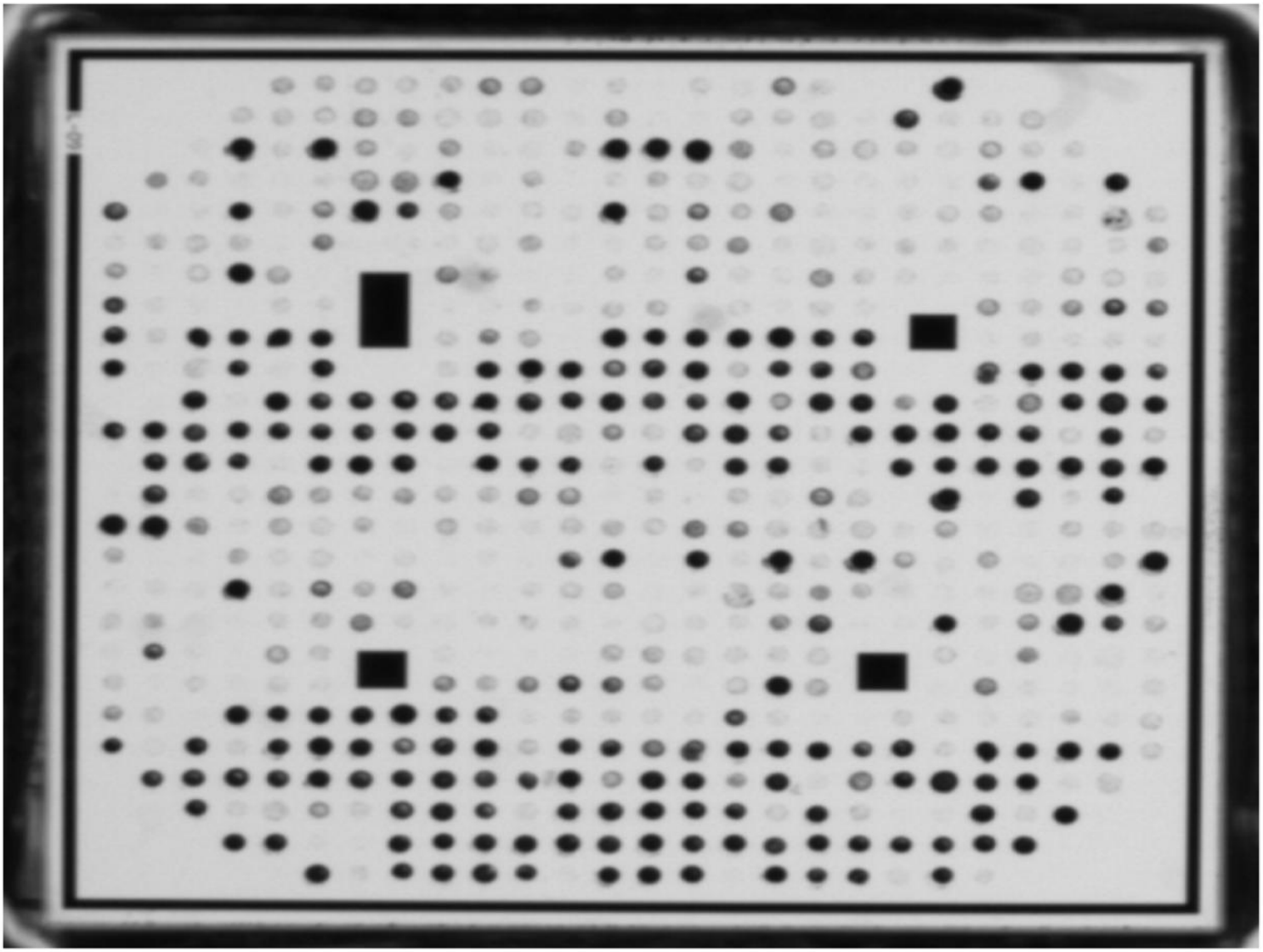

Rui Filipe Ramos Figueiredo

MICROARRAY-BASED DETECTION OF ANTIBIOTIC RESISTANCE AND VIRULENCE FACTORS GENES OF *SALMONELLA* SPP. ISOLATED FROM FOOD-PRODUCING ANIMALS AND PROCESSED FOOD

