, carbapenems are similar to penicillins in structure but have the widest spectrum of antibacterial activity of all the ß-lactams that are currently available. Iminipenem and meropenem are the only carbapenems that are available clinically and are effective against many Gram-negative and Gram-positive bacteria (Livermore and Williams, 1996).

The ß-lactamase-inhibitors include clavulanic acid, sulbactam and tazobactam. These three agents have poor antimicrobial activity on their own but act synergistically with penicillins and cephalosporins by protecting them against some ß-lactamases. Clavulanic acid is a natural product of *Streptomyces clavuligerus* that structurally resembles penicillin and thus it can interact with some ß-lactamases, especially the molecular class A ß-lactamases (see section 1.4.1.3) produced by staphylococci and many Gram-negative bacteria, to form an irreversible acyl-enzyme complex which inactivates the ß-lactamase and allows penicillin to interfere with bacterial cell wall synthesis (Yao and Moellering, 1995). Sulbactam and tazobactam, which belong to the second group of ß-lactamase inhibitors, are semisynthetic penicillanic acid sulfones. Like clavulanic acid, sulbactam and tazobactam have inhibitory activity towards the molecular class A ß-lactamases that may be plasmid- and chromosomally- encoded. However, clavulanic acid, sulbactam and tazobactam have very poor activity against the class C inducible chromosomal ß-lactamases such as those produced by *Enterobacter* and *Pseudomonas* spp.

1.4.1.2 Mode of action of ß-lactams.

ß-lactams interfere with the biosynthesis of the peptidoglycan (or murein) layer of the bacterial cell wall that is essential for protecting the integrity of the cytoplasmic membrane against osmotic forces (Park, 1996). The peptidoglycan macromolecule consists of glycan chains that are made of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues that are cross-linked to each other via the pentapeptide side-chains of the MurNAc moieties (Nikaido, 1994). The sequence of the MurNAc pentapeptide differs among bacterial species except for the two terminal D-alanine residues and the free amino group that is attached to the third amino acid (Livermore and Williams, 1996). The cross-linking of pentapeptide side-chains, or transpeptidation, is catalyzed by D-alanyl-D-alanine transpeptidase and is the final step of peptidoglycan synthesis (Ghuysen, 1997; Livermore and Williams, 1996).

Transpeptidases, carboxypeptidases and transglycosylases are enzymes responsible for the assembly, regulation and maintenance of the peptidoglycan layer of the bacterial cell wall and are collectively called the penicillin-binding proteins (PBPs). Once ß-lactam antibiotics that have entered Gram-positive bacteria through the cell wall or Gram-negative bacteria through their porins, they bind covalently to PBPs to inhibit their catalytic activity and thereby interfere with cell wall metabolism (Ghuysen, 1997). This PBP inactivation, however, may or may not lead to cell death since not all PBPs are essential for the growth and survival of bacteria. In Gram-negative bacilli, for example, there are three major types of PBPs- PBP1, PBP 2 and PBP 3, which are all transpeptidases/ transglycosylases that are essential for the viability of this group of bacteria and their inactivation by ß-lactams would be lethal to the cell (Livermore and

Williams, 1996; Park 1996). The inhibition of the D-alanyl-D-alanine carboxypeptidases designated PBP-4,-5 and -6, however, is not lethal to Gram-negative bacilli since these PBPs are not crucial for cell survival (Quintiliani and Courvalin, 1995; Park, 1996).

The principal method by which ß-lactam antibiotics inhibit bacterial growth is by inactivating the D-alanyl-D-alanine transpeptidases which are essential for the construction of peptidoglycan (Frère, 1995; Guysen, 1997; Livermore and Williams, 1996) The D-alanyl-D-alanine transpeptidase becomes inactivated as the ß-lactam acylates the hydroxyl group of the serine residue in the active site of the enzyme to form a stable acyl-enzyme (Livermore and Williams, 1996). This covalent drug-enzyme interaction is due to the structural similarity of the amide bond of ß-lactams to the configuration of D-alanyl-D-alanine and therefore, the ß-lactam serves as an alternate substrate for the D-alanyl-D-alanine transpeptidase (Livermore and Williams, 1996).

The bactericidal action of ß-lactams is poorly understood but is known to be accelerated by "autolysins", or peptidoglycan hydrolases, that are triggered by the presence of ß-lactams (Yao and Moellering, 1995). Though the main role of autolysins is to cleave peptidoglycan at various sites to permit the insertion of newly synthesized peptidoglycan it is possible that the inactivation of PBPs by ß-lactams triggers the de-regulation of the autolysins resulting in cell lysis (Dever and Dermody, 1991; Livermore and Williams, 1996).

1.4.1.3 Mechanisms of bacterial resistance to ß-lactams

There are four mechanisms by which bacteria become resistant to ß-lactam antibiotics: (a) the inactivation of the drugs by ß-lactamases, (b) modifications in pre-existing PBPs, (c) production of novel PBPs and finally, (d) changes in outer membrane permeability in Gram-negative bacteria (Livermore, 1998). Of these resistance mechanisms, ß-lactamases are the principal and most prevalent mediators of clinically significant resistance to ß-lactams in Gram-negative bacteria (Gur 2002; Livermore, 1998; Nordmann, 1998; Williams, 1999). In Gram-positive bacteria, however, resistance to ß-lactams is primarily due to the modification and bypassing of PBPs (Livermore, 1998). Only ß-lactamases will be discussed here since they are the focus of the work presented in this thesis.

ß-lactamases. ß-lactamases are a diverse family of enzymes that are primarily penicillinases or cephalosporinases. Most are active-site serine peptidases that interact similarly with ß-lactam drugs as do PBPs by forming an ester-linked acylenzyme intermediate (Quintiliani and Courvalin, 1995). Unlike PBPs, however, once a ß-lactamase inactivates the ß-lactam drug, water efficiently hydrolyzes the ester bond and causes the release of the damaged ß-lactam antibiotic (acyl protein) with subsequent reactivation of the ß-lactamase. ß-lactamases may have a narrow, broad or extended spectrum of action, may be constitutive or inducible, and can be encoded by chromosomes or by plasmids (Berkowitz, 1995). In Gram-negative bacteria, ß-lactamases are secreted into the periplasmic space whereas in Gram-positive bacteria, they are secreted into the growth medium (Livermore and Williams, 1996).

Though the origin of ß-lactamases in unknown, bacteria collected prior to the antibiotic era were found to carry chromosomally-encoded ß-lactamases (Livermore, 1998). It is speculated that these ß-lactamases may have played a minor role in cell wall metabolism or may have protected them against ß-lactam-producing fungi in the environment (Livermore, 1998). Throughout our antibiotic era, however, the armament of ß-lactam antibiotics used to fight bacterial infections has caused the necessary selective pressure for the rapid evolution and diversification of the ß-lactamase family (Petrosino *et al*, 1998). A single base change is all that is necessary to change the substrate specificity of ß-lactamases and thus, it is not surprising that a series of point mutations at different sites within the gene has resulted in a wider spectrum of resistance against ß-lactam antibiotics (Davies, 1994; Livermore and Williams, 1996). Pharmaceutical companies have used two strategies to overcome the growing problem of resistance due to ß-lactamases: (a) the development of new extended-spectrum ß-lactam drugs, such as third-generation cephalosporins, that are less susceptible to ß-lactamases and (b) the production of synergistic ß-lactam / ß-lactamase inhibitor combination drugs using clavulanic acid, sulbactam or tazobactam (Petrosino *et al*, 1998; Randegger and Hächler, 2001). Unfortunately, since the 1980s, the clinical use of these newer extended-spectrum ß-lactams and ß-lactam/inhibitor combinations has also selected for new variants of ß-lactamases capable of inactivating these newer drugs (Livermore, 1998; Madeiros, 1997; Randegger and Hächler, 2001). As early as 1983, the first extended-spectrum ß-lactamase (ESBL)-producing clinical isolate was identified in Germany and since then, more than 100 different ESBL variants have been identified worldwide (Gniadkowski, 2001). In addition, since the 1990s, inhibitor-resistant ß-lactamases (IRBLs) have emerged and have compromised the use of ß-lactamase inhibitors as

therapeutic alternatives for ESBL-producing pathogens (Randegger and Hächler, 2001).

Several classification schemes have been proposed for ß-lactamases based on one or more of the following characteristics: molecular structure, substrate profile, susceptibility to inhibitors and isoelectric point (Ambler, 1980; Bush *et al,* 1995; Philippon *et al*, 1989). As there are over 200 ß-lactamases identified to date, it has become increasingly important to have a standard classification scheme (Gur, 2002). A review published by Bush *et al.* (1995) offers a complete synopsis of ß-lactamase classifications presented to date and introduces the widely-accepted Bush-Jacoby-Madeiros (BJM) classification scheme that encompasses all the aforementioned structural and functional characteristics. In addition, to prevent confusion regarding the nomenclature of TEM ß-lactamases, Bush and Jacoby (1997) strongly urged the scientific community to use the TEM numbering system to identify novel ß-lactamases rather than assigning subjective phenotypic names such as "CAZ-1".

The BJM scheme classifies ß-lactamases into four main groups and eight subgroups. ß-lactamase groups 1, 2b, 2be, 2br and 2d of the BJM scheme are the most important groups involved in ß-lactam resistance in *Enterobacteriaceae* and are the focus of the work presented in this thesis (Table 3). The ß-lactamases TEM-1, TEM-2 and SHV-1 belonging to the BJM group 2b, are considered to be the classical ß-lactamases and are broad-spectrum ß-lactamases that are inhibited by clavulanic acid. The TEM-1 ß-lactamase, which is encoded by the $bla_{\text{TEM-1}}$ gene and is often found on the Class II Tn3 transposon, is the most common plasmid-encoded ß-lactamase in Gram-negative bacteria (Livermore, 1998; Petrosino *et al*, 1998). In addition, since the 1970s, TEM-1 has been the most widespread ß-lactamase among the *Enterobacteriaceae* in particular (Petrosino *et al*, 1998). Plasmid-encoded TEM-2 and SHV-1 have also spread throughout the

Enterobacteriaceae though they remain less prevalent than TEM-1 (Livermore, 1998). A complete listing of all TEM, SHV and other ß-lactamases can be found at http://www.lahey.hitchcock.org/studies/webt.htm.

According to the BJM classification scheme, ESBLs are group 2be and 2d ß-lactamases that have the ability to inactivate the newer extended-spectrum ß-lactam compounds such as cefpodoxime, cefotetan and cephalothin (Bush *et al*, 1995). The vast majority of ESBL-producing bacteria are *Enterobacteriaceae* isolated from the nosocomial environment (Gniadkowski, 2001). Although at present the most prevalent ESBL-producers are *Klebsiella pneumoniae* and *Escherichia coli,* there is a growing number ESBL-producing *Salmonella* spp. being isolated in Latin America, Asia, Europe, Africa and Canada (Shannon and French, 1998; Bradford *et al*, 1998; Tassios *et al*, 1999; Mulvey *et al*, 2003). Most ESBLs are derivatives of the classical plasmid-mediated TEM-1, TEM-2 and SHV-1 ß-lactamases and differ from these parental enzymes by one to four amino acid substitutions (Livermore, 1998; Gniadkowski, 2001). There are specific positions in the amino acid sequence of ß-lactamases that when altered, can result in an extended spectrum of activity. In 1995, Knox described the various structural and functional changes that these spectrum-extending amino acid substitutions can cause in ß-lactamases. For example, amino acid substitutions that occur in position 164 in TEMs, 179 in SHVs and 238 in both, expand the ß-lactam-binding site enough to permit interactions with extended-spectrum ß-lactams possessing large oxyimino side-chains such as ceftazidime and aztreonam (Knox, 1995; Madeiros, 1997). Although the activity of ESBLs is inhibited by clavulanic acid, only urinary tract infections may be treated effectively with ß-lactam/ ß-lactamase inhibitor combinations since the concentration of

the inhibitor attained is high enough to inactivate ESBLs. For bloodstream and tissue infections, the treatment options for infections caused by ESBL-producing pathogens is limited to carbapenems and cephamycins such as cefoxitin or cefotetan (Nordmann, 1998).

There exists another group of ESBLs, the non-TEM and non-SHV ESBLs such as CTX-M1 and PER-1, that are plasmid-encoded class A ß-lactamases that are inhibited by clavulanic acid. They share very low amino acid homology (about 30-60%) with TEM-derived ß-lactamases and their corresponding non-ESBL parental enzymes have yet to be isolated (Nordmann, 1998). Like the TEM- and SHV-derived ESBLs, these unusual ESBLs have spread worldwide and are predominantly found in *Enterobacteriaceae* (Bonnet et al, 2000; Bradford, 1998; Sabaté et al, 2000; Silva, 1999). In Brazil, for example, CTX-M ß-lactamases were detected in *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter amalonaticus* and *Proteus mirabilis* clinical strains that were isolated between 1996 and 1997(Bonnet et al, 2000). In Spain, CTX-M-9 was detected in an *E. coli* clinical isolate in 1996 (Sabaté et al, 2000) and in 1998, a Latvian hospital detected CTX-M-5 in an outbreak strain of *Salmonella* Typhimurium (Bradford et al, 1998).

Table 3. A selection of ß-lactamases belonging to groups 1 to 2d of the Bush-Jacoby-Madeiros classification scheme. [a]

Bush-Jacoby-Madeiros Group	Preferred substrates	Molecular class[b]	Inhibited by clavulanic acid	Representative ß-lactamases
1	cephalosporins	C	No	AmpC from Gram-bacteria
2a	penicillins	A	Yes	Penicillinases from Gram-positive bacteria
2b	penicillins, cephalosporins	A	Yes	TEM-1,TEM-2,SHV-1
2be	narrow-/extended spectrum penicillins,cephalosporins; monobactams	A	Yes	TEM-3 to TEM- 26 SHV-2 to SHV-6
2br	penicillins	A	Variable[c]	TEM-30 to TEM-36
2c	penicillins, carbenicillin	A	Yes	PSE-1, PSE-3, PSE-4
2d	penicillins, cloxacillin	D	Variable	OXA-1 to OXA-11

[a] Adapted from Bush et al., 1995.
[b] Molecular class of ß-lactamases as described in Ambler (1980)
[c] Inhibitor resistant ß-lactamases have varied resistance levels to clavulanic acid, sulbactam and tazobactam

The increased used of ß-lactams has encouraged the growth of bacteria that not only produce large quantities of chromosomal ß-lactamases but that possess additional plasmid- or -transposon encoded ß-lactamases. Of particular concern are the recently emerged plasmid-mediated Ambler class C (BJM group 1) cephalosporinases (Ambler, 1980) that confer resistance to cephamycins, oxymino-cephalosporins and aztreonam (Nordmann, 1998). These enzymes are related to the chromosomally-encoded AmpC that is found in most Gram-negative bacilli that is resistant to clavulanic acid and tazobactam (except for MOX-1) (Nordmann, 1998). In *E. coli* and *Shigella spp.*, chromosomal AmpC does not play a significant role in resistance to ß-lactams since the *ampC* gene is uninducible and thus is only expressed in trace amounts (Livermore and Williams, 1998). In other Gram-negative bacilli, such as *Enterobacter spp.* and *Pseudomonas aeruginosa,* the *ampC* gene is inducible and therefore confers resistance to ß-lactams when it is de-

repressed. Unlike chromosomally-encoded AmpC, the plasmid-mediated AmpC-type ß-lactamases are usually constitutively expressed (Nordmann, 1998; Barnaud, 1998). Now that plasmid-mediated AmpC-type ß-lactamases have emerged, a growing number of clinical isolates of *Salmonella spp* and *Klebsiella pneumoniae*, which normally do not possess the chromosomal *ampC* gene, now possess these inhibitor-resistant cephalosporinases (Barnaud, 1998).

1.4.2 Aminoglycosides

1.4.2.1 Structure and classification of Aminoglycosides

The aminoglycoside antimicrobials are a large group of broad-spectrum drugs that are used extensively to treat many serious infections including those caused by Gram-negative and Gram-positive bacteria in the nosocomial environment (Davies, 1996; Davies and Wright, 1997). Since the discovery of the first aminoglycoside streptomycin in 1944, there are more than 50 different natural and semi-synthetic aminoglycoside drugs available for therapeutic use (Davies, 1996). Below is a summary of the structure and mode of action of aminoglycosides as well as the mechanisms bacteria have developed to protect themselves against them.

Aminoglycoside antimicrobials are bactericidal compounds that are produced naturally by *Streptomyces* spp., *Bacillus* spp., *Micromonospora* spp. and *Pseudomonas* spp. Aminoglycosides are composed of amino-sugars linked through glycosidic bonds and are polycationic at physiological pH. The full name of this group of antimicrobials is "aminoglycoside-aminocyclitols" since there are some compounds, such as

spectinomycin, that lack amino-sugar residues but still retain the same mechanism of action of this group. For simplicity, the term "aminoglycosides" will be used throughout this thesis to describe the "aminoglycoside-aminocyclitol" group of antimicrobials.

Although the aminoglycosides are not classified as distinctly as the ß-lactams (see section 1.4.1.1), they can be subdivided into the 4,5-disubstituted deoxystreptamines, the 4,6-disubstituted deoxystreptamines and a third group which includes all those which do not have the 2-deoxystreptamine core that the first two classes possess (Davies, 1996; Recht and Puglisi, 2001). The structural diagram of one 4,6-disubstituted deoxystreptamine, kanamycin, is shown in Figure 3 in section 1.4.2.3. The aminoglycosides that are relevant to the research presented in this thesis are summarized in Table 4.

Table 4: The source and general structure of aminoglycosides mentioned in this study.

Aminoglycoside	Structural Type[ab]	Source[c]
Kanamycin	4,6-disubstituted deoxystreptamine	*Streptomyces kanamyceticus*
Amikacin	4,6-disubstituted deoxystreptamine	Semi-synthetic derivative of Kanamycin
Gentamicin	4,6-disubstituted deoxystreptamine	*Micromonospora purpurea*
Streptomycin	Other (streptidine)	*Streptomyces griseus*
Spectinomycin	Other (actinamine)	*Streptomyces spectabilis*

[a] Cyclitol moiety of aminoglycosides in the "other" structural category are noted in parentheses
[b] Adapted from reference Davies, 1996.
[c] Adapted from reference Davies and Wright,1997.

Although aminoglycosides have greater activity against aerobic Gram-negative bacilli than do other groups of antimicrobials, they have varying degrees of nephro- and otoxicity and are, therefore, usually reserved for the treatment of serious infections (Davies, 1996; Davies and Wright, 1997).

1.4.2.2 Mode of action of Aminoglycosides

Aminoglycosides are protein synthesis inhibitors that act by binding irreversibly to rRNA in the 30S subunit of the bacterial ribosome (Davies, 1996; Davies and Wright, 1997; Recht and Puglisi, 2001). Unlike the ß-lactams that have one specific target, recent evidence has shown that the three classes of aminoglycosides bind to different sites on the 16S rRNA (Carter *et al*, 2000; Recht and Puglisi, 2001). How do the highly positively charged aminoglycosides enter through the negatively charged cell surface of bacteria to reach their ribosomal target? Although the full details regarding bacterial uptake of aminoglycosides have not been determined, the following steps are known to occur: a) adsorption of the drug to the outer layer of the cell through ionic interactions; b) diffusion through an aqueous channel or porin-associated adsorption; c) electrophoretic entry of the aminoglycoside through the cytoplasm via an energy-dependent process which establishes a proton gradient between the inner and outer surfaces of the cytoplasm; and finally, e) rapid influx of the aminoglycoside into the cell, binding of the drug to ribosomes and subsequently blocking protein synthesis (Bryan and Kwan, 1983; Davies, 1996; Taber *et al*, 1987). Oxidative energy production is essential for the uptake of aminoglycosides since the absence of a proton gradient across the cytoplasm prevents the entry of these antimicrobials (Schlessinger, 1988). These findings explain why

aminoglycosides are less effective against anaerobic bacteria as well as facultative bacteria growing in an anaerobic environment (Davies, 1996).

Aminoglycosides are the only bactericidal protein synthesis inhibitors and their mode of action is related to their ability to bind ribosomes irreversibly (Davies, 1996). Although it is known that the primary target of aminoglycosides is the ribosome, it is still unknown exactly how this drug group kills bacteria due to the fact that aminoglycosides cause pleiotropic effects in susceptible bacteria (Davis, 1987; Davies, 1996). The four most important effects aminoglycosides exert on bacteria are: ribosomal blockage, membrane damage, misreading mRNA during translation and irreversible uptake of the drug (Davis, 1987). The type of physiological outcome is dependant on the concentration of the aminoglycoside and on the growth rate of the bacterium (Davies, 1996). Davis (1987) described a plausible yet unproven theory explaining the bactericidal action of aminoglycosides. Briefly, the proposed model states that a small amount of aminoglycosides first enter the cell, bind to polysomal ribosomes and cause the misreading of mRNA followed by the production of abnormal proteins. Abnormal membrane proteins become incorporated into the membrane and increase the influx of molecules. As numerous aqueous channels form, large quantities of aminoglycoside molecules enter the cell, saturate all the ribosomes, all protein synthesis in the cell stops and cell death occurs. Though Davis' theory is logical, it is based on experimentation done with streptomycin only and does not explain all of the effects aminoglycosides have on bacteria. To date, there is no unifying theory that explains the bactericidal mode of action of aminoglycosides as well as all the pleiotropic effects these drugs have on bacteria (Davies, 1996).

1.4.2.3 Bacterial resistance to Aminoglycosides

Bacterial resistance to aminoglycosides can occur through the following mechanisms: a) mutations in the 30S ribosome that decrease aminoglycoside-ribosome binding; b) reduced permeability of the bacterial membrane to the aminoglycoside and finally, c) the enzymatic modification of the aminoglycoside. Enzymatic inactivation of aminoglycosides is the most common mechanism of resistance in both Gram-negative and Gram-positive bacteria and will therefore be the only mechanism discussed here.

Aminoglycoside-modifying enzymes. There are three classes of aminoglycoside-modifying enzymes: acetyltransferases (AAC), nucleotidyltransferases (or adenyltransferases) (ANT) and phosphotransferases (APH) (Davies, 1996; Shaw *et al*, 1993). The AAC enzymes inactivate aminoglycosides by modifying their amino groups via N-acetylation which is an acetyl-CoA-dependant process (Davies and Wright, 1997). The ANT and APH enzymes, however, modify aminoglycoside hydroxyl groups by ATP-dependant O-adenylation and O-phosphorylation, respectively (Davies and Wright, 1997). Figure 3 shows the chemical structure of kanamycin and indicates sites of enzymatic inactivation by AAC, ANT and APH enzymes. Aminoglycoside-modifying enzymes are found in the cytoplasm of resistant bacteria where they inactivate aminoglycoside molecules as they enter the cell and thereby, prevent these antibiotics from binding to the ribosome (Davies, 1996). Genes encoding aminoglycoside-modifying enzymes are frequently plasmid- or integron-encoded which has contributed to their dissemination throughout many bacterial species (Shaw *et al*, 1993). Bacterial resistance to a particular aminoglycoside increases if the bacterium carries multiple copies of the

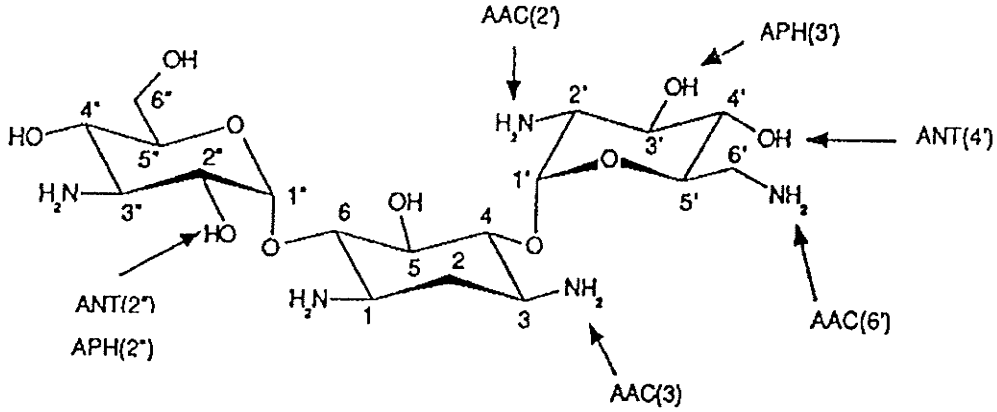
same aminoglycoside-modifying enzyme or if it expresses more than one type of modifying enzyme that inactivates the antibiotic (Davies, 1996).

More than 50 different aminoglycoside-modifying enzymes have been identified and each enzyme can inactivate different aminoglycosides due to a varied and often broad substrate range. Shaw *et al* (1993) have published a review describing the nomenclature, classification and characteristics of each class of aminoglycoside-modifying enzymes. The nomenclature used to describe members of the three main classes of aminoglycoside-modifying enzymes, namely the AAC, ANT and APH enzymes, provides information about their site of modification, their unique aminoglycoside resistance profiles and their amino acid sequence (Shaw *et al*, 1993). APH(3')-Ia, for example is an aminoglycoside phosphotransferase ("APH") that modifies the hydroxyl group attached to the 3' carbon of the target aminoglycoside "(3')", has a resistance profile type "I" and has a protein sequence type "a". The nomenclature used to designate the gene encoding APH(3')-Ia is simply "*aph(3')Ia*". Table 5 lists subclasses of aminoglycoside-modifying enzymes that are most frequently found in resistant Gram-negative bacteria.

Figure 3:

Chemical structure of kanamycin and sites of enzymatic inactivation by bacterial aminoglycoside-modifying enzymes. Arrows indicate sites which are susceptible to the following enzymatic modifications: N-acetylation of amino groups at positions 2', 3 and 6' by acetyltransferases (AAC), O-adenylation of hydroxyl groups attached to carbon at positions 2"and 4' by adenyltransferases (ANT), and O-phosphorylation of hydroxyl groups at positions 3' and 2" by phosphotransferases (APH). (Figure reproduced from Courvalin and Quintiliani, 1995).

(A) **Kanamycin**



(B) **Streptomycin**

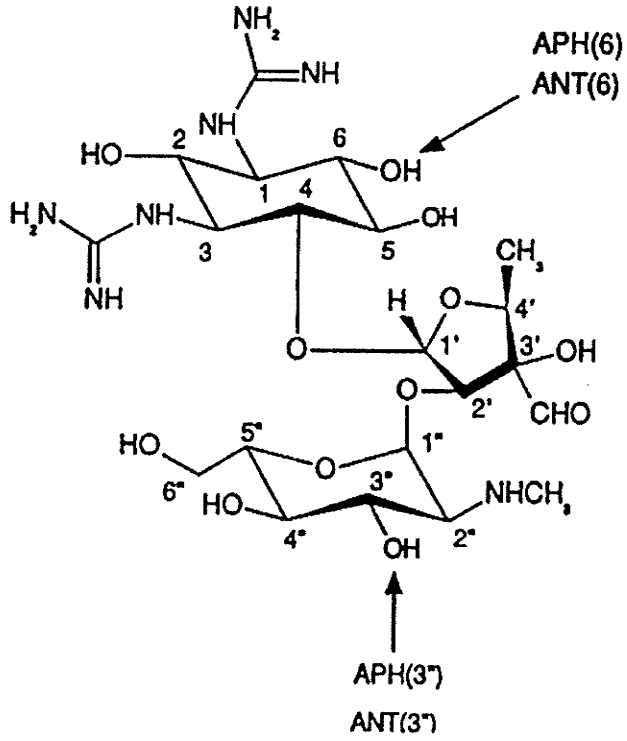


Table 5: Aminoglycoside-modifying enzymes most frequently isolated from resistant Gram-negative clinical isolates[a]

Enzyme class	Substrate Profile	Comment
AAC (N-acetyltransferases)		
AAC(3)-I	gentamicin, fortimicin	Widespread among the *Enterobacteriaceae*
AAC(3)-II	gentamicin, tobramycin, dibekacin, netilmicin, 2- and 6-N-ethylnetilmicin, sisomicin	Widespread among the *Enterobacteriaceae*
AAC(6')-I	amikacin, tobramycin, dibekacin, netilmicin, 2- N-ethylnetilmicin, 5-episisomicin, sisomicin	Often found in combination with other aminoglycoside-modifying enzymes in *Enterobacteriaceae*[b]
AAC(6')-II	gentamicin, tobramycin, dibekacin, netilmicin, 2-N-ethylnetilmicin, sisomicin	Observed only in *Pseudomonas* spp.
ANT (O-adenyltransferases)		
ANT(2")-I	kanamycin, gentamicin, tobramycin, dibekacin, sisomicin	Widespread among all Gram-negative bacteria
ANT(3")-I	streptomycin, spectinomycin	ANT(3")Ia[c] is ubiquitous among Gram-negative bacteria
ANT(4')-II	tobramycin, amikacin, isepamicin	
APH (O-phosphotransferases)		
APH(3')-I	kanamycin, neomycin, paromomycin, ribostamycin, lividomycin, gentamicin B	

[a] Adapted from Shaw *et al*, 1993
[b] Miller *et al*, 1995
[c] Alternate name for the *ant(3")Ia* gene is *aadA1*

1.4.3 Chloramphenicol

1.4.3.1 Structure and mode of action of chloramphenicol

Chloramphenicol, a broad-spectrum antibiotic that was widely used in the 1950s, was originally isolated from *Streptomyces venezuelae* in 1947 but is now produced synthetically. Since the 1960s, however, the use of chloramphenicol has declined as it causes a few unusual life-threatening syndromes such as grey baby syndrome, aplastic anemia and bone marrow suppression in a small number of patients (Yao and Moellering, 1995; Lam et al., 2002). Chloramphenicol use is therefore reserved for serious infections caused by most Gram-negative and Gram-positive bacteria, *Rickettsia*, *Mycoplasma* and *Chlamydia* that are resistant to other antimicrobials and for infections in patients who are allergic to other, less toxic antimicrobials (Lam et al., 2002).

Chloramphenicol is a nitrobenzene derivative that is structurally unrelated to other antibiotics and only has one other derivative- thiamphenicol (Yao and Moellering, 1995). Florfenicol is a fluorinated structural analog of thiamphenicol that is only used for veterinary purposes (White et al., 2000). Chloramphenicol is a small, amphiphilic molecule with three biologically active functional groups (see Figure 4) which permit it to traverse through cellular membranes due to its high lipid solubility while also maintaining a moderate degree of solubility in aqueous environments (Shaw and Leslie, 1991).

Chloramphenicol inhibits the elongation step of bacterial protein synthesis by binding reversibly to the peptidyltransferase centre of the 50S subunit of the bacterial

ribosome (Gale et al., 1981). By binding to peptidyltransferase, chloramphenicol inhibits the enzyme's ability to catalyze peptide bond formation between the growing polypeptide chain and the next amino acid to be added to the elongating polypeptide (Gale et al., 1981). Bacterial growth is therefore inhibited in the presence of chloramphenicol but once removed, protein synthesis may resume. Chloramphenicol may also be bactericidal with prolonged exposure due to irreparable damage to the cell wall and subsequent cell lysis (Yao and Moellering, 1995).

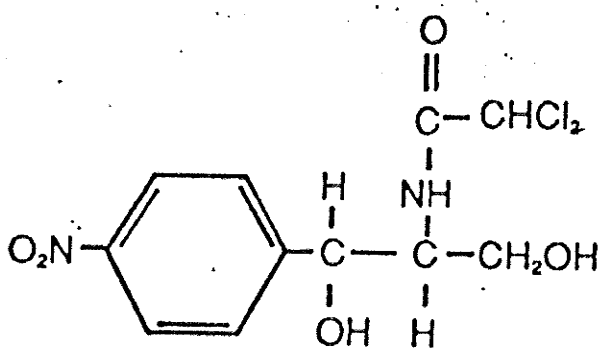
1.4.3.2 Bacterial resistance to chloramphenicol

The two most important mechanisms of resistance to chloramphenicol in bacteria are: a) enzymatic inactivation of the antibiotic by chloramphenicol acetyltransferases, or "CATs"; and (b) a non-enzymatic mechanism, that is a putative efflux pump encoded by a *cmlA* gene, that pumps chloramphenicol out of the cell (Bischoff et al., 2002; Murray and Shaw, 1997). Only CATs will be mentioned here since high level clinical resistance to chloramphenicol is attributed to chloramphenicol acetyltransferase enzymes that inactivate chloramphenicol by O-acetylation as is shown in Figure 4 (Dever et al., 1991; Murray and Shaw, 1997; Gaffney et al., 1981).

Genes encoding for chloramphenicol acetyltransferases, or *cat* genes, are widespread among both Gram-positive and Gram-negative bacteria (Murray and Shaw, 1997). The three structural variants of CATs are designated CAT_I , CAT_{II} and CAT_{III} that have a size range from 24 kDa to 26 kDa (Murray and Shaw, 1997). Only 23 amino acids are conserved among CAT variants which represent residues that are essential for correct tertiary structure and catalysis (Murray and Shaw, 1997). CAT_I and CAT_{II} are the

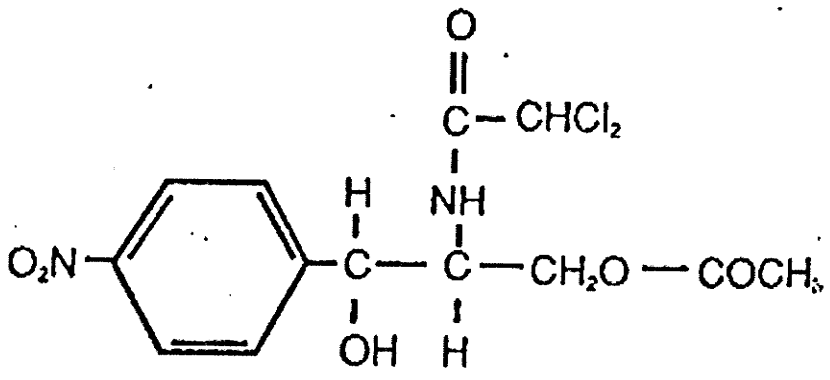
Figure 4:

The inactivation of chloramphenicol by chloramphenicol acetyltransferases. Chloramphenicol has three biologically active functional groups attached to its benzene ring: the p-NO_2 group, the dichloroacetyl moiety and the hydroxyl group attached to the C-3 of the propanediol chain. Chloramphenicol acetyltransferase catalyzes acetylation at the C-3 hydroxyl of chloramphenicol using acetyl-CoA as the acyl donor. (Figure reproduced from Dever et al., 1991)



Chloramphenicol

Acetyl CoA



3-*O*-Acetyl Chloramphenicol

two CAT variants most commonly found in Gram-negative bacteria and are often plasmid-mediated though they can also be found on transposons, integrons and the chromosome (Alton and Vapnek, 1979; Murray and Shaw, 1997; Shaw and Leslie, 1991).

1.4.4 Tetracyclines

1.4.4.1 Structure and mode of action of tetracycline

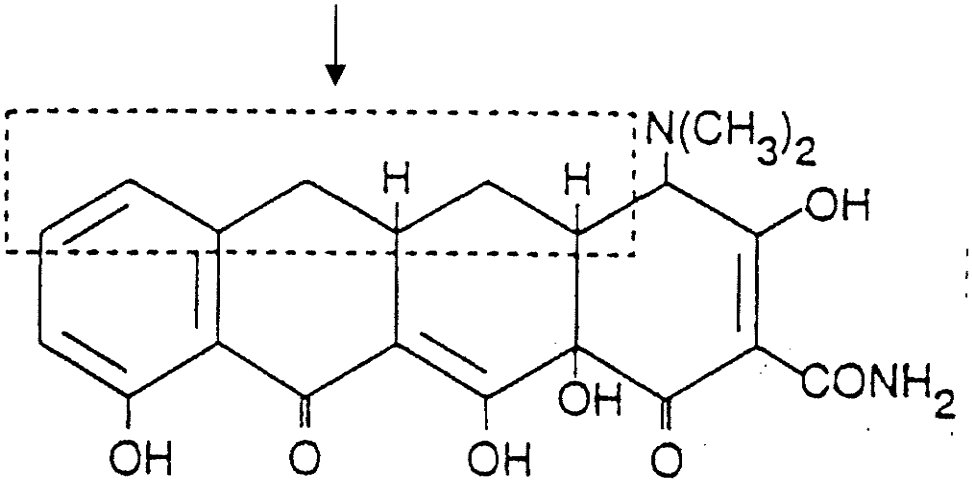
Tetracyclines, a family of broad-spectrum antimicrobials, are active against a wide range of Gram-positive and Gram-negative aerobic and anaerobic bacteria as well as many other intra- and extracellular pathogens (Roberts, 1996). Chlortetracycline, a natural product of *Streptomyces aureofaciens*, was the first tetracycline to be used therapeutically in 1947 (Roberts, 1996). Since then, many other derivatives were discovered including oxytetracycline and tetracycline which were heavily used in the 1950s and 1960s (Speer et al., 1992). Tetracycline and its derivatives are all composed of a fused four-ring structure (see Figure 5). Today, tetracycline use has decreased due to the emergence of tetracycline resistance in many different bacterial species (Roberts, 1996). A new class of semi-synthetic tetracyclines, the glycylcyclines, are minocycline derivatives that are active against most tetracycline-resistant Gram-positive and Gram-negative bacteria including *Enterobacteriaceae* (Sum et al., 1998; Tally et al., 1995).

There are two groups of tetracyclines that differ in their mode of action: the "typical" tetracyclines that exert bacteriostatic activity and the "atypical" tetracyclines that are bactericidal (Schnappinger and Hillen, 1996). The exact mode of action of both groups of tetracyclines is still undetermined though it is known that "typical"

Figure 5:

Chemical structure of tetracyclines. The conserved four-ring structure of tetracyclines is shown. Different tetracycline derivatives possess various functional groups, such as CH_3 ,OH^- and Cl^- groups, in the region that is indicated with a box. (Figure adapted from Speer et al., 1992)

Region of structural variation



Conserved structure of Tetracyclines

tetracyclines inhibit protein synthesis whereas the "atypical" tetracyclines target the cytoplasmic membrane (Roberts, 1996; Schnappinger and Hillen, 1996). The "atypical" tetracyclines, which include chelocardin, anhydrotetracycline and 6-thiatetracycline are not used therapeutically as they cause severe side-effects (Schnappinger and Hillen, 1996).

The "typical" tetracyclines, such as chlortetracycline, tetracycline, minocycline and doxycycline inhibit bacterial growth by binding reversibly to the 30S subunit of the bacterial ribosome (Roberts, 1996). There is one site on the 30S ribosomal subunit to which tetracycline binds with high-affinity but there are also several low-affinity tetracycline binding sites on both the 30S and 50S subunits (Schnappinger and Hillen, 1996). The 30S proteins that are important for high-affinity binding of tetracycline are: S7, S3, S8, S14 and S19 (Buck and Cooperman, 1990). There is evidence that tetracycline binds directly to the S7 protein and it is speculated that this binding prevents the attachment of the aminoacyl-tRNA to the acceptor site thereby inhibiting protein synthesis (Taylor and Chau, 1996). However, the exact mechanism of protein synthesis inhibition by tetracycline has yet to be proven experimentally.

1.4.4.2 Bacterial resistance to tetracycline

There are three tetracycline resistance mechanisms used by bacteria: (a) active efflux of tetracycline; (b) ribosomal protection by preventing antibiotic-ribosome interactions; and (c) enzymatic inactivation of tetracycline (Roberts, 1996; Schnappinger and Hillen, 1996). Although all three mechanisms have been observed in clinical isolates (Speer et al., 1992), only active efflux and ribosomal protection proteins confer clinically significant resistance to tetracycline (Roberts, 1996). Enzymatic inactivation of

tetracycline has only been observed in *Bacteroides* spp. and does not confer clinical resistance in this organism (Speer et al., 1992). Ribosomal protection proteins are frequently found in tetracycline-resistant Gram-positive bacteria as well as some non-enteric Gram-negative bacteria such as *Neisseria* spp. but have not been found in enteric Gram-negative bacteria (Roberts, 1996). This review will focus on the active efflux mechanism since it alone causes high-level clinical resistance to tetracycline in both Gram-negative and Gram-positive bacteria (Schnappinger and Hillen, 1996).

Tetracycline-specific efflux pumps are cytoplasmic membrane proteins that transport tetracycline from the bacterial cytoplasm to the periplasm (Schnappinger and Hillen, 1996). By pumping tetracycline out of the cytoplasm, bacteria decrease the intracellular concentrations of tetracycline and thereby limit access of this antibiotic to the ribosomes. In Gram-negative bacteria, there are seven classes of resistance genes that encode tetracycline-specific efflux pump proteins: *tet(A)* to *tet(E)*, *tet(G)* and *tet(H)* (Roberts, 1996; Schnappinger and Hillen, 1996). Each *tet* gene encodes for a membrane-bound efflux protein, approximately 46 kDa, that has 12 lipophilic domains separated by short regions of hydrophilic amino acid residues (Roberts, 1996). The range of amino acid sequence identity between these seven Tet efflux proteins is 43 to 78% (Roberts, 1996). Structural analysis of the efflux proteins suggest that the 12 lipophilic domains span the cytoplasmic membrane while the hydrophobic loops extend into the cytoplasm as well as the periplasm (Eckert and Beck, 1989; Yamaguchi et al., 1992). The pH gradient across the cytoplasmic membrane provides energy for the efflux pump to transport a positively charged tetracycline complex (tetracycline chelates metal divalent cations) in exchange for a proton from the periplasm (Schnappinger and Hillen, 1996).

TetA, TetC, TetD, TetE, TetG and TetH efflux pumps confer resistance to tetracycline but not minocycline whereas TetB confers resistance to both (Chopra et al., 1992). Fortunately, neither of these seven efflux pumps confers resistance to the new glycylcycline compounds (Oliva and Chopra, 1992). Genes encoding TetA, TetB, TetC, TetD, TetE, TetG and TetH efflux proteins are frequently associated with large conjugative plasmids and are widespread among Gram-negative bacteria (Jones et al., 1992; Roberts, 1996).

1.4.5 Sulfonamides and Trimethoprim

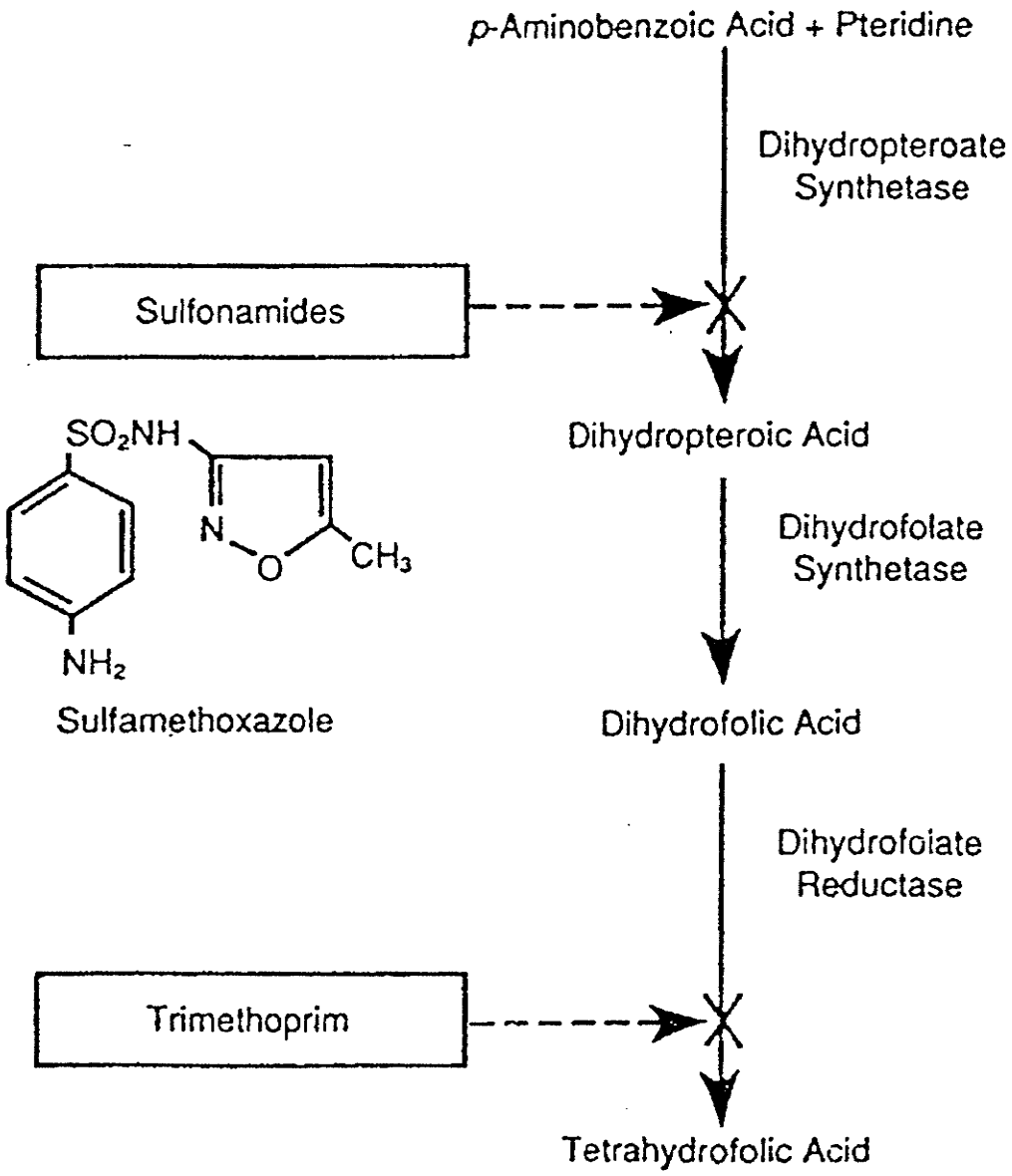
1.4.5.1 Structure and mode of action of sulfonamides and trimethoprim

Sufonamides and trimethoprim are broad-spectrum synthetic antimicrobial agents that are active against a variety of Gram-positive and Gram-negative pathogens that cause urinary, respiratory and gastrointestinal tract infections (Huovinen et al., 1995). Sulfonamides were first used in human and veterinary medicine in 1932 whereas trimethoprim was first used therapeutically in 1962 (Huovinen, 1987). Since 1968, however, sulfonamides were frequently used in combination with trimethoprim due to the emergence of bacterial resistance to sulfonamides (Huovinen et al., 1995).

Sulfonamides and trimethoprim inhibit bacterial growth by interfering with their folic acid biosynthetic pathway using three modes of action: competitive inhibition, false synthesis and sequential inhibition. Tetrahydrofolic acid (THF), the end-product of the bacterial folate biosynthetic pathway, is essential for nucleic acid and protein synthesis in bacteria since it donates a methyl group in the conversion of dUMP to thymidine and

Figure 6:

Mode of action of sulfonamides and trimethoprim. Sulfonamides inhibit the folate biosynthetic pathway by acting as alternative substrates for DHPS and produce a sulfa-containing pteroate analog instead of the normal dihydropteroic acid. Trimethoprim prevents the reduction of DHF to THF by competitively inhibiting the DHFR, thus reducing the quantities of THF needed for nucleic acid synthesis.


p-Aminobenzoic Acid + Pteridine
Dihydropteroate Synthetase
Sulfonamides
Dihydropteroic Acid
Dihydrofolate Synthetase
Dihydrofolic Acid
Dihydrofolate Reductase
Trimethoprim
Tetrahydrofolic Acid
SO2NH
N
O
CH3
NH2
Sulfamethoxazole

in the synthesis of the purine ring (see Figure 6) (Huovinen et al., 1995; Yao and Moellering, 1995). The action of sulfonamides and trimethoprim depletes THF by acting at two enzymatic steps in the folate pathway. Sulfonamides are structural and competitive analogs of the folic acid precursor *p*-aminobenzoic acid (PABA) (Yao and Moellering, 1995). When an adequate concentration of sulfonamide is present in the bacterium, the sulfonamide becomes a substrate for dyhydropteroate synthase (DHPS) more often then does the normal substrate PABA and thus a false analog of dihydropteroic acid is synthesized which then inhibits subsequent steps in the pathway (Huovinen et al., 1995; Yao and Moellering, 1995). Trimethoprim is a dihydrofolic acid (DHF) analog that competitively inhibits the reduction of DHF to THF by binding to the bacterial dihydrofolate reductase (DHFR) (Huovinen, 1987; Huovinen et al., 1995). Trimethoprim's affinity for the bacterial DHFR is 10 000 times greater than for the human and animal DHFR and thus, it maintains its selective toxicity towards bacteria (Yao and Moellering, 1995; Huovinen et al., 1995). When used together, sulfonamides and trimethoprim sequentially inhibit the folic acid pathway by preventing the *de novo* synthesis of DHF and the reduction of existing DHF to THF, respectively.

1.4.5.2 Bacterial resistance to sulfonamides and trimethoprim

Bacterial resistance to sulfonamides and trimethoprim can be achieved by using one or more of the following mechanisms: (a) expression of a mutated DHPS or DHFR that has a low binding affinity for sulfonamides and trimethoprim, respectively; (b) over-production of the wild-type or sulfonamide-resistant DHPS or trimethoprim-resistant DHFR; (c) over-production of PABA as a means to out-compete sulfonamides as a substrate for DHPS (sulfonamide resistance only); and (d) decreased permeability of

sulfonamides and trimethoprim into the cell (Courvalin and Quintiliani, 1995). Sulfonamide and trimethoprim resistance genes are found on the chromosome, on plasmids as well as on transposons (Huovinen et al., 1995). High-level clinical resistance to sulfonamides in enteric Gram-negative bacteria is most frequently due to plasmid-mediated *sulI* and *sulII* genes which encode a mutant DHPS that is sulfonamide-resistant (Swedberg and Sköld, 1980). The two *sul* variants have 57% amino acid identity and both are found at approximately the same frequency in sulfonamide-resistant Gram-negative clinical isolates (Rådström et al., 1991). The *sulI* variant is usually located on transposons of the Tn21 family, such as integrons, whereas the *sulII* variant is commonly found on small multi-copy plasmids such as those belonging to the IncQ family, pBP1, and some conjugative plasmids such as pGS05 (Rådström et al., 1991; Rådström and Swedberg, 1988; van Treeck et al., 1981). There is also a third *sul* variant, *sulIII*, that was originally isolated from *Mycobacterium fortuitum* (Martin et al., 1990). The *sulIII* variant is a deletion mutant of *sulI* and is not commonly found in enteric Gram-negative clinical isolates (Martin et al., 1990).

A high level of clinical resistance to trimethoprim is achieved by bacteria possessing trimethoprim-resistant DHFR enzymes frequently encoded by plasmids and transposons (Huovinen et al., 1995). Table 5 lists the three groups of transferable trimethoprim-resistant DHFRs that consist of a total of seventeen DHFR variants that are categorized according to amino acid sequence similarities (Huovinen et al., 1995). Sixteen trimethoprim-resistant DHFRs were isolated from members of the *Enterobacteriaceae* (Amyes et al., 1992; Huovinen et al., 1995) and one was found in staphylococci (S1 DHFR) (Burdeska et al., 1990). Group 1 DHFRs (also called "family

1") have 64% to 88% amino acid sequence identity and have a polypeptide length of 157 amino acids whereas Group 2 (or "family 2") DHFRs are unrelated to other prokaryotic and eukaryotic DHFRs, are 78 amino acids in length and are 78% to 86% identical to each other (Flensburg and Steen, 1986; Huovinen et al., 1995). The third group of DHFRs consists of all the other DHFRs that do not belong to the first two groups and have only 20% to 50% amino acid identity. All of the DHFRs in families 1 and 2 confer resistance to very high trimethoprim concentrations ($\geq$ 1 g/liter) whereas DHFRs belonging to the third group mediate various levels of trimethoprim resistance (Huovinen et al., 1995). For example, the Group 3 DHFR enzymes of types III, IIIb, IIIc, IV and IX confer lower levels of trimethoprim resistance (< 1g/liter) than do other enzymes in Group 3 (Huovinen et al., 1995).

One of the most widespread transferable DHFR genes among Gram-negative bacteria is the Group 1 *dhfrI* gene cassette carried by integrons (Chang et al, 1992; Heikkilä et al, 1990). The spread of integron-borne *dhfrI* genes is most likely attributed to their integration into a carrier transposon, Tn7, that inserts itself with high-frequency into a preferred site on the chromosome of *E. coli* and a range of other bacterial species (Lichtenstein and Brenner, 1981; Craig, 1991). The *dhfrI* gene cassette has also been found in integrons located on Tn*21* and other trimethoprim-resistant *dhfr* genes have been found on Tn7-like transposons such as Tn4132 (Young et al., 1994; Sundström and Sköld, 1990). As shown in Table 6, all Group 1 and 2 trimethoprim-resistant DHFR genes are found on gene cassettes whereas only one Group 3 gene, *dhfrXII*, is cassette-borne (Heikkilä et al, 1993).

Table 6: Classification of transferable trimethoprim-resistant DHFRs in Gram-negative bacteria[a]

Group	*dhfr* gene	DHFR type	Cassette-encoded
1 (Family 1)	*dhfrI*	I	Yes
	dhfrIb	Ib	Yes
	dhfrV	V	Yes
	dhfrVI	VI	Yes
	dhfrVII	VII	Yes
2 (Family 2)	*dhfrIIa*	IIa	Yes
	dhfrIIb	IIb	Yes
	dhfrIIc	IIc	Yes
3	*dhfrIII*	III	No
	dhfrVIII	VIII/IIIc[b]	No
	dhfrIX	IX	No
	dhfrX	X	No
	dhfrXII	XII	Yes
		IIIb	ND[c]
		IV	ND

[a] Adapted from reference Huovinen et al., 1995

[b] Barg et al. (1995) determined that the gene encoding the DHFR enzyme type VIII was identical to the gene encoding the type IIIc DHFR.

[c] ND, not determined

1.4.6 Quinolones and Fluoroquinolones

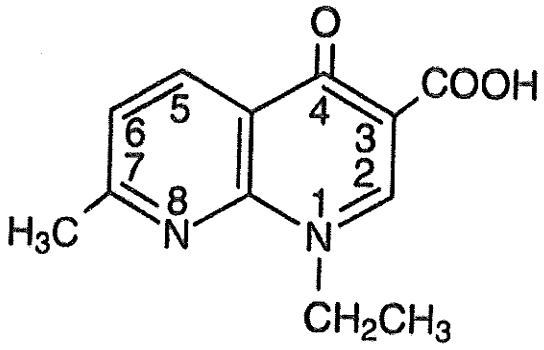
1.4.6.1 Structure and mode of action of quinolones

The quinolones and fluoroquinolones are synthetic antimicrobials that inhibit bacterial DNA topoisomerase II (DNA gyrase) and topoisomerase IV activity (Ng et al., 1996). Quinolones are carboxylic acids that have in common a 4-quinolone structure (4-oxo-1,4-dihydroquinolone) and are divided into four groups: naphthyridines, cinnolines,

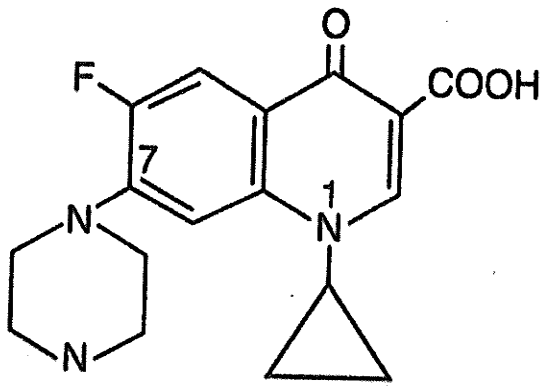
Figure 7:

The chemical structures of nalidixic acid and ciprofloxacin. Nalidixic acid is a naphthyridine derivative whereas ciprofloxacin is a fluorinated quinoline derivative. (Figure adapted from Andriole, 1998)

(A) **Nalidixic Acid**



(B) **Ciprofloxacin**



pyridopyrimidines and quinolines (Andriole, 1991; Schentag and Scully, 1999). The quinolones that were first used in the 1960s, such as nalidixic acid (see Figure 7), are only active against *Enterobacteriaceae* whereas the newer quinolones, the fluoroquinolones, have a much broader spectrum of activity against Gram-negative and Gram-positive bacteria (Schentag and Scully, 1999). First introduced in the 1980s, fluoroquinolones such as ciprofloxacin (see Figure 7) have improved activity against *Enterobacteriaceae* and are also active against staphylococci, *P. aeruginosa*, *Mycoplasma*, *Chlamydia* and some streptococci (Schentag and Scully, 1999; Rosen 1990).

It is postulated that fluoroquinolones inhibit bacterial DNA replication by binding covalently to the single stranded region of the DNA duplex created by the two A subunits of DNA gyrase (Schentag and Scully, 1999). A stable quinolone-gyrase-DNA complex is formed whereby each DNA strand is covalently linked to the Gyr A subunits (Hooper, 1998; Ng et al., 1996). The subsequent sequence of events leading to cell death have not been determined though inhibition of replication and induction of the SOS response triggers various potentially deleterious responses within the cell (Hooper, 1998).

1.4.6.2 Bacterial resistance mechanisms to quinolones

Although the exact mechanisms underlying quinolone resistance are not completely understood, quinolone resistance in bacteria is caused by: (a) mutations in chromosomal genes encoding DNA gyrase and topoisomerase IV and (b) changes in permeability (Schentag and Scully, 1999; Hooper, 1998) . Mutations in *gyrA*, which encodes the "A" subunits of the bacterial DNA gyrase, reduce binding of quinolones to

the gyrase-DNA complex thereby allowing replication to occur (Hooper, 1998). This mechanism, however, confers low-level resistance on its own and thus does not cause clinical resistance in bacterial pathogens (Wilson and Hooper, 1985). In some Gram-negative bacteria such as *E. coli*, however, GyrA mutations accompanied by Par C (a GyrA homolog) mutations in Topoisomerase IV confer high-level resistance (Crumplin, 1990). Mutations in DNA gyrase B subunits, GyrB, have also been described but they only confer low-level quinolone resistance and are generally not the predominant mutation found in quinolone-resistant clinical isolates (Power et al., 1992)

1.5. Thesis: Characterization of antimicrobial resistance mechanisms in multi-drug resistant *Salmonella* and *Shigella* strains from Brazil and Colombia

This project is an extension of the Pan American Health Organization (PAHO) antimicrobial susceptibility surveillance program. Since there is currently no data available in the literature regarding resistance genes encoded by *Salmonella* and *Shigella* strains from either Brazil or Colombia, the focus of this study was to identify and characterize some of the antimicrobial resistance genes found in multi-drug resistant *Salmonella* spp and *Shigella* spp from these two developing countries. The objectives of this research project were the following: (a) to determine whether the multi-resistant *Salmonella* and *Shigella* strains obtained from Brazil and Colombia encoded some of the antimicrobial resistance genes previously detected in *Salmonella* and *Shigella* strains around the world; (b) to investigate the correlation between resistance genes found in animal/environmental/food samples of *Salmonella* versus clinical samples; (c) to compare the antimicrobial resistance gene profiles of different *Salmonella* and *Shigella*

serotypes within the same country and between both countries; (d) to screen for the presence of extended-spectrum ß-lactamases in a subset of strains; (e) to determine if a subset of strains encoded integrons with antimicrobial resistance gene cassettes; and finally, (f) to determine plasmid content of a subset of strains and to subsequently determine whether some antimicrobial resistance genes were located on plasmids.

This study was carried out in two stages. In general, Stage 1 involved the screening of the 501 Brazilian and Colombian isolates for multi-drug resistance. Susceptibility data submitted along with the strains from the collaborating Brazilian and Colombian institutions on the antimicrobial susceptibility profiles, serotypes and epidemiology of the 501 isolates to the six antimicrobials routinely tested (ampicillin, chloramphenicol, ciprofloxacin, gentamicin, SXT and cefotaxime) were compiled. Strains resistant to at least one of the six antimicrobials mentioned above were chosen for and their susceptibilities were verified by the disk diffusion method (NCCLS, 2000) using the same antimicrobials listed above. The results between the sender laboratories and ours were compared. Strains which did not have reproducible antibiograms between laboratories for these six antimicrobials were excluded from this study whereas isolates with reproducible results were further tested for susceptibility to tetracycline, streptomycin, kanamycin, sulfisoxazole, trimethoprim and ceftazidime by the disk diffusion method. Intermediate results were verified by the agar dilution method (see section 2.2.1). Isolates resistant to three or more groups of antimicrobials were considered to be multi-resistant. Isolates possessing resistance to two groups of antimicrobials and intermediate resistance to at least one group of antimicrobials were also considered to be possible candidates for Stage 2 experimentation involving the

molecular analysis of antimicrobial resistance genes. The second last screening step of isolates that were being considered for Stage 2 analysis was the confirmation of their serotypes. Although the serotypes of all Brazilian and Colombian isolates were initially determined by the institutions of their respective countries of origin, the serotypes of the majority of final test strains were re-confirmed by Health Canada (D. Woodward, NLEP, NML). The phage types of 23 *Salmonella* Typhimurium isolates were also determined (R. Ahmed, NLEP, NML). Finally, most isolates obtained from FIOCRUZ and INS were accompanied by epidemiological data. At least one of the following categories of information was known about each isolate to be tested in Stage 2: the source of isolation, the date of isolation and the region/institute from which it originated (see Appendix section 6). The final number of test strains included in Stage 2 molecular experiments is listed in Table 8 of the Results section 3.1.

In Stage 2, a subset of 10 multi-drug resistant strains were tested for the presence of known resistance determinants by PCR amplification. The resistance genes that were detected in the small subset of MDR strains were used to make probes for the detection of resistance genes in all 242 test strains by dot blot hybridizations. PCR amplification of integrons was performed on 82 strains and 13 of those were sequenced in order to determine the type of antimicrobial resistance gene cassettes inserted within. Isoelectric focusing (IEF) was also used to screen for putative ESBLs in 24 strains. Plasmid profiles were determined for another 21 strains and Southern hybridizations were performed in order to determine if bla_{TEM}, *tetB* and *catI* were plasmid-encoded.

The antimicrobial resistance gene profiles of MDR *Salmonella* and *Shigella* strains revealed that similar resistance mechanisms were present in all strains analyzed

from both Brazil and Colombia. Putative ESBLs were also detected in a subset of strains as well as nine different integron-borne antimicrobial gene cassette combinations encoding resistant to ß-lactams, aminoglycosides and trimethoprim. All 21 plasmid profiles observed were varied and Southern hybridizations indicated the presence of all three bla_{TEM} , *tetB* and *catI* genes on large plasmids in Brazilian MDR *Salmonella* and *Shigella* strains.

2. MATERIALS AND METHODS

2.1 Bacterial strains

In this study, a total of 501 *Salmonella* and *Shigella* strains from Brazil and Colombia with varied susceptibilities to antimicrobials were obtained from the P.A.H.O./N.M.L strain collection and were screened for multi-drug resistance. Of these 501 isolates, 243 were Brazilian *Salmonella* isolates (designated by prefix BR-SA-) from human, animal, environmental and food sources collected by our collaborator at the Fundação Oswaldo Cruz (Daliá Dos Prazeres, National Reference Center for Cholera and Enteric Disease) in Rio de Janeiro, Brazil. Brazilian *Salmonella* strains from animal sources were isolated from the stool, liver, spleen and blood of both healthy and unhealthy poultry, cattle, swine and horses living on farms, in zoos or in the wilderness. Environmental sources of Brazilian *Salmonella* include river and well water, raw sewage and sand from beaches. Food sources of Brazilian *Salmonella* isolates were from meat sold in markets (beef, chicken and pork), salad, mayonnaise and vegetables. Also collected from Brazil were 176 *Shigella* (designated by prefix BR-SH-) clinical isolates that were screened in this study. All of the Brazilian strains were isolated between 1986-1999 from different geographical regions in Brazil. The 58 Colombian *Salmonella* (designated by prefix CO-SA-) and 24 Colombian *Shigella* (designated by prefix CO-SH) clinical isolates were obtained from our collaborator (Nelida Muñoz) at the Instituto Nacional de Salud (INS) in Bogotá, Colombia and were originally isolated between 1997-1999 from various regions in Colombia. The final panel of test strains is listed in Appendix-I (section 6).

2.2 Antimicrobial susceptibility testing

2.2.1 Disk diffusion and agar dilution methods.

Antimicrobial resistance profiles were determined by the Kirby-Bauer disk diffusion method according to the National Committee for Clinical Standards (NCCLS) guidelines except for the standardization of inoculum that was carried out using a Vitek colorimeter (Hack Company, Colorado, U.S.A) (NCCLS, 2000). The concentration of antimicrobial disks used (Oxoid, Unipath, Nepean, Ontario, Canada) was the following: chloramphenicol (30 μg), ciprofloxacin (5 μg), ampicillin (10 μg), gentamicin (10 μg), sulfamethoxazole/trimethoprim(19:1) (25 μg), cefotaxime (30 μg), streptomycin (10 μg), tetracycline(30 μg), kanamycin (30 μg), trimethoprim (5 μg), sulfisoxazole (300μg) and ceftazidime (30 μg). Isolates with an intermediate susceptibility to a drug were tested further by the agar dilution method following NCCLS standard M7-A4 (NCCLS, 2000). Minimum inhibitory concentration (MIC) values were interpreted using Table 2A in the NCCLS standard M7-A4 except for streptomycin (Breakpoint >16 μg /ml) (CA-SFM, 1996).

2.2.2 Screening for ESBLs: double disk diffusion

Test strains fully or intermediately resistant to cefotaxime and/or ceftazidime were tested for the presence of ESBLs by double disk diffusion with cephalosporin and cepholosporin/clavulanic acid combination disks using a Mast Diagnostics ESBL detection kit (Merseyside, United Kingdom). The sets of disks used were: cefpodoxime (CPD) (30μg) and cefpodoxime (30μg) plus 10 μg clavulanic acid (CPD/CLAV);

ceftazidime (CAZ) (30μg) and ceftazidime (30μg) plus 10 μg clavulanic acid (CAZ/CLAV); and finally, cefotaxime (30μg) (CTX) and cefotaxime (30μg) plus 10 μg clavulanic acid (CTX/CLAV). Zone of inhibition diameter ratios were determined for each set of disks by dividing the zone diameters for the combination disks by those obtained for the individual cephalosporin disks. Isolates having zone diameter ratios greater than or equal to 1.5 for any or all of the disk sets were considered to be positive for ESBLs according to the manufacturer's interpretation guidelines (Mast Diagnostics ESBL detection kit, Merseyside, United Kingdom).

2.2.2.1 Preparation of crude protein extracts and iso-electric focusing (IEF)

ESBL-positive isolates were grown in 2 ml of Mueller Hinton broth at 37°C overnight and cells were harvested by centrifugation at 14,000 rpm for 2 minutes. After discarding the supernatant, cells were resuspended in 250 μl of 1% glycine/30% glycerol and were sonicated for 2 x 30 seconds while cooling the cells on ice between sonications. Cell lysates were centrifuged at 14,000 rpm for 15 minutes. Supernatants were collected into clean tubes and were stored at -20°C. Prior to iso-electric focusing, cell extracts were tested for ß-lactamase activity by adding 50 μl of 50μg/ml nitrocefin stock solution (Oxoid Limited, Hampshire, England) to 17 μl of extract and then recording the time required for the reaction to turn dark pink. The optimal reaction time was 30-120 seconds. For reaction times of 5 seconds or less, the extract was diluted with phosphate buffer and was re-tested. For isolates with reaction times of 5 minutes or more, another extract was prepared from a culture of greater density and then was re-tested. For iso-electric focusing, pre-cast polyacrylamide IEF mini-gels (pH 3-10) (Bio-Rad

Laboratories, Hercules, CA) were assembled in a vertical Bio-Rad Mini-Protean II electrophoresis unit. Cathode buffer (20mM lysine/20mM arginine)(Bio-Rad) was added to the middle chamber, the wells were flushed and then 10 µl of crude extract was loaded in every second well. An IEF standard (Bio-Rad) and a marker composed of beta-lactamases of known iso-electric point (Ip) were used. Approximately 200 ml of anode buffer (7mM phosphoric acid) (Bio-Rad) were added to the outer buffer chamber. The electrophoresis unit was placed on a tray and was surrounded with ice. Electrophoresis was performed in three steps: 100V for 1 hour; 250 V for another hour; and finally, at 500V for 30 minutes. IEF gels were then dismantled from the unit and the glass plates were separated while leaving the gel on one glass plate. To visualize ß -lactamase activity, 1 ml of nitrocefin stock solution (1 mg/ml) was added to 6 ml of molten 3% agarose in 50mM phosphate buffer (pH 7.5) (cooled to 50-60°C), was mixed by inversion and then was poured evenly over the gel. The presence of pink/red lines on the gel indicated ß -lactamase activity. Pictures of IEF gels were taken using a dark green filter and the gels were illuminated from behind with white light.

2.3 PCR detection of antimicrobial genes

Test strains were grown at 35°C on Mueller Hinton agar containing antimicrobials. Genomic DNA was extracted with a Puregene kit (Gentra Systems, Inc., Minneapolis, Minnesota). All primers and reference strains used for PCR are found in Table 7. The PCR reaction mix for the detection of *bla*$_{TEM}$, *catI, sulI, aph3'Ia* and integrons included 1.0 µM of forward and reverse primers, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide dATP, dCTP, dGTP and dTTP (Gibco BRL, Burlington, Ontario), 0.025 U/µl *Taq* polymerase (Gibco BRL, Burlington, Ontario) and approximately 1 µg of

template DNA. The multiplex PCR mix for the detection of *tetB, tetC, and tetD* included 0.25 μM each primer, 1X PCR buffer, 3.0 mM $MgCl_2$, 300 μM of each deoxynucleotide, 0.025 U/μl *Taq* polymerase and approximately 1 μg of template DNA. Amplification conditions for *catI, sulI, aph3'Ia* and *tetB,C,D* PCR were: 1 cycle at 94°C for 5 min, 35 cycles (94°C for 1 min, 55°C for 1 min, and 72°C 1 min 30s). An annealing temperature of 48°C was used for amplification of bla_{TEM}. Integron amplification involved 1 cycle at 94°C for 12 min and 35 cycles (94°C for 1 min, 55°C for 1 min, and 72°C 5 min). All PCR reactions had a final volume of 50 ul. Finally, 10 ul of each PCR product were analyzed by gel electrophoresis in a 1% agarose gel run at 100V for 1 hour. To visualize band migration the gel was stained with ethidium bromide and observed under UV light. A 100bp or 1kb ladder (Gibco BRL) was used to estimate amplicon size.

Table 7: PCR primers and reference strains used in the detection of antimicrobial resistance genes in Brazilian and Colombian *Salmonella* and *Shigella* strains

Gene	Primer Sequence 5'-3'	Reference Strain (plasmid)	Source and Reference
tet A	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	*E. coli* D20-15 (pSL18)	S. Levy (Mendez *et al*, 1980)
tet B	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	*E. coli* D20-16 (pRT11)	S. Levy (Marshall *et al*, 1983)
tet C	CTT GAG AGC CTT CAA CCC AG ATG GTC GTC ATC TAC CTG CC	*E. coli* D20-6 (pBR322)	S. Levy (Marshall *et al*, 1983)
tet D	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	*E. coli* D22-2 (pSL106)	S. Levy (Marshall *et al*, 1983)
tet E	AAA CCA CAT CCT CCA TAC GC AAA TAG GCC ACA ACC GTC AG	*E. coli* D22-14 (pSL1504)	S. Levy (Marshall *et al*, 1986)
tet G	CAG CTT TCG GAT TCT TAC GG GAT TGG TGA GGC TCG TTA GC	*E. coli* HB101 (pJA8122)	T. Aoki (Zhao and Aoki, 1992)
tet H	CCT GAA AAC CAA ACT GCC TC ACA GAC CAT CCC AAT AAG CG	*Pasteurella multocida* (pVM112)	M. Roberts (Hansen *et al*, 1993)
cat I	TCA GCT GGA TAT TAC GGC CT CAT TCT GCC GAC ATG GAA G	LK 169 (pBR329)	(Alton and Vapnek, 1979)
cat II	ATT CAG CCT GAC CAC CAA AC CTT CCT GCT GAA ACT TTG CC	*E. coli* J52 (pSA)	M. Roberts (Murray *et al*, 1988)
cat III	CCC ACA ATT CAC CGT ATT CC GAA CCT GTA CTG AGA GCG GC	*E. coli* J53 (R387)	M. Roberts (Murray *et al*, 1990
sul I	CAC CGC GGC GAT CGA AAT GC GGT TTC CGA GAA GGT GAT	820 *Proteus mirabilis*	P. H. Roy (Lévèsque *et al*, 1995)
sul II	ATC GCT CAT CAT TTT CGG CA CTC GTG TGT GCG CAT GAA GT	*S.* Typhimurium CO-8861	C. Clark (Raadstroem and Swedberg, 1988)
dhfrI	CGA AGA ATG GAG TTA TCG GG TAA ACA TCA CCT TCC GGC TC	C600 (R483)	Sundström and Sköld (1990)
aadA1	GCG CTA AAT GAA ACC TTA AC TCG CCT TTC ACG TAG TGG AC	*E. coli* JE 2571 (pHH1457)	D. Taylor (Bradley *et al*, 1980)
aadA2	TGT TGG TTA CTG TGG CCG TA GCT GCG AGT TCC ATA GCT TC	*S.* Typhimurium PT104 96-5227	D. Taylor (Bito and Susani, 1994)
aph3'Ia	TTA TGC CTC TTC CGA CCA TC GAG AAA ACT CAC CGA GGC AG	*E. coli* JE 2571 (pHH1457)	D. Taylor (Bradley *et al*, 1980)
aac6'Iq	GCT GGA AAT GAA TCA TGG GT TAA TTC CCC TAC CCT TCG CT	BR-SA-97-368	D. Rodrigues (This study)
bla_{TEM-1}	ATA AAA TTC TTG AAG ACG AAA GAC AGT TAC CAA TGC TTA ATC A	*Neisseria gonorrhoeae* 18795	(Edelstain and Stratchounski, 1998)
Integron 5'CS/3'CS	GGC ATC CAA GCA GCA AG AAG CAG ACT TGA CCT GA	*S.* Typhimurium PT104 96-5227	D. Taylor (Lévèsque *et al*, 1995)
Integrase (*intI*)	GAT GCC TGC TTG TTC TAC GG GCC TTG CTG TTC TTC TAC GG	*S.* Typhimurium PT104 96-5227	D. Taylor (Lévèsque *et al*, 1995)

2.4 DNA Sequencing

Amplicons resulting from PCR reactions using the primers specific to the 5'conserved and 3' semi-conserved segments of integrons and amplicons generated with universal *bla*$_{TEM}$ primers were purified by gel extraction and were sequenced in both directions using and ABI Prism,377 DNA Sequencer (Applied Biosystems Division of Perkin-Elmer, Foster City, California). DNA sequences were compared to those in the GenBank database (National Center for Biotechnology Information) using the BLAST suite of sequence similarity searching programs (Altschul *et al.,* 1990; 1997)

2.5 Dot blot hybridizations

Test strains were grown at 35°C on Mueller Hinton agar containing antimicrobials. Genomic DNA was extracted with Puregene (Gentra Systems, Inc., Minneapolis, Minnesota). Approximately 900ug of genomic DNA and 100ng of amplicon DNA (positive controls for probes) were transferred to Hybond-N^+ nylon membrane (Amersham Life Science, Amersham, United Kingdom) using a dot-blot vacuum manifold (BioRad Laboratories, Hercules, California). Prior to blotting, the concentration of DNA samples was estimated by electrophoresis in a 1% agarose gel run in 1x TAE and a representative number of samples with varying band intensities were quantified by spectrophotometer. After dilution of DNA samples in TE buffer, all samples were denatured in 0.5 M NaOH for 5 min., chilled on ice for 5 min. and then one volume of 20x SSC was added before they were passed through the dot-blot vacuum manifold according to the manufacturer's instructions. After blotting, the nylon membranes were

denatured a second time in 1.5M NaCl / 0.5 M NaOH for 5 min. and then neutralized for 1 min. in 1.5 M NaCl / 0.5 M Tris-HCl (pH 7.2)/ 1mM EDTA. Blots were then air dried for 5 min., baked for 10 min. at 80 °C and were then exposed to UV at 312 nm for 5 min to crosslink the DNA to the membrane.

Probe labelling, hybridizations,washings and exposure to XRAY film were carried out using the ECL random prime labelling system (Amersham Life Science, Amersham, United Kingdom) according to manufacturer's instructions. The only modification that was made was changing the pre-hybridization and hybridization temperatures from 60°C to 55 °C. Probe DNA was prepared by amplifying antimicrobial resistance genes using primers and reference strains listed in Table 7 and then the amplicons were gel purified prior to labelling. To remove probe from the membranes, the blots were placed in 0.4 M NaOH at 45°C for 30 min., then boiling 0.5% SDS was poured over the blots. Once the 0.5% SDS cooled to room temperature, the blots were placed neutralized in 0.1X SSC /0.1 % SDS/ 0.2 M Tris-HCl (pH 7.5) for 15 min at 45°C. Blots were then rinsed in 2X SSC at room temperature prior to pre-hybridization.

2.6 Plasmid DNA extractions

Isolates with various antibiograms were chosen for plasmid analysis. Plasmids were extracted according to Birnboim and Doly (1979) with some modifications. Briefly, isolates were grown for 12-14 hours at 35°C in 3ml of Mueller Hinton broth with the appropriate antimicrobials (ampicillin (20 µg/ml), chloramphenicol (25 µg/ml),

streptomycin (8 μg/ml), tetracycline (25 μg/ml), kanamycin (10 μg/ml), gentamicin (8 μg/ml), sulfamethoxazole (10 μg/ml) and trimethoprim (4 μg/ml). Cells were harvested by centrifugation at 14,000 rpm for 10 min, the supernatant was removed and the sample tubes were placed on ice. All centrifugations in this method were performed at 14,000 rpm in a microcentrifuge (Applied Biosystems Division of Perkin-Elmer, Foster City, California). Cell pellets were resuspended in 100 μl of Solution 1 (24 mM Tris pH 8.0, 0.2% glucose, 10mM EDTA, 0.5 mg/ml RNase and 2mg/ml lysozyme), were vortexed and were left at room temperature for 5 minutes. Cells were lysed with 200 μl of Solution 2 (0.2 N NaOH, 1% SDS) for 5 minutes at room temperature and then neutralized with 150 μl of 3M sodium acetate. Samples were chilled on ice for 10 min, were centrifuged and the supernatant transferred to a fresh 1.5 ml microcentrifuge tube. One volume of phenol was added to the supernatant, was mixed, and then the same volume of chloroform was added to the mixture. Samples were centrifuged for 5 minutes. The aqueous layer was transferred to a fresh tube followed by two volumes of 95% ethanol and 1/10 volume of 3M sodium acetate. To precipitate the DNA, samples were stored at –80°C for 10 minutes and then centrifuged for another 10 minutes. The supernatant was discarded, the DNA pellets were washed with 70% ethanol and centrifuged for 2 minutes. After removing the ethanol, the DNA pellets were air-dried for 10 minutes and then re-hydrated with 30 μl of 10 mM Tris, 1 mM EDTA (pH 8.0). Plasmid samples were analyzed by electrophoresis in 0.75% agarose in 1X TBE at 6V/cm for 4.5 hours. A supercoiled DNA ladder (Gibco BRL, Burlington, Ontario) was used as a size marker and plasmid sizes were determined by plotting ladder and plasmid band migration distances on semi-logarithmic graph paper. The molecular mass of plasmids was determined by

converting their sizes in kilobases to megadaltons (MDa) using the following formula: 1 MDa = 1.54 kb (Helling and Lomax, 1978). The plasmid gel was stained with ethidium bromide and was observed under UV light.

2.7 Southern hybridizations

After electrophoresis, the plasmid gel was exposed to UV (60mJ) for 5 minutes to nick the DNA and then was transferred downwards onto Hybond-N+ nylon membrane (Amersham Life Science, Amersham, United Kingdom) using the Turboblotter apparatus (Schleicher and Schuell, Keene, New Hampshire). The DNA from the plasmid gel was allowed to transfer for 12 hours in 0.4 M NaOH according to the Turboblotter manufacturer's instructions. Once the transfer was completed, the efficiency of DNA transfer out of the agarose gel was verified by re-staining the plasmid gel with ethidium bromide and observing it under UV light. The nylon membrane was neutralized in 1.5 M NaCl/ 0.5 M Tris HCl (pH 7.2)/ 1mM EDTA for 5 minutes and then air-dried for 10 minutes. The same bla_{TEM-1}, *tetB*, *catI* and *dhfrI* probes used in the dot blot hybridizations were used for hybridizations with plasmid DNA. Hybridizations and membrane stripping methods used were exactly as described in section 2.5 above.

3. RESULTS

3.1 Stage I. Serotypes and antibiograms of multi-drug resistant *Salmonella* and *Shigella* strains from Brazil and Colombia

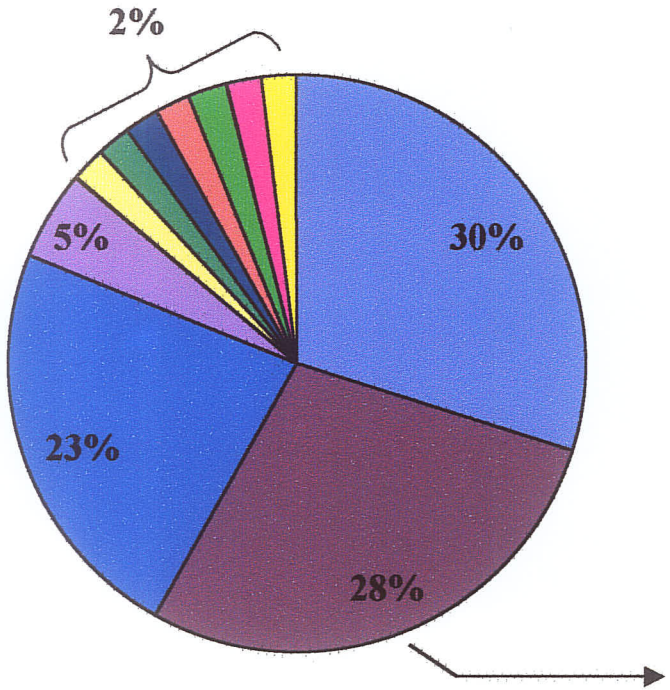
Of the 501 *Salmonella* and *Shigella* strains that were screened for resistance to antimicrobials in this study, 236 strains were resistant to three or more groups of antimicrobials and were thus considered to be multi-drug resistant. In addition, 6 strains which were resistant to two groups and intermediately resistant to one or more groups of antimicrobials were selected for further study. Together, these 242 strains comprise the final panel of test strains which were used to study the mechanisms of antimicrobial resistance in *Salmonella* and *Shigella* strains from Brazil and Colombia. Table 8 summarizes the final numbers of Brazilian and Colombian strains that have successfully passed each screening step in Stage 1 as described in section 1.5. It was, therefore, possible to obtain consistent disk diffusion results for all 242 isolates using ampicillin, chloramphenicol, ciprofloxacin, SXT, gentamicin and cefotaxime in comparison to those obtained previously by the institution from which the strains originated and those obtained by Health Canada's National Microbiology Laboratory (data not shown).

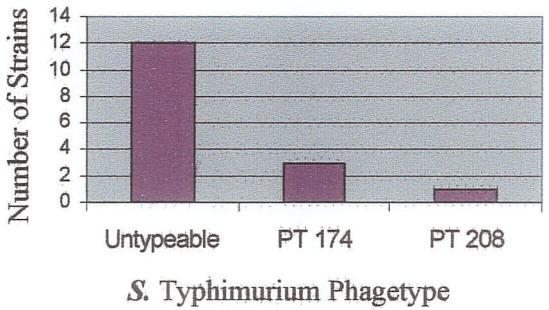
Eleven different serotypes were identified within the 56 Brazilian *Salmonella* strains used in this study (see Figure 8a) and four different serotypes were identified among the 28 Colombian *Salmonella* strains (see Figure 8b). The serotypes of the majority of Brazilian *Salmonella* strains are *S.* Agona (30%), *S.* Typhimurium (28%) and *S.* Infantis (23%) whereas the majority of Colombian *Salmonella* strains are *S.* Typhimurium (88%).

Figure 8:

(A) Serotypes of multi-drug resistant Brazilian *Salmonella* strains used in this study. Phage types of *S.* Typhimurium strains are also shown. The number of strains with the same serotype is expressed as a percentage out of a total of 56 isolates. "*Salmonella* spp." is a *Salmonella* strain that has a monophasic serotype 4,5,12:i:-. (B) Serotypes of multi-drug resistant Colombian *Salmonella* strains used in this study. The number of strains with the same serotype are expressed as a percentage out of a total of 28 isolates.

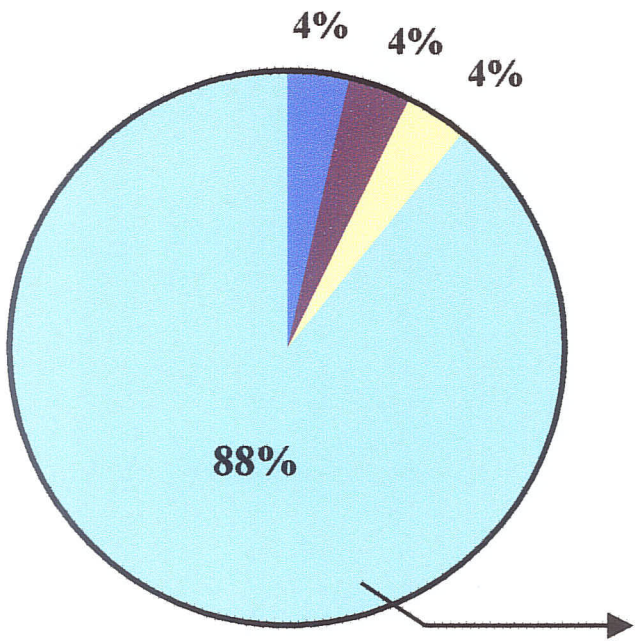
(A)

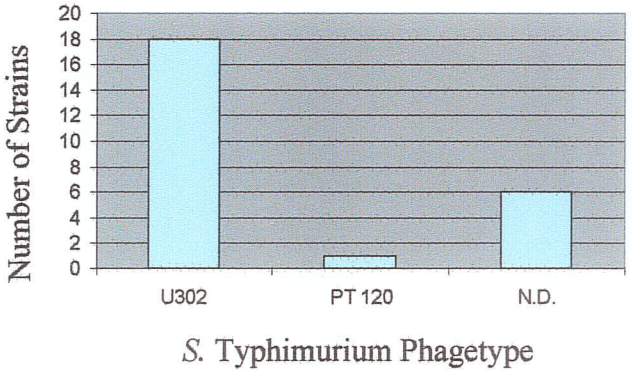




S. Agona	S. Typhimurium	S. Infantis	S. Enteritidis
S. Brandenberg	S. Bredeney	S. Derby	S. Heidelberg
S. Panama	S. Schwarzengrund	*Salmonella* spp.	

(B)





S. Enteritidis S. Infantis S. Typhi S. Typhimurium

Table 8: Total number of multi-drug resistant *Salmonella* and *Shigella* strains from Brazil and Colombia used in this study.

Country	Organism	Number of Strains Screened	Number of MDR Strains	
			Clinical	Other
Brazil	*Salmonella*	243	41	15
	Shigella	176	136	N.A.
Colombia	*Salmonella*	58	28	0
	Shigella	24	22	N.A.

Total MDR strains: 242

As is shown in Table 9, twenty-two different antibiograms were detected in *Salmonella* strains isolated from human patients in Brazil. Out of the 41 clinical *Salmonella* strains from Brazil, 61% are resistant to five groups of antimicrobials, 19.5% are resistant to four groups and another 19.5% are resistant to three groups of antimicrobials. The most multi-resistant strains that were resistant to five antimicrobial groups consisted of four serotypes: *S.* Agona (10 strains), *S.* Typhimurium (4 strains), *S.* Infantis (10 strains) and *S.* Schwarzengrund (1 strain). Clinical *S.* Agona strains had eight different antibiograms and thus had the greatest variety of antibiograms out of all the Brazilian *Salmonella* serotypes tested. Five out of the eight different antibiograms that *S.* Agona strains possessed indicated resistance to five groups of antimicrobials which included resistance to: β-lactams (to all penicillins and/or to some cephalosporins), chloramphenicol, some aminoglycosides, sulfonamides and/or trimethoprim and tetracyclines. Table 9 also shows that *S.* Typhimurium and *S.* Infantis strains each had six different antibiograms all of which are mutually exclusive. Only the ACSSuTKGTm

Table 9: Antibiograms of 41 clinical *Salmonella* strains from Brazil

Antibiogram[ab]	Serotype and Number of Strains					
	S. Agona	*S.* Typhimurium	*S.* Infantis	*S.* Enteritidis	*S.* Schwarzengrund	*S.* spp[c]
ACSSuTKGCTXCAZTm			7			
ACSuTKGCTXCAZTm			1			
ACSSuTKGCTXCAZ		1				
ACSSuTKGCTXTm			2			
ACSuTKGCTXCAZ	2					
ACSSuTKGTm	1	3				
ACSuTKGCAZ	1					
ACSuTKGTm	2					
ACSSuTTm					1	
ACSuTKG	4					
ACSuKCTXCAZTm	1					
ASSuTKGCAZ			1			
ACSSuKG						1
ASuTGTm				1		
ASuTG			1			
ACSuT			1			
ASSuT		1				
ATKTm		1				
ASSuKG	2					
ASuKCTX	4					
ASSuTm		1				
SSuTTm		1				

[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); three antimicrobial groups (green).

[b] Abbreviations used: A: ampicillin; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; K:kanamycin; G: gentamicin; CTX: cefotaxime; CAZ: ceftazidime; Tm: trimethoprim

[c] This *Salmonella* strain is monophasic of serotype 4,5,12:i:-.

antibiogram is found in both *S.* Agona and *S.* Typhimurium strains. The single *S.* Enteritidis and *Salmonella* spp. 4,5,12:i:- strains were both resistant to penicillins, sulfonamides and at least one of the aminoglycosides tested. The *S.* Enteritidis strain, however, was susceptible to the cephalosporins CTX and CAZ, to chloramphenicol and to the aminoglycosides streptomycin and kanamycin while the *Salmonella* spp. 4,5,12:i:- strain was susceptible to tetracyclines, CTX, CAZ and trimethoprim. Resistance to three groups of antimicrobials was found only in six *S.* Agona and two *S.* Typhimurium strains. Clinical *Salmonella* strains from Brazil were all sensitive to ciprofloxacin.

Table 10: Antibiograms of 15 Brazilian *Salmonella* strains isolated from animals, the environment and food

Antibiogram[ab]	Source	Serotype and Number of Strains						
		S. Typhimurium	*S.* Enteritidis	*S.* Brandenberg	*S.* Bredeney	*S.* Derby	*S.* Heidelberg	*S.* Panama
ACSSuTKTm	Animal				1			
ACSSuTKG	Animal	1						
ACSuKGCTX	Food		1					
CSSuT	Animal	1						1
CSSuT	Environment			1				
CSSuTmK	Animal	1						
SSuTG	Environment						1	
SSuTTm	Animal		1					
SSuT	Animal	5						
SuTTm	Animal					1		

[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); three antimicrobial groups (green); two antimicrobial groups (black)

[b] Abbreviations used: A: ampicillin; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; K:kanamycin; G: gentamicin; CTX: cefotaxime; Tm: trimethoprim

Table 10 lists the Brazilian *Salmonella* strains that were isolated from non-clinical sources such as animal feces, meat products and the environment (see Materials and Methods section 2.1). Nine different antibiograms were detected in the non-clinical

Salmonella strains and only one antibiogram, SSuTTm, was common to both a Brazilian veterinary strain (S. Enteritidis) and one clinical S. Typhimurium strain (as shown in Table 9). In contrast to the 61% of clinical *Salmonella* strains from Brazil that were resistant to five groups of antimicrobials, only 13% of *Salmonella* strains isolated from non-clinical samples were resistant to five antimicrobial groups. The penta-resistant veterinary strains, one *S.* Typhimurium and one *S.* Bredeney strain, were both resistant to ACSSuTK plus one other antimicrobial: gentamicin in the case of *S.* Typhimurium and trimethoprim in the *S.* Bredeney strain. The antibiograms of these two penta-resistant veterinary strains are similar to the four clinical *S.* Typhimurium, nine clinical *S.* Infantis, one clinical *S.* Agona and one clinical *S.* Schwarzengrund strains from Brazil (see Table 9) that were also resistant to ACSSuTK plus one or more of the following antimicrobials: G, CTX, CAZ and Tm.

The Brazilian *S.* Enteritidis isolated from food (see Table 10) and the Brazilian *S.* Enteritidis isolated from human stool (refer to Table 9) were both resistant to four antimicrobial groups though their antibiograms were different. Both the foodborne and clinical strains of *S.* Enteritidis were resistant to ASuG but the foodborne strain possessed additional resistance to C, K and CTX while the clinical strain was also resistant to T and Tm. Resistance to four antimicrobial groups can also be seen in strains isolated from all three sources: food (*S.* Enteritidis resistant to ACSuKGCTX), animals (CSSuT-resistant *S.* Typhimurium and *S.* Panama) and the environment (CSSuT-resistant *S.* Brandenberg). Resistance to three groups of antimicrobials was observed in six veterinary *S.* Typhimurium strains, in one strain of *S.* Heidelberg isolated from the environment, and in one veterinary *S.* Enteritidis isolate. Only one veterinary *S.* Derby strain was resistant to

two groups of antimicrobials- SuTTm. All non-clinical *Salmonella* strains from Brazil were sensitive to ciprofloxacin.

Table 11: Antibiograms of 28 clinical *Salmonella* strains from Colombia

Antibiogram [ab]	Serotype and Number of Strains			
	S. Typhimurium	*S.* Infantis	*S.* Typhi	*S.* Enteritidis
ACSSuTTm			1	
ASSuTKTm	4			
ASSuTTm	5			
ASuTKTm	1	1		
ASSuKTmCTXCAZ				1
ASSuKTm	2			
ASuKTm	1			
ASuTTm	9			
SSuTTm	1			
SuTTm	2			

[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); three antimicrobial groups (green); two antimicrobial groups (black).

[b] Abbreviations used: A: ampicillin; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; K:kanamycin; CTX: cefotaxime; CAZ: ceftazidime; Tm: trimethoprim

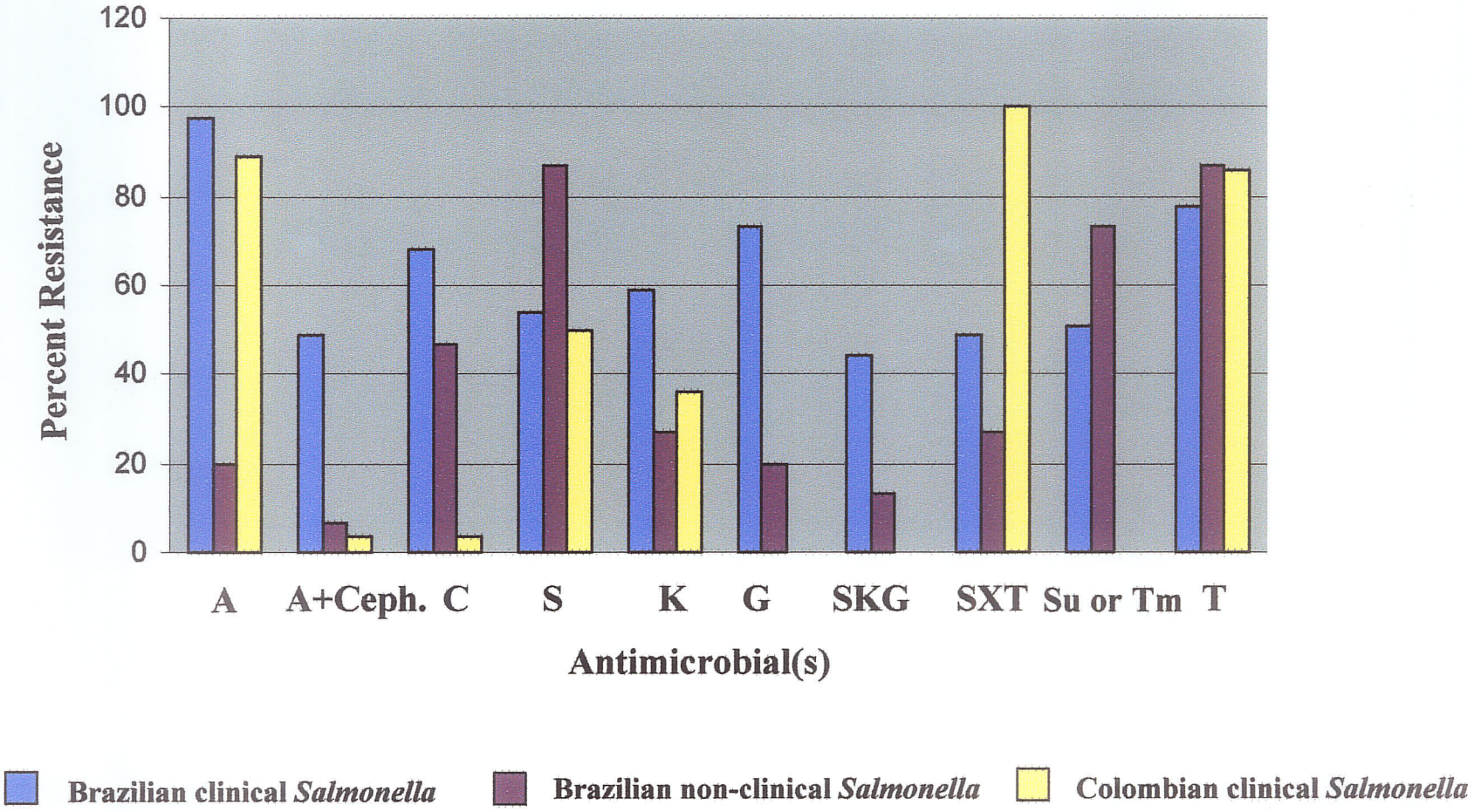
As is shown in Table 11, ten different antibiograms were observed in clinical *Salmonella* strains from Colombia. Seven of these antibiograms were unique to Colombian *Salmonella* strains whereas ACSSuTTm, SSuTTm and SuTTm were also observed in clinical and/or veterinary *Salmonella* strains from Brazil. Penta-resistance in clinical *Salmonella* strains from Colombia was seen only in *S.* Typhi that was resistant to ACSSuTTm. As was shown previously in Table 9, the ACSSuTTm antibiogram was also found in a clinical strain of S. Schwarzengrund from Brazil. Table 11 clearly demonstrates how the Colombian *S.* Typhimurium strains used in this study had the most variety regarding antibiograms. The only antibiogram that was common to two serotypes

was ASuTKTm that was observed in both *S.* Infantis strain and *S.* Typhimurium. Both the Colombian and Brazilian clinical *S.* Enteritidis strains were resistant to ASuTm except that the former was also resistant to S, K, CTX and CAZ whereas the latter had additional resistance to T and G. All Colombian *Salmonella* strains were susceptible to ciprofloxacin.

The comparison of the percentage of strains resistant to different antimicrobials for all three groups of *Salmonella* strains studied is shown in Figure 9. For Brazilian clinical and non-clinical *Salmonella* strains as well as for Colombian clinical *Salmonella* strains, the antimicrobial to which the highest percentage of strains showed resistance were to A, S, SXT and T. In comparison to the clinical *Salmonella* strains from Brazil and Colombia that were on average 93% ampicillin-resistant, only 20% of the non-clinical *Salmonella* strains were resistant to ampicillin. Only a very low percentage of Brazilian non-clinical and Colombian clinical *Salmonella* strains were resistant to ampicillin plus a cephalosporin (ceftazidime and/or cefotaxime) whereas half of the clinical Brazilian *Salmonella* were resistant to the two types of ß-lactams. Chloramphenicol resistance was found mostly in the Brazilian *Salmonella* strains, especially in the clinical strains that were 68% resistant, but only a small 3.6% of Colombian strains were chloramphenicol resistant.

Figure 9:

Comparison of the percentage of *Salmonella* strains from both Brazil and Colombia that were resistant to various antimicrobial groups. Percentage calculations are based on the following total number of strains: Brazilian clinical *Salmonella* (41 strains), non-clinical Brazilian *Salmonella* (15 strains) and Colombian clinical *Salmonella* (28 strains). Abbreviations used: A: ampicillin; A+ Ceph.: ampicillin plus one or both cephalosporins cefotaxime or ceftazidime; C: chloramphenicol; S: streptomycin; K: kanamycin; G: gentamicin; Su: sulfonamides, Tm= trimethoprim, SXT: sulfamethoxazole/ trimethoprim; T= tetracycline.


120
100
80
60
40
20
0
Percent Resistance
A
A+Ceph.
C
S
K
G
SKG
SXT
Su or Tm
T
Antimicrobial(s)
Brazilian clinical *Salmonella*
Brazilian non-clinical *Salmonella*
Colombian clinical *Salmonella*

The majority of all *Salmonella* tested were resistant to streptomycin with the non-clinical *Salmonella* from Brazil being the most resistant. Kanamycin resistance was found mostly in Brazilian clinical strains whereas less than 40% of non-clinical Brazilian and clinical Colombian *Salmonella* strains were resistant. In contrast to the Colombian *Salmonella* strains which were all susceptible to gentamicin, over 70% of clinical Brazilian *Salmonella* were gentamicin-resistant. Over 40% of clinical *Salmonella* strains from Brazil were resistant to all three aminoglycosides tested whereas none of the Colombian strains were SKG-resistant. All Colombian *Salmonella* are resistant to both sulfonamides and trimethoprim while Brazilian *Salmonella* have a higher percentage of strains resistant to only one of the two antimicrobials. The only antimicrobial to which all three *Salmonella* groups had a similar percent resistance (approximately 80%) was to tetracycline.

Figure 10 (a) displays the four different serotypes which were identified within the 136 *Shigella* strains from Brazil and the two serotypes within the 22 Colombian strains are shown in Figure 10 (b). The majority of Brazilian and Colombian strains are *S. flexneri* and *S. sonnei.*

Shigella strains from both Brazil and Colombia tested for multi-drug resistance in this study were susceptible to ciprofloxacin. Twelve antibiograms were found within Brazilian *Shigella* strains as is listed in Table 12. Over half of the *Shigella* strains were resistant to five groups of antimicrobials, 8% were resistant to four groups, 37% were resistant to three groups and 2% were resistant to two groups of antimicrobials. Nearly

Figure 10:

(A) Serotypes of multi-drug resistant Brazilian *Shigella* isolates used in this study. Strains with the same serotype are expressed as a percentage out of a total of 136 strains.

(B) Serotypes of multi-drug resistant Colombian *Shigella* isolates used in this study. Strains with the same serotype are expressed as a percentage out of a total of 22 strains.

75% of Brazilian *S. flexneri* were resistant to ACSSuTTm whereas only16% of *S. sonnei* were ACSSuTTm-resistant. The majority of Brazilian *S. sonnei* (65%) were resistant to SSuTTm. Each of the *S. flexneri* and *S. sonnei* groups of strains had seven different antibiograms and only two of these antibiograms, ACSSuTTm and SSuTTm, were found in both serotypes. Similarly to Brazilian *S. flexneri* strains, the majority of Colombian *S. flexneri* strains were ACSSuTTm-resistant (Table 13). There were only three antibiograms found in the 22 Colombian *Shigella* strains tested and the only antibiogram shared by both *S. flexneri* and *S. sonnei* was ASSuTTm.

Table 12: Antibiograms of 136 *Shigella* strains from Brazil

Antibiogram [ab]	Serotype and Number of Strains			
	S. flexneri	*S. sonnei*	*S. boydii*	*S. dysenteriae*
ACSSuTTm	62	8		
ACSTTm	1			
ACSSuTm	2			
ASSuTTm		1		
ASTTm	1			
CSSuTG		1		
CSSuTTm	6			
CSSuT		1		
ASSuTm		4		2
SSuTTm	11	32	1	
SSuTm		2		
CTm	1			

[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); three antimicrobial groups (green); two antimicrobial groups (black).

[b] Abbreviations used: A: ampicillin; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; G: gentamicin; Tm: trimethoprim.

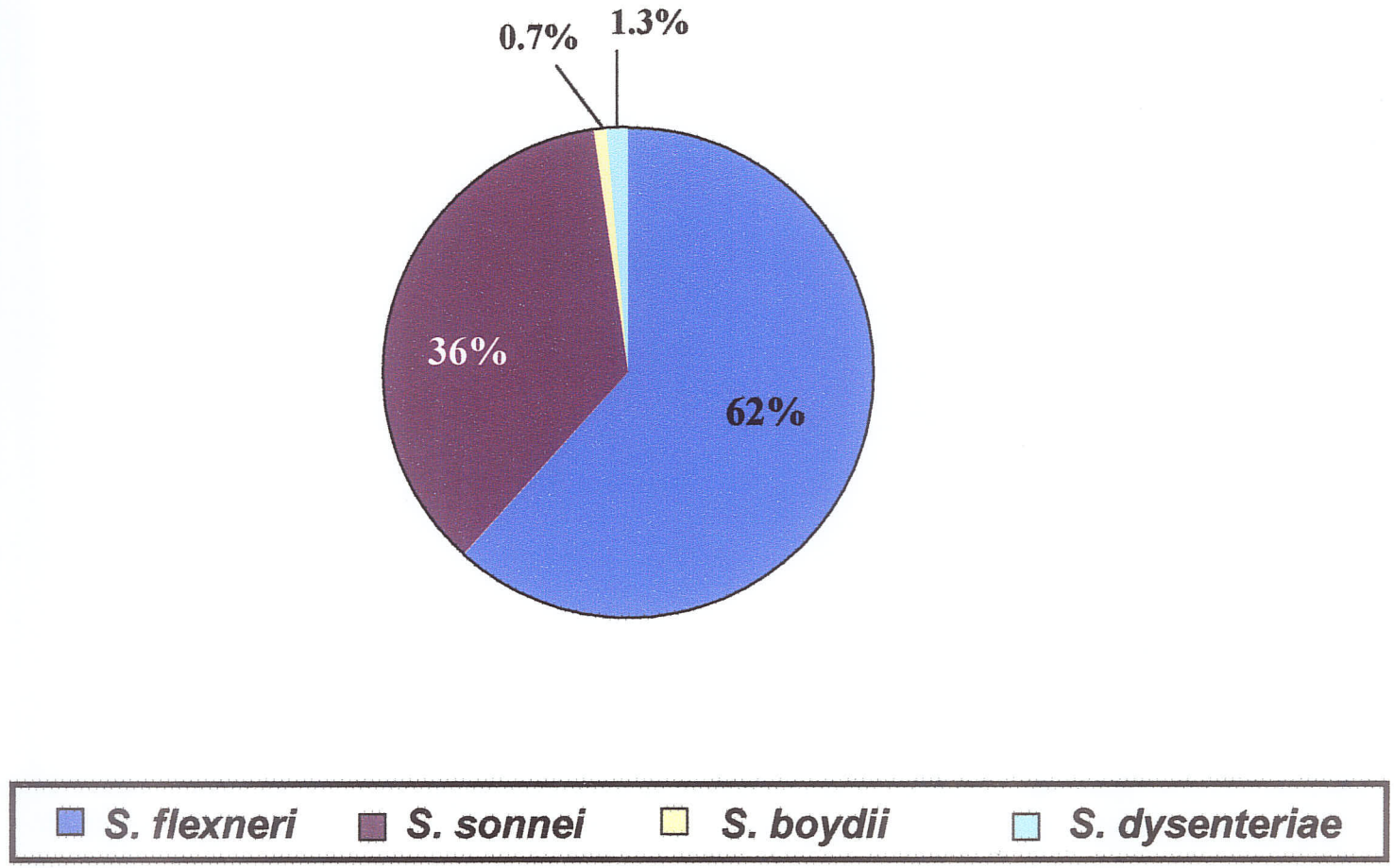
Table 13: Antibiograms of *S. flexneri* and *S. sonnei* from Colombia

Antibiogram [ab]	Number of Strains	
	S. flexneri	*S. sonnei*
ACSSuTTm	13	
ASSuTTm	1	2
SSuTTm		6

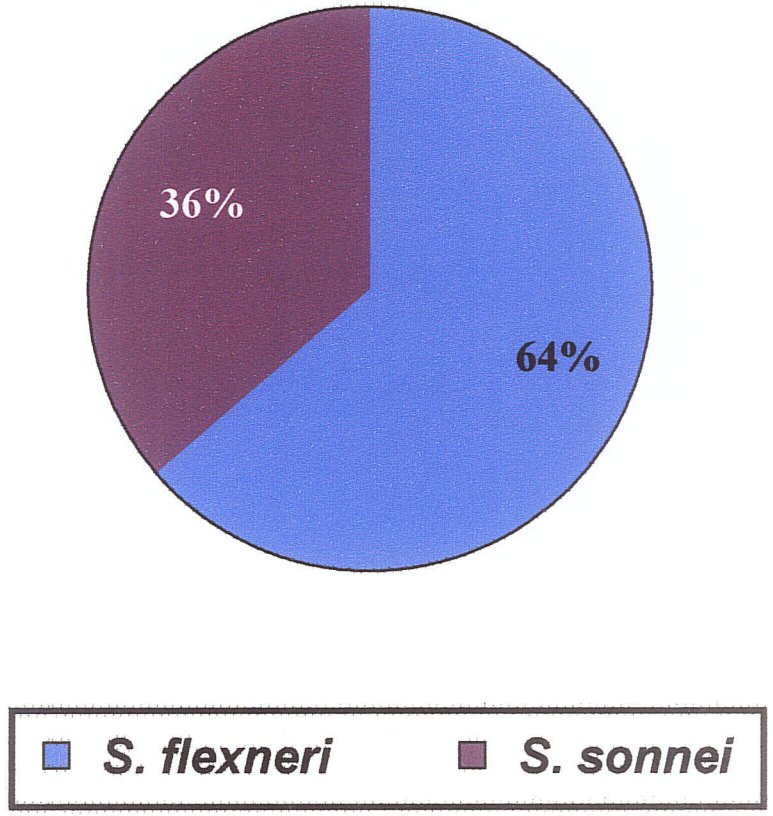
[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); three antimicrobial groups (green).
[b] Abbreviations used: A: ampicillin; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; Tm: trimethoprim

The comparison of the percentage of *Shigella* strains from both Brazil and Colombia that were resistant to different antimicrobials is shown in Figure 11. A similarity was observed between the *Salmonella* strains mentioned above which showed the highest percent resistance against A, S, SXT and T (Figure 9) and *Shigella* strains from both countries which also had the highest percentage resistance to S, SXT and T and at least 60% of the strains were resistant to ampicillin. The same percentage of *Shigella* strains were resistant to chloramphenicol from both countries (60%) which is comparable to the 68% of chloramphenicol-resistant clinical *Salmonella* strains from Brazil. However, unlike all *Salmonella* strains used in this study, none of the *Shigella* strains were resistant to either kanamycin or the cephalosporins CTX and CAZ.

(A)



(B)



75% of Brazilian *S. flexneri* were resistant to ACSSuTTm whereas only16% of *S. sonnei* were ACSSuTTm-resistant. The majority of Brazilian *S. sonnei* (65%) were resistant to SSuTTm. Each of the *S. flexneri* and *S. sonnei* groups of strains had seven different antibiograms and only two of these antibiograms, ACSSuTTm and SSuTTm, were found in both serotypes. Similarly to Brazilian *S. flexneri* strains, the majority of Colombian *S. flexneri* strains were ACSSuTTm-resistant (Table 13). There were only three antibiograms found in the 22 Colombian *Shigella* strains tested and the only antibiogram shared by both *S. flexneri* and *S. sonnei* was ASSuTTm.

Table 12: Antibiograms of 136 *Shigella* strains from Brazil

Antibiogram [ab]	Serotype and Number of Strains			
	S. flexneri	*S. sonnei*	*S. boydii*	*S. dysenteriae*
ACSSuTTm	62	8		
ACSTTm	1			
ACSSuTm	2			
ASSuTTm		1		
ASTTm	1			
CSSuTG		1		
CSSuTTm	6			
CSSuT		1		
ASSuTm		4		2
SSuTTm	11	32	1	
SSuTm		2		
CTm	1			

[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); three antimicrobial groups (green); two antimicrobial groups (black).

[b] Abbreviations used: A: ampicillin; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; G: gentamicin; Tm: trimethoprim.

Table 13: Antibiograms of *S. flexneri* and *S. sonnei* from Colombia

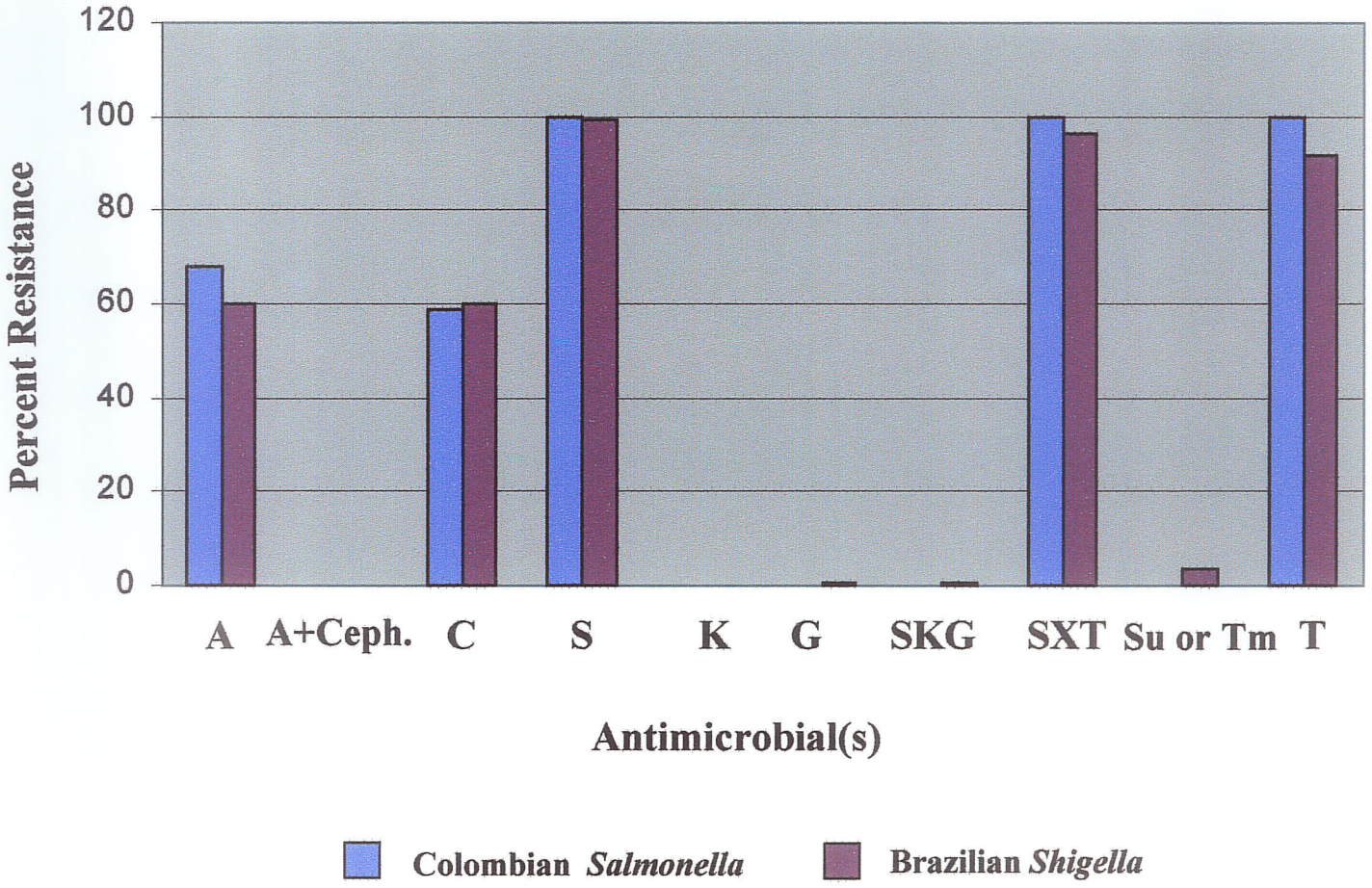
Antibiogram [ab]	Number of Strains	
	S. flexneri	*S. sonnei*
ACSSuTTm	13	
ASSuTTm	1	2
SSuTTm		6

[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); three antimicrobial groups (green).
[b] Abbreviations used: A: ampicillin; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; Tm: trimethoprim

The comparison of the percentage of *Shigella* strains from both Brazil and Colombia that were resistant to different antimicrobials is shown in Figure 11. A similarity was observed between the *Salmonella* strains mentioned above which showed the highest percent resistance against A, S, SXT and T (Figure 9) and *Shigella* strains from both countries which also had the highest percentage resistance to S, SXT and T and at least 60% of the strains were resistant to ampicillin. The same percentage of *Shigella* strains were resistant to chloramphenicol from both countries (60%) which is comparable to the 68% of chloramphenicol-resistant clinical *Salmonella* strains from Brazil. However, unlike all *Salmonella* strains used in this study, none of the *Shigella* strains were resistant to either kanamycin or the cephalosporins CTX and CAZ.

Figure 11:

Comparison of the percentage of *Shigella* strains from both Brazil and Colombia that were resistant to various antimicrobial groups. Percentage calculations are based on a total of 136 Brazilian *Shigella* and 22 Colombian *Shigella* strains. Abbreviations used: A: ampicillin; A+ Ceph.: ampicillin plus one or both cephalosporins cefotaxime or ceftazidime; C: chloramphenicol; S: streptomycin; K: kanamycin; G: gentamicin; Su: sulfonamides, Tm= trimethoprim, SXT: sulfamethoxazole/trimethoprim; T= tetracycline.


120
100
80
60
40
20
0
Percent Resistance
A
A+Ceph.
C
S
K
G
SKG
SXT
Su or Tm
T
Antimicrobial(s)
Colombian Salmonella
Brazilian Shigella

3.2 Stage II: Molecular analysis of antimicrobial resistance genes of multi-drug resistant *Salmonella* and *Shigella* strains from Brazil and Colombia

3.2.1 Antimicrobial resistance gene profiles of MDR *Salmonella* and *Shigella* strains from Brazil and Colombia

Antimicrobial resistance gene profiles were determined for all test strains by dot blot hybridizations with the following probes (see Table 7): *tetA, tetB, tetC, tetD, tetE, tetG, tetH, catI, catII, catIII, sulI, sulII, dhfrI, aadA1, aadA2, aph3'Ia, aac6'Iq and* bla_{TEM-1} . The antimicrobial resistance gene profiles that were detected in clinical Brazilian *Salmonella* strains by dot blot hybridizations are shown in Table 14. (A sample *tetB* dot blot hybridization is shown in Appendix III). The three groups of strains that had identical antibiograms (ACSSuTKGTm, ACSuTKGTm and ASuKCTX) also had nearly identical resistance gene profiles. For instance, the ACSSuTKGTm-resistant *S.* Typhimurium and *S.* Agona strains possessed identical resistance mechanisms to the antimicrobials tested and differed only by the resistance gene variant for streptomycin (*aadA1* [*ant(3")Ia*] and/or *aadA2* [*ant(3")Ib*]), sulfonamides (*sulI* and/or *sulII*) and tetracyclines (*tetB* or *tetC* alone or in combination). The majority of strains had similar resistance mechanisms regardless of differences in their antibiograms. For example, 36 out of 40 ampicillin-resistant strains carried a bla_{TEM} ß-lactamase, all chloramphenicol-resistant *Salmonella* encoded the *catI* gene while *catII* and *catIII* were absent, streptomycin resistance genes *aadA1* and/or *aadA2* were detected all *Salmonella* strains including those that were susceptible to streptomycin and finally, the tetracycline resistance genes *tetB*, *tetC* and *tetD* were detected alone or in combination in all tetracycline-resistant strains. The kanamycin and trimethoprim

Table 14: Antimicrobial resistance genes detected in clinical strains of *Salmonella* from Brazil isolated from 1987 to 1999

Antibiogram[ab]	Serotype	# of strains	Antimicrobial Resistance Genes[d]						
			A(BL)	C	S	Su	T	K	Tm
ACSSuTKGCTXCAZTm	*S.* Infantis	7	bla_{TEM}	*catI*	*aadA1*	*sulI,sulII*	*tetD*		
ACSuTKGCTXCAZTm	*S.* Infantis	1	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*	*tetD*		
ACSSuTKGCTXCAZ	S. Typhimurium	1	bla_{TEM}	*catI*	*aadA1*	*sulI*	*tetC,tetD*		---
ACSSuTKGCTXTm	*S.* Infantis	2	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*	*tetD*		
ACSuTKGCTXCAZ	*S.* Agona	2	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*	*tetD*		---
ACSSuTKGTm	*S.* Agona	1	bla_{TEM}	*catI*	*aadA2*	*sulI,sulII*	*tetB*	*aph3'Ia*	
ACSSuTKGTm	S. Typhimurium	1	bla_{TEM}	*catI*	*aadA1*	*sulI*	*tetC*		
ACSSuTKGTm	S. Typhimurium	2	bla_{TEM}	*catI*	*aadA1*	*sulI*	*tetB, tetC*	*aph3'Ia*	
ACSuTKGCAZ	*S.* Agona	1	bla_{TEM}	*catI*	***aadA1, aadA2***	*sulI,sulII*	*tetD*		---
ACSuTKGTm	*S.* Agona	1	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*	*tetD*		
ACSuTKGTm	*S.* Agona	1	bla_{TEM}	*catI*	*aadA1*	*sulI,sulII*	*tetD*		
ACSSuTTm	S. Schwarzengrund	1	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*	*tetC*	---	*dhfrI*
ACSuTKG	*S.* Agona	4	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*	*tetD*		---
ACSuKCTXCAZTm	*S.* Agona	1		*catI*	***aadA1, aadA2***	*sulI,sulII*	---		
ASSuTKGCAZ	*S.* Infantis	1	bla_{TEM}	---	*aadA1, aadA2*	*sulII*	*tetD*		---
ACSSuKG	*Salmonella* spp.[c]	1	bla_{TEM}	*catI*	*aadA2*	*sulI*	***tetB***	*aph3'Ia*	---
ASuTGTm	*S.* Enteritidis	1	bla_{TEM}	---	*aadA2*	*sulI,sulII*	*tetD*	---	
ASuTG	*S.* Infantis	1	bla_{TEM}	---	*aadA1, aadA2*	*sulII*	*tetD*	---	---
ACSuT	*S.* Infantis	1	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulII*	*tetD*	---	---
ASSuT	S. Typhimurium	1	bla_{TEM}	---	*aadA1, aadA2*	*sulII*	*tetB*	***aph3'Ia***	---
ATKTm	S. Typhimurium	1	bla_{TEM}	---	*aadA1*	---	*tetB*	*aph3'Ia*	
ASSuKG	*S.* Agona	2	bla_{TEM}	---	*aadA1, aadA2*	*sulII*	---		---
ASuKCTX	*S.* Agona	1		---	*aadA1*	*sulI*	---		---
ASuKCTX	*S.* Agona	3		---	*aadA1, aadA2*	*sulI*	---		---
ASSuTm	S. Typhimurium	1		***catI***	*aadA1*	*sulII*	***tetB***	---	*dhfrI*
SSuTTm	S. Typhimurium	1	---	---	*aadA1, aadA2*	*sulI,sulII*	*tetC*	---	*dhfrI*

[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); and, three antimicrobial groups (green).

[b] Abbreviations used: A: ampicillin; A(BL): ampicillin and other β-Lactams; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; K:kanamycin; G: gentamicin; CTX: cefotaxime; CAZ: ceftazidime; Tm: trimethoprim

[c] This *Salmonella* strain is monophasic of serotype 4,5,12:i:-.

[d] Bold genes indicate the presence of the resistance gene in a strain that is susceptible to the antimicrobial the gene encodes resistance to. Genes highlighted in purple indicate the presence of a resistance gene in a strain that was intermediately resistant to the antimicrobial the gene encodes resistance to. These strains that were intermediately resistant to an antimicrobial by disk diffusion were re-tested by the agar dilution method and were found to be sensitive to the antimicrobial. Blank spaces indicate that none of the antimicrobial resistance genes tested were detected in a strain resistant to this antimicrobial group.. The "—" symbol indicates that strains are susceptible to that particular group of antimicrobials.

resistance genes, *aph3'Ia* and *dhfrI* respectively, were not detected in most strains and thus the resistance mechanisms for these two antimicrobials are unknown.

Within several of the resistance gene profiles found for Brazilian *Salmonella* strains shown in Table 14, there were several streptomycin resistance genes, *aadA1* and/or *aadA2*, that were present in the genome of streptomycin sensitive *Salmonella* strains. Likewise, there was one chloramphenicol- and tetracycline-sensitive *S.* Typhimurium (ASSuTm-resistant) that encoded *catI* and *tetB* and other strains that encoded tetracycline resistance and kanamycin resistance while remaining clinically susceptible.

Most non-clinical *Salmonella* strains from Brazil (Table 15) possessed similar resistance genes to the ones found in clinical strains. The most noticeable differences were the *catII* chloramphenicol resistance gene found in *S.* Enteritidis isolated from food and the *tetA* tetracycline resistance gene found in *S.* Panama. Also, there were two CSSuT-resistant strains isolated from animals, one *S.* Panama and one *S.* Typhimurium strain, that had a chloramphenicol resistance mechanism that was not *cat*-encoded. No *sul*-encoded sulfonamide resistance genes were detected in that same S. Panama strain. Three SSuT-resistant S. Typhimurium strains isolated from animals possessed a tetracycline resistance mechanism that was not encoded by *tetA* to *tetE, tetG* or *tetH.* Similarly to the clinical *Salmonella* strains from Brazil that were sensitive to

Table 15: Antimicrobial resistance genes detected in Brazilian *Salmonella* strains isolated from animal, environmental and food samples

Antibiogram[ab]	Serotype	Source	# of strains	Antimicrobial Resistance Genes[c]						
				A(BL)	C	S	Su	T	K	Tm
ACSSuTKTm	*S.* Bredeney	Animal	1	bla_{TEM}	*catI*	*aadA1*	*sulII*	*tetB, tetC*	*aph3'Ia*	
ACSSuTKG	S. Typhimurium	Animal	1	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*	*tetB*	*aph3'Ia*	---
ACSuKGCTX	*S.* Enteritidis	Food	1	bla_{TEM}	*catII*	***aadA1, aadA2***	*sulI*	---		---
CSSuT	*S.* Brandenberg	Environment	1	---	*catI*	*aadA1*	*sulI*	*tetB*	---	---
CSSuT	*S.* Panama	Animal	1	---		*aadA2*		*tetA, tetC*	---	---
CSSuT	S. Typhimurium	Animal	1	---		*aadA1, aadA2*	*sulII*	*tetB*	---	---
CSSuTmK	S. Typhimurium	Animal	1	---	*catI*	*aadA1, aadA2*	*sulI*	---	*aph3'Ia*	*dhfrI*
SSuTG	S. Heidelberg	Environment	1	---	---	*aadA1, aadA2*	*sulI*	*tetB*	---	---
SSuTTm	*S.* Enteritidis	Animal	1	---	---	*aadA1, aadA2*	*sulII*	*tetB*	---	
SSuT	S. Typhimurium	Animal	3	---	---	*aadA2*	*sulI*		---	---
SSuT	S. Typhimurium	Animal	2	---	---	*aadA1, aadA2*	*sulII*	*tetB*	---	---
SuTTm	S. Derby	Animal	1	---	---	*aadA1, aadA2*	*sulII*	*tetB*	---	

[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); three antimicrobial groups (green); two antimicrobial groups (black).

[b] Abbreviations used: A: ampicillin; A(BL): ampicillin and other β-Lactams; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; K:kanamycin; G: gentamicin; CTX: cefotaxime; Tm: trimethoprim

[c] Bold genes indicate the presence of the resistance gene in a strain that is susceptible to the antimicrobial the gene encodes resistance to. Genes highlighted in purple indicate the presence of a resistance gene in a strain that was intermediately resistant to the antimicrobial the gene encodes resistance to. These strains that were intermediately resistant to an antimicrobial by disk diffusion were re-tested by the agar dilution method and were found to be sensitive to the antimicrobial. Blank spaces indicate that none of the antimicrobial resistance genes tested were detected in a strain resistant to this antimicrobial group. The "—" symbol indicates that strains are susceptible to that particular group of antimicrobials.

streptomycin but possessed *aadA1* and/or *aadA2* genes, a non-clinical *S*.Enteritidis isolated from food and one *S*. Derby animal isolate were streptomycin-susceptible but encoded streptomycin-resistance genes.

Unlike the majority of Brazilian *Salmonella* strains that carry a bla_{TEM} ß-lactamase gene, only two clinical *Salmonella* strains from Colombia had the bla_{TEM} gene (refer to Table 16). The only chloramphenicol-resistant *Salmonella* strain from Colombia, an ACSSuTTm-resistant *S*. Typhi, did not encode any *cat* genes whereas one susceptible S. Typhimurium strain did possess a *catI* gene. Table 16 also highlights several streptomycin-susceptible strains that encoded *aadA1* and/or *aadA2* as was also seen in Brazilian clinical and non-clinical *Salmonella* strains above. All Colombian *Salmonella* possessed two variants of the *sul* gene, *sulI* and *sulII,* which is similar to sulfonamide resistance in Brazilian *Salmonella* where more than half of the clinical strains encoded both *sulI* and *sulII.* The tetracycline resistance genes that were detected in Colombian *Salmonella* strains were the same three that were found in Brazilian non-clinical strains: *tetA, tetB* and *tetC.* The kanamycin-resistance gene *aph3'Ia* was detected in all but two Colombian *Salmonella* strains and 40% of all trimethoprim resistant strains were *dhfrI*-positive. The four groups of strains that had identical antibiograms (refer to Table 16 for antibiogram categories ASSuTKTm, ASSuTTm and ASuTKTm and ASuTTm) had similar streptomycin and sulfonamide resistance genes but differed in their *tet* genes and in the presence or absence of *aph3'Ia*, *dhfrI* and bla_{TEM}.

Table 16: Antimicrobial resistance genes detected in clinical strains of *Salmonella* from Colombia

Antibiogram[ab]	Serotype	# of strains	Antimicrobial Resistance Genes						
			A(BL)	C	S	Su	T	K	Tm
ACSSuTTm	*S.* Typhi	1			*aadA1, aadA2*	*sulI,sulII*		---	
ASSuTKTm	S. Typhimurium	1	bla_{TEM}	---	*aadA1, aadA2*	*sulI,sulII*	*tetB*	*aph3'Ia*	
ASSuTKTm	S. Typhimurium	2		---	*aadA2*	*sulI,sulII*	*tetA, tetC*	*aph3'Ia*	*dhfrI*
ASSuTKTm	S. Typhimurium	1		***catI***	*aadA2*	*sulI,sulII*	*tetC*	*aph3'Ia*	
ASSuTTm	S. Typhimurium	1		---	*aadA1, aadA2*	*sulI,sulII*	*tetC*	---	*dhfrI*
ASSuTTm	S. Typhimurium	3		---	*aadA2*	*sulI,sulII*	*tetC*	---	*dhfrI*
ASSuTTm	S. Typhimurium	1		---	*aadA2*	*sulI,sulII*	*tetA, tetC*	---	*dhfrI*
ASuTKTm	S. Typhimurium	1		---	*aadA1, aadA2*	*sulI,sulII*		*aph3'Ia*	
ASuTKTm	S. Infantis	1		---	*aadA1, aadA2*	*sulI,sulII*	*tetB, tetC*		*dhfrI*
ASSuKTmCTXCAZ	S. Enteritidis	1	bla_{TEM}	---	*aadA1, aadA2*	*sulI,sulII*	---		*dhfrI*
ASSuKTm	S. Typhimurium	2		---	*aadA2*	*sulI,sulII*	---	*aph3'Ia*	*dhfrI*
ASuKTm	S. Typhimurium	1		---	*aadA1, aadA2*	*sulI,sulII*	---	*aph3'Ia*	
ASuTTm	S. Typhimurium	3		---	*aadA1, aadA2*	*sulI,sulII*	*tetC*	---	
ASuTTm	S. Typhimurium	5		---	*aadA1, aadA2*	*sulI,sulII*		---	
ASuTTm	S. Typhimurium	1		---	*aadA2*	*sulI,sulII*		---	
SSuTTm	S. Typhimurium	1	---	---	*aadA1, aadA2*	*sulI,sulII*		---	
SuTTm	S. Typhimurium	2	---	---	*aadA1, aadA2*	*sulI,sulII*		---	

[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); three antimicrobial groups (green); two antimicrobial groups (black).

[b] Abbreviations used: A: ampicillin; A(BL): ampicillin and other β-Lactams; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; K:kanamycin; Tm: trimethoprim

[c] Bold genes indicate the presence of the resistance gene in a strain that is susceptible to the antimicrobial the gene encodes resistance to. Genes highlighted in purple indicate the presence of a resistance gene in a strain that was intermediately resistant to the antimicrobial the gene encodes resistance to. These strains that were intermediately resistant to an antimicrobial by disk diffusion were re-tested by the agar dilution method and were found to be sensitive to the antimicrobial. Blank spaces indicate that none of the antimicrobial resistance genes tested were detected in a strain resistant to this antimicrobial group.. The "---" symbol indicates that strains are susceptible to that particular group of antimicrobials.

The antimicrobial resistance genes that were detected in the majority of Brazilian *Shigella* strains were: *catI*, *aadA1* and *aadA2*, *sulII*, *tetB* and *dhfrI* (see Table 17). However, the bla_{TEM} gene was found in only 20% of ampicillin-resistant *Shigella.* There were also other instances where some *Shigella* strains had resistance mechanisms other than the ones that were probed for (see Section 2.5 of Materials and Methods). For example, two *S. flexneri* strains did not have *cat*-encoded chloramphenicol resistance, several sulfonamide-resistant strains did not encode *sul* genes, *tet* genes encoding efflux proteins were not detected in some strains and *dhfrI* was not present in all trimethoprim resistant strains. Within the five groups of *Shigella* strains with the same antibiogram, four groups displayed various differences in their corresponding resistance gene profiles (Table 17). Within the ACSSuTTm-resistant strains, for example, the following variations were found: bla_{TEM} and *dhfrI* were either present or absent; *sulI* and *sulII* were found singly, together or not at all; and, finally, *tet* genes were either absent, *tetB* or *tetC* were detected alone or as a *tetB/tetC* or *tetA/tetC* combination.

The majority of ACSSuTTm-resistant Colombian *Shigella* strains have the exact same resistance gene profile as do most of the Brazilian *Shigella* with the ACSSuTTm antibiogram: *catI*, *aadA1*and *aadA2*, *sulII*, *tetB* and *dhfrI* (see Tables 17 and 18). Another similarity between Brazilian and Colombian ACSSuTTm-resistant *Shigella* strains is the absence of bla_{TEM} and *tet* genes in some strains. The ASSuTTm-resistant *Shigella* strains from both countries carry the bla_{TEM} and the *aadA1/aadA2* genes but there are a few differences in their resistance gene profiles: the Brazilian *S. sonnei* strain carried *sulII* and *tetC,* the two Colombian *S. sonnei* had *sulII*, *tetB* and *dhfrI* whereas the Colombian *S. flexneri* carried *tetB* but no *sul* or *dhfrI* genes were detected. The resistance

Table 17: Antimicrobial resistance genes detected in *Shigella* strains from Brazil

Antibiogram[ab]	Serotype	# of strains	Antimicrobial Resistance Genes						
			A(BL)	C	S	Su	T	K	Tm
ACSSuTTm	*S. flexneri*	48		*catI*	*aadA1, aadA2*	*sulII*	*tetB*	---	*dhfrI*
ACSSuTTm	*S. flexneri*	2		*catI*	*aadA1, aadA2*	*sulII*	*tetB, tetC*	---	*dhfrI*
ACSSuTTm	*S. flexneri*	4		*catI*	*aadA1, aadA2*		*tetB*	---	*dhfrI*
ACSSuTTm	*S. sonnei*	1		*catI*	*aadA1, aadA2*		*tetB*	---	*dhfrI*
ACSSuTTm	*S. flexneri*	2	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*	*tetB*	---	*dhfrI*
ACSSuTTm	*S. flexneri*	2	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*		---	
ACSSuTTm	*S. sonnei*	1	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*		---	
ACSSuTTm	*S. sonnei*	2	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*	*tetC*	---	
ACSSuTTm	*S. flexneri*	1	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*	*tetC*	---	
ACSSuTTm	*S. sonnei*	1	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI*	*tetA, tetC*	---	
ACSSuTTm	*S. sonnei*	1		*catI*	*aadA1, aadA2*	*sulI*		---	
ACSSuTTm	*S. flexneri*	1		*catI*	*aadA1, aadA2*			---	*dhfrI*
ACSSuTTm	*S. flexneri*	1		*catI*	*aadA1, aadA2*		*tetB*	---	
ACSSuTTm	*S. flexneri*	1		*catI*	*aadA1, aadA2*			---	*dhfrI*
ACSTTm	*S. flexneri*	1		*catI*	*aadA1, aadA2*	---	*tetB*	---	*dhfrI*
ACSSuTm	*S. flexneri*	1	bla_{TEM}	*catI*	*aadA1, aadA2*		***tetB***	---	*dhfrI*
ACSSuTm	*S. flexneri*	1			*aadA1, aadA2*	*sulII*	---	---	
ASSuTTm	*S. sonnei*	1	bla_{TEM}	---	*aadA1, aadA2*	*sulII*	*tetC*	---	
ASTTm	*S. flexneri*	1		---	*aadA1, aadA2*		*tetB*	---	*dhfrI*
CSSuTG	*S. sonnei*	1	---	*catI*	*aadA2*	*sulI,sulII*	*tetA, tetC*	---	*dhfrI*
CSSuTTm	*S. flexneri*	5	---	*catI*	*aadA1, aadA2*	*sulI*	*tetB*	---	*dhfrI*
CSSuTTm	*S. flexneri*	1	---		*aadA1, aadA2*	*sulII*	*tetB*	---	*dhfrI*
CSSuT	*S. sonnei*	1	---	*catI*	*aadA1, aadA2*	*sulI*	*tetC*	---	—
ASSuTm	*S. sonnei*	4	bla_{TEM}	---	*aadA1, aadA2*	*sulII*	---	---	
ASSuTm	*S. dysenteriae*	2	bla_{TEM}	---	*aadA1, aadA2*	*sulII*	---	---	
SSuTTm	*S. flexneri*	9	---	---	*aadA1, aadA2*	*sulII*	*tetB*	---	*dhfrI*
SSuTTm	*S. flexneri*	1	---	---	*aadA1*	*sulII*	*tetB*	---	
SSuTTm	*S. flexneri*	1	---	---	*aadA2*	*sulII*	*tetB*	---	*dhfrI*
SSuTTm	*S. sonnei*	29	---	---	*aadA1, aadA2*	*sulII*	*tetB*	---	*dhfrI*
SSuTTm	*S. sonnei*	1	---	---	*aadA1, aadA2*	*sulII*	*tetB*	---	
SSuTTm	*S. sonnei*	1	---	---	*aadA1, aadA2*			---	
SSuTTm	*S. sonnei*	1	***bla***$_{TEM}$	---	*aadA1, aadA2*	*sulII*	*tetB*	---	*dhfrI*
SSuTTm	*S. boydii*	1	---	---	*aadA1, aadA2*		*tetB*	---	*dhfrI*
SSuTm	*S. sonnei*	2	---	---	*aadA1, aadA2*	*sulII*	*tetB*	---	*dhfrI*
CTm	*S. flexneri*	1	---		***aadA1, aadA2***	---	---	---	

[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); three antimicrobial groups (green); two antimicrobial groups (black).

[b] Abbreviations used: A: ampicillin; A(BL): ampicillin and other β-Lactams; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; K:kanamycin; G: gentamicin; CTX: cefotaxime; CAZ: ceftazidime; Tm: trimethoprim

[c] Bold genes indicate the presence of the resistance gene in a strain that is susceptible to the antimicrobial the gene encodes resistance to. Blank spaces indicate that none of the antimicrobial resistance genes tested were detected in a strain resistant to this antimicrobial group. The "—" symbol indicates that strains are susceptible to that particular group of antimicrobials.

gene profile of the 6 Colombian SSuTTm-resistant *S. sonnei* strains was identical to that of the 29 Brazilian *S. sonnei* and 9 Brazilian *S. flexneri* that were SSuTTm-resistant.

Table 18: Antimicrobial resistance genes detected in *Shigella* strains from Colombia[b]

Antibiogram[a]	Serotype	# of strains	Antimicrobial Resistance Genes[c]						
			A(BL)	C	S	Su	T	K	Tm
ACSSuTTm	*S. flexneri*	11		*catI*	*aadA1, aadA2*	*sulII*	*tetB*		*dhfrI*
ACSSuTTm	*S. flexneri*	2	bla_{TEM}	*catI*	*aadA1, aadA2*	*sulI,sulII*			*dhfrI*
ACSSuTTm	*S. flexneri*	1		*catI*	*aadA2*	*sulII*	*tetB*		*dhfrI*
ASSuTTm	*S. flexneri*	1	bla_{TEM}	---	*aadA1, aadA2*		*tetB*		
ASSuTTm	*S. sonnei*	2	bla_{TEM}	---	*aadA1, aadA2*	*sulII*	*tetB*		*dhfrI*
SSuTTm	*S. sonnei*	6	---	---	*aadA1, aadA2*	*sulII*	*tetB*		*dhfrI*

[a] Antibiogram colours indicate resistance to: five antimicrobial groups (blue); four antimicrobial groups (red); three antimicrobial groups (green).

[b] Abbreviations used: A: ampicillin; A(BL): ampicillin and other β-Lactams; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; Tm: trimethoprim

[c] Blank spaces indicate that none of the antimicrobial resistance genes tested were detected in a strain resistant to this antimicrobial group. The "---" symbol indicates that strains are susceptible to that particular group of antimicrobials.

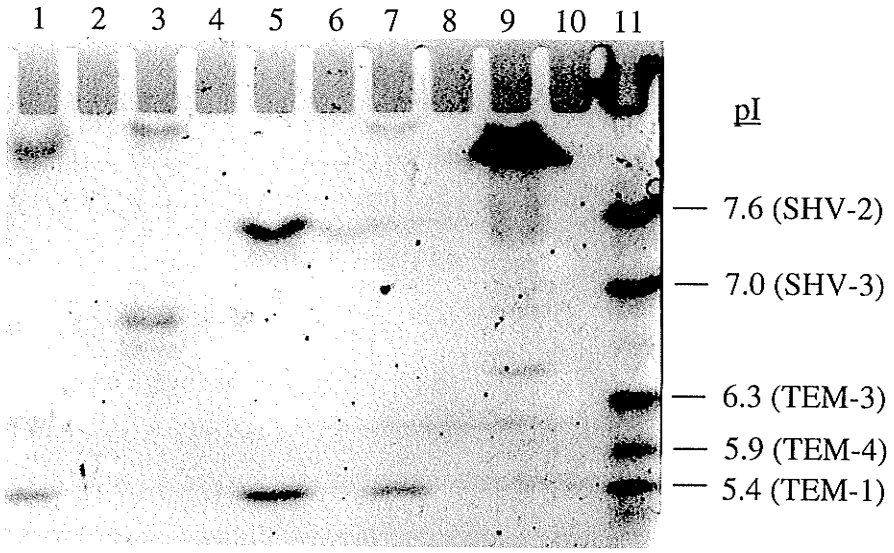
3.2.2 Detection of putative extended-spectrum ß-lactamases in MDR *Salmonella* and *Shigella* strains from Brazil and Colombia

Several types of ß-lactamases were detected by isoelectric focusing (IEF) in the 23 Brazilian and 1 Colombian *Salmonella* strains that were tested (Table 19). An example of IEF results is shown in Figure 12. Since different ß-lactamases can have the same pI values, the results obtained in Table 19 may represent more than one type of ß-lactamase. For example, all 21 strains that were positive for the $bla_{\text{TEM-1}}$ gene by dot blot hybridizations expressed a ß-lactamase with a pI of 5.4 which further suggests the presence of this non-ESBL but could also, according to Bush et al. (1995), indicate the presence of ESBLs such as TEM-7 and TEM-20. The ß-lactamases with a pI of 6.3 that were detected in ACSSuTKGCTXCAZTm- and ACSSuTKGCTXTm-resistant *S.* Infantis from Brazil could be TEM-3 (CTX-1), TEM-16 (CAZ-7) and/or TEM-22. Putative SHV class ESBLs were also detected in strains with ß-lactamase pI values of 7.0 (SHV-3) and 7.6 (SHV-2, SHV-6 and/or the non-ESBL SHV-1). The ESBLs with a pI in the range of 8 to 9, as seen in Figure 12 as the bands that migrated just below the well of the IEF gel, were most likely CTX-M1 (pI 8.9), SHV-5 (pI 8.2) and/or CTX-M2 (pI 7.9) according to Bush *et al.* (1995).

Figure 12:

Detection of ESBLs in Brazilian *Salmonella* strains by isoelectric focusing. Panel (A): Lane 1: BR-SA-97-018; Lane 3: BR-SA-97-291; Lane 5: BR-SA-97-368; Lane 7: BR-SA-97-369; Lane 9: BR-SA-97-377; Lane 11: IEF standards. Panel (B): Lane 1: IEF standards; Lane 2: BR-SA-97-378; Lane 4: BR-SA-97-379; Lane 6: BR-SA-97-380; Lane 8: BR-SA-97-383; Lane 10: BR-SA-97-384; Lane 12: IEF standards.

(A)


1 2 3 4 5 6 7 8 9 10 11
pI
7.6 (SHV-2)
7.0 (SHV-3)
6.3 (TEM-3)
5.9 (TEM-4)
5.4 (TEM-1)

(B)

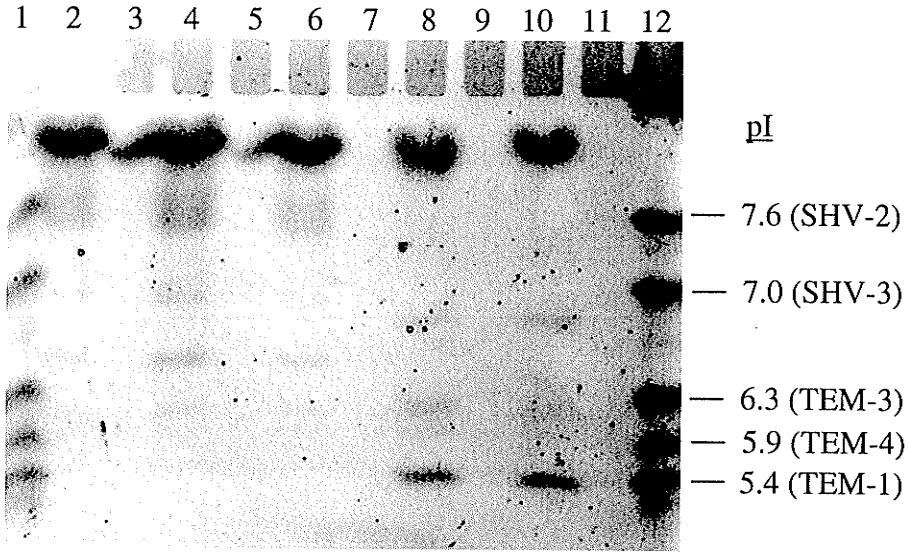

1 2 3 4 5 6 7 8 9 10 11 12
pI
7.6 (SHV-2)
7.0 (SHV-3)
6.3 (TEM-3)
5.9 (TEM-4)
5.4 (TEM-1)

Table 19: Putative ESBLs detected by isoelectric focusing in *Salmonella* strains from Brazil and Colombia

Antibiogram[a]	Serotype[b]	# of Strains	bla_{TEM-1} positive[d]	Approximate pI values[e]	# of Putative ESBLs
ACSSuTKGCTXCAZTm	*S.* Infantis	1	Y	8/9, 5.4	2
ACSSuTKGCTXCAZTm	*S.* Infantis	6	Y	8/9,6.9,6.3,5.4	4
ACSSuTKGCTXCAZ	*S.* Typhimurium[c]	1	Y	8/9, 5.4	2
ACSSuTKGCTXTm	*S.* Typhimurium[c]	1	Y	7.6,5.4	2
ACSSuTKGCTXTm	*S.* Infantis	2	Y	8/9,6.9,6.3,5.4	4
ACSuKGCTX	*S.* Enteritidis[g]	1	Y	8/9,5.4	2
ACSuKCTXCAZTm	*S.* Agona	1	N	8/9,6.8	2
ACSuTKG	*S.* Agona[f]	1	Y	6.9,5.4	2
ACSuTKGCAZ	*S.* Agona[f]	1	Y	6.9,5.4	2
ACSuTKGCTXCAZ	*S.* Agona	2	Y	8/9,6.9,5.4	3
ACSuTKGCTXCAZTm	*S.* Infantis	1	Y	8/9,6.9,5.4	3
ASSuKCTXCAZTm	*S.* Enteritidis	1	Y	8/9,5.4	2
ASSuTKGCAZ	*S.* Infantis[f]	1	Y	8/9,6.9,5.4	3
ASuKCTX	*S.* Agona[g]	1	N	8/9,6.5	2
ASuKCTX	*S.* Agona[g]	3	N	8/9,7.6,7.0, 6.4,6.0	5

[a] Abbreviations used: A: ampicillin; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; K:kanamycin; G: gentamicin; CTX: cefotaxime; CAZ: ceftazidime; Tm: trimethoprim

[b] All strains were from Brazil except for the ASSuKCTXCAZTm-resistant *S.* Enteritidis that was isolated from human saliva in Colombia. All Brazilian strains were isolated from human stool except for the ACSuKGCTX-resistant *S.* Enteritidis that was isolated from food and the ACSSuTKGCTXCAZ-resistant S. Typhimurium that was isolated from human blood.

[c] The *S.* Typhimurium strains were both PT174.

[d] Strains that carried the bla_{TEM-1} gene as was detected by dot blot hybridizations were indicated as "Y" (yes) and those that did not were labelled as "N" (no) (see Tables 14 to 16).

[e] pI (isolelectric point) values as determined by isoelectric focusing (see Figure 12). The 8/9 value indicates a B-lactamase with a pI that is approximately between 8 and 9.

[f] These strains were intermediately resistant to CTX as was determined by disk diffusion and confirmed by agar dilution.

[g] These strains were intermediately resistant to CAZ as was determined by disk diffusion and confirmed by agar dilution.

3.2.3 Determination of the location of resistance genes in MDR *Salmonella* and *Shigella* strains from Brazil and Colombia: integron detection, plasmid profiling and hybridizations

Of the 82 strains that were screened for the presence of integrons by PCR amplification, seventeen different integron profiles were detected in a total of 68 MDR *Salmonella* and *Shigella* strains from Brazil and Colombia (Table 20). Many strains shared the same integron profile regardless of differences in serotype, source and/or the antibiogram of the strains. For instance, integron profile "K" was detected in human and animal *S.* Typhimurium isolates, an environmental strain of *S.* Heidelberg and in clinical *Shigella* strains from both Brazil and Colombia- most of which had different antibiograms. A total of thirteen different integron sizes was observed and thus, one integron of each size was sequenced in order to determine the presence of antimicrobial resistance cassettes. Various antimicrobial resistance gene cassettes and ORFs were identified within 11 integrons that were sequenced successfully and are shown schematically in Figure 13. In general, the integrons that encoded resistance gene cassettes were to aminoglycosides, β-lactams and trimethoprim. Figure 13 illustrates the different combinations of resistance genes present in tandem within the integrons carrying two resistance gene cassettes.

Aminoglycoside resistance gene cassettes were detected in 8 different integrons and these included the streptomycin/spectinomycin resistance genes *aadA1* and *aadA2,* the amikacin resistance gene, *aac(6')-Iq,* a partial streptothricin resistance gene *sat-1* and

Table 20: Integron profiles detected in MDR *Salmonella* and *Shigella* strains from Brazil and Colombia

Integron Profile	# of Integrons	Integron size (bp)[a]	Number of Strains	Serotypes[b]	Country
A	2	3000, 700	1	*S.* Bredeney[c]	BR
B	2	2000, 200	4	*S.* Agona[d]	BR
B	2	2000, 200	1	*Salmonella* 4,5,12:i:-	BR
C	1	>3000	4	*S.* Typhimurium	CO
C	1	>3000	1	*S.* Enteritidis[g]	CO
D	1	1800	2	*S.* Enteritidis[h]	BR
E	2	1800, 200	1	*S.* Agona	BR
F	2	1750, 200	10	*S.* Agona	BR
G	1	1700	4	*S.* Typhimurium[i]	BR
H	1	1600	1	*S.* Schwarzengrund[j]	BR
H	1	1600	1	*S.* Typhimurium	BR
I	1	1250	10	*S.* Infantis	BR
J	2	1250, 200	1	*S.* Typhimurium	BR
K	1	1000	4	*S.* Typhimurium[c]	BR
K	1	1000	1	*S.* Heidelberg[f]	BR
K	1	1000	1	*S. flexneri*	BR
K	1	1000	1	*S. sonnei*	BR
K	1	1000	1	*S. flexneri*	CO
L	2	1000, 300	1	*S. sonnei*	BR
M	2	1000, 200	1	*S.* Brandenberg[f]	BR
N	1	700	3	*S.* Infantis	BR
O	2	700, 200	2	*S.* Agona[d]	BR
P	1	600	1	*S.* Panama[c]	BR
Q	1	300	2	*S. flexneri*	BR
Q	1	300	2	*S. sonnei*	CO
Q	1	300	1	*S. sonnei*	BR
R	1	200	4	*S.* Typhimurium[e]	BR
R	1	200	1	*S.* Derby[c]	BR
R	1	200	1	*S.* Enteritidis[c]	BR

[a] The integron sizes are approximate and are based on agarose gel electrophoresis (data not shown). See Figure 13 for accurate sizes of the integrons that were sequenced.
[b] Strains were isolated from human stool unless otherwise noted.
[c] Strains were isolated from animals.
[d] One *S.* Agona strain was isolated from human blood.
[e] Three *S.* Typhimurium strains were isolated from animals and one from human stool.
[f] Strains were isolated from the environment.
[g] Strain was isolated from human saliva.
[h] One *S.* Enteritidis strain was isolated from food.
[i] Three *S.* Typhimurium strains were isolated from human blood.
[j] Strain was isolated from a skin wound (human).

a novel aminoglycoside 6'-N-acetyltransferase gene *aac(6')-I30.* The *sat-1* gene cassette that was 98% identical to |gb|AY090896 had a total of seven nucleotide (nt) changes within the *sat-1* ORF: five nucleotide changes resulted in synonymous amino acid (AA) changes while two nucleotide changes resulted in two non-conservative changes- a T to A nt substitution resulted in a Leu to His AA substitution at position 23 and another T to A nt substitution caused a Val to Asp substitution at AA position 155. In addition, the partially sequenced integron shown in Figure 13(f) encoded a 59-base element that was 95% identical to the 59-base element belonging to an *aac(6')-Ib* gene cassette (Accession no. |gb| AJ311891). Two out of four nucleotide substitutions in the *aac(6')-Ib*-like 59-base element sequenced for this project resulted in synonymous AA changes while the other two nt substitutions (G to A at position 450 and G to T at position 451) resulted in a non-conservative Gly to Ile AA substitution at position 184. Unfortunately, due to a technical error, a region of approximately 600 bp in the integron shown in Figure 13(f) was not sequenced and thus it is unknown if this integron encoded a *aac(6')-Ib* gene upstream of the 59-base element mentioned above.

Integrons shown in Figure 13 (c), (d) and (f) carried three different variants of the gene encoding a trimethoprim-resistant DHFR*: dhfrI*, *dhfrVII* and *dhfrXII.* The *dhfrI* cassette in Figure 13 (c) had a one nucleotide difference in comparison to |gb| AF382145: an A to T nt substitution at position 496 of the *dhfrI* ORF resulted in a conservative Ile to Phe substitution at position 166. The integron shown in Figure 13 (c) also encoded an *aadA1* cassette that had two nucleotide differences compared to the GenBank sequence |gb| AF382145: a G to T nt substitution at positions 82 and 83 of the *aadA1* ORF resulted in a non-conservative Gly to Leu substitution at AA position 28. The *dhfrVII* gene

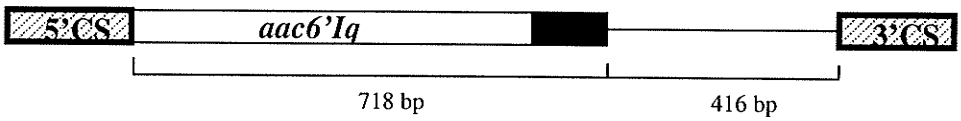
cassette in Figure 13 (f) matched only three segments of the *dhfrVII* gene cassette sequence in GenBank (Accession no. |gb| AY139596). The regions of unknown sequence that "interrupted" the *dhfrVII* gene cassette sequence had the exact same length as the missing *dhfrVII* gene segments that would be in their place if the *dhfrVII* gene was identical to the entire *dhfrVII* sequence shown in |gb| AY139596.

β-lactam resistance genes were detected in three integrons that were found together with another gene cassette encoding resistance to streptomycin/ spectinomycin (Figure 13 (h) and (i)) or trimethoprim (Figure 13 (f)). The bla_{OXA-2} gene cassette shown in Figure 13 (f) is a BJM Group 2d β-lactamase that encodes resistance to cloxacillin, oxacillin and all penicillins. The bla_{PSE-1} gene cassette illustrated in Figure 13(h), however, encodes the BJM Group 2c PSE-1 β-lactamase that confers resistance to carbenicillin and all penicillins including ampicillin. The bla_{OXA-45} gene cassette that was detected in the same integron as the novel *aac(6')-I30* gene cassette (shown in Figure 13(i)), is a novel β-lactamase that has yet to be characterized.

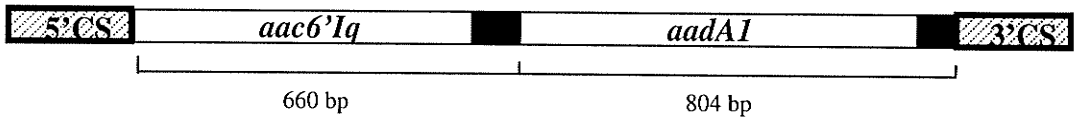
Figure 13: (Part 1)

Schematic diagrams of antimicrobial gene cassettes and ORFs detected within the variable region of integrons from 11 different *Salmonella* strains from Brazil and Colombia. Refer to Table 21 for details regarding each strain listed below. Note: the diagrams are not to scale. Black boxes represent the "59-base element" (59-be) of gene cassettes and are of variable size. Dotted boxes represent segments of one gene cassette that are interrupted by regions of unknown sequence. The "?" represents an unsequenced region. (a) BR-SA-99-1540 encoded an amikacin resistance gene cassette *aac(6')-Iq* that displayed 100% identity to the GenBank sequence |gb|AF047556. The 416 bp sequence downstream of the *aac(6')-Iq* gene cassette did not match any sequencein the GenBank database. (b) BR-SA-97-373 also encoded the *aac(6')-Iq* gene cassette as above with 100% identity to |gb|AF047556 as well as the streptomycin/spectinomycin resistance gene *aadA1* that was 100% identical to |gb|071413. (c) The entire integron detected within BR-SA-97-212B was 99.8% identical to gb|AF382145 which encoded both a trimethoprim resistance cassette, *dhfrI,* and *aadA1*. (d) The integron detected in BR-SA-97-283 was 100% identical to gb|AF284063 and encoded the trimethoprim resistance gene variant *dhfrXII,* an unknown ORFX and *aadA2* in tandem. (e) BR-SA-99-1573 has an integron encoding *aadA2* that is 100% identical to gb|AF261825.2. (f) A partial sequence of the large integron detected in CO-SA-99-1427 that contains 437bp that does not match any sequences in GenBank, a 59-base element (90 bp in length), an ORF that displayed 100% identity to three segments of a *dhfrVII* gene cassette (Accession no. |gb| AY139596) that are separated by regions that do not match any other sequence in GenBank, an unsequenced middle region of approximately 600 bp, a 59-base element that is 95% identical to that of an *aac(6')-Ib* 59-base element (Accession no. |gb| AJ311891) and a complete bla_{OXA-2} gene cassette that is 100% identical to |gb| AY046276.

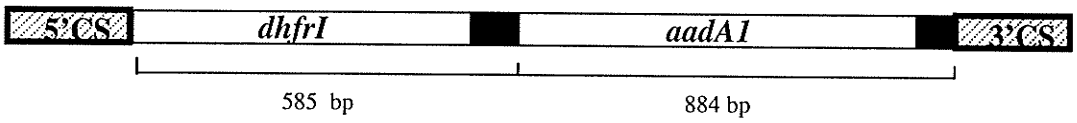
(a) BR-SA-99-1540 (1269 bp)



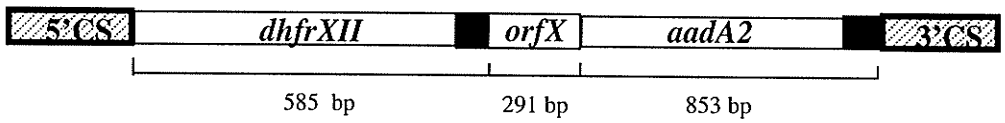
(b) BR-SA-97-373 (1721 bp)



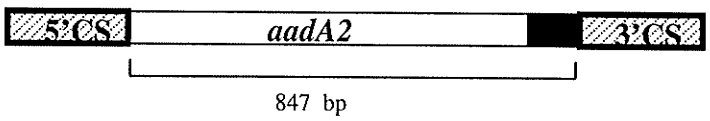
(c) BR-SA-97-212B (1586 bp)



(d) BR-SA-97-283 (1913 bp)



(e) BR-SA-99-1573 (1009 bp)



(f) CO-SA-99-1427 (> 3000 bp)

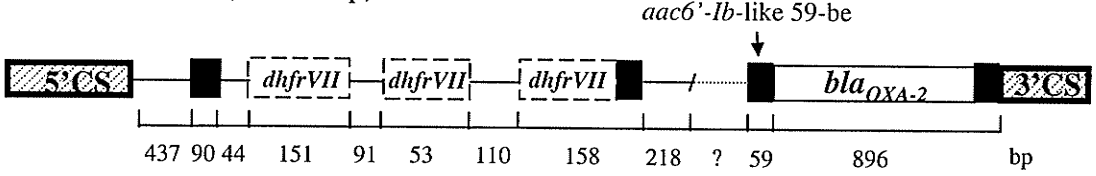
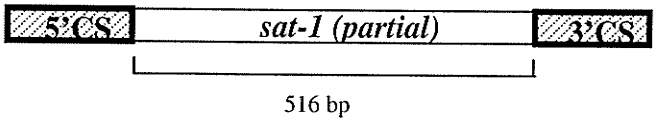


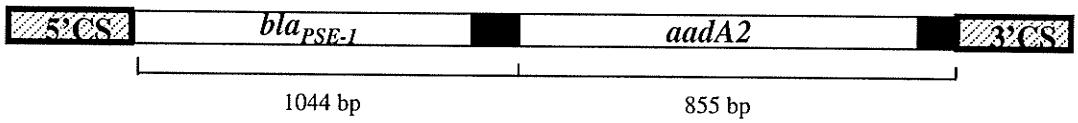
Figure 13: (Part 2)

Schematic diagrams of antimicrobial gene cassettes and ORFs detected within the variable region of integrons from 11 different *Salmonella* strains from Brazil and Colombia. Refer to Table 21 for details regarding each strain tested below. Note: the diagrams are not to scale. Black boxes represent the"59-base element" (59-be) of gene cassettes and are of variable size. (g) A partial streptothricin acetyl-transferase (*sat-1*) gene cassette was detected in BR-SA-99-1538. Only 516 bp of the 858 bp *sat-1*cassette was amplified but was 98% identical to the *sat-1* gene cassette sequence in |gb|AY090896. (h) An integron with two resistance gene cassettes was detected in BR-SA-97-367: the β-lactamase gene cassette bla_{PSE-1} that was 100% identical to the one in S. Typhimurium PT104 (|gb| 071555) followed by an *aadA2* cassette with 100% identity to |gb|AY259085. (i) BR-SA-97-285 encoded an integron with two resistance gene cassettes bla_{OXA-45} and *aac(6')-I30.* The entire integron was 100 % identical to the GenBank sequence |gb| AY289608. (j) A 182 bp fragment was ampified from BR-SA-99-1567 which was 99% identical to a small fragment of the *purG* gene, which encodes phosphoribosylformylglycinamidine synthetase (Accession number: |gb|AF151984). The entire 197 bp sequence, including the complete 5'CS/3'CS primer sequences at each end, was 99% identical to the GenBank sequence |gb|AF151984. (k) A 697 bp fragment was amplified from BR-SA-97-265 and 686 bp of that sequence had 95% identity to the GenBank sequence |gb|AE016845 which encodes a small segment (282 bp) of the *codB* gene (cytosine permease). The |gb|AE016845 sequence did not contain complete 5'CS or 3'CS sequences but only a few bases that matched each primer used to amplify these specific regions (see Table 7 for primer sequences). Note: A few attempts were made to sequence the >3000 bp integron (integron profile C) and the 300 bp integron (integron profile Q) but neither could be sequenced successfully.

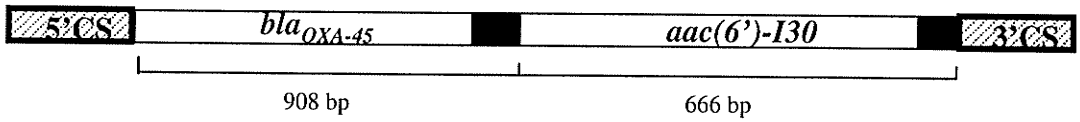
(g) BR-SA-99-1538 (638 bp)



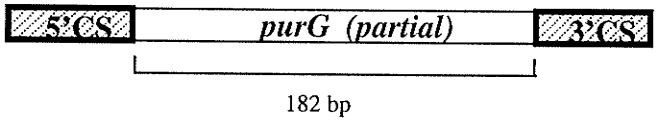
(h) BR-SA-97-367 (2033 bp)



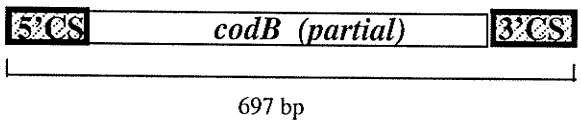
(i) BR-SA-97-285 (1755 bp)



(j) BR-SA-99-1567 (197 bp)



(k) BR-SA-97-265 (697 bp)



Two of the integrons that were sequenced, shown schematically in Figures 13 (j) and Figure 13 (k), contained small segments of the *purG* and *codB* genes that are not antimicrobial resistance genes. Within the full sequences of both *purG* (Accession number: |gb|AF151984) and *codB* (Accession number: |gb|AE016845) there are regions that are complementary to the 3' ends of the 5'CS and 3'CS oligonucleotides that were used to sequence the integrons.

Table 21 summarizes all the details pertaining to the strains that encoded the integrons shown in Figure 13. The presence of class I integrons was confirmed by PCR amplification of the *intI1* gene (data not shown). The strains that tested positive for *intI1* all encoded *sulI* as well. As expected, strains that encoded *sulII* only were *intI1*-negative and thus, did not possess a class I integron. The Colombian S. Typhimurium CO-SA-99-1427 that was bla_{TEM-1} negative by dot blot hybridizations was found to be bla_{OXA-2} positive by integron analysis. Integron sequencing also revealed that the streptomycin-susceptible *S.* Enteritidis strain BR-SA-97-283 (though found to be intermediately resistant to streptomycin by disk diffusion) encoded an integron with an *aadA2* gene cassette which encodes resistance to streptomycin and spectinomycin. Also, the mechanism of resistance to trimethoprim in *dhfrI*-negative BR-SA-97-283 was found to be *dhfrXII*, instead. Two different ß-lactamase genes were detected in the *Salmonella* (4,5,12:i:-) strain BR-SA-97-367 (bla_{TEM-1} and bla_{PSE-1}) and the *S.* Agona strain BR-SA-97-285 (bla_{TEM-1} and bla_{OXA-45}). The only integron that was successfully amplified from an animal strain was the class I *aadA2*-encoding integron from S. Typhimurium (BR-SA-99-1573).

Table 21: Summary of characteristics of the *Salmonella* and *Shigella* strains encoding integron-borne antimicrobial resistance gene cassettes [d]

Strain	Antibiogram	Serotype	Source	Integron size/ Profile (bp)[f]	*intI1*	Integron-encoded genes	Antimicrobial Resistance Genes (dot blot hybridization results)[e]			
							A(BL)	S	Su	Tm
CO-SA-99-1427	ASSuKTm	*S. Typhimurium* [c]	Stool	>3000/ C	+	*dhfrVII-?-* bla_{OXA-2}		*aadA2*	*sulI,II*	*dhfrI*
BR-SA-99-1532	ACSSuTKTm	*S. Bredeney*	Animal	~3000/ A	-	N.D.	bla_{TEM}	*aadA1*	*sulII*	
BR-SA-97-367	ACSSuKG	*Salmonella spp.* [a]	Blood	2,033/ B	+	bla_{pse-1}*-aadA2*	bla_{TEM}	*aadA2*	*sulI*	---
BR-SA-97-283	ASuTG Tm	*S. Enteritidis*	Stool	1913/ D	+	*dhfrXII-orfX-aadA2*	bla_{TEM}	*aadA2*	*sulI,II*	
BR-SA-97-285	ACSuTKG	*S. Agona*	Stool	1755/ F	+	bla_{OXA-45}*–aac6'I30*	bla_{TEM}	*aadA1,A2*	*sulI,II*	---
BR-SA-97-373	ACSSuTKGTm	*S. Typhimurium* [b]	Blood	1721/ G	+	*aac6'Iq-aadA1*	bla_{TEM}	*aadA1*	*sul1*	
BR-SA-97-212B	ACSSuTTm	*S. Schwarzengrund*	Skin Wound	1586/ H	+	*dhfrI-aadA1*	bla_{TEM}	*aadA2*	*sulI,II*	*dhfrI*
BR-SA-99-1540	ACSuTKGCTXCAZTm	*S. Infantis*	Stool	1269/ I	-	*aac6'Iq*	bla_{TEM}	*aadA1,A2*	*sulI,II*	
BR-SA-99-1573	SSuT	*S. Typhimurium*	Animal	1009/ K	+	*aadA2*	---	*aadA2*	*sulI*	---
BR-SA-97-265	ASuTG	*S. Infantis*	Stool	697/ N	-	*codB*	bla_{TEM}	*aadA1,A2*	*sulII*	---
BR-SA-99-1538	CSSuT	*S. Panama*	Animal	638/ P	+	*sat-1*	---	*aadA2*		---
BR-SH-97-234	ASSuTm	*S. sonnei*	Stool	~300/ Q	N.D.	N.D.	bla_{TEM}	*aadA1,A2*	*sulII*	
BR-SA-99-1567	ASSuT	*S. Typhimurium*	Stool	197/ R	N.D.	*purG*	bla_{TEM}	*aadA1,A2*	*sulII*	---

[a] *Salmonella* spp is a monophasic *Salmonella* (4,5,12:i:-)

[b] *S.* Typhimurium PT174

[c] *S.* Typhimurium U302

[d] Abbreviations used: A: ampicillin; A(BL): ampicillin and other β-Lactams; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; Tm: trimethoprim; G: gentamicin; CTX:cefotaxime; CAZ:ceftazidime; K: kanamycin; N.D.: not determined; A2: *aadA2*; *II: sul II; intI1:* integrase 1.

[e] Genes highlighted in purple indicate the presence of a resistance gene in a strain that was intermediately resistant to the antimicrobial the gene encodes resistance to. These strains that were intermediately resistant to an antimicrobial by disk diffusion were re-tested by the agar dilution method and were found to be sensitive to the antimicrobial. Blank spaces indicate that none of the antimicrobial resistance genes tested were detected in a strain resistant to this antimicrobial group. The "—" symbol indicates that strains are susceptible to that particular group of antimicrobials.

[f] For strains that encoded more than one integron, only the integron of specified size was sequenced and listed in this table.

Plasmid profiles were obtained for 21 *Salmonella* and *Shigella* from Brazil and Colombia that had similar antibiograms but different antimicrobial resistance gene profiles. Table 22 summarizes the plasmid profiles obtained for all 21 strains and the corresponding plasmid gel is shown in Panel A of Figure 14. The molecular mass of plasmids ranged from 1 MDa to approximately 26 MDa and strains possessed from zero to eight different plasmids. Different plasmid profiles were observed in all strains that had identical antibiograms. There were several Brazilian strains that did not possess small plasmids from 1.0 MDa to 6.5 MDa: three highly MDR clinical *S.* Infantis strains, three *S.* Typhimurium, one *S.* Panama and one *S.* Bredeney veterinary strains, one environmental *S.* Brandenberg strain and finally one *S. flexneri* strain. Large plasmids of approximately 23MDa and 26MDa were found in all strains except for a Brazilian *S. flexneri* strain (BR-SA-97-750), one Brazilian S. Typhimurium veterinary isolate (BR-SA-99-1581) and a clinical *S.* Typhi strain from Colombia (CO-SA-99-1694). The Brazilian ACSSuTKG-resistant S. Typhimurium veterinary strain BR-SA-99-1581 did not possess any plasmids whereas the SSuTTm-resistant *S.* sonnei BR-SH-98-1070 from Brazil had the highest number of plasmids.

The plasmids shown in Figure 14, Panel A, were transferred to a membrane and Southern hybridizations were performed in order to determine whether the following three genes were plasmid-encoded: bla_{TEM-1} (Figure 14, Panel B), *tetB* (Figure 14, Panel C) and *catI* (Figure 14, Panel C) . All three probes hybridized to the larger, 23 MDa or the 26 MDa plasmids. The hybridization results that are summarized in Table 22 indicate that the highly MDR S. Infantis, S. Typhimurium PT174 and PT208 clinical strains from

Table 22: Plasmid profiles of 21 MDR *Salmonella* and *Shigella* strains from Brazil and Colombia

Strain #	Serotype (Source[a])	Antibiogram[c]	Plasmid Profile[b] (MDa)
BR-SA-99-1573	*S.* Typhimurium (V)	SSuT	23
BR-SA-99-1586	*S.*Typhimurium (V)	SSuT	**23** *5.6* *4.9* 3.0 *2.7*
BR-SA-98-1038	*S.*Heidelberg (E)	SSuTG	23? 4.5 4.2 *2.9* *2.2* *2.0* 1.4
BR-SH-97-750	*S. flexneri*	SSuTTm	*4.9* 4.3 2.7 2.0 1.7 1.0
BR-SH-98-1070	*S. sonnei*	SSuTTm	*26* **23** *6.5* 5.5 3.5 *2.0* 1.7 1.3
BR-SA-99-1531	*S.* Brandenberg (E)	CSSuT	23
BR-SA-99-1538	*S.* Panama (V)	CSSuT	23 18
BR-SA-99-1570	*S.* Typhimurium (V)	CSSuTmK	23
BR-SH-97-744	*S. flexneri*	CSSuTTm	26
BR-SA-97-369	*S.* Typhimurium 174 (B)	ACSSuTKGCTXCAZ	26 23 *5.2* *4.9* 2.9 1.8
BR-SA-97-018	*S.* Infantis	ACSSuTKGCTXCAZTm	26 23
BR-SA-97-384	*S.* Infantis	ACSSuTKGCTXCAZTm	26
BR-SA-99-1542	*S.* Infantis	ACSSuTKGCTXTm	26
BR-SA-97-371	*S.* Typhimurium 208 (B)	ACSSuTKGTm	26 23 *2.9* 1.8
BR-SA-99-1581	*S.* Typhimurium (V)	ACSSuTKG	
BR-SA-97-212B	*S.* Schwarzengrund (W)	ACSSuTTm	*4.7* 2.5
BR-SH-97-737	*S. flexneri*	ACSSuTTm	26 **23** 2.7 2.2
CO-SH-98-1126	*S. flexneri*	ACSSuTTm	26 4.7 2.7 2.2
CO-SA-99-1694	*S.* Typhi	ACSSuTTm	4.5 2.5 2.4 1.4 1.1
BR-SA-99-1532	*S.* Bredeney (V)	ACSSuTKTm	**26**
BR-SH-97-519	*S. flexneri*	ACSSuTm	26 **23** 4.9 3.6 2.6 2.1

[a] Strains were isolated from human stool unless otherwise noted by the following: V: from animals; E: from the environment; B: from human blood; and, W: from a human skin wound

[b] Plasmids in italics were faintly visible in the plasmid gel as shown in Figure 14. Plasmids highlighted in colours indicate that they hybridized with the following probes (as seen in Figure 14): bla_{TEM-1} only (blue); *tetB* only (purple); bla_{TEM-1} and *tetB* (red); bla_{TEM-1} , *tetB* and *catI*.(green)

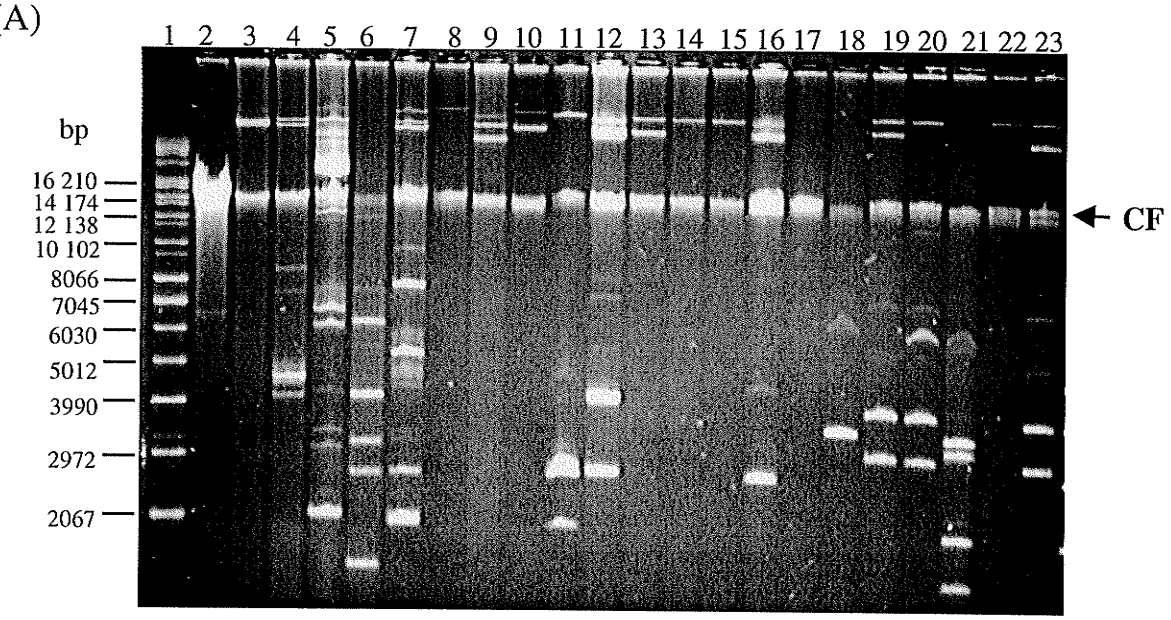
[c] Abbreviations used: A: ampicillin; A(BL): ampicillin and other β-Lactams; C: chloramphenicol; S: streptomycin; Su: sulfamethoxazole; T: tetracycline; Tm: trimethoprim; G: gentamicin; CTX:cefotaxime; CAZ:ceftazidime; K: kanamycin.

Brazil have all of the bla_{TEM}, *tetB* and *catI* genes on the 26 MDa plasmid. The only discrepancy here is that none of the strains with their 26 MDa plasmids hybridizing to all three probes have a *tetB* gene according to dot blot results; they only encoded *tetC* and *tetD* genes. The 26 MDa plasmid from the *S.* Bredeney veterinary strain, however, only hybridized to the bla_{TEM-1} probe and not to the *tetB* or *catI* probes which this strain was known to encode according to dot blot results (see Table 15). Despite the fact that BR-SA-99-1581 did not possess any plasmids, dot blot results indicated that it did encode all three genes bla_{TEM-1}, *tetB* and *catI* . If Southern hybridization and dot blot hybridization results for these three genes are both considered for all 21 strains analyzed, the results can be summarized as follows: all three bla_{TEM-1}, *tetB* and *catI* genes were found on plasmids in some strains and on the chromosome for others in different combinations.

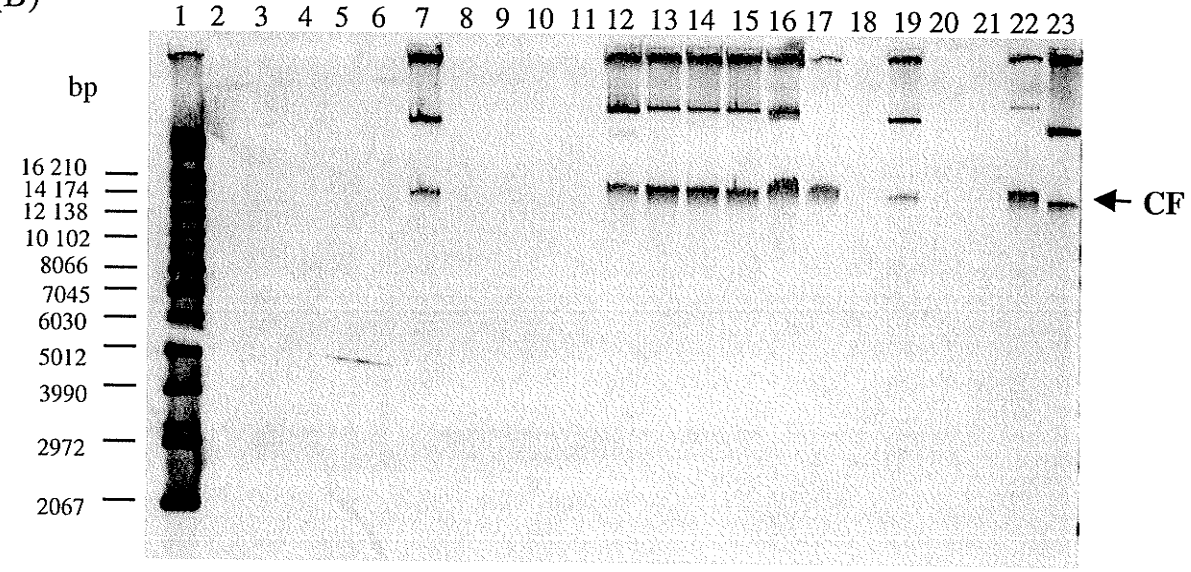
Figure 14:

Hybridization of bla_{TEM-1} , *tetB* and *catI* probes to plasmid DNA of 21 MDR *Salmonella* and *Shigella* strains from Brazil and Colombia. **Panel A**: Agarose gel electrophoresis of plasmid DNA extracted from MDR strains as described in Materials and Methods section 2.6. Lane1: supercoiled DNA ladder; Lane2: *tetB* positive control strain; Lane 3: BR-SA-99-1573; Lane 4: BR-SA-99-1586; Lane 5: BR-SA-98-1038; Lane 6: BR-SH-97-750; Lane 7: BR-SH-98-1070; Lane 8: BR-SA-99-1531; Lane 9: BR-SA-99-1538; Lane 10: BR-SA-99-1570; Lane 11: BR-SH-97-744; Lane12: BR-SA-97-369; Lane 13: BR-SA-97-018; Lane 14: BR-SA-97-384; Lane 15: BR-SA-99-1542; Lane 16: BR-SA-97-371; Lane 17: BR-SA-99-1581; Lane18: BR-SA-97-212B; Lane 19: BR-SH-97-737; Lane 20: CO-SH-98-1126; Lane 21: CO-SA-99-1694; Lane 22: BR-SA-99-1532, Lane 23: BR-SH-97-519. CF= non-specific chromosomal fragments. Refer to Table 22 for details pertaining to plasmid profiles and additional information about each strain. **Panel B**: Southern hybridization of bla_{TEM-1} probe to the plasmids shown in Panel A (5 min exposure). **Panel C**: Southern hybridization of a *tetB* probe to the plasmids shown in Panel A (1 hr exposure). **Panel D**: Southern hybridization of a *catI* probe to the plasmids shown in Panel A (20 min exposure). The DNA in the well and CF band in lane 22 were more visible in the overnight exposure (not shown).

(A)


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
bp
16 210
14 174
12 138
10 102
8066
7045
6030
5012
3990
2972
2067
CF

(B)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bp
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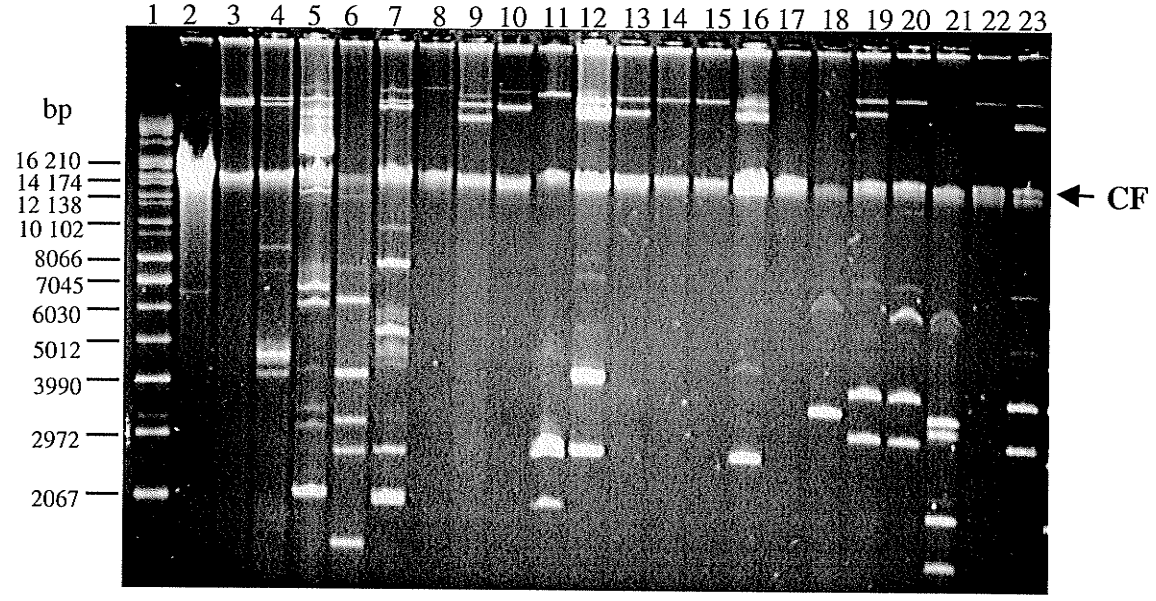
2972
2067
CF

(A)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5012
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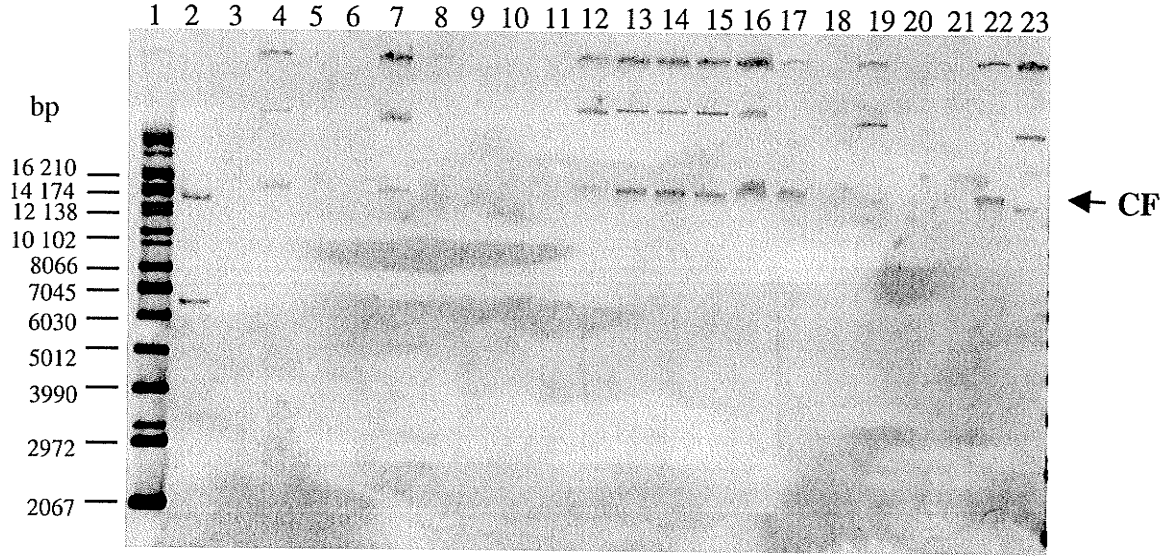
CF

(C)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16 210
14 174
12 138
10 102
8066
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5012
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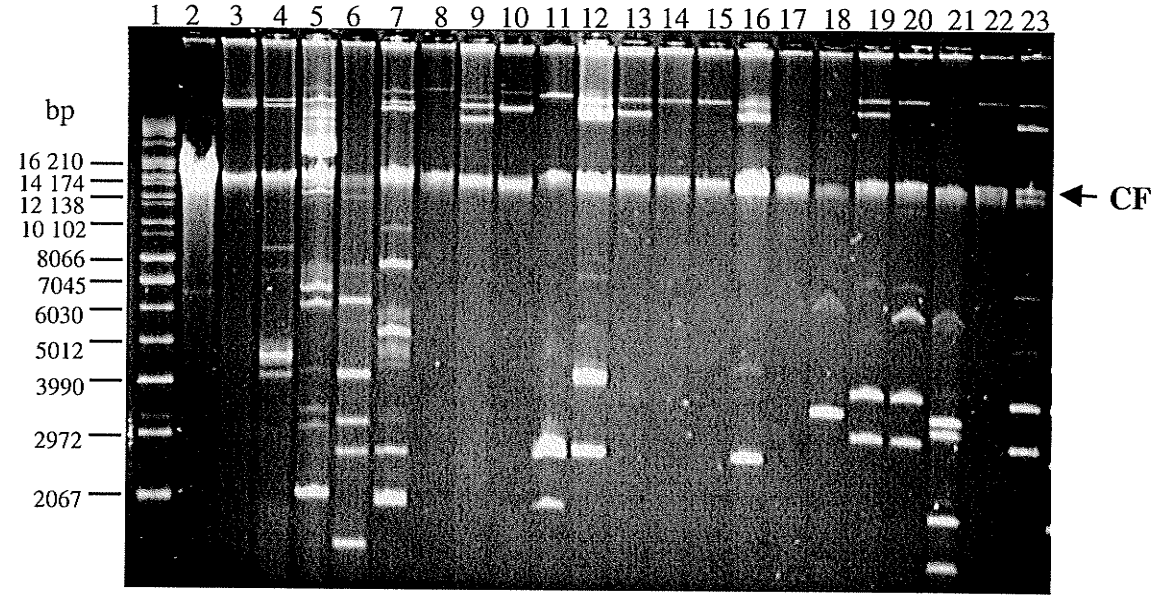
(A)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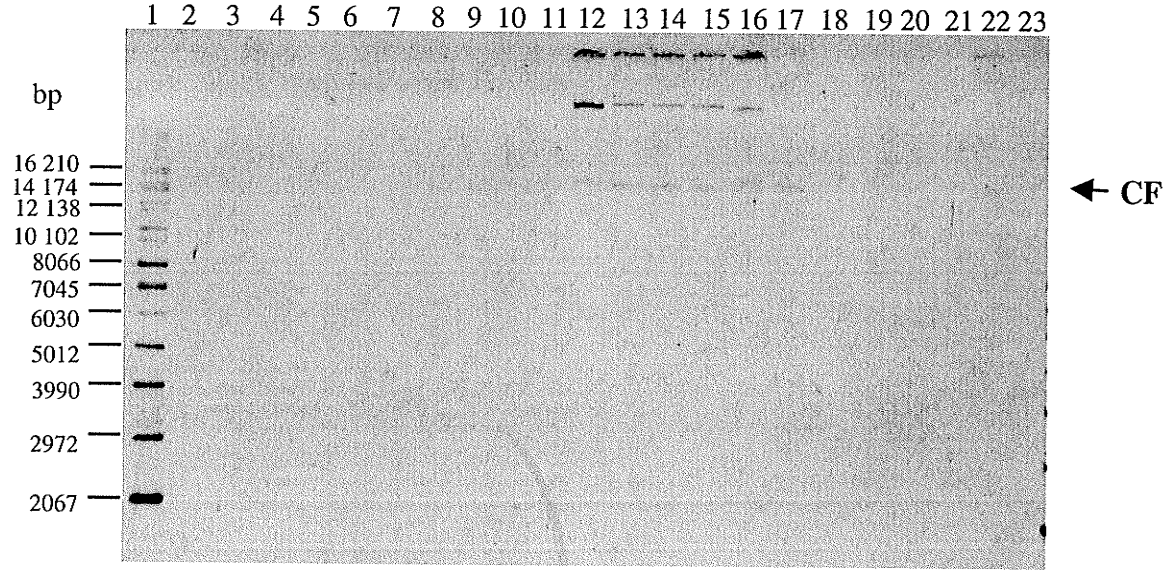
(D)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4. DISCUSSION

Multi-drug resistance in enteric pathogens such as *Salmonella* and *Shigella* spp. are threatening the economical treatment of severe diarrheal infections in developing countries. Although most *Salmonella* and *Shigella* infections are self-limiting in healthy individuals, the use of antimicrobials is necessary to control potentially life-threatening infections that can occur in individuals with weakened immune systems such as immuno-compromised patients, the young and the elderly. Once conventional antimicrobials such as ampicillin become ineffective due to resistant bacteria, physicians must turn to more expensive second-line or third-line antimicrobials to cure patients with severe cases of salmonellosis and shigellosis. Unfortunately, in developing countries, therapeutic options are already severely limited for economic reasons and thus it is often impossible to treat serious infections caused by resistant bacteria. To further complicate matters, empirical treatment is used in health care facilities of impoverished nations due to the lack of financial resources necessary to properly diagnose patients and to check for antimicrobial resistance (Lima et al., 1995; WHO, 2000). One of the most important factors contributing to multi-drug resistance in developing countries is the fact that antimicrobials are available over-the-counter (WHO, 2000). Surveillance of antimicrobial resistance in developing countries is especially important if control measures are to be effective.

Although a surveillance system named "RESISTNET" has been implemented in Latin American countries since 1998, the focus of this system is the collection of epidemiological data and thus does not include the molecular analysis of antimicrobial

resistance mechanisms in *Enterobacteriaceae* such as *Salmonella* and *Shigella* (Oplustil et al., 2001). To date, neither Brazil or Colombia have published data regarding the antimicrobial resistance gene content of MDR *Salmonella* and *Shigella* isolates. For this reason, the objective of this study was to determine some but not all of the clinically significant resistance mechanisms in *Salmonella* and *Shigella* strains from Brazil and Colombia. The antimicrobial resistance mechanisms that were determined in this study did not represent all the resistance mechanisms found within each test strain. Instead, by screening for a small selection of antimicrobial resistance genes representing some of the most common resistance mechanisms found in clinical strains of *Salmonella* and *Shigella*, it was possible to obtain a "skeleton" of significant resistance mechanism profiles for all test strains.

Antibiograms and antimicrobial resistance gene profiles. The majority of non-PT104 ACSSuT- resistant *S.* Typhimurium in this study had different antimicrobial resistance gene profiles to those known to be encoded by the well-characterized ACSSuT-resistant *S.* Typhimurium PT104 (MDR PT104). As described by Boyd et al. (2001 and 2002), the majority of MDR PT104 strains encode the following resistance genes in a 13 kb MDR region of the chromosomal *Salmonella* genomic island 1 (SGI1): $bla_{PSE\text{-}1}$, *floR* , *aadA2*, *sulI*, and *tetG.* (According to Arcangioli et al., (1999) the *floR* gene in MDR PT104 encodes an MDR efflux pump that confers resistance to both chloramphenicol and florfenicol). The clinical *S.* Typhimurium PT174 and PT 208 strains from Brazil that were ACSSuT-resistant (as well as to other antimicrobials) characterized in this study encoded: $bla_{TEM\text{-}1}$, *catI*, *aadA1*, *sulI*, *tetC* (alone or in combination with *tetB* and *tetD).* Also the Brazilian ACSSuT-resistant *S.* Typhimurium veterinary strain encoded an

additional *aadA2* and *sulII*. Although the strains in this study were not screened for bla_{PSE-1} and *floR*, the presence of the aforementioned genes indicates that there are differences in the chloramphenicol-, ß-lactam-, aminoglycoside-, sulfonamide- and tetracycline- resistance gene determinants that were detected in clinical and veterinary ACSSuT-resistant non-PT104 S. Typhimurium strains from Brazil versus those found in MDR PT104.

The antimicrobial resistance gene patterns detected in most Brazilian *Shigella* strains (Table 12) were comparable to those reported by Lima et al. (1995 and 1997) in their study of multi-drug resistant *Shigella* strains isolated from hospitalized and non-hospitalized individuals suffering from diarrhea in Northeastern Brazil between 1988 and 1993. Out of the 39 strains they studied, >50% of them were resistant to A and SXT, >64% were CST-resistant and >82% were resistant to 4 or more antimicrobial groups though, unfortunately, the authors did not specify these MDR antibiograms. The sixteen *Shigella* strains in this study that were isolated from Northeastern Brazil were all isolated from 1998 to 1999 (see Table 23 in Appendix I) and were mostly ACSSuTTm- and SSuTTm-resistant. The resistance genes detected in the three ACSSuTTm-resistant *S. flexneri* from this region were *cat-1, aadA1* and *aadA2, sulII, tetB* and *dhfr1* for all three strains. Interestingly, this same gene profile was detected in 11 out of the 13 ACSSuTTm-resistant Colombian *S. flexneri* analyzed in this study (see section 3.2). The 9 SSuTTm-resistant *S. sonnei* from northeastern Brazil also encoded resistance gene profiles similar to the ones in ACSSuTTm- resistant S. flexneri from the same region: *aadA1* and *aadA2, sulII, tetB* and *dhfr1*. Although bla_{TEM-1} was detected in only one

ASSuTTm-resistant *S. sonnei* from northeastern Brazil, four *S. sonnei* and two *S. flexneri* that were ACSSuTTm-resistant and were isolated by the IEC insitution in northern Brazil, did encode bla_{TEM-1}. Since the northern and northeastern parts of Brazil are the poorest areas that are in greatest need of sanitation, the presence of MDR *Shigella* carrying clinically significant resistance determinants poses the greatest threat to young children since diarrhea is a major cause of child mortality in these regions (Orlandi et al., 2001)

Detection of putative ESBLs. ESBL-producing isolates pose a serious clinical problem since they are often resistant to other groups of antimicrobials, including aminoglycosides (especially AAC(6')), chloramphenicol, quinolones and sulfamethoxazole/trimethoprim (Gniadkowski, 2001; Nordmann, 1998). The results obtained in this project further support this statement as can be seen from the multi-resistance antibiograms of the putative ESBL-producers in Table 19. Using IEF to detect ESBLs is limited due to the fact that pI values are not unique for each known ESBL. IEF was used in this project only to obtain a general idea of the number and type of ß-lactamases carried by some of the MDR *Salmonella* strains from Brazil and Colombia. In order to fully elucidate the exact ESBL content of strains the next step would be to amplify specific ESBLS using PCR with primers specific to each ESBL or ESBL family and perform DNA sequencing of the amplified products. It would be particularly beneficial to pursue the true identity of the putative CTX-M-type ESBLs that were detected in Brazilian and Colombian MDR *Salmonella* strains and to compare those findings to the ones reported by Orman et al., (2002) describing the presence of $bla_{CTX-M-2}$ in Argentinian *Salmonella* strains. In addition, the novel ß-lactamase CTX-M-8 should also be screened for since Bonnet et al.

(2000) reported that CTX-M-8 was detected in Brazilian *Enterobacter aerogenes* and *Enterobacter cloacae* nosocomial isolates.

Integron analysis in MDR *Salmonella* and *Shigella* strains. Eight different integrons were found carrying different antimicrobial gene cassettes and gene cassette combinations: *aac(6')-Iq*, *aac(6')-Iq-aadA1*, *aadA2*, *dhfrI-aadA1*, *dhfrXII-orfX-aadA2*, bla_{PSE-1} - *aadA2*, bla_{OXA-45} – *aac(6')-I30*, and a partially sequenced *dhfrVII* (partial) - bla_{OXA-2} cassettes with a putative *aac(6')Ib* gene cassette located in between these two cassettes. In addition, one partial streptothricin resistance gene cassette, *sat-1*, and two gene fragments, *purG* and *codB*, were also obtained as products of PCR amplification using integron-specific 5'CS and 3'CS primers. These results are discussed below.

The integron encoding the two gene cassettes *aac(6')-Iq- aadA1* in tandem was found in 4 clinical *S.* Typhimurium strains from Brazil: one PT174 and one PT208 ACSSuTKGTm-resistant strains isolated from blood, one ACSSuTKGTm-resistant PT174 from stool and finally, one ACSSuTKGCTXCAZ-resistant PT174 strain isolated from blood. The *aac(6')-Iq* gene cassette was first characterized in an Argentinian *Klebsiella pneumoniae* isolate by Centrón and Roy (1998) and the nucleotide sequence that they submitted to GenBank (accession no. AF047556), which was 100% identical to the first 1600 bp of the sequenced integron in this study, included the *aac(6')-Iq* gene cassette followed by only a partial sequence of the *aadA1* gene cassette. The is the first occurrence of clinical *S.* Typhimurium strains encoding an integron with inserted *aac(6')-Iq-aadA1* gene cassettes and we have obtained the entire 1721 bp sequence. A

surveillance study conducted by Gales et al. (2002) on the antimicrobial resistance profiles of *Salmonella* spp. isolated from blood in Latin American countries including Brazil and Colombia, led to the conclusion that MDR in blood-borne *Salmonella* spp. did not pose a serious problem in Latin American medical centers. In contrast to the conclusions drawn by Gales et al., the results obtained in this study indicate that blood-borne infections caused by MDR *Salmonella* may become a serious problem in Brazil if MDR strains such as the S. Typhimurium PT174 and PT208 strains characterized in this study disseminate throughout nosocomial environments. Empirical therapy of systemic salmonellosis must be practised with caution and with the full knowledge that MDR *Salmonella* do exist in Brazil.

Another novel integron was detected in a clinical, ACSuTKG-resistant *S.* Agona strain that was isolated from stool in 1996 from Brazil. There were two gene cassettes inserted in tandem within this novel integron: bla_{OXA-45} – *aac(6')-I30,* encoding for a ß-lactamase and an aminoglycoside 6'-N- acetyltransferase, respectively. This strain should be characterized further in order to determine the preferred substrates of these two enzymes. In addition, the other nine *S.* Agona isolates that had the integron profile "F", the same as for the *S.* Agona strain that was characterized, should also have their integrons sequenced in order to verify the presence of this novel integron. All 9 *S.* Agona strains are at least ACSuTKG-resistant with some encoding additional resistance to Tm, CTX and CAZ. The fact that this novel integron has been found may indicate that antimicrobial resistance genes are still evolving in the MDR organisms that possess them.

The *bla*$_{PSE-1}$ - *aadA2* integron detected in the ACSSuKG-resistant *Salmonella* spp. (4,5,12;i;-) is intriguing due to the fact that only the *aadA2*- *bla*$_{PSE-1}$ combination has been reported to date. For example, in 2003, Lindstedt et al. described an ACSSuT-resistant S. Typhimurium U302 from Portugal encoding *aadA2*- *bla*$_{PSE-1}$. In contrast, MDR PT104 has two integrons- one encodes *bla*$_{PSE-1}$ and the other, *aadA2* (Carattoli et al., 2002). The integron that most closely resembles *bla*$_{PSE-1}$ - *aadA2* is the *bla*$_{PSE-4}$ – *aadA1* that is encoded on integron In33 in the *Pseudomonas aeruginosa* Dalgleish strain (Partridge et al., 2002). The four Brazilian *S.* Agona strains listed in Table 20 that also had the same integron profile "B" as did *Salmonella* spp. (4,5,12;i;-), could encode the *bla*$_{PSE-1}$ - *aadA2* integron but this has yet to be verified. Despite the fact that all four S. Agona were ASuKCTX-resistant, there is a possiblility that they do encode the *bla*$_{PSE-1}$ - *aadA2* integron since they were *aadA1*- and *aadA2*-positive and they were all intermediately resistant to streptomycin by disk diffusion which could indicate a low-level of *aadA1* and/or *aadA2* expression.

An unusual integron was detected in the ASSUKTm-resistant *S.* Typhimurium U302 clinical strain from Colombia encoding *dhfrVII* (partial) (or "*dhfrVII*-like") and *bla*$_{OXA-2}$ with a putative third gene cassette inserted in between. No integrons of this nature were found in the literature. Unfortunately, this large integron was only partially sequenced (one more round of primer walking towards the middle of the integron should have been performed) and was therefore missing approximately 600bp of sequence between the *dhfrVII*-like and *bla*$_{OXA-2}$ cassettes. What is interesting is that immediately downstream of the 600 bp of missing sequence, there was a 59-base element with 95% identity to an *aac(6')-Ib* 59-base element and coincidentally, the expected size of an

aac(6')-Ib gene is approximately 600 bp. If complete sequencing was to reveal the presence of the entire *aac(6')-Ib* gene cassette, however, it would then be expected to confer high levels of resistance to both amikacin and gentamicin (Casin et al, 2003). The CO-SA-99-1427 strain, however, was susceptible to gentamicin and therefore, the presence of a functional *aac(6')-Ib* gene cassette within this large integron is unlikely. Nevertheless, the full integron content of CO-SA-99-1427 and the remaining 4 Colombian strains with integron profile "B" is worthy of future investigation as this would be the first report of integron-encoded resistance in Colombian *Salmonella* strains.

The *dhfrI-aadA1* gene cassette that was found within the 1.5 kb integron detected in the ACSSuTTm-resistant *S.* Schwarzengrund strain from a patient's skin wound (Figure 13 C) was also reported in numerous other clinical *Salmonella* isolates around the world: in an ACSSuTTm- and nalidixic acid (Na)-resistant *S.*Typhimurium DT204 from Belgium (Lindstedt et al., 2003); in MDR *S.* Panama, *S.* Ohio, *S.* Brandenberg and *S.* Virchow from Spain (Guerra et al., 2000); and, in MDR *S.* Enteritidis isolates originating from Indonesia, Rwanda, India, Kenya and Thailand (Lindstedt et al., 2003). In Ireland, the *dhfrI-aadA1* integron was also detected in two ASSuTTm-resistant S. Typhimurium strains: one DT193 food isolate and one DT170a veterinary isolate (Daly and Fanning, 2000).

The integron detected in the clinical *S.* Enteritidis strain from Brazil with inserted *dhfrXII-orfX-aadA2* gene cassettes has also been detected in MDR *Salmonella* in different parts of the world. In Spain, it was located on a 90 MDa multi-resistance plasmid in a *Salmonella* spp. (4,5,12:i:-) (Guerra et al., 2001). In a study published by

Lindstedt et al., (2003) other plasmid-borne *dhfrXII-orfX-aadA2* integrons were identified in an *S.* Typhimurium PT104a isolate originating from Australia, an *S.* Enteritidis strain from Asia and from South America (country not specified). In addition, 33 MDR clinical *E. coli* isolates from Taiwan also possessed this integron and it too was plasmid-encoded (Chang et al., 2000). The *dhfrXII-orfX-aadA2* integron was also found in two *S. sonnei* strains from Australia (McIver et al., 2002). The fact that this *dhfrXII-orfX-aadA2* integron was found in S. Enteritidis from a Brazilian patient is worrisome not only because DHFRXII confers a 10-fold higher level of resistance to trimethoprim than does DHFRI (Heikkilä et al., 1993) but also due to the epidemic increase in S. Enteritidis isolation in São Paulo since 1994 (Fernandes et al., 2003).

The partial *sat-1* streptothricin resistance gene that was detected in the CSSuT-resistant animal isolate *S.* Panama BR-SA-99-1538 could be an indication that MDR *Salmonella* found in Brazilian food animals carry transferable streptothricin resistance. Werner et al. (2001) described the emergence of streptothricin resistance in Gram-negative bacteria following the use of nourseothricin as an antimicrobial feed additive on industrial animal farms in Germany. The integron-borne *sat-1* resistance gene cassette has been found in Australian *Shigella sonnei* strains in a *dhfrI-sat1-aadA1* combination (McIver et al., 2002). It is possible that the entire *sat-1* gene was present within the integron detected in BR-SA-99-1538 although only one third of the gene was amplified by PCR. The streptothricin resistance gene cassette appeared to be truncated most likely because of the presence of 7bp of the 3'CS primer within the *sat-1* gene. Interestingly, a partial *sat-1* amplification product was also reported by Lindstedt et al., (2003) during their study of integrons encoded by *Salmonella* spp. from Norwegian patients.

The 200 bp and 697 bp integrons that contained partial *purG* and *codB* genes, respectively, were suspected to be "false" integrons and were amplified accidentally due to regions of complementarity within the *purG* and *codB* genes to the 5'CS and 3'CS oligonucleotides used for amplification. The partial *purG* sequence was also detected by Daly et al. (2000) and was confirmed as a PCR product artefact by Lindstedt et al. (2003). As a result, the integron profiles shown in Table 20 should not have included the 200 bp amplicon and should be disregarded from this point forward. The same situation applies to the 697 bp "*codB*" PCR artefact. This false amplification might also be the reason why the two integrons of approximately 3kb and 0.3 kb could not be sequenced successfully despite several attempts.

Plasmid profiles and Southern hybridizations with *bla*$_{TEM-1}$, *tetB* and *catI*. The varied plasmid profiles obtained for the MDR *Salmonella* and *Shigella* strains from Brazil were expected since numerous plasmid profiles have been previously observed in Brazilian *Salmonella* and *Shigella* isolates (Asensi et al., 1995; Lima et al., 1997). The 60MDa virulence plasmid that is normally associated with S. Typhimurium was not observed in any of the strains studied. In a recent study published by Guerra *et al.* (2002) they failed to detect the 60 MDa plasmid in clinical *S.* Typhimurium isolates, therefore the absence of a 60 MDa plasmid is not necessarily unusual. It must be noted, however, that it is also possible that such large plasmids had difficulty entering the gel during electrophoresis, making their detection more technically challenging. The fact that the 26 MDa plasmids hybridized to *tetB* was also expected since *tet* genes are usually associated with large plasmids (Roberts, 1996).

This study was designed to first survey the antimicrobial resistance gene profiles in a large collection of *Salmonella* and *Shigella* strains obtained from Brazil and Colombia and to then further characterize their resistance mechanisms by screening for ESBLs, by integron analysis, plasmid profiling and Southern hybridizations. The large number of possible resistance mechanisms found for any given class of antimicrobials precluded comprehensive testing of all possible variants. It is therefore important to bear in mind that resistant strains testing negative for any of resistance mechanisms studied must have their resistance determinant encoded by a different resistance mechanism, which could also be non-specific mechanisms, such as MDR efflux pumps. While the list of resistance mechanisms tested is not all encompassing, specific resistance mechanisms were selected for testing based on clinical prevalence and based on the results of a pilot study involving 10 strains showing resistance towards most of the 12 antimicrobials used in this study. The resulting data is sufficiently comprehensive to provide an accurate representation of the most common resistance mechanisms found in strains of *Salmonella* and *Shigella*.

Brazilian and Colombian MDR *Salmonella* and *Shigella* strains encoded similar resistance mechanisms. The homogeneous distribution of these resistance genes in the large collection of strains analyzed in this study would suggest that horizontal dissemination is occurring at a high rate. The presence of novel combinations of resistance determinants within this data set also suggests a high rate of "recombination" within the pool of resistance genes dispersed among the populations studied in this work.

An initial panel of 501 Colombian and Brazilian strains were initially obtained for this study and of these, nearly 60% were resistant to at least one of the 6 "first-line" antimicrobials commonly administered in a clinical setting. Of these, nearly 80% showed resistance to two or more antimicrobials. Currently most Latin American countries allow nearly unregulated access to antimicrobials and have only recently began to pursue more aggressive surveillance. The results of this study suggest a high rate of multi-drug resistance along with a high rate of horizontal dissemination. These findings underscore the need for increased regulation over use of antimicrobials in both a clinical and agricultural setting in Latin American countries.

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6. APPENDIX-I

Epidemiological information for all strains used in this study

Table 23: Multi-drug resistant *Salmonella* strains from Brazil used in this study

Serotype	Isolate Number	Sample type[a]	Originating Institution[bc]	Date of Isolation
S. Agona	BR-SA-97-017	Stool	Fiocruz	1996- Sept.
S. Agona	BR-SA-97-284	Stool	IAL	1996- Feb.
S. Agona	BR-SA-97-285	Stool	Fiocruz	1996- Aug.
S. Agona	BR-SA-97-286	Stool	Fiocruz	1996- July
S. Agona	BR-SA-97-291	Stool	Fiocruz	1996- July
S. Agona	BR-SA-97-377	Stool	IEC	1994- Oct.
S. Agona	BR-SA-97-378	Stool	IEC	1994- Oct.
S. Agona	BR-SA-97-379	Stool	IEC	1994- Aug.
S. Agona	BR-SA-97-380	Stool	IEC	1994- Oct.
S. Agona	BR-SA-97-484	Stool	Fiocruz	1991
S. Agona	BR-SA-97-497	Stool	Fiocruz	1996- Sept.17
S. Agona	BR-SA-97-498	Stool	Fiocruz	1996- Sept.17
S. Agona	BR-SA-97-499	Stool	Fiocruz	1996- Sept. 17
S. Agona	BR-SA-97-501	Stool	Fiocruz	1996- Sept. 2
S. Agona	BR-SA-97-502	Stool	Fiocruz	1996- Sept. 17
S. Agona	BR-SA-97-510	Stool	IAL	1993- Aug. 7
S. Agona	BR-SA-97-692	Blood	IAL	
S. Brandenberg	BR-SA-99-1531	Environment	Fiocruz-SC	1997- Dec. 31
S. Bredeney 4,12:lv:1,7	BR-SA-99-1532	Animal	Fiocruz-SC	1998- Dec. 28
S. Derby 4,12:f,g:-	BR-SA-98-1040	Animal	Fiocruz	1997- June 12
S. Enteritidis	BR-SA-97-283	Stool	Fiocruz	
S. Enteritidis	BR-SA-98-1046	Animal	Fiocruz	1997- Oct. 24
S. Enteritidis	BR-SA-99-1566	Food	Fiocruz-SC	1999- Feb. 4
S. Heidelberg 4,5,12:r:1,2	BR-SA-98-1038	Environment	Fiocruz	1997- May 15
S. Infantis	BR-SA-97-018	Stool	Fiocruz	1996- June
S. Infantis	BR-SA-97-019	Stool	Fiocruz	1996- June
S. Infantis	BR-SA-97-265	Stool	Fiocruz	
S. Infantis	BR-SA-97-383	Stool	Fiocruz	1997- Mar. 4
S. Infantis	BR-SA-97-384	Stool	Fiocruz	1997- Mar. 4
S. Infantis	BR-SA-97-385	Stool	Fiocruz	1996- June 18
S. Infantis	BR-SA-97-386	Stool	Fiocruz	1996- June 18
S. Infantis	BR-SA-97-387	Stool	Fiocruz	1996- July 17
S. Infantis	BR-SA-97-388	Stool	Fiocruz	1996- Aug. 13
S. Infantis	BR-SA-99-1540	Stool	Fiocruz-RJ	1998- Feb. 17
S. Infantis	BR-SA-99-1541	Stool	Fiocruz-RJ	1997- Feb. 25
S. Infantis	BR-SA-99-1542	Stool	Fiocruz-RJ	1998- Mar. 4
S. Infantis	BR-SA-99-1543	Stool	Fiocruz-RJ	1997- Jan. 14
S. Panama	BR-SA-99-1538	Animal	Fiocruz-SC	1997- Oct. 10
S. Schwarzengrund	BR-SA-97-212(B)	Skin Wound	ISDF	1996- Aug.
Salmonella spp. 4,5,12:i:-	BR-SA-97-367	Blood	Fiocruz	1987- May
S. Typhimurium PT174	BR-SA-97-368	Stool	Fiocruz	1991
S. Typhimurium PT174	BR-SA-97-369	Blood	Fiocruz	1991- Apr.
S. Typhimurium PT174	BR-SA-97-373	Blood	Fiocruz	1990- Apr.
S. Typhimurium PT208	BR-SA-97-371	Blood	Fiocruz	1990-July
S. Typhimurium[d]	BR-SA-97-372	Blood	Fiocruz	1990

Table 23: (Continued)

Serotype	Isolate Number	Sample type[a]	Originating Institution[bc]	Date of Isolation
S. Typhimurium[d]	BR-SA-97-505	Stool	Fiocruz	1995- Nov. 7
S. Typhimurium[d]	BR-SA-97-508	Stool	IAL	1995- Dec. 19
S. Typhimurium[d]	BR-SA-99-1567	Stool	Fusam-AL	1998- Jan. 13
S. Typhimurium[d]	BR-SA-99-1570	Animal	Fiocruz-SC	1994- May. 30
S. Typhimurium[d]	BR-SA-99-1573	Animal	Fiocruz-SC	1997- Apr. 3
S. Typhimurium[d]	BR-SA-99-1574	Animal	Fiocruz-SC	1997- Apr. 3
S. Typhimurium[d]	BR-SA-99-1575	Animal	Fiocruz-SC	1997- Apr. 3
S. Typhimurium[d]	BR-SA-99-1581	Animal	Fiocruz-SC	1998- May. 8
S. Typhimurium[d]	BR-SA-99-1584	Animal	Fiocruz-SC	1995- Aug. 24
S. Typhimurium[d]	BR-SA-99-1585	Animal	Fiocruz-SC	1995- Aug. 24
S. Typhimurium[d]	BR-SA-99-1586	Animal	Fiocruz-SC	1995- Nov. 16

[a]Sources identified as "stool", "blood" and "skin wound" were isolated from humans. See Materials and Methods section for detailed descriptions of animal, environmental and food sources of *Salmonella* isolates.
[b]Abbreviations used for the primary isolation laboratory in Brazil: Fiocruz: Fundação Ozwaldo Cruz (Rio de Janeiro); AL: Alagoas; SC: Santa Catarina; RJ: Rio de Janeiro; IEC: Instituto Evandro Chagas (Para); ISDF: Instituto de Saude Distrito Federal (Brasilia); IAL: Instituto Adolfo Lutz (Sao Paolo)
[c]Institution names that are followed by the name of a district indicate that isolates originated from that district but were sent to and were processed by the institution.
[d]Phage types were not determined for these *S.* Typhimurium isolates.

Table 24: Multi-drug resistant *Shigella* isolates from Brazil used in this study

Serotype	Isolate Number	Source[a]	Institution[bc]	Date of Isolation
Shigella boydii	BR-SH-97-205	Stool	ISDF	No Data
Shigella dysenteriae 5	BR-SH-97-530	Stool	Fiocruz	1996-Nov 5
Shigella dysenteriae 5	BR-SH-97-531	Stool	Fiocruz	1996-Jun 6
Shigella flexneri	BR-SH-97-252	Stool	ISDF	1996-May
Shigella flexneri	BR-SH-98-1282	Stool	IEC-N	1998-Aug 17
Shigella flexneri	BR-SH-97-204	Stool	ISDF	1997-Mar
Shigella flexneri	BR-SH-97-213	Stool	ISDF	1997-Mar
Shigella flexneri 1	BR-SH-97-220	Stool	Fiocruz	No Data
Shigella flexneri 1	BR-SH-97-226	Stool	ISDF	No Data
Shigella flexneri 1	BR-SH-97-238	Stool	ISDF	No Data
Shigella flexneri 1	BR-SH-97-741	Stool	IAL	No Data
Shigella flexneri 1	BR-SH-97-742	Stool	IAL	No Data
Shigella flexneri 1	BR-SH-97-747	Stool	IAL	No Data
Shigella flexneri 1	BR-SH-97-748	Stool	IAL	No Data
Shigella flexneri 1	BR-SH-97-750	Stool	IAL	No Data
Shigella flexneri 1b	BR-SH-99-1529	Stool	Fiocruz-NE	1999-Feb 24
Shigella flexneri 2	BR-SH-97-206	Stool	IAL	1997
Shigella flexneri 2	BR-SH-97-221	Stool	Fiocruz	1986-Apr
Shigella flexneri 2	BR-SH-97-222	Stool	Fiocruz	1986-Apr
Shigella flexneri 2	BR-SH-97-224	Stool	ISDF	No Data
Shigella flexneri 2	BR-SH-97-225	Stool	IAL	1996

Table 24: (Continued)

Serotype	Isolate Number	Source[a]	Institution[bc]	Date of Isolation
Shigella flexneri 2	BR-SH-97-227	Stool	ISDF	1996
Shigella flexneri 2	BR-SH-97-228	Stool	ISDF	No Data
Shigella flexneri 2	BR-SH-97-229	Stool	ISDF	1996
Shigella flexneri 2	BR-SH-97-231	Stool	ISDF	1996
Shigella flexneri 2	BR-SH-97-232	Stool	Fiocruz	97-May
Shigella flexneri 2	BR-SH-97-233	Stool	Fiocruz	No Data
Shigella flexneri 2	BR-SH-97-235	Stool	Fiocruz	1997-May
Shigella flexneri 2	BR-SH-97-237	Stool	Fiocruz	1997-Mar
Shigella flexneri 2	BR-SH-97-250	Stool	IEC	No Data
Shigella flexneri 2	BR-SH-97-358	Stool	ISDF	1996-Feb
Shigella flexneri 2	BR-SH-97-366	Stool	Fiocruz	1997-Feb 17
Shigella flexneri 2	BR-SH-97-517	Stool	IEC	1997-Mar 12
Shigella flexneri 2	BR-SH-97-519	Stool	IEC	1997-Mar 4
Shigella flexneri 2	BR-SH-97-520	Stool	Fiocruz	1997-Feb 17
Shigella flexneri 2	BR-SH-97-734	Stool	IEC	1996
Shigella flexneri 2	BR-SH-97-735	Stool	IEC	1996
Shigella flexneri 2	BR-SH-97-737	Stool	IEC	1996
Shigella flexneri 2	BR-SH-97-739	Stool	IAL	1996
Shigella flexneri 2	BR-SH-97-743	Stool	IAL	1996
Shigella flexneri 2	BR-SH-97-745	Stool	ISDF	No Data
Shigella flexneri 2	BR-SH-97-746	Stool	ISDF	1997-Feb
Shigella flexneri 2	BR-SH-97-751	Stool	IAL	1997-Feb
Shigella flexneri 2	BR-SH-97-752	Stool	IAL	No Data
Shigella flexneri 2	BR-SH-97-754	Stool	IAL	No Data
Shigella flexneri 2	BR-SH-97-755	Stool	ISDF	No Data
Shigella flexneri 2	BR-SH-97-756	Stool	ISDF	No Data
Shigella flexneri 2	BR-SH-97-757	Stool	ISDF	No Data
Shigella flexneri 2	BR-SH-97-758	Stool	ISDF	No Data
Shigella flexneri 2	BR-SH-97-759	Stool	ISDF	No Data
Shigella flexneri 2	BR-SH-98-1076	Stool	IEC-PA	1998-Mar 17
Shigella flexneri 2	BR-SH-98-1082	Stool	ISDF	1998-Apr 16
Shigella flexneri 2	BR-SH-98-1084	Stool	ISDF	1998-Apr 16
Shigella flexneri 2	BR-SH-98-1086	Stool	Fiocruz	1998-Apr 16
Shigella flexneri 2	BR-SH-98-1087	Stool	Fiocruz	1998-Apr 16
Shigella flexneri 2	BR-SH-98-1088	Stool	Fiocruz	1998-Apr 16
Shigella flexneri 2	BR-SH-98-1089	Stool	Fiocruz	1998-Apr 16
Shigella flexneri 2	BR-SH-98-1090	Stool	Fiocruz	1998-Apr 16
Shigella flexneri 2	BR-SH-98-1091	Stool	Fiocruz	1998-Apr 16
Shigella flexneri 2a	BR-SH-97-024	Stool	Fiocruz	1997-Mar
Shigella flexneri 2a	BR-SH-97-522	Stool	IAL	1997-Jun 9
Shigella flexneri 2a	BR-SH-97-524	Stool	IAL	1997-Jun 4
Shigella flexneri 2a	BR-SH-97-525	Stool	Fiocruz	1996-Jul 24
Shigella flexneri 2a	BR-SH-97-526	Stool	Fiocruz	1996-Jul 29
Shigella flexneri 2a	BR-SH-97-527	Stool	Fiocruz	1996-Jul 30
Shigella flexneri 2a	BR-SH-98-1276	Stool	IEC-N	1998-Aug 17
Shigella flexneri 2a	BR-SH-98-1279	Stool	IEC-N	1998-Aug 17
Shigella flexneri 2a	BR-SH-98-1280	Stool	IEC-N	1998-Aug 17

Table 24: (Continued)

Serotype	Isolate Number	Source[a]	Institution[bc]	Date of Isolation
Shigella flexneri 2a	BR-SH-98-1281	Stool	IEC-N	1998-Aug 17
Shigella flexneri 2a	BR-SH-98-1283	Stool	IEC-N	1998-Aug 17
Shigella flexneri 2a	BR-SH-98-1284	Stool	IEC-N	1998-Aug 17
Shigella flexneri 2a	BR-SH-98-1287	Stool	IEC-N	1998-Aug 17
Shigella flexneri 2a	BR-SH-98-1289	Stool	IEC-N	1998-Aug 17
Shigella flexneri 2a	BR-SH-98-1290	Stool	IEC-N	1998-Aug 17
Shigella flexneri 2a	BR-SH-98-1294	Stool	IEC-N	1998-Aug 17
Shigella flexneri 2a	BR-SH-98-1296	Stool	IEC-N	1998-Aug 17
Shigella flexneri 2a	BR-SH-98-1300	Stool	Fiocruz-NE	1998-Aug 21
Shigella flexneri 2a	BR-SH-98-1305	Stool	Fiocruz-NE	1998-Aug 21
Shigella flexneri 2a	BR-SH-98-1307	Stool	Fiocruz-NE	1998-Sept 11
Shigella flexneri 2a	BR-SH-99-1527	Stool	Fiocruz-NE	1999-Jan 6
Shigella flexneri 2a	BR-SH-99-1528	Stool	Fiocruz-NE	1999-Feb 24
Shigella flexneri 6	BR-SH-97-236	Stool	IAL	No Data
Shigella flexneri 6	BR-SH-97-518	Stool	IEC	1997-Jun 13
Shigella flexneri 6	BR-SH-97-744	Stool	IAL	No Data
Shigella flexneri 6	BR-SH-98-1079	Stool	IEC-PA	1998-Apr 16
Shigella flexneri 6	BR-SH-98-1278	Stool	IEC-N	1998-Aug 17
Shigella flexneri 6	BR-SH-98-1291	Stool	IEC-N	1998-Aug 17
Shigella sonnei	BR-SH-97-210	Stool	ISDF	No Data
Shigella sonnei	BR-SH-97-211	Stool	ISDF	No Data
Shigella sonnei	BR-SH-97-219	Stool	ISDF	No Data
Shigella sonnei	BR-SH-97-230	Stool	Fiocruz	No Data
Shigella sonnei	BR-SH-97-234	Stool	Fiocruz	No Data
Shigella sonnei	BR-SH-97-273	Stool	Fiocruz	No Data
Shigella sonnei	BR-SH-97-351	Stool	ISDF	No Data
Shigella sonnei	BR-SH-97-359	Stool	IEC	1997-Mar 4
Shigella sonnei	BR-SH-97-361	Stool	IEC	1996-Mar 18
Shigella sonnei	BR-SH-97-362	Stool	IEC	1997-Jul 6
Shigella sonnei	BR-SH-97-363	Stool	Fiocruz	1997-Feb 17
Shigella sonnei	BR-SH-97-529	Stool	Fiocruz	1996-Oct 21
Shigella sonnei	BR-SH-97-533	Stool	Fiocruz	1997-Jul 29
Shigella sonnei	BR-SH-97-728	Stool	IEC	No Data
Shigella sonnei	BR-SH-97-730	Stool	IEC	1996
Shigella sonnei	BR-SH-97-732	Stool	IEC	No Data
Shigella sonnei	BR-SH-97-733	Stool	IEC	No Data
Shigella sonnei	BR-SH-97-740	Stool	IAL	No Data
Shigella sonnei	BR-SH-98-1075	Stool	IEC-PA	1998-Mar 17
Shigella sonnei	BR-SH-98-1077	Stool	IEC-PA	1998-Mar 17
Shigella sonnei	BR-SH-98-1078	Stool	Fiocruz	1998-Mar 17
Shigella sonnei	BR-SH-98-1080	Stool	IEC-PA	1998-Apr 16
Shigella sonnei	BR-SH-98-1081	Stool	IEC-PA	1998-Apr 16
Shigella sonnei	BR-SH-98-1083	Stool	ISDF	1998-Apr 16
Shigella sonnei	BR-SH-98-1085	Blood	Fiocruz	1998-Apr 16
Shigella sonnei	BR-SH-98-1093	Stool	Fiocruz	1998-Apr 16
Shigella sonnei	BR-SH-98-1094	Stool	Fiocruz	1998-Apr 20
Shigella sonnei	BR-SH-98-1095	Stool	Fiocruz	1998-Apr 16
Shigella sonnei	BR-SH-98-1285	Stool	IEC-N	1998-Aug 17
Shigella sonnei	BR-SH-98-1286	Stool	IEC-N	1998-Aug 17

Shigella sonnei	BR-SH-98-1292	Stool	IEC-N	1998-Aug 17
Shigella sonnei	BR-SH-98-1293	Stool	IEC-N	1998-Aug 17
Shigella sonnei	BR-SH-98-1295	Stool	IEC-N	1998-Aug 17
Shigella sonnei	BR-SH-98-1297	Stool	IEC-N	1998-Aug 17
Shigella sonnei	BR-SH-98-1298	Stool	Fiocruz-NE	1998-Aug 21
Shigella sonnei	BR-SH-98-1299	Stool	Fiocruz-NE	1998-Aug 21
Shigella sonnei	BR-SH-98-1301	Stool	Fiocruz-NE	1998-Aug 21
Shigella sonnei	BR-SH-98-1302	Stool	Fiocruz-NE	1998-Aug 21
Shigella sonnei	BR-SH-98-1303	Stool	Fiocruz-NE	1998-Aug 21
Shigella sonnei	BR-SH-98-1304	Stool	Fiocruz-NE	1998-Aug 21
Shigella sonnei	BR-SH-98-1308	Stool	Fiocruz-NE	1998-Sept 11
Shigella sonnei	BR-SH-99-1518	Stool	IEC-N	1998-Oct 19
Shigella sonnei	BR-SH-99-1520	Stool	Fiocruz-NE	1998-Nov 23
Shigella sonnei	BR-SH-99-1521	Stool	Fiocruz-NE	1998-Oct 14
Shigella sonnei	BR-SH-99-1522	Stool	Fiocruz-NE	1999-Feb 24
Shigella sonnei 1	BR-SH-98-1070	Stool	IEC-PA	1998-Mar 17
Shigella sonnei 1	BR-SH-98-1072	Stool	IEC-PA	1998-Mar 17
Shigella sonnei 1	BR-SH-98-1073	Stool	IEC-PA	1998-Mar 17
Shigella sonnei 1	BR-SH-98-1074	Stool	IEC-PA	1998-Mar 17

[a]Sources identified as "stool" and "blood" were isolated from humans only.

[b]Abbreviations used: Fiocruz: Fundação Ozwaldo Cruz; ; IEC: Instituto Evandro Chagas (Para); PA: Para; ISDF: Instituto de Saude Distrito Federal (Brasilia); IAL: Instituto Adolfo Lutz (Sao Paolo); N: Northern Brazil and NE: Northeastern Brazil.

[c]Institution names that are followed by the name of a district indicate that isolates originated from that district but were sent to and were processed by the institution.

Table 25: Colombian multi-drug resistant *Salmonella* isolates used in this study

Serotype	Isolate Number	Source[a]	Institution/Region[b]	Date of Isolation
S. Enteritidis	CO-SA-98-823	Saliva	HURGV	1997-Jan 27
S. Infantis 6,7:r:1,5	CO-SA-99-1695	Blood	HSB	1999-Mar 11
S. Typhi 1,4,12:i:1,2	CO-SA-99-1694	Stool	HI	1999-Mar 4
S. Typhimurium	CO-SA-98-837	Stool	LSP	1997-Apr 16
	CO-SA-99-1420	Stool	HSB	1997-Jan
S. Typhimurium PT 120	CO-SA-97-03	LCR	Cundinomorca	1997-Jan
S. Typhimurium (4,5,12:i:1,2)	CO-SA-98-1117	Stool	SFB	1998-May 5
	CO-SA-98-1119	Stool	SFB	1998-May 5
	CO-SA-98-1120	Stool	Antioquia	1998-Feb 23
	CO-SA-98-1121	Stool	SFB	1998-Apr 8
	CO-SA-98-1311	Stool	No Data	No Data
	CO-SA-99-1421	Stool	No Data	1997
	CO-SA-99-1423	Stool	No Data	1997
	CO-SA-99-1424	Stool	No Data	1997
	CO-SA-99-1425	Stool	No Data	1997
	CO-SA-99-1426	Stool	No Data	1997
	CO-SA-99-1427	Stool	No Data	1997
	CO-SA-99-1428	Stool	No Data	1997
	CO-SA-99-1429	Stool	No Data	1997
	CO-SA-99-1430	Stool	No Data	1997
	CO-SA-99-1431	Stool	No Data	1997
	CO-SA-99-1432	Stool	No Data	1998
	CO-SA-99-1433	Stool	No Data	1998
	CO-SA-99-1434	Stool	No Data	1998
	CO-SA-99-1435	Stool	No Data	1998
	CO-SA-99-1436	Stool	No Data	1998
	CO-SA-99-1437	Stool	No Data	1998
	CO-SA-99-1438	Stool	No Data	1998

[a] Sources identified as "stool" and "blood" were isolated from humans only.
[b] Abbreviations used: HURGV: Hospital Universitano Ramon Gonzalez Valencia, Santander, Bucaramanga; HI: Hospital Infantil, Medellin; LSP: Laboratorio de Salud Publica, Antioquia, Rio Negro; HSB: Hospital Simon-Bolivar, Cundinomorca, Bogota and SFB: Santa Fe de Bogota.

Table 26: Colombian multi-drug resistant *Shigella* isolates used in this study

Serotype	Isolate Number	Source[a]	Institution[b]	Date of Isolation
Shigella flexneri	CO-SH-98-1314	Stool	No data	No data
	CO-SH-98-1315	Stool	No data	No data
	CO-SH-98-1318	Stool	No data	No data
	CO-SH-98-1319	Stool	No data	No data
Shigella flexneri 2	CO-SH-98-839	Stool	HURGV	1997-Apr 30
	CO-SH-98-842	Stool	LSP	1997-Mar 14
	CO-SH-98-838	Stool	LSP	1997-Mar 14
Shigella flexneri 2a	CO-SH-97-07	Stool	HK	1997-Feb12
	CO-SH-98-1124	Stool	SS	1998-Jan 22
	CO-SH-98-1125	Stool	HURGV	1998-Mar 5
	CO-SH-98-1126	Stool	HK	1998-Mar 1
	CO-SH-99-1698	Stool	HURGV	1999-Jan 15
Shigella flexneri 3a	CO-SH-97-06	Stool	HSB	1997-Feb12
Shigella flexneri 6	CO-SH-98-840	Stool	HSB	1997-Mar 13
Shigella sonnei	CO-SH-97-08	Stool	HLV	1997-Feb
	CO-SH-97-09	Stool	LC	1996-Dec
	CO-SH-97-10		LC	1996-Jun13
	CO-SH-98-1316	Stool	HSFA	1998-Aug 10
	CO-SH-98-1317	Stool	FVL	1998-Aug 19
	CO-SH-99-1699	Stool	HK	1999-Mar 12
	CO-SH-99-1701	Stool	SJ	1999-Apr 16
	CO-SH-99-1702	Blood	FHA	1999-May 4

[a] Sources identified as "stool" and "blood" were isolated from humans only.

[b]Abbreviations used: HURGV: Hospital Universitano Ramon Gonzalez Valencia, Santander, Bucaramanga; LSP: Laboratorio Salud Publica, Antioquia, Rio Negro; HK: Hospital Kennedy, Cundinomorca, Bogota; SS: Sector Salud, Risaralda; HSB: Hospital Simon-Bolivar, Cundinomorca, Bogota; HLV: Hospital La Victoria, Cundinomorca, Bogota; LC: Laboratorio Central, Cundinomorca, Bogota; HSFA: Hospital Santa Fe de Antioquia, Antioquia, Rio Negro; FVL: Fundacion Valle de Lili, Valle del Cauca; SJ: San Jorge, Risaralda and FHA: Federico Heras Acosta, Ibague.

7. Appendix-II:

Table 27. Integron profiles detected in MDR *Salmonella* and *Shigella* strains from Brazil and Colombia

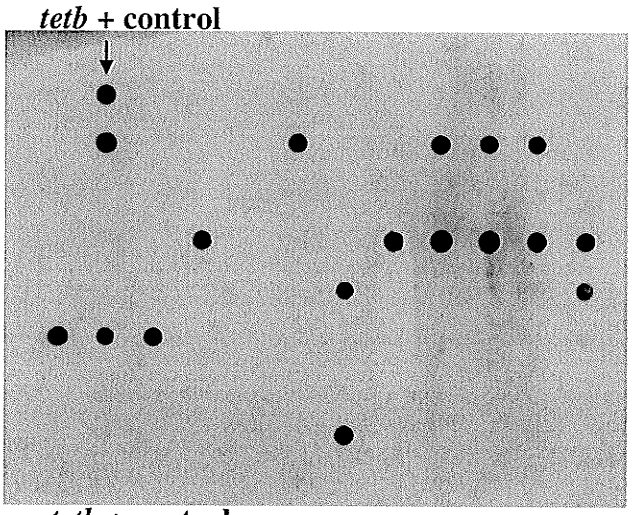
Integron Profile	Integron size (bp)	Number of Strains	Antibiogram	Serotypes	Country
A	3000, 699	1	ACSSuTKTm	*S.* Bredeney	BR
B	2017, 200	4	ASuKCTX	*S.* Agona	BR
B	2017, 200	1	ACSSuKG	*Salmonella* 4,5,12:i:-	BR
C	>3000	1	ASSuKTm	*S.* Typhimurium	CO
C	>3000	1	ASSuTTm	*S.* Typhimurium	CO
C	>3000	1	ASSuTKTm	*S.* Typhimurium	CO
C	>3000	1	AsuTTm	*S.* Typhimurium	CO
C	>3000	1	ASSuKTmCTXCAZ	*S.* Enteritidis	CO
D	1878	1	ASuTG Tm	*S.* Enteritidis	BR
D	1878	1	ACSuKGCTX	*S.* Enteritidis	BR
E	1878, 200	1	ACSSuTKGTm	*S.* Agona	BR
F	1756, 200	4	ACSuTKG (1)	*S.* Agona	BR
F	1756, 200	2	ACSuTKGTm	*S.* Agona	BR
F	1756, 200	1	ACSuKCTX CAZTm	*S.* Agona	BR
F	1756, 200	1	ACSuTKGCAZ	*S.* Agona	BR
F	1756, 200	2	ACSuTKGCTXCAZ	*S.* Agona	BR
G	1686	2	ACSSuTKGTm (1)	*S.* Typhimurium PT 174	BR
G	1686	1	ACSSuTKGTm	*S.* Typhimurium PT 208	BR
G	1686	1	ACSSuTKGCTXCAZ	*S.* Typhimurium PT 174	BR
H	1589	1	ACSSuTTm	*S.* Schwarzengrund	BR
H	1589	1	SSuTTm	*S.* Typhimurium	BR
I	1269	7	ACSSuTKGCTXCAZTm	*S.* Infantis	BR
I	1269	2	ACSSuTKGCTXTm	*S.* Infantis	BR
I	1269	1	ACSuTKGCTXCAZTm	*S.* Infantis	BR
J	1269, 200	1	ATKTm	*S.* Typhimurium	BR
K	1002	3	SSuT	*S.* Typhimurium	BR
K	1002	1	ACSSuTKG	*S.* Typhimurium	BR
K	1002	1	SSuTG	*S.* Heidelberg	BR
K	1002	1	CSSuTTm	*S. flexneri*	BR
K	1002	1	CSSuTG	*S. sonnei*	BR
K	1002	1	ACSSuTTm	*S. flexneri*	CO
L	1002, 300	1	CSSuT	*S. sonnei*	BR
M	1002, 200	1	CSSuT	*S.* Brandenberg	BR
N	699	1	ASuTG	*S.* Infantis	BR
N	699	1	ACSuT	*S.* Infantis	
N	699	1	ASSuTKGCAZ	*S.* Infantis	
O	699, 200	2	ASSuKG	*S.* Agona	
P	624	1	CSSuT	*S.* Panama	
Q	300	1	ASSuTm	*S. sonnei*	BR
Q	300	1	SSuTTm	*S. sonnei*	CO
Q	300	1	ASSuTTm	*S. sonnei*	CO
Q	300	1	ASTTm	*S. flexneri*	BR
Q	300	1	ACSTTm	*S. sonnei*	BR

R	201	1	ASSuT	*S.* Typhimurium	BR
R	201	2	SSuT	*S.* Typhimurium	BR
R	201	1	CSSuT	*S.* Typhimurium	BR
R	201	1	SuTTm	*S.* Derby	BR
R	201	1	SSuTTm	*S.* Enteritidis	BR

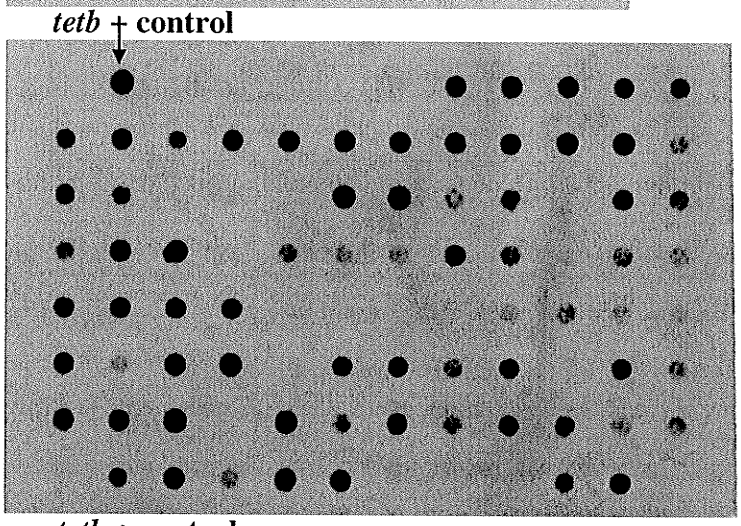
8. Appendix-III:

Example of a dot blot hybridization: genomic DNA from all test strains (listed in Appendix I) were blotted onto 3 panels and were hybridized to a *tetB* probe

panel a



panel b



panel c