



CATÓLICA
UNIVERSIDADE CATÓLICA PORTUGUESA
ESCOLA SUPERIOR DE BIOTECNOLOGIA

MOLECULAR TYPING OF PORTUGUESE CLINICAL ISOLATES
OF
Salmonella spp., *S. Enteritidis* AND *S. Typhimurium*

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to
fulfill the
requirements of Master of Science degree in Food Innovation

by
Diana Luísa Campos Ferreira Ramos

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Resumo

A *Salmonella* spp. é um importante patógeno associado a doença humana. Permanece uma das maiores causas de surtos de origem alimentar em todo o mundo. Na Europa, dois serotipos permanecem no topo da lista de número de casos por ano, Enteritidis e Typhimurium. A salmonelose é usualmente uma doença manifestada por diarreia e que dispensa tratamento médico, embora a terapia com antibióticos seja necessária em casos de doença invasiva ou infeções com complicações associadas. A resistência a antibióticos tem sido um problema crescente mundialmente. *Salmonella enterica* proveniente de uma variedade de alimentos e animais, tem sido extensamente estudada e caracterizada quanto a fenótipos de resistência e vários serotipos revelaram resistência a múltiplos antibióticos.

Na Europa, o ECDC (European Center of Disease Control) é o responsável, desde 2007, pela rede de vigilância de infeções gastrointestinais humanas. Em Portugal o INSA (Instituto Nacional de Saúde Dr. Ricardo Jorge) foi designado como laboratório de referência para fornecer dados epidemiológicos ao ECDC.

Neste estudo 216 isolados clínicos de *Salmonella*, provenientes do Hospital de São João no Porto (HSJ) e Hospital de São Marcos em Braga (HSM) ambos na região Norte de Portugal, foram testados quanto à resistência a antibióticos e analisados por PFGE, como implementado pelo CDC nos Estados Unidos. A amostra é constituída por isolados de *S. Enteritidis* e *S. Typhimurium*, assim como isolados identificados como *Salmonella* spp..

A análise de suscetibilidade a antibióticos confirmou as tendências Europeias, baixa resistência de isolados de *S. Enteritidis* exceto ao ácido nalidíxico, e elevada resistência em isolados de *S. Typhimurium* à maioria dos antibióticos testados. Alguns dos isolados de *S. Typhimurium* testados neste estudo também foram testados em trabalhos prévios no laboratório quanto a resistência a sulfonamidas, a maioria mostrou ter um perfil de resistência típico da *Salmonella* DT104 (ACSSuTe).

A análise de PFGE dos isolados permitiu a diferenciação de Enteritidis e Typhimurium assim como a identificação do serotipo para a maior parte dos isolados identificados como *Salmonella* spp. Um isolado de *Salmonella* spp. não pertence a nenhum dos outros serotipos. . As amostras provenientes dos dois hospitais têm uma similaridade elevada entre si. A discriminação dos isolados de Enteritidis provou ser baixa usando o enzima XbaI levando à conclusão de que um painel diferente de enzimas poderia ajudar. Os isolados de Typhimurium foram bem discriminados pelo PFGE porém não foi possível relacionar essa discriminação quer com a resistência a antibióticos quer com a informação disponível dos isolados.

Abstract

Salmonella spp. is an important pathogen associated with human disease. It remains one of the major causes of food-borne outbreaks all over the world. In Europe two serovars are at the top of the list in number of cases per year, Enteritidis and Typhimurium. Salmonellosis is usually a self-limiting diarrheal disease requiring little or no medical intervention. However, in cases of invasive disease or infections with added complications, antimicrobial treatment may be required. Resistance to antimicrobials has been an increasing problem worldwide. *Salmonella enterica* from a variety of food and animal sources has been extensively characterized in terms of resistance phenotypes and numerous serotypes revealed multiple antimicrobial resistance determinants (strains resistant to two or more antimicrobials).

In Europe the ECDC (European Center of Disease Control) has been in charge of the international surveillance network for human gastrointestinal infections since 2007. In Portugal the INSA (Instituto Nacional de Saúde Dr Ricardo Jorge) was the designated reference laboratory to report the epidemiological data, and though an effort was made still the actual data is not available or clearly reported.

In this study 216 clinical *Salmonella* isolates, from Hospital de São João in Oporto (HSJ) and Hospital de São Marcos in Braga (HSM) both from the Northern region of Portugal, were tested for antimicrobial resistance and by PFGE analysis, as implemented by the CDC in the USA. The sample contained *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* spp.

The antimicrobial susceptibility analysis to a panel of antimicrobials has confirmed European tendencies, low resistance in *S. Enteritidis* isolates except for nalidixic acid, and high resistance in *S. Typhimurium* isolates to most of the tested antimicrobials. Some of the *S. Typhimurium* isolates tested in this study were also tested in previous works in the lab for sulphonamides resistance and proved to have the typical DT104 resistance profile (ACSSuT).

The PFGE analysis allowed the differentiation of Enteritidis and Typhimurium isolates as well as the identification of the serotype for most isolated identified has *Salmonella* spp. One *Salmonella* spp. Isolate didn't belong to any of the two serotypes. The samples from the two hospitals showed high similarity between themselves. The discrimination of Enteritidis isolates was insufficient using XbaI enzyme leading to the conclusion that it would benefit with the analysis using a different panel of enzymes. Typhimurium isolates were well discriminated with PFGE though no correlation was possible with the resistance profile or the isolates available information.

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Introduction

Salmonella spp. classification

Salmonella spp. are Gram-negative, rod-shaped bacteria belonging to the Enterobacteriaceae family (Le Minor and Popoff, 1987; Darwin and Miller, 1999). *Salmonella* was named after an American bacteriologist, Daniel E. Salmon, who first isolated *Salmonella* Choleraesuis from porcine intestine in 1884 (Su, 2007). The genus *Salmonella* comprises a large and closely related population of medically important pathogens and consists of three species, *Salmonella bongori*, *Salmonella enterica* (Su *et al.*, 2007) and *Salmonella subterranean*, the last recognized in 2005 (Tankouo-Dandjong, 2007). *Salmonella enterica* is divided into the following six subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI) (Grimont and Weill, 2007).

More than 2,500 serovars of zoonotic *Salmonella* exist and the prevalence of the different serovars changes over time (EFSA, 2009). *Salmonella* serotypes are closely related evidencing their clonal origin, and based on the degree of sequence divergence, it can be estimated that a common ancestor of the genus existed about 25 to 40 million years ago (Bäumler *et al.*, 1998).

While some serovars of *Salmonella* such as *S. Typhi* and *S. Pullorum* have a restricted host range, most serovars infect a broad range of warm-blooded animals and are capable of causing disease in humans. The majority that causes disease in humans belong to subgroup I (Darwin and Miller 1999).

Salmonella is one of the most extensively studied bacterial genera in terms of its physiology, genetics, cell structure, and development (Darwin and Miller, 1999). The reasons for this are that they cause significant morbidity and mortality worldwide; they have broad host ranges, infections result in different diseases in different hosts; they are able to establish persistent infections, which serve as reservoirs for transmission/shedding; and they are increasingly resistant to many antibiotics (Boyle *et al.*, 2007).

Even though more than 2,500 serovars of *Salmonella enterica* have thus far been recognized, few appear to cause most foodborne illnesses (Gebreyes *et al.*, 2006). Food-borne outbreaks are infections or intoxications in humans caused by the consumption of a common contaminated food (EFSA, 2009).

In 2007, a total of 5,609 food-borne outbreaks were reported by the European Union Member States (EU MSs) of which 38.7% were verified and where 1,475 people were affected, hospitalizing 55 and causing 5 deaths. *Salmonella* was as in previous years, one of the most commonly reported cause of food-borne outbreaks in the EU, second only in numbers caused in 2007 by *Campylobacter*. A total of 155,540 confirmed cases of human salmonellosis were reported via TESSy (The European Surveillance System) from 30 countries, including the 27 EU MSs and three non-MSs, and directly to the European Food Safety Agency (EFSA) from Switzerland. The number of confirmed cases in the EU has decreased since 2005 (fig 1). Overall, total case counts of salmonellosis have decreased since 2004.

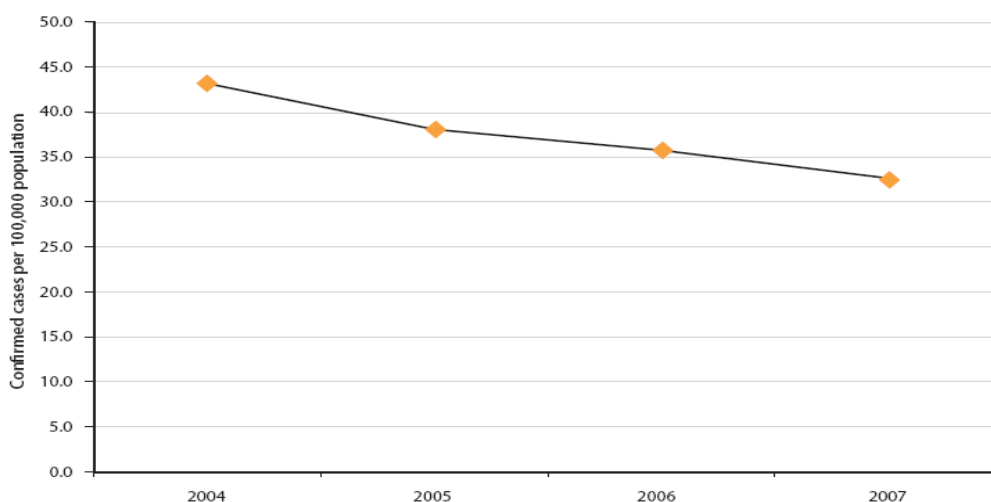


Figure 1 - Notification rates of reported confirmed cases of human salmonellosis in the EU, 2004-2007 (in EFSA, 2009).

Since the EFSA was established in 2002, no data was found on *Salmonella* for the years 2002 and 2003. General *Salmonella* Quarterly reports can be found on the ECDC's Enter-net (Enter- Net 2002-2003 http://ecdc.europa.eu/en/publications/surveillance_reports/fwd)

The age distribution of *Salmonella* cases in 2007 closely parallels those seen in 2006. The distribution by age group is depicted in the following figure (Fig.2). The highest rate was observed for 0 to 4 years old group (EFSA, 2009). Children especially those <1 year of age , and adults over 60, are most susceptible to disease and tend to have more severe infections (Darwin and Miller, 1999).

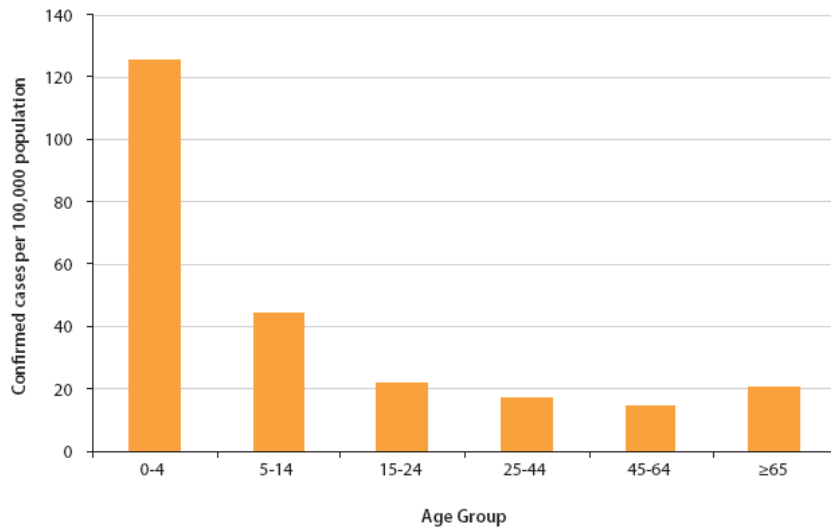


Figure 2 - Age distribution of reported confirmed cases of human salmonellosis in the EU, 2004-2007; data from all Member States, N=131,229. (in EFSA, 2009)

A seasonal effect is notable in the numbers of reported cases of salmonellosis throughout the year, usually higher in the summer and autumn and with a rapid decline in winter months. This pattern supports the influence of temperature and behavior, and has been observed in earlier reports (EFSA, 2002, 2007). When further analyzed by specific serovar counts per month, serovar Enteritidis shows a more prominent summer/autumn peak than other serovars. The two most common *Salmonella* serovars are Enteritidis and Typhimurium, representing 81%, in 2007, of all identified types in human cases (fig 3).

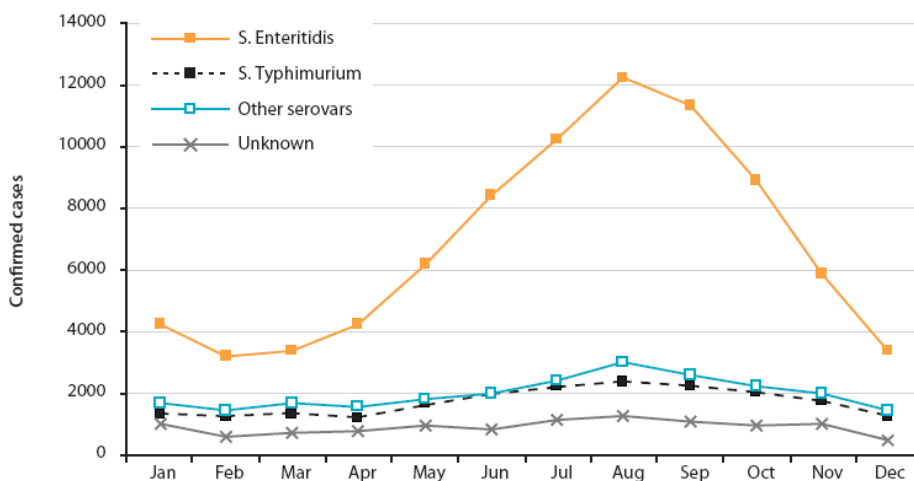


Figure 3 - Specific serovars counts per month in the EU, 2007 (in EFSA-2009)

***Salmonella* spp. Transmission and Disease**

Transmission of *Salmonella* to humans is usually by consumption of contaminated food, but human-to-human transmission and direct animal-to-human transmission can occur (Darwin, 1999). The common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals which results in a variety of foodstuffs of animal and plant origin as sources of infections (EFSA, 2009). The foodstuffs most frequently associated with *Salmonella* outbreaks in humans are meat, especially poultry, eggs and egg products.

Human *S. Enteritidis* cases are most commonly associated with the consumption of contaminated eggs and broiler meat, while *S. Typhimurium* cases are most often associated with the consumption of contaminated pig, poultry and bovine meat. Transmission often occurs when organisms are introduced into food preparation areas and are allowed to multiply in food, e.g. due to inadequate storage temperatures, inadequate cooking or cross-contamination of ready-to-eat food (EFSA, 2009).

Salmonella enterica serovar Enteritidis is the cause of the foodborne salmonellosis pandemic in humans, in part because it has the unique ability to contaminate eggs without causing discernible illness in the birds infected (Guard-Petter, 2001). *Salmonella* serovar Enteritidis is the only human pathogen that contaminates eggs routinely, even though the on-farm environment of the chicken is a rich source of a number of *Salmonella* serotypes. Human illness caused by infection with *Salmonella enterica* serovar Enteritidis increased worldwide beginning as early as the mid-1970s and, by 1990, this serovar displaced *Salmonella enterica* serovar Typhimurium as the primary cause of salmonellosis in the world (Guard-Petter, 2001).

Salmonella is capable of causing a variety of disease syndromes: enteric fever, bacteremia, enterocolitis, and focal infections. Enterocolitis is by far the most common manifestation of disease caused by *Salmonella*, but bacteremia and focal infections can accompany or follow enterocolitis (Darwin, 1999). Salmonellosis has also been associated with long-term and sometimes chronic sequelae, e.g. reactive arthritis. The incubation period is from 6 to 72 hours, usually about 12-36 hours (EFSA, 2009). It is estimated from volunteer studies that 10^5 to 10^{10} bacteria are required to initiate an infection, but the exact amount needed varies with the strain, what is consumed with the bacteria, and the physiological state of the host (Darwin, 1999). Salmonellosis is usually a self-limiting diarrhoeal disease requiring little or no medical intervention. *S. enterica* is a facultative intracellular pathogen which preferentially resides inside macrophages, yet requires both antibodies and a cellular

immune response for clearance (Kaufmann, 2001). However, in cases of invasive disease or infections with added complications, such as at the extremities of age or in the presence of underlying disease, antimicrobial treatment may be required.

***Salmonella* spp. Evolution and Pathogenicity**

It has been postulated that in the genus *Salmonella*, virulence evolved in three phases. The first phase involved acquisition of *Salmonella* pathogenicity island 1 (SPI1) by plasmid or phage mediated horizontal transfer (Marcus et al., 2000). Pathogenicity islands are distinct regions of the chromosome where virulence functions are clustered; they are characteristically unstable and have a high deletion frequency (Maskell, 2006). SPI1 was likely obtained by a lineage ancestral to all *Salmonella* serotypes, since it is present in all phylogenetic lineages of the genus *Salmonella* but absent from *Escherichia coli* and other related organisms. SPI1 encodes virulence factors that mediate mechanisms used by *Salmonella* serotypes during the intestinal phase of infection, including invasion of intestinal epithelial cells, induction of neutrophil recruitment, and secretions of intestinal fluid (Bäumler, 1998).

The formation of the two species *S. enterica* and *S. bongori* could be considered a second phase in the evolution of virulence in the genus *Salmonella*, since it involved not only divergence of their lineages by point mutation but also acquisition of new virulence determinants by horizontal gene transfer. Serovars belonging to *S. enterica* possess a second pathogenicity island, designated SPI2, which is not present in *S. bongori* serotypes (Bäumler, 1998).

The formation of *S. enterica* subspecies *I* involved a dramatic expansion in host range: while *S. bongori* and *S. enterica* subspecies *II*, *IIIa*, *IIIb*, *IV*, and *VII* are mainly associated with cold-blooded vertebrates, members of *S. enterica* subspecies *I* are most frequently isolated from avian and mammalian hosts. The host adaptation of *S. enterica* subspecies *I* to warm-blooded vertebrates characterized a third phase in the evolution of virulence in the genus *Salmonella*. In addition to horizontal gene transfer, deletion events and sequence divergence by point mutation likely contributed to changes in the host ranges of *S. enterica* serovars (Bäumler, 1998).

Salmonella serotypes initiate infection by attaching to the intestinal mucosa of the host. Upon penetration of the intestinal mucosa of mammals, *Salmonella* serotypes are confronted by an effective barrier to further spread, namely macrophages that line the lymphatic sinuses of regional lymph nodes. In mammals this host defense mechanism can successfully limit

bacterial expansion to the intestine, the gut-associated lymphoid tissue, and the mesenteric lymph node. It has therefore been speculated that *Salmonella* serotypes evolved in the alimentary tract of reptiles, where they developed from pathogens into commensal organisms. The ability of *S. enterica* serotypes to cause systemic disease is directly related to the capability to withstand an assault by the macrophages of a given host (Bäumler, 1998). Pathogens that lack host specificity, such as *S. enterica* serotype Typhimurium and *S. enterica* serotype Enteritidis, tend to be more frequently associated with disease in young animals than in adults, suggesting that they are not optimally adapted to cope with a fully mature immune system.

Non-typhoidal salmonellosis has been characterized by a pattern of dissemination of clonal *Salmonella enterica*. Epidemic clones may remain largely restricted to the animal reservoir for years before their incidence rises among human infections (Davis *et al.*, 2007).

Antimicrobial treatment and resistance mechanisms in *Salmonella*

Food animals have been worldwide exposed to antimicrobials to treat or prevent infectious diseases or to promote growth. Many of these antimicrobials are similar or identical to the ones used to treat infections in humans. As a consequence, antimicrobial resistance has emerged in zoonotic enteropathogens (e.g. *Salmonella* spp., *Campylobacter* spp.), commensal bacteria (e.g. *E. coli*, enterococci), and bacterial pathogens of animals (e.g. *Pasteurella*, *Actinobacillus* spp.), with variable prevalence. Resistance to antimicrobials emerges from the use in animals and subsequent transfer of resistance genes and bacteria among animals and animal products and the environment (McEwen *et al.* 2002). Antimicrobial resistance is the best-known example of rapid adaptation of bacteria to a new ecosystem (Carattoli *et al.*, 2003).

In 1969, the Swann Committee was asked by the UK Government to report on antimicrobial use in both human and veterinary practice. The Committee concluded that there was a significant problem and made several recommendations. For example, animal feed antibiotics should not be those used in human medicine as this could compromise efficacy in man. Swann also recommended that the UK Government establish a committee that should have overall responsibility for the whole field of antibiotic use (Wise, 2007). In 1986 Sweden was the first country to ban all the food animal growth promoting antibiotics. Other European countries followed this decision and in 1997 and 1999 the European Union banned the remaining four growth promoting antibiotics under the “Precautionary Principle”. This action posed many questions about how to deal with the antimicrobial resistant bacteria that are

already in the environment and whether the removal of the antimicrobial agents could have adverse effects (Casewell *et al.*, 2003). To help the European Countries tackle this antimicrobial resistance problem the World Health Organization published a booklet in 2011. This booklet contains instructions and recommendations destined to European Member States to consult in order to establish an “intersectoral and multifaceted approach with effective coordination of action and exchange of information among the agricultural, food, veterinary and health sectors” (WHO, 2011).

Antimicrobial treatment of salmonellosis is rare and only required in cases of generalized and invasive infection with added complications. Surveillance data of antimicrobial resistance in *Salmonella* was reported by European Member States for the 2004-2007 periods. In general, in *Salmonella* susceptibility tested isolates, resistance percentages range from 8 to 42%. Nalidixic acid resistance increased in 2007 as well as ampicillin resistance; on the other hand chloramphenicol resistance has decreased to the level observed in 2005. In *S. Enteritidis* isolates, low levels of resistance were reported for most antimicrobials tested; however, the percentages of resistant isolates reported for nalidixic acid were at a higher level and varied from 7 to 26%. For *S. Typhimurium* isolates, medium to high levels of resistance were reported for all antimicrobials tested except for nalidixic acid, during the reporting years (EFSA, 2010).

Salmonella enterica from a variety of food and animal sources has been extensively characterized in terms of resistance phenotypes and numerous serotypes revealed multiple antimicrobial resistance determinants. Multidrug-resistant (MDR) strains are defined as resistant to two or more antimicrobial agents. MDR *S. enterica* strains may carry the resistance determinants on chromosomal locations and/or on resistance plasmids.

Resistance to broad-spectrum cephalosporin ceftriaxone, and to the quinolone nalidixic acid, are of particular importance to the medical community since they are the drug of choice for the treatment of pediatric and adult salmonellosis, respectively (Brichta-Harhay, 2011).

In this work we determined the resistance to a panel of commonly tested antimicrobials, namely tetracycline, chloramphenicol, ampicillin, streptomycin, nalidixic acid, and trimethoprim-sulphamethoxazole, having different modes of action and therefore subject of different resistance mechanisms. The compiled resistance percentages reported for some antimicrobials in the European Member states in the years 2004 to 2007 are depicted in table 1. No data is shown for the years 2002 and 2003 because they were not available at the EFSA. Quarterly reports from ECDC only contain general numbers for all *Salmonella* isolates (ECDC 2004).

Table 1 – Resistance (%) to tetracycline, chloramphenicol, ampicillin, sulfonamide and nalidixic acid among tested *Salmonella* spp. isolates , 2004 to 2007 (EFSA 2010)

	2004		2005		2006		2007		
	N	%	N	%	N	%	N	%	
<i>Salmonella</i> spp.	Tetracycline	1694	37,8	2357	28,4	2463	40,9	6769	42,3
	Chloramphenicol	1683	11,6	2285	8,4	2439	15,8	6669	10,9
	Ampicillin	1690	21,1	2066	18,4	2137	25,2	5974	33,1
	Sulfonamide	1565	36,1	2294	25,0	2330	36,0	6549	38,5
	Nalidixic Acid	1599	10,5	2292	8,8	2375	13,6	6787	18,1

	2004		2005		2006		2007		
	N	%	N	%	N	%	N	%	
<i>S. Enteritidis</i>	Tetracycline	181	0,0	869	0,0	437	2,0	808	2,0
	Chloramphenicol	182	1,0	867	0,0	437	1,0	783	0,0
	Ampicillin	183	4,0	842	1,0	432	4,0	685	2,0
	Sulfonamide	167	3,0	866	1,0	436	6,0	685	2,0
	Nalidixic Acid	156	26,0	875	7,0	417	22,0	808	22,0

	2004		2005		2006		2007		
	N	%	N	%	N	%	N	%	
<i>S. Typhimurium</i>	Tetracycline	1342	27,4	1779	25,2	1557	63,1	1941	58,8
	Chloramphenicol	1340	24,8	1800	27,5	1557	35,0	1939	28,5
	Ampicillin	1326	38,7	1761	46,6	1522	38,2	1920	52,3
	Sulfonamide	1260	36,5	1801	54,8	1557	63,4	1923	58,8
	Nalidixic Acid	1341	1,3	1800	3,0	1557	2,0	1943	4,3

Ampicillin is a β -lactam antibiotic with action against Gram negative and Gram positive bacteria. It is a peptidoglycan synthesis inhibitor and has a bacteriolytic effect only in

growing bacteria or in hypotonic environment. The β -lactam antibiotics stop the peptidoglycan synthesis (bacteriostatic effect) and act by activation of the bacterial endogenous autolytic system. These antibiotics bind the cellular membrane through the Penicillin-binding-proteins (PBPs). The bacterial resistance is mediated by the hydrolysis of the β -lactam ring by the β -lactamase enzyme. These enzymes are of chromosomal or plasmid origin and are excreted to the environment by Gram-positive bacteria and to the periplasm by Gram-negative bacteria.

Chloramphenicol is considered a broad spectrum antibiotic with effective action against Gram-positive and Gram-negative bacteria. It is used in the therapy of meningitis, typhoid fever and eye infections, among others, and has a high penetration rate of the blood-brain barrier. It is a protein synthesis inhibitor that binds to the 50S ribosomal subunit resulting in a bacteriostatic effect. Several Gram-positive and Gram-negative bacterial strains are resistant to chloramphenicol and its derivatives; these strains often show cross resistance to fusidic acid. High-level resistance is conferred by the *cat*-gene. A mechanism of low-level resistance is the reduction of membrane permeability by alteration of available porin 1 α channels. Rare resistance mechanisms to chloramphenicol are the mutation of the ribosomal target, and the activation of efflux-pumps in *E.coli* strains. The *cat*-gene, responsible for acetyltransferase production, is located in plasmids or in chromosomal DNA and is expressed constitutively. The resistance plasmids can be co-transferred to other bacterial species by genetic recombination (i.e. chloramphenicol resistance transference from *S. typhi* to *E. coli*).

Streptomycin is an aminoglycoside antibiotic and was the first antibiotic remedy for tuberculosis. It is effective against Gram-positive and Gram-negative bacteria and therefore considered a broad spectrum antibiotic. It is a protein synthesis inhibitor that crosses the bacterial envelope altering the permeability of the cell and inhibits the DNA replication. It has a bactericidal and bacteriolytic effect in Gram-positive bacteria. For these antibiotics to be effective they have to accumulate inside the cell in its native form, after crossing the cellular wall and membrane. The selective pressure, especially in hospital environments, has led to the emergence of resistant bacterial strains. The bacterial resistance is mediated by plasmids, transposons and integrons, which disseminate easily between bacterial strains of the same and different species. The most frequently observed resistance mechanism in clinical isolates is the enzymatic modification of the $-\text{NH}_2$ groups, and the $-\text{OH}$ groups, among others. Other described resistance mechanisms are the mutation of ribosomal protein genes and the alteration of cell wall permeability.

Tetracycline is a broad spectrum antibiotic with bacteriostatic effect, and bactericidal in high concentrations. It is a protein synthesis inhibitor, acting in the 30S ribosomal subunit. As other protein synthesis inhibitors, tetracycline has to cross the outer membrane through porin channels, in order to reach the periplasm and the cytoplasmic membrane of the bacterial cell. Tetracycline also binds to the DNA, proteins and tRNA. The incidence of tetracycline resistance is very high and broad in bacterial strains. The genetic determinants responsible for the resistance can be of chromosomal origin, the most frequently found is in plasmids or transposons. The resistance mechanisms to tetracycline, in a general way, are due to: outer membrane impermeabilization in Gram-negative bacteria; ribosome mutation; low intracellular concentration due to efflux of the antibiotic; ribosomal protection. The most frequent mechanism is mediated by efflux pumps that keep the tetracycline intracellular concentration low. The strong efflux system is determined by genes *tet*, which codify the TET proteins that actively transport the tetracycline out of the cell.

Nalidixic acid is a first generation antibiotic of the quinolone group with activity against Gram-negative bacteria, and is usually used in urinary infection treatment. The first generation quinolones are DNA gyrase inhibitors. The inhibition of the DNA gyrase will affect the DNA coiling and consequently the replication and transcription. Therefore quinolones have a bactericidal effect. The resistance to quinolones can be due to the impermeabilization of the outer membrane, antibiotic efflux or mutation of the target enzymes.

Trimethoprim-sulfamethoxazole is a combination of two broad spectrum antibiotics, active against Gram-positive and negative bacteria. When trimethoprim is used in association with sulfonamids they can have a synergistic bactericidal effect, not being linear for all associations. The trimethoprim-sulfamethoxazole association decreases mostly the development of bacterial resistance, compared to monotherapy. Different mechanisms can explain the bacterial resistance to these antibiotics. The permeability barrier against sulfonamides and trimethoprim is generally associated with energy dependent efflux pumps. Chromosomal mutations in the *dhps* gene also confer resistance to sulfonamides. Point mutations in the *dhfr* gene can cause trimethoprim resistance. The resistance can also be acquired in plasmids that codify the DHPS and DHPR production, resistance to sulfonamides and trimethoprim, respectively. The sulfonamide resistance in Gram-negative enteric bacteria is generally mediated by horizontal gene transfer of external *sul* genes.

Molecular typing – Pulsed Field Gel Electrophoresis

Typing methods fall into two broad categories; phenotypic methods and genotypic methods.

Phenotypic methods are those that characterize the products of gene expression in order to differentiate strains. Properties such as biochemical profiles, bacteriophage types, antigens present on the cell's surface, and antimicrobial susceptibility profiles, all are examples of phenotypic properties that can be determined in the laboratory. Because they involve gene expression, these properties all have a tendency to vary, based on changes in growth conditions, growth phase, and spontaneous mutation. Phenotypic methods, such as antimicrobial susceptibility profiles, biochemical profiles, bacteriophage susceptibility patterns, multilocus enzyme electrophoresis (MLEE) profiles, and immunoblot fingerprinting, have allowed investigators to describe the epidemiology of some nosocomial and community acquired infections. However, the phenotypic methods discriminate poorly among strains, they frequently require intensive labour, long elapsed times, and they often produce variable results (Pfaller, 1999).

Genotypic methods are those that are based on the analysis of the genetic structure of an organism and include polymorphisms in DNA restriction patterns based on cleavage of the chromosome by enzymes that cleave the DNA into hundreds of fragments (frequent cutters), or into 10 to 30 fragments (infrequent cutters), and the presence or absence of extra-chromosomal DNA. Genotypic methods are less subject to natural variation, although they can be affected by insertions or deletions of DNA in the chromosome, the gain or loss of extra-chromosomal DNA, or random mutations that may create or eliminate restriction endonuclease sites (Tenover *et al.*, 1997).

In the characterization of methods, four criteria must be considered: rapidity, reproducibility, ease of use, and the ability to differentiate strains with similar phenotypes which are genetically unrelated (Lim *et al.*, 2005).

Salmonella comprises a large genus, and serotyping is widely used to classify isolates into serogroups according to their surface antigen variability. Serotyping is based on the immunological classification of the lipopolysaccharide (O antigen), the flagellar protein (H antigen), and the capsular polysaccharide moieties (Vi antigen). The Kauffmann-White scheme, generally used for the classification of *Salmonella* serotypes, recognizes 2523 characterized serotypes (Kaufmann, 1952; Tankouo-Sandjong *et al.*, 2007). However, the

complexity of the system and difficulty of laboratory to laboratory comparison of results limit the application of serotyping to reference laboratories (Lim *et al.*, 2005).

Among the most frequently isolated serovars are *S. enterica* subsp. *enterica* serovars Typhimurium and Enteritidis. Serotyping is not efficient enough when trying to track the source of common outbreaks of these two serotypes, as well as other frequently seen serovars. To further discriminate within *S. Typhimurium* and *S. Enteritidis*, phage typing is the primary sub-typing technique (Torpdahl *et al.*, 2005). Phage typing requires a well-maintained phage-library, precise methodology, and experience in interpretation of results (Ross and Heuzenroeder, 2005). Phage typing of *Salmonella* can be subjective and fail to provide sufficient discrimination between isolates and it does not necessarily reflect a close genetic relationship between isolates (Ross and Heuzenroeder, 2005).

Several DNA-based typing methods have been developed in an attempt to improve the reproducibility and discriminatory ability in typing of *Salmonella* (Torpdahl *et al.*, 2005). The most widely used molecular typing methods are the DNA-based methods, such as plasmid profiling, restriction endonuclease analysis of plasmid and genomic DNA, Southern hybridization analysis using specific DNA probes, and chromosomal DNA profiling using either pulsed-field gel electrophoresis or polymerase chain reaction-based methods. The various molecular typing methods may be applied to the investigation of outbreaks of infections or may be used in the context of epidemiological surveillance. For outbreak investigation, typing methods are used to compare isolates from a suspected outbreak to delineate clonally related and unrelated strains with the goal of short-term control of transmission. In the context of epidemiological surveillance, molecular typing methods may be used to monitor geographic spread and prevalence shifts of epidemic and endemic clones with the goal of long-term evaluation of preventive strategies or for the detection and monitoring of emerging and re-emerging infections (Pfaller, 1999).

Investigators have used a variety of DNA-based methods to genotype microbial pathogens. All these methods use electric fields to separate DNA, either restriction endonuclease digestion fragments, amplified DNA fragments, or whole chromosomes or plasmids, into unique patterns or fingerprints that are visualized by staining the DNA with ethidium bromide or by nucleic acid hybridization. Advances in genomic analysis, such as automated nucleic acid sequencing and the use of DNA chip technology, offer the potential

for extremely sensitive, high throughput analysis of microorganisms that may be applicable to simultaneous detection and characterization of organisms in clinical material (Pfaller, 1999).

In 1996, the Centers for Disease Control and Prevention established the PulseNet National Database (USA), consisting of pulsed-field gel Electrophoresis (PFGE) patterns obtained from isolates of food-borne pathogens and textual information about the isolates. Though many molecular methods are available, macrorestriction analysis by PFGE has been shown to be particularly useful for the clustering and differentiation of many bacterial pathogens. Although the sensitivity and discriminatory power of PFGE depend on the organism being sub-typed and the restriction enzyme used, its high epidemiological relevance, has made this technique the “gold standard” for sub-typing food-borne bacterial pathogens.

Pulsed-field gel electrophoresis was first described in 1984 as a tool for examining the chromosomal DNA of eukaryotic organisms. Subsequently, PFGE has proven to be a highly effective molecular typing technique for many different bacterial species (Tenover *et al.*, 1995). In this method, the bacterial genome, typically 2000 to 5000 kb pairs in size, is digested with a restriction enzyme that has relatively few recognition sites generating approximately 10 to 30 restriction fragments ranging from 10 to 800 kb. The fragments can be resolved as a pattern of distinct bands using a contour-clamped homogeneous electric field (CHEF). The CHEF apparatus consists of an array of electrodes positioned around the gel (on a contour) and clamped to specific voltages to produce a nearly homogeneous electric field inside the contour. CHEF was developed to bypass two problems of the PFGE apparatus existing then, one being the inhomogeneous electric fields that caused the DNA molecules to migrate with curvilinear, arc-like, or even wavelike trajectories. The second was the non-linear shapes made it difficult to compare samples across the gel (Levene, 1992). The CHEF produces an electric field that causes DNA to wiggle through the gel and the alternation of pulses from the three sets of electrodes produces a back and forward movement that results in a higher level of fragment resolution.

All species are typable by PFGE, although the isolation of intact chromosomal DNA is technically difficult for some species. In general, PFGE is one of the most reproducible and highly discriminatory typing techniques available and is currently the typing method of choice for many species. The major difficulties associated with PFGE relate to the technical demands of the procedure and initial cost of the equipment. However, once the method is operational in

a laboratory, it can be applied readily to a wide range of species with only minimal modifications. The interpretation of PFGE is relatively straightforward, and consensus guidelines for correlating variations in restriction profiles with epidemiological relatedness were published (Tenover *et al.*, 1997). Patterns that are distinctly different from the outbreak patterns are considered unrelated types. Patterns that differ from the outbreak pattern by two or three fragment differences are considered to be subtypes of the outbreak pattern (Tenover *et al.*, 1995).

Objectives

The aim of this work was to characterize *Salmonella* spp. clinical isolates collected in two Portuguese hospitals from geographically distinct areas. Characterization was based on susceptibility of the isolates to a panel of common antimicrobials, and genetic profiling of a smaller group by PFGE DNA macrorestriction analysis. Correlation of resistance profiles with source of sample, sex and age of patient was assessed and how they relate with trends described by the EFSA for the time period of the sample collection was described. Comparison of PFGE patterns from the two areas was used to assess the clonality of *Salmonella* serovars.

Materials and Methods

Bacterial Strains

This study included 216 *Salmonella* isolates (35 *Salmonella* spp., 147 *S. Enteritidis* and 34 *S. Typhimurium*) collected and primarily identified in two hospitals, Hospital de São João – Porto (HSJ) and Hospital São Marcos – Braga (HSM). The isolates were collected in the period of 2002 to 2007 from HSM patients, and only in 2007 from HSJ patients; a great percentage of the isolates came from young patients (97 from 0 to 4 years, 95 from > 4 to 15 years). Most of the isolates were collected from faeces samples (197) and some (14) were collected from blood samples. The isolates were transferred from the hospitals and were stored in Tryptone Soy Broth (TSB – LabM, Lancashire, UK) with 30% glycerol at -20 °C. Prior to the testing the isolates were always streaked on Tryptone Soy Agar (TSA) and incubated at 37 °C for 24 h.

Analyzing the sample per hospital, the isolates retrieved from the HSJ hospital were in the majority identified only as *Salmonella* spp. whereas the isolates retrieved from HSM the majority was identified as serovar Enteritidis. Though the sample from HSJ is visibly smaller than from HSM the tendencies are a match. The distribution of the sample according to sex shows a higher number in male isolates than female; the majority of isolates are from children age 0-4 or 4-15; around 90% of both samples are isolated from faeces, isolates from blood are present in both samples; most isolates come from urgency and pediatric patients.

The presence of both serovars in blood samples is not unexpected as all *Salmonella* spp. can cause blood stream infections although the main invasive organisms are *S. enterica* serovar Typhi, Paratyphi, Choleraesuis and Dublin. The occurrence of blood stream infection caused by *Salmonella* spp. is more common in the elderly, the very young and in immunocompromised patients (Stephen et al, 2002). In our sample the blood isolates are evenly distributed in all age ranges, but because we don't have access to the full medical history of each patient we cannot state that it does not follow the expected trend.

Table 2 – Distribution of the 216 isolates per hospital, strain, date of isolation, sex and age of the patients, and source of sample, in percentage.

n (%)															
Hospital		Serovar		date of isolation		Sex of the patients		Age of the patients		Source of samples		Case			
HSJ	23 (10.6%)	S. Enteritidis	8 (34.8%)	2007	23 (100%)	Female	8 (34.8%)	0-4	13 (56.5%)	Faeces	20 (87.0%)	urgency	6 (26.1%)		
		S. Typhimurium	2 (8.7%)			Male	15 (65.2%)	>4-15	7 (30.4%)	Urine	1 (4.3%)	pediatric	10 (43.5%)		
		Salmonella spp.	13 (56.5%)					>15-65	3 (13.0%)	Blood	2 (8.7%)	ICU	2 (8.7%)		
												general	3 (13.0%)		
												infecto-contagious	1 (4.3%)		
												no info	1 (4.3%)		
HSM	193 (89.4%)	S. Enteritidis	140 (72.5%)	2002	6 (3.1%)	Female	92 (47.7%)	0-4	84 (43.5%)	Faeces	177 (91.7%)	urgency	76 (39.4%)		
		S. Typhimurium	31 (16.2%)	2003	50 (25.9%)	Male	101 (52.3%)	>4-15	88 (45.6%)	Urine	2 (1.0%)	pediatric	76 (39.4%)		
		Salmonella spp.	13 (11.4%)	2004	31 (16.1%)					>15-65	16 (8.3%)	Blood	12 (6.2%)	ICU	1 (0.5%)
				2005	50 (25.9%)					>65-90	5 (2.6%)	other	2 (1.0%)	general	9 (4.7%)
				2006	45 (23.3%)									gastroenterology	1 (0.5%)
				2007	11 (5.7%)									no info	30 (15.5%)

N=216

Antimicrobial susceptibility testing

The Minimal Inhibitory Concentration (MIC) of six antimicrobial agents: ampicillin (A), chloramphenicol (C), streptomycin (S), tetracycline (T) and nalidixic acid (Nx) were determined by the agar dilution method as described by the Clinical Laboratory Standards Institute (CLSI, 2007), and of trimethoprim/sulfamethoxazole determined by E-test (ABBIODISK/ BioMérieux, France).

The CLSI manual defines three categories for the results, as quoted:

Susceptible – this implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the recommended dosage is used for the site of infection;

Intermediate – this category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated or when a higher than normal dosage of a drug can be used.

Resistant – this category implies that isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or that demonstrate MICs that fall in the range where specific microbial resistance mechanisms are likely, and clinical efficacy of that agent against the isolate has not been reliably shown in treatment studies.

Table 3- MIC Interpretative standards according to CLSI/NCCLS manual.

	MIC ($\mu\text{g/ml}$)		
	S	I	R
Ampicillin	≤ 8	16	≥ 32
Chloramphenicol	≤ 8	16	≥ 32
Streptomycin	-	-	≥ 16
Tetracycline	≤ 4	8	≥ 16
nalidixic acid	≤ 16	-	≥ 32
Trimethoprim-sulfamethoxazole*	≤ 2	-	≥ 4

* for E-test was considered resistant if MIC ≥ 32

The isolates were streaked on TSA plates and incubated for 24 hours at 37 °C. Cell suspensions were prepared in sterile Ringer's solution (LabM) and adjusted to the 0.5 McFarland standard. The suspensions were then spotted, using a calibrated 1 μl loop, on

Mueller-Hinton agar (MHA; BioMerieux, Marcy l'Etoile, France) plates containing serial dilutions of all antibiotics, except ampicillin that was in Muller-Hinton agar cation-adjusted plates. The plates were incubated for 18 hours at 37 °C and the results were recorded in terms of the highest concentration with bacterial growth (CLSI/NCCLS, 2007).

The E-test was performed according to the manufacturer's instructions. The E-test strip contained a serial dilution of the antimicrobial; a single organism suspension was used to inoculate a Mueller-Hinton agar plate, on which the e-test strip was placed. The cultures were incubated at 37 °C for 18 hours. For all isolates the breakpoints used were those defined by the CLSI for Enterobacteriaceae (Table 2). *Staph. aureus* ATCC 29213 was used as the control strain.

Pulsed-field gel electrophoresis (PFGE)

Sixty five isolates were subjected to DNA macro-restriction analysis by pulsed-field gel electrophoresis, following *Xba*I digestion, according to the CDC PulseNet one-day standardized laboratory protocol for molecular subtyping of *E.coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* (PulseNet, 2004).

The *Salmonella* isolates were grown on Trypticase Soy Agar with 5% (v/v) defibrinated Sheep blood (Liofilchem, Teramo, Italy) at 37 °C overnight. A bacterial suspension was prepared in 3 ml of Cell Suspension Buffer (100 mM Tris: 100 mM EDTA pH8) using a sterile cotton swab, cell density was adjusted to 1.35 OD₆₁₀ nm in a helios spectrophotometer (Spectronic Unicam, Leeds, UK). 400 µl of adjusted suspension were transferred to a 2 ml microcentrifuge tube and 20 µl of Proteinase K (PK; BIORON GmbH, Ludwigshafen Germany) solution (20 mg/ml) was added, then mixed by pipetting up and down. A volume of 400 µl of melted 1% w/v SeaKem® Gold agarose (Lonza, Rockland, ME USA) 1% w/v Sodium Dodecyl Sulfate (Bio-Rad, CA USA) prepared in sterile TE buffer and maintained at 55 to 60 °C, was added to the cell suspension and mixed gently by pipetting up and down. The mixture was immediately dispensed into plug mold wells (sample reusable plug mold Bio-Rad) and allowed to solidify for 10 to 15 min at room temperature. The agarose plugs were transferred to 15 ml tubes containing 5 ml of Cell Lysis Buffer (50 mM Tris 50 mM EDTA pH8, 1% w/v Sarcosyl, 0.1mg/ml PK) and incubated at 54 °C for 2hours at 170 rpm. After proteolysis the CLB was removed and the plugs were washed twice with 15 ml of sterile ultrapure water at 50 °C (water bath) for 10 min each wash, followed by four washes with 15 ml of TE buffer at the same temperature for 15 min. After the TE wash, the plugs were sliced (2 mm: 6 mm), on a microscope glass slide using a sterile blade, and stored in 1.5 ml TE at 4 °C until restriction. The agarose embedded DNA plug slices were restricted with 50 U of *Xba*I (Fermentas, Ontario Canada) at 37 °C for 5 hours.

The digested DNA plug slices were separated by PFGE through 1% SeaKemGold agarose in 0.5X TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) at 14 °C using CHEF DR-III system (Bio-Rad Laboratories). The electrophoresis conditions used were as follows: initial switch time 2.2 s; final switch time, 63.8 s; voltage, 6 V; included angle, 120°; run time, 18 to 19 hours. Genomic DNA from *Salmonella* serotype Braenderup H9812 was also restricted with *Xba*I and used as a molecular size marker. After electrophoresis the gel was stained with ethidium bromide (40 µl of [1 mg/ml] in 400 ml of deionized water) for 15

min and washed twice for 30 min in water (PulseNet, 2004). The gel was then visualized and recorded using Gel-Doc 2000 (Bio-Rad laboratories) under UV transillumination. Band patterns were analyzed with Gelcompar software (Applied Maths, Kortrijk, Belgium) using Dice similarity coefficients with 1.5% band position tolerance. Isolates relatedness was determined by the unweighted-pair group method using average linkages (UPGMA).

Results

Antimicrobial susceptibility testing

The frequency of resistance and susceptibility of the isolates to individual antimicrobial agents is shown in Table 4. The highest resistance percentage observed was of 42% for nalidixic acid, resistance percentages of 20 to 25 were observed for ampicillin, tetracycline and streptomycin. Most of the isolates were sensitive to trimethoprim-sulfamethoxazole, chloramphenicol, streptomycin, tetracycline and ampicillin.

Table 4- Susceptibility to antibiotics of the *Salmonella* isolates tested.

Antibiotic	%S*	%I**	%R***
Ampicillin	73	1	25
Chloramphenicol	84	0	15
Streptomycin	79	0	20
Tetracycline	76	0	23
Nalidixic acid	52	0	42
Trimethoprim-sulfamethoxazole	95	0	3

*,Sensible; **, Intermediate; ***,Resistant

Of the isolates displaying resistance, 44% showed resistance to more than one antibiotic of which 42% were resistant to four and 27% to two. The resistance profiles found in the tested isolates are presented in Table 5. MIC50 and MIC90 values by serovar per year are presented in Table 6 and Figure4, 5, and 6.

Table 5- Frequency of *Salmonella* isolates resistance patterns by serovar.

S. Enteritidis (N=148)		S. Typhimurium (N=33)		Salmonella spp. (N=35)	
Resistance to	Isolates (%)	Resistance to	Isolates (%)	Resistance to	Isolates (%)
no resistance	67 (45.3%)	no resistance	1 (3.03%)	Te	1 (2.86%)
A	2 (1.35%)	A	2 (6.06%)	Nx	5 (14.3%)
C	1 (0.68 %)	Tmp	1 (3.03%)	ANx	1 (2.86%)
Nx	63 (42.6 %)	AS	2 (6.06%)	STe	1 (2.86%)
Te	2 (1.35 %)	ASTe	4 (12.1%)	SNx	1 (2.86%)
ANx	6 (4.05 %)	ANxTmp	1 (3.03%)	ACS	2 (5.71%)
SNx	1 (0.68 %)	ACSTe	15 (45.5%)	ASTe	1 (2.86%)
TeNx	2 (1.35 %)	ASTeNx	1 (3.03%)	ATeTmp	1 (2.86%)
TeTmp	1 (0.68 %)	ACSTeNx	5 (15.5%)	ACSTe	6 (17.1%)
ATeNx	1 (0.68 %)	ACSTeTmp	1 (3.03%)	ACSTeNx	1 (2.86%)
STeNxTmp	1 (0.68 %)			ATeNxTmp	2 (5.71%)
ACSTeNx	1 (0.68 %)				

A-ampicillin, C-chloramphenicol, S-streptomycin, Te-tetracycline, Nx- nalidixic acid, Tmp- trimethoprim/sulfamethoxazole,

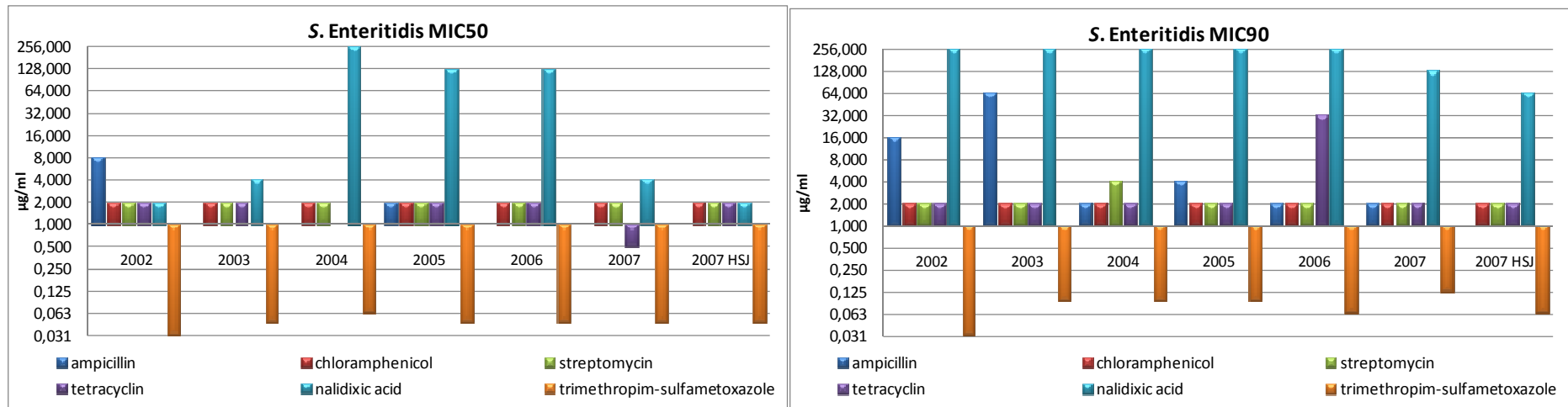


Figure 4 - Graphic representation of the MIC50 and MIC90 for *S. Enteritidis* per year.

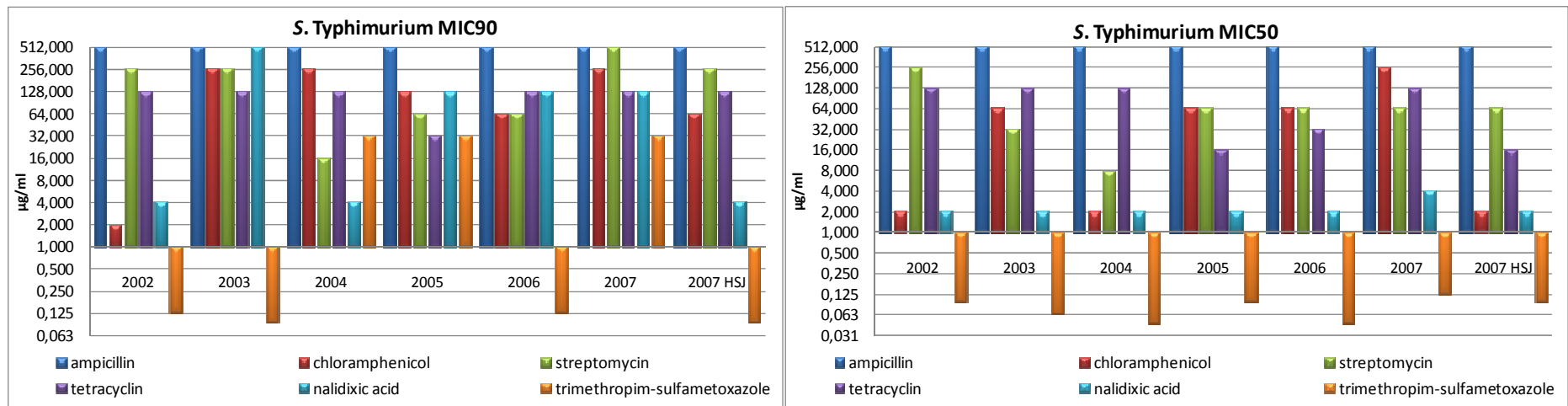


Figure 5 - Graphic representation of the MIC50 and MIC90 for *S. Typhimurium* per year.

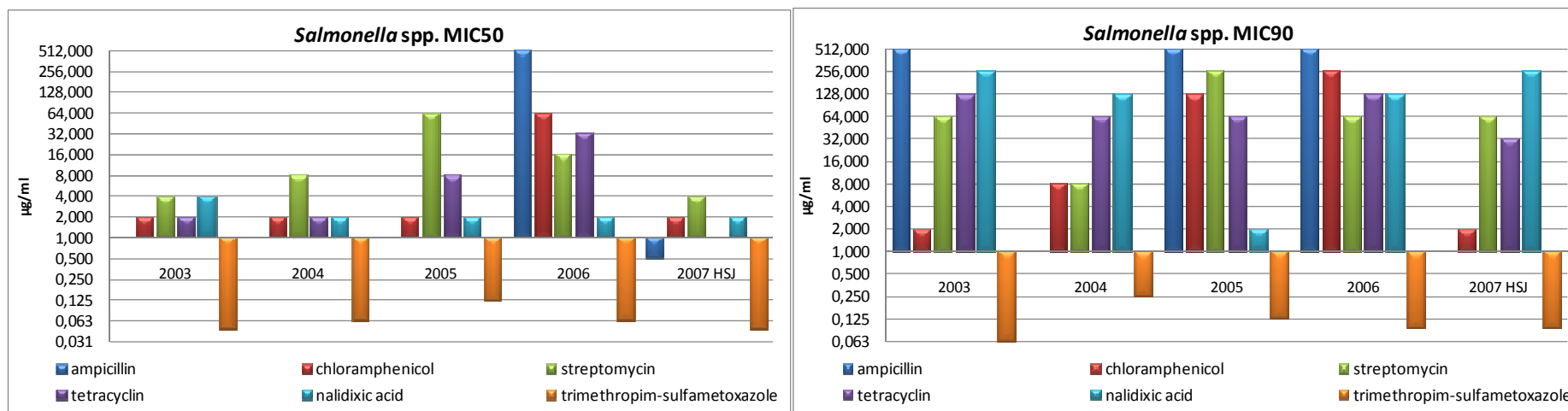


Figure 6 - Graphic representation of the MIC50 and MIC90 for *Salmonella* spp. per year.

For *S. Enteritidis* the MIC50 values for all the tested antimicrobials have in the majority maintained. The MIC50 for Nalidixic acid which have increased significantly from 2004 to 2006 has in 2007 decreased to the value observed in 2003. Values from 2007 for the HSJ isolates are similar to the ones observed for the HSM isolates. The MIC90 values for this serovar are mostly stable, ampicillin and tetracycline values oscillate more though the tendency is to decrease. The MIC90 values for Nalidixic acid have been fairly stable until 2006 and show a decrease in 2007.

For *S. Typhimurium* the MIC50 and MIC90 values for ampicillin are the same for all years. The values for the other antimicrobials show a steady increase. Trimethopim-sulphametoxazole MIC90 values show higher values for 2004, 2005 and 2007. The MIC values for HSJ isolates are mainly lower than for HSM isolates.

For *Salmonella* spp. the MIC50 and MIC 90 values are more difficult to analyze given that this is a diverse sample of isolates.

Pulsed-field gel electrophoresis (PFGE)

Clusters were defined using an 80% similarity index according to Foley *et al.* (2006). Using restriction enzyme XbaI, six clusters were found, A to E (fig. 7).

Dice (Opt:1.38%) (Tol 0.9%-0.9%) (H>0.0% S>0.0%) [0.0%-100.0%]

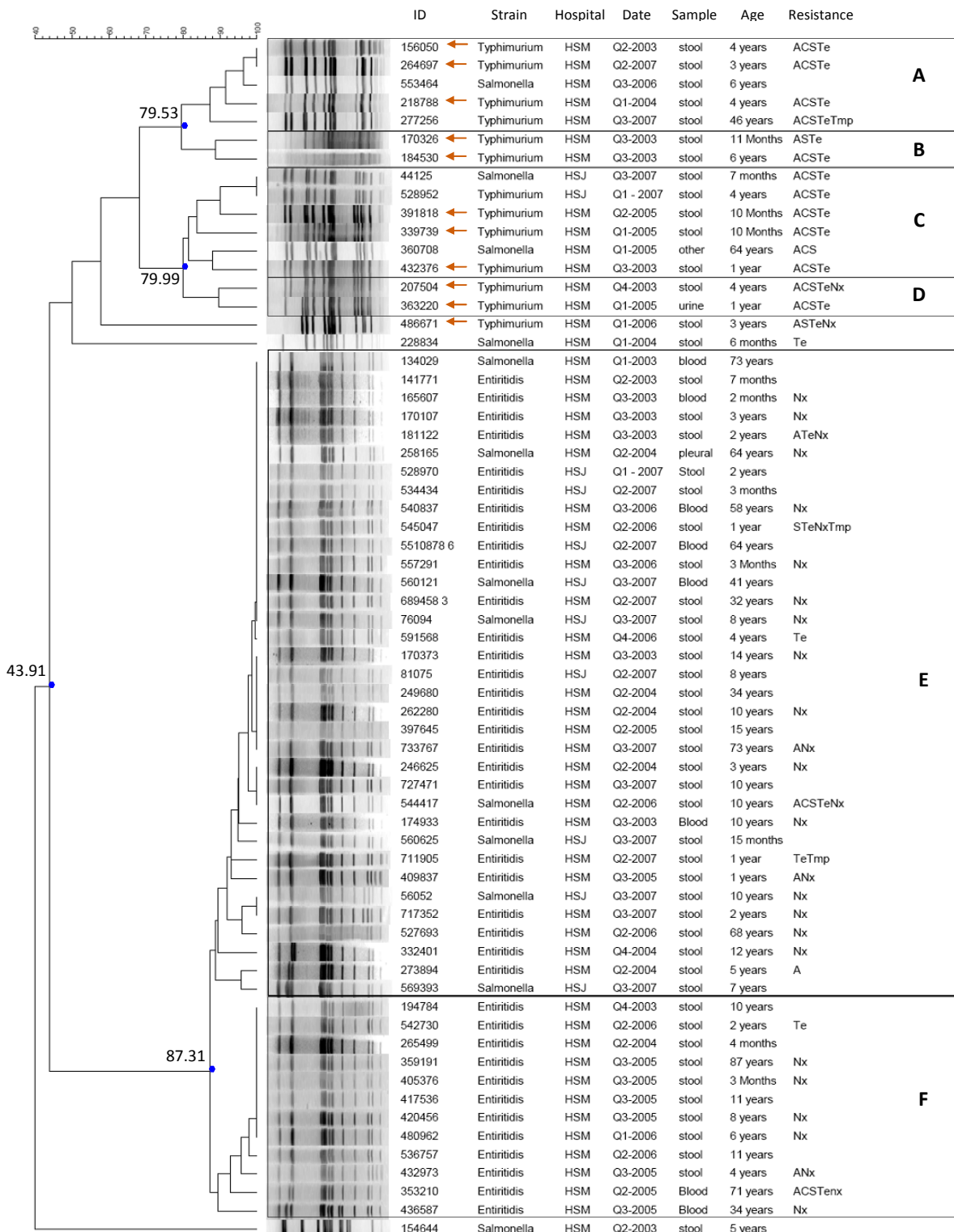


Figure 7 - Dendrogram of similarity illustrating the genetic relationships between the XbaI profiles using the Dice coefficient, and clustering by UPGMA. (←: isolates tested positive for sulphonamides resistance in Freixo *et al.*, 2011)

Discussion

Analyzing the frequency of resistance to 1 up to 5 antimicrobials, there is a noted difference between the tested serovars. *S. Enteritidis* isolates are either sensitive to all the antimicrobials tested (45.3%) or resistant to just one (45.98%), mainly Nalidixic acid. The frequency of multiresistant *Enteritidis* isolates is very low, 1.35% are resistant to four or more antimicrobials. On the other hand, *S. Typhimurium* shows opposite results, 63.4% of the *Typhimurium* isolates, in this study, are resistant to 4 or more antimicrobials. This is a higher number than the 39.7% reported by the EFSA for the year 2006, but no data from Portugal isolates was available and variability between countries is expected (EFSA,2010). The *Salmonella* spp. isolates show mixed results, the majority of these isolates are either sensitive to all the antimicrobials or secondly, resistant to 4 of the antimicrobials tested. Since these isolates were only identified as *Salmonella* spp., and no further strain identification was performed, some diversity is expected and therefore the results are diverse.

The analysis of the PFGE profiles of a small panel of the isolates resulted in six clusters when a cut-off of around 80% similarity is applied. Some of the samples only primarily identified as *Salmonella* spp. are distributed in *Enteritidis* and *Typhimurium* clusters as well as unique clones. This validates the assay demonstrating that the discriminatory power of the PFGE tool allows us to differentiate the two serotypes and further identify the samples. Also, isolate 154644 identified as *Salmonella* spp. was neither similar to *Enteritidis* or *Typhimurium* isolates (43.91% similarity), which can be seen as a control that the assay had no identification bias toward the other two serotypes. The samples from both hospitals are mixed indicating high similarity between isolates from the two areas (Figure 7).

Salmonella serovar *Enteritidis* shows a general predominant resistance to nalidixic acid (63 out of 81 resistant isolates). Multidrug-resistant isolates are rare and most are resistant to a second antimicrobial in combination with the resistance to nalidixic acid. The PFGE analysis shows two large groups (E and F) with high similarity (87.31%). Samples from HSJ are all present in cluster E. No differentiation of isolates with different resistance patterns was possible with the PFGE using the *Xba*I enzyme. Enforcing the evident need to use a different restriction approach. Other works with PFGE profiling of *S. Enteritidis* show the same difficulty in discrimination power, this can be overcome by the use of a second or third enzyme (Zheng *et al.*, 2007). For some of the isolates extra bands are visible, this can be explained by the acquisition of genetic material containing an extra *Xba*I restriction site or by point mutation. An explanation to the high similarity can be that the genetic variation may not impact the restriction fragments in a visible way with restriction using *Xba*I enzyme.

Salmonella serovar Typhimurium isolates are mainly resistant to two or more antimicrobials. The most frequent resistance profile is ACSTe counting for 45% of the isolates. The sample analyzed by PFGE profiling showed high diversity. The isolates are grouped in four clusters, A, B, C and D. Though the discrimination is higher than for the Enteritidis isolates, it is difficult to establish correlation between the isolates of the same group based solely on the isolation information and resistance profile to a small panel of antimicrobials. The different clusters do not present specific characteristics and the available information is fairly distributed. Isolate 486671 presents a very distinctive PFGE profile, with 58% similarity to the rest of the isolates. Also isolate 228834, identified as *Salmonella* spp. has a very distinct PFGE profile and is resistant only to Te, of the tested panel. The isolates marked with the orange arrow in figure 7 were also tested for sulphadiazine resistance in previous works in the laboratory, and were positive (Freixo *et al.*, 2011) thus displaying the resistance profile of *Salmonella* Typhimurium DT104. Two isolates present the ACSSUTE (ampicillin, chloramphenicol, streptomycin, sulphadiazine and tetracycline) resistance profile in combination to nalidixic acid resistance. The added resistance to a sixth antimicrobial is not uncommon for DT104 isolates and has been observed for gentamicin, trimethoprim and fluoroquinolones (Baggensen *et al.* 2000). The Typhimurium isolates from the different hospitals are similar; the only two isolates from HSJ are 100% similar in PFGE profile, resistance profile, isolation date and source and present 90% similarity to the rest of the isolates in the same cluster.

Conclusion

In this study, two hundred and sixteen isolates identified as *Salmonella* spp., *Salmonella* Enteritidis and Typhimurium, were tested for antimicrobial resistance against a panel of five antibiotics and a smaller panel was characterized using PFGE. Results showed that resistance to five antimicrobials is commonly found in Typhimurium isolates while Enteritidis shows a specific emergent resistance to nalidixic acid. High resistance is distributed in the isolates from the younger and older age groups (babies, children and the elderly), as has been described by the EFSA (2009). A panel of sixty-five isolates was subjected to PFGE analysis with *Xba*I restriction enzyme to assess the clonality. The analysis revealed that the discriminatory power of PFGE is higher for Typhimurium isolates than for Enteritidis. While PFGE is considered the “gold standard” molecular typing method, if genetic variation does not significantly impact the size or mobility of a specific restriction fragment during electrophoresis, then the change may not be identified as a separate pulsotype. This limitation can be overcome by the use of a second enzyme, at least, or even a different panel of restriction enzymes for the analysis. Though the discriminatory power was higher for Typhimurium isolates it is hard to elaborate on the clonality of the isolates since the available information is not sufficient to establish a correlation between isolates from the same cluster and between different clusters.

The two analyses performed with this set of isolates are only part of a more extensive characterization usually performed on outbreak isolates. To complete the information on this library of Portuguese clinical isolates, we would need access to epidemiological data for the two regions to establish correlations, and the availability of resources to extend the characterization and construct a database.

This work allowed the “Laboratório de Bactérias Lácticas e Pescado” of the Superior School of Biotechnology, of the Portuguese Catholic University, to implement and optimize the PFGE Standard Protocol used by the CDC and to further characterize the existing library of clinical isolates of *Salmonella*.

Future work

The antimicrobial susceptibility testing was limited to the antimicrobials available, more antimicrobials typically tested in Salmonella, like sulphonamides, could be used in further works.

The PFGE analysis of the isolates could be improved with the use of an additional panel of restriction enzymes to further differentiate the *S. Enteritidis* isolates. Also since only a small sample of the isolates were able to be tested by PFGE, this analysis could be applied to a larger sample in order to verify the tendencies observed.

Salmonella isolates are target of much analysis like phage typing and gene profiling for virulence and resistance, an interesting follow up would be to build a database for these isolates so that all the information collected in the ESB-UCP research projects could be searched and correlated easily. This could be a useful tool for matching new isolates with previously tested ones in order to assess the recurrence of these isolates in food quality control tests and clinical outbreaks.

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Annex

Table i- MIC50 and MIC90 values by serovar per year.

	HSM												HSJ		
	2002		2003		2004		2005		2006		2007		2007		
	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	
S. Enteritidis	A	8	16	1	64	1	2	2	4	1	2	1	2	1	1
	C	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	S	2	2	2	2	2	4	2	2	2	2	2	2	2	2
	Te	2	2	2	2	1	2	2	2	2	32	1	2	2	2
	Nx	2	256	4	256	256	256	128	256	128	256	4	128	2	64
	Tmp	0,032	0,032	0,047	0,094	0,064	0,094	0,047	0,094	0,047	0,064	0,047	0,125	0,047	0,064
S. Typhimurium	A	512	512	512	512	512	512	512	512	512	512	512	512	512	512
	C	2	2	64	256	2	256	64	128	64	64	256	256	2	64
	S	256	256	32	256	8	16	64	64	64	64	64	512	64	256
	Te	128	128	128	128	128	128	16	32	32	128	128	128	16	128
	Nx	2	4	2	512	2	4	2	128	2	128	4	128	2	4
	Tmp	0,094	0,125	0,064	0,094	0,047	>32	0,094	>32	0,047	0,125	0,125	>32	0,094	0,094
Salmonella spp.	A	-	-	1	512	1	1	1	512	512	512	-	-	1	1
	C	-	-	2	2	2	8	2	128	64	256	-	-	2	2
	S	-	-	4	64	8	8	64	256	16	64	-	-	4	64
	Te	-	-	2	128	2	64	8	64	32	128	-	-	1	32
	Nx	-	-	4	256	2	128	2	2	2	128	-	-	2	256
	Tmp	-	-	0,047	0,064	0,064	0,250	0,125	0,125	0,064	0,094	-	-	0,047	0,094

A-ampicillin, C-chloramphenicol, S-streptomycin, Te-tetracycline, Nx- nalidixic acid, Tmp- trimethoprim/sulfamethoxazole,