Tese de doutoramento em Ciências Farmacêuticas, na área de especialização em Microbiologia e Parasitologia, orientada pela Professora Doutora Gabriela Conceição Duarte Jorge da Silva, pelo Doutor Nuno Ricardo Furtado Dias Mendonça, e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro/2015

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FFUC FACULDADE DE FARMÁCIA
UNIVERSIDADE DE COIMBRA

Rui Filipe Ramos Figueiredo

Microarray-based detection of antibiotic resistance and virulence factor genes in *Salmonella* spp. isolated from food-producing animals and processed food

Dissertation presented to obtain a Ph.D. degree in Pharmaceutical Sciences, speciality Microbiology/Parasitology in University of Coimbra, Faculty of Pharmacy.

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A meus pais e meu irmão

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Abbreviations

AAC	Aminoglycoside Acetyltransferases
bla	β-Lactamase encoding gene
CAT	Chloramphenicol Acetyltransferases
CDT	Cytolethal Distending Toxin
CLSI	Clinical and Laboratory Standards Institute
DHFR	Dihydrofolate Reductase
DHPS	Dihyrdropteroate Synthetase
DNA	Deoxyribonucleic Acid
DT104	definitive phage type 104
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ESBLs	Extended-Spectrum β-Lactamases
EU	European Union
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
IS	Insertion Sequence
ISCR	Insertion Sequence Common Region
Lpf	Long-Polar Fimbriae
LPS	Lipopolysaccharide
MIC	Minimal Inhibitory Concentration
NTS	Non-Typhoidal *Salmonella*
ORFs	Open Reading Frames
PBP	Penicillin-Binding Proteins
Pef	Plasmid-encoded fimbriae
PCR	Polymerase Chain Reaction

QRDR	Quinolone Resistance-Determining Region
RT-PCR	Reverse transcriptase - Polymerase Chain Reaction
SCV	*Salmonella*-Containing Vacuole
SGI1	*Salmonella* Genomic Island 1
Sifs	*Salmonella*-Induced Filaments
SPIs	*Salmonella* Pathogenicity Islands
SPV	*Salmonella* Plasmid Virulence
USA	United States of America

Abstract

Salmonella enterica is a foodborne pathogen that infects human with a worldwide distribution. In some cases, *Salmonella* infection requires antimicrobial treatment. Antimicrobial resistance is a current problem around the world, and causes bacterial treatment failure. Few studies have been performed in Portugal of antimicrobial and heavy metal resistance as well as scarce *Salmonella* virulence studies were previously done in *Salmonella* of animal and food origin. This work aimed to study phenotypic and genotypic determinants of antimicrobial and heavy metals resistance, and to screen and characterize virulence genes. A new virulence microarray was designed and validated, and then tested in *Salmonella* panel of animal and food origin. A total of 14 serotypes were identified, from 258 isolates recovered, being *S.* Typhimurium (32.6%) and *S.* Enteritidis (10.1%) the most common. The majority of isolates (70.2%) were resistant at least to one class of antimicrobial agent and showed high frequency of resistance to tetracycline (47.7%) and ampicillin (36.0%). Resistance to fluoroquinolones was found in 8% of isolates. ESBL-producer *S.* Enteritidis strain carrying $bla_{\text{SHV-12}}$ gene on IncI conjugative plasmid was isolated from poultry. A subset of 106 *S. enterica* isolates were selected, that included *S.* Typhimurium, *S.* Enteritidis, and other antimicrobial resistant (AMR) strains belonging to less common serotypes. AMR profile in 106 *Salmonella* strains was assessed by AMR microarray. Phenotypic and genotypic AMR testing of this isolates subset detected high levels of resistance to tetracycline (65.1%) and ampicillin (58.8%), mainly due to presence of *tet*(B), $bla_{\text{TEM-type}}$ and $bla_{\text{PSE-type}}$. Heavy metal resistance and associated genetic elements were also detected by whole genome sequencing (WGS). Their transfer was confirmed by conjugation. Multidrug resistant *S.* Typhimurium Sal25, showing heavy metal resistance, carried $bla_{\text{CTX-M-1}}$ gene on a conjugative IncHI2 plasmid that also harboured

a numerous genes encoding resistance to tellurite, zinc, copper, cobalt, arsenic, mercury, silver, and cadmium. This work describes the first report of a *bla*$_{\text{CTX-M-1}}$ in *S.* Typhimurium in Portugal. AMR *S.* Typhimurium Sal368, showed for the first time in *Salmonella*, the presence of a *tet*(M) gene inserted within Tn*3* transposase in an IncF plasmid that also harboured *bla*$_{\text{TEM-1}}$ gene and resistance genes to arsenic, mercury, and silver. IncHI2 and IncF plasmids were transferred by conjugation to *Escherichia coli*. On virulence approach, the same 106 *Salmonella* strains were studied. *Salmonella* Pathogenicity islands (SPIs) and adherence genes were highly conserved, while prophages and virulence plasmid genes were variably present. Atypical microarray results lead to WGS of *S.* Infantis Sal147, which identified deletion of thirty-five SPI-1 genes. Adhesion/invasion studies were performed in human colon cells, HT-29, and Sal147 showed capacity to adhere but not to invade HT-29. Further studies performed *in vivo* in *Galleria mellonella* caterpillar showed reduced mortality in comparison to a SPI-1 harbouring *S.* Infantis. Microarray and WGS of *S.* Typhimurium Sal199 established for the first time in *S.* Typhimurium the presence of *cdtB* and other *S.* Typhi-related genes. Characterization of Sal199 showed *cdtB* genes were upstream of transposase *IS*911 and co-expressed with other Typhi-related genes. In HeLa cells infected by Sal199, morphological cells' assays showed cell cycle arrest, cytoplasmic distension and nuclear enlargement was detected, but not with *S.* Typhimurium LT2 control. Studies *in vivo* showed increased mortality of *G. mellonella* with Sal199 infection compared to LT2.

This work allowed to know that in case of human infection the antibiotherapy can be ineffective, and the *Salmonella* infection can be more severe. Identification of strains with high virulence potential and infectivity in the food at an early stage may help facilitate interventions reducing the risk of dissemination of epidemic strains. So,

tools like DNA microarray and WGS can be used to trace *Salmonella* outbreaks, and perform epidemiological analysis. These methods for *Salmonella* AMR and virulence studies can help assess the potential risk associated with certain *Salmonella* to humans.

Resumo

Salmonella enterica é uma bactéria patogénica de origem alimentar que infecta seres humanos pelo mundo inteiro. Nalguns casos, as infeções por *Salmonella* requerem tratamento com antibióticos. A resistência a agentes antimicrobianos é um problema global e leva ao insucesso do tratamento de infeções bacterianas. Alguns estudos têm sido realizados em Portugal na resistência a antimicrobianos e metais pesados em *Salmonella* de origem animal e alimentar. E poucos estudos de virulência têm sido efetuados até ao momento em Portugal. Este trabalho teve assim como objetivo o estudo fenotípico e genotípico de genes de resistência a antibióticos e metais pesados. Por outro lado, um novo DNA microarray foi desenhado e validado para avaliar a virulência de isolados de *Salmonella* spp. recolhidos de animais e alimentos de origem animal. Um total de 14 serótipos foi identificado, dos 258 isolados recolhidos, sendo *S.* Typhimurium (32.6%) e *S.* Enteritidis (10.1%) os mais comuns. A maioria dos isolados (70.2%) era resistente a pelo menos uma classe de antibióticos, apresentando uma maior frequência de resistência à tetraciclina (47.7%) e ampicilina (36.0%). A resistência às fluoroquinolonas foi verificada em 8% dos isolados. *S.* Enteritidis possuía ESBL $bla_{\text{SHV-12}}$ num plasmídeo conjugativo IncI. Um subgrupo de 106 isolados de *S. enterica* foi selecionado, que incluía *S.* Typhimurium, *S.* Enteritidis, e outros isolados resistentes a antibióticos pertencentes a serótipos menos comuns. O perfil genético de resistência a antibióticos foi efetuado com recurso a microarrays para genes de resistência a antibióticos. Verificou-se que 65.1% destes isolados apresentavam resistência à tetraciclina conferida por *tet*(B), e 58.8% à ampicilina, principalmente devido à presença de $bla_{\text{TEM-type}}$ e $bla_{\text{PSE-type}}$. A resistência a metais pesados foi detetada pela sequenciação completa do genoma, e estudos fenotípicos e sua capacidade de disseminação foi feita por conjugação com *Escherichia coli*. Um isolado

multirresistente *S.* Typhimurium Sal25, apresentava resistência aos metais pesados, e assim como a presença da ESBL *bla*$_{\text{CTX-M-1}}$ num plasmídeo conjugativo IncHI2, plasmídeo esse que possuía vários genes de resistência a metais pesados como telurite, zinco, cobre, cobalto, arsénio, mercúrio, prata e cádmio. Este trabalho descreveu pela primeira vez *S.* Typhimurium com *bla*$_{\text{CTX-M-1}}$ em Portugal. A estirpe *S.* Typhimurium Sal368 mostrou também pela primeira vez em *Salmonella*, a presença do gene *tet*(M) inserido na transposase Tn*3* num plasmídeo IncF que também albergava os genes de resistência a arsénio, mercúrio e prata. Os plasmídeos IncHI2 e IncF foram facilmente transferidos por conjugação para *E. coli*. Nos estudos de virulência, os mesmos 106 isolados foram estudados. Genes das ilhas de patogenicidade (SPI) e genes de adesão muito conservados enquanto genes de profágos e plasmídeos de virulência eram variáveis. Resultados atípicos de microarrays levaram à sequenciação do genoma do isolado *S.* Infantis Sal147, onde se identificou a deleção de 35 genes da SPI-1. Ensaios de adesão/invasão foram efetuados em células de cólon humano, HT-29, e o isolado mostrou capacidade para aderir mas não para entrar nas células HT-29. Estudos subsequentes efetuados *in vivo* em larvas de *Galleria mellonella* demonstraram reduzida capacidade de mortalidade em comparação com uma estirpe de *S.* Infantis com SPI-1 completa. A análise de microarray e da sequenciação do genoma de *S.* Typhimurium Sal199 demonstrou pela primeira vez a presença do gene *cdtB* e outros genes relacionados com *S.* Typhi, em *S.* Typhimurium. A caracterização de Sal199 mostrou a localização a montante de uma transposase IS*911* e a co-expressão de outros genes relacionados com *S.* Typhi. Em células HeLa infetadas com Sal199, verificou-se paragem de divisão celular, distensão do citoplasma, e enlargamento do núcleo, resultados não observados aquando da infeção pelas células com controlo *S.* Typhimurium LT2. Estudos *in vivo* mostraram um aumento na mortalidade de *G.*

mellonella infetadas com Sal199 em comparação com LT2. Em conclusão, este estudo permitiu uma primeira abordagem a *Salmonella* que podem infetar seres humanos. Em caso de infeção o tratamento poderia ser ineficaz, e as infeções por *Salmonella* poderiam ser mais severas. A identificação de estirpes com grande potencial de virulência em alimentos numa fase inicial facilita a intervenção precoce reduzindo o risco de disseminação de estirpes epidémicas. Assim, métodos como DNA microarrays e sequenciação completa do genoma podem ser usados em análises epidemiológicas e surtos de *Salmonella*. Assim, o uso de métodos de rastreamento de resistência a antibióticos e virulência podem ajudar a perceber os potenciais riscos associados a infeção de humanos por *Salmonella*.

Thesis outline

Salmonella enterica is a food borne pathogen that infects animals and humans worldwide. Antimicrobial resistance is of extreme importance leading to the bacterial infection treatment failure. The plasticity of *Salmonella* spp. genome could allow the emergence of strains more virulent. So, the main goal of this PhD thesis is the characterization of antimicrobial resistance and virulence among *Salmonella enterica* isolated from food-animal samples and food products. Heavy metal tolerance, usually used as additives or biocides, was also studied due to the hypothesis of co-selection of antimicrobial resistance determinants.

Chapter 1 is the introduction of the work and describes the state of art of *Salmonella* spp. epidemiology. Antimicrobial resistance (AMR) and heavy metal (HM) tolerance in *Salmonella* spp., and mechanisms associated were also described. Transmission of AMR and HM tolerance and mobile genetic elements were revised. Pathogenicity and virulence factors involved in host cell adherence, invasion and survival of *Salmonella* spp. are reviewed, as well *Salmonella* spp. genome comparison tools.
The objectives of this thesis were pointed out in this chapter too.

The findings to answer to the specific aims yielded 3 publications in peer review journals and one manuscript under submission. Such results have been organized with the help of the published papers, according of the follow rationale:

1. **Insights in the antimicrobial resistance of *S. enterica* isolated from food-producing animals and processed food.**

Chapter 2 and **3** reports of AMR phenotype of *Salmonella enterica* strains isolated in this work. Extended-Spectrum Beta-Lactamases detected are also characterized. Results were compared with to previous studies before the ban of antibiotic as growth promoter in animal feed, and data before the implementation of National Plan of *Salmonella* control in poultry.

Chapter 2

Figueiredo R, Henriques A, Sereno R, Mendonça N, da Silva GJ. 2013. Resistência a antibióticos em isolados de *Salmonella enterica* em alimentos de origem animal. Revista Portuguesa de Ciências Veterinárias. **108:** 39-43.

Chapter 3

Figueiredo R, Henriques A, Sereno R, Mendonça N, da Silva GJ. 2015. Antimicrobial Resistance and Extended-Spectrum β-Lactamases of *Salmonella enterica* Serotypes Isolated from Livestock and Processed Food in Portugal: An Update. Foodborne Pathogen Disease. **12:** 110-117.

2. **Characterization of AMR in selected 106 *S. enterica* isolated from food-producing-animals and processed food by microarrays and evaluation of heavy metal tolerance**

Chapter 4 reports antimicrobial and heavy metal resistance genes characterization by microarrays and whole genome sequence. Phenotypic evaluation of AMR and HM tolerance is also presented, as well their transmission to other bacteria.

Figueiredo R, Card R, Nunez J, Mendonça N, Anjum MF, da Silva GJ. 2015. Multidrug and heavy metal resistance in *Salmonella enterica* isolated from food-

producing animals and food products. **Submitted to Journal of Antimicrobial Chemotherapy**

3. Design of new microarray for evaluation of *S. enterica* virulence and evaluation of CDT toxin in *S*. Typhimurium

Chapter 5 describes a design and validation of a new DNA microarray for evaluation of virulence *S. enterica*. Microarray predictions of virulence differences between *Salmonella* spp. strains are explored using *in vitro* cells assays, and *in vivo* assays using *Galleria mellonella*.

Figueiredo R, Card R, Nunes C, AbuOun, Bagnall MC, Nunez J, Mendonça N, Anjum MF, da Silva GJ. 2015. Virulence characterization of *Salmonella enterica* by a new microarray: detection and evaluation of the cytolethal distending toxin gene activity in the unusual host *S.* Typhimurium. PlosOne **10(8)**:e0135010.

Final chapters **(Chapter 6, 7, 8, 9)** presents general discussion, general conclusions, future perspectives and supplementary date, respectively, of all results obtained during this PhD dissertation, as well several questions raised along this PhD thesis.

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Chapter 1

General Introduction

Salmonella enterica is one of the primary causes of bacterial food-borne infection in humans worldwide. Every year, thousands of cases of salmonellosis are reported all over the world. In the United States of America (USA), *S. enterica* produces the highest mortality among zoonotic food-borne disease, with an annual estimated number of more than 1.2 million illness, 23,000 hospitalizations, and 450 deaths (CDC, 2014), and has the highest cost burden (Batz *et al*., 2012). In Europe, infections caused by *S. enterica* are the second leading cause of bacterial food borne illness (EFSA & ECDC, 2014). In the European Union (EU), over 100,000 human cases are reported each year, corresponding to 45.1% hospitalizations of the confirmed salmonellosis (EFSA & ECDC, 2014). European Food Safety Authority (EFSA) has estimated that the overall economic burden of human salmonellosis could be high as 3 billion Euros a year. Due to the elevated costs and number of infection cases, a wide range of studies have been undertaken with the goal to understand *S. enterica* virulence and resistance mechanisms to antimicrobial agents. The molecular characterization of antimicrobial resistance and virulence-associated genes is important to identify *S. enterica* pathogenicity, to understand the potential transfer of resistance mechanisms, and to develop an accurate and fast detection method to help in disease control. The development of a DNA array-based assay for the rapid and parallel detection of antimicrobial resistance genes and virulence factors in *Salmonella* spp. is of crucial importance. This will improve the knowledge of the phenotypic antimicrobial resistance, as well genetic factors associated to antimicrobial resistance and virulence of strains isolated from food producing animals and processed food.

1.1. *Salmonella* spp. taxonomy and serological classification

The genus *Salmonella* was first identified as a distinct group of pathogens by Theobald Smith, stemming from his work on the etiologic agent of hog cholera. The genus is named after Daniel Salmon, at the time Smith's supervisor in the Veterinary Division at the U. S. Department of Agriculture, who first reported the discovery in 1886 (Sanderson & Nair, 2013). However, it was Smith who carried out the work while at the Department of Agriculture, in an early application of Koch's postulates. *Salmonella* is a rod-shaped, Gram-negative bacteria member of the *Enterobacteriaceae* family, in the Gamma-proteobacteria subdivision (Sanderson & Nair, 2013). Two species are currently recognized, *S. bongori* and *S. enterica*. *S. enterica* is subdivided in six subspecies (subsp.), being *S. enterica* subsp. *enterica* (designated subspecies I), associated to 99% of all human infections. According to the White-Kauffman-Le Minor scheme (Grimont & Weill, 2007), the subspecies are further divided into serotypes by serological testing with monovalent antisera. Serotyping is widely used as an epidemiological and standardised typing method for the genus *Salmonella*. There are more than 2.600 serotypes described for *S. enterica* based on scheme White-Kauffmann-Le Minor (Issenhuth-Jeanjean *et al*., 2014). Each antigenically distinguishable *Salmonella* possesses a specific O (cell wall) and H (flagellar) antigen; many express alternate phase flagella of two antigenic types (H1 and H2), and a few produce Vi (capsular) antigen (Sanderson & Nair, 2013).

1.2. Epidemiology

The genus *Salmonella* is widely distributed in nature, colonizing a range of warm and cool-blooded animals, including mammals, amphibians, reptiles, birds, and insects. Human infection is mainly caused by ingestion of contaminated food and direct

contact with animals (Hoelzer *et al*., 2011; EFSA & ECDC, 2014). In industrialized countries, food animals are the main source for human infections, the majority of which originate from contaminated meat products and eggs (Table 1.1.). Other food vehicles include unpasteurized milk and cheese, fish, shell-fish, fresh fruits and juice, spices, chocolate, and vegetables (McDermott, 2006). Host adaptations vary among *S. enterica* serotypes and affect the pathogenicity for man and animals. Ubiquitous serotypes, such as *S. enterica* serotype Enteritidis (hereafter *S.* Enteritidis) and *S. enterica* serotype Typhimurium, (hereafter *S.* Typhimurium) which infect both man and animals, generally cause gastrointestinal infections with less severity than *S.* Typhi, causing septic typhoid syndrome. *S.* Enteritidis, mainly associated with poultry derived products, and *S.* Typhimurium related to poultry, swine and cattle, are the most common serotypes in European countries associated with human disease, representing 63.4% of all serotypes (Coburn *et al.*, 2007; EFSA & ECDC, 2014), and are responsible for 79% of all human infections worldwide (WHO, 2006). Other frequently isolated *S. enterica* serotypes in European countries are Typhimurium monophasic 1,4,[5],12:i:-, Infantis, Stanley, Thompson, Newport, Derby, Panama, and Kentucky (EFSA & ECDC, 2014). Serotypes adapted to man, such as Typhi, usually cause severe systemic disease in humans. Others serotypes are highly adapted to animal hosts, such as Gallinarium (poultry) (Wu *et al*., 2005) or Abortusovis (sheep) (Pardon *et al*., 1990) and may only produce very mild symptoms in humans. However, serotype Cholerasuis, which its primary host is swine, also causes severe systemic illness in man. Similarly, serotype Dublin, which has a preference for cattle as the host, causes systemic salmonellosis in humans.

Table 1.1. Occurrence of *S. enterica* in selected food categories in the European Union in 2009-2012.

Food Category	2009			2010			2011			2012		
	MSs[a]	*N*	Positive (%)	MSs	*N*	Positive (%)	MSs	*n*	Positive (%)	MSs	*n*	Positive (%)
Poultry meat, fresh	19	26 591	5.4	19	21 539	4.8	19	25 611	5.9	19	51 093	4.1
Turkey meat, fresh	9	3 953	8.7	11	4 329	9.0	9	4 400	4.8	11	6 412	4.4
Swine, fresh	18	83 797	0.7	17	69 005	0.9	-	-	-	19	85 000	0.7
Beef, fresh	15	44 359	0.2	15	34 236	0.2	19	25 497	0.3	17	47 279	0.2
Table eggs	14	15 966	0.5	13	19 142	0.3	-	-	-	16	18 843	0.1
Fruit, vegetables, and herbs	18	13 466	0.6	15	8 312	0.6	20	11 998	0.3	21	12 841	0.7

[a]Member states

(Adapted from EFSA & ECDC, 2011, 2012; 2013, 2014)

The prevalence of *S. enterica* in food-producing animals differs widely amongst the species; there is also a difference in the prevalence of serotypes between countries. In Portugal, a recent report by *Salmonella* National Control Programme in food-producing animals and food of animal origin identified three main serotypes in live poultry: *S.* Enteritidis (32.8%), Havana (18.3%) and Mbandaka (16.5%). From swine samples, the serotypes Rissen and Typhimurium (21.8%), Derby and London (10.9%), were the most common. Considering food products per animal group (swine, poultry and bovine), serotype 1,4,[5],12:i:-, as the most common serotype recovered from food products of swine (32.6%) and bovine (30%) origin, followed by *S.* Rissen (26% in swine and 24% in bovine) and *S.* Typhimurium (18.2% in swine and 19% in bovine). In poultry products, *S.* Enteritidis was the most frequently recovered serotype (62.7%), followed by *S.* Mbandaka (10.2%) and *S.* Derby (8.5%) (Clemente *et al*., 2013).

1.3. Salmonellosis

S. enterica can colonize the gastrointestinal lumen asymptomatically, but in the majority of infections cause a number of different diseases, depending of serotype. The most common mode of *S. enterica* infection is acute gastroenteritis, mostly associated to non-Typhi *Salmonella*. The incubation period may vary from 4 to 72 h after the ingestion of contaminated food or water. However, the severity of infections differ according to serotype, strain, infectious dose, properties of the contaminated food (e.g. fat content) and the host's state of health. Symptoms are acute onset of diarrhoea, usually self-limiting, lasting 3–7 days, nausea and vomiting, abdominal cramping, and sometimes chills and fever (Hohmann, 2001; Foley & Lynne, 2008). Bacteraemia occurs in 5–10% of infected persons, and some of them may progress to have focal infection, such as meningitis, and bone and joint infection (Chen *et al*., 2013).

1.4. Antimicrobial therapy and antimicrobial resistance

Antimicrobial therapy is usually not required in acute gastroenteritis caused by non-typhoid *S. enterica,* which is usually a self-limiting disease, and treatment should consist mainly of fluid/electrolyte replacement. A meta-analysis demonstrated that there is no evidence of any clinical benefit of antibiotic therapy in healthy children and adults with non-severe *S. enterica* gastroenteritis. However, while the risk of developing bacteraemia is low (< 5% of all patients), certain patients, such as young infants < 3 months old, patients suspected extra-intestinal infection, immunocompromised patients, and patients with severe colitis, would benefit from empirical antibiotic treatment (Hohmann, 2001). The proportion of *S. enterica* infections caused by antimicrobial-resistant strains has steadily increased around the world and represents 20 to 40% of all isolates from human infections (McDermott, 2006).

If bacteraemia or an extra intestinal focal infection is confirmed, the choice of the antimicrobials would depend on the susceptibility pattern of the strain and the clinical condition. Options may include ampicillin, trimethoprim–sulfamethoxazole, fluoroquinolones or third-generation cephalosporins, such as ceftriaxone, depending on the results of *in vitro* susceptibility testing (Hohmann, 2001; Chen *et al*., 2013).

The contribution of agricultural antimicrobial use to resistance in food-borne pathogens is a long-standing controversy, but nowadays there is a consensus in their contribution towards pathogens acquiring resistance. Antimicrobials have been used in livestock and poultry since the early 1950s to improve feed efficacy, prevent disease, and treat infections. The animal production industry relies heavily on these agents to protect animal health and believes that they are needed to provide cheap, high-quality products. While it is accepted that drug use selects for resistant pathogens in the animal environment, and that these resistant variants are transmitted to humans via the food

supply, there is a great deal of debate on the magnitude of the public health impact (McDermott, 2006). The use of antimicrobial agents as growth promoters have been totally banned from EU countries since 2006 (Guardabassi & Courvalin, 2006). Resistance genes against antimicrobial agents that are or have only been used in animals were soon found after their introduction not only in bacteria isolated from animals, but also in zoonotic pathogens such as *Escherichia coli* that inhabit the human flora , and in strictly human pathogens, such as *Shigella* (Rashid & Rahman, 2014). Therefore, not only may the clonal spread of resistant strains occur, but there is also a transfer of resistance genes via zoonotic bacteria between humans and animals (Angulo *et al*., 1994). Several antimicrobial agents are described so far, as well their resistance mechanisms.

Antimicrobial resistance in non-typhoid *Salmonella* serotypes is a global problem. Surveillance data demonstrate an obvious increase in overall antimicrobial resistance among salmonellae from 20–30% in the early 1990s to as high as 70% in some countries at the turn of the century (Su *et al*., 2004). The resistance rate, however, varies with different serotypes and different antimicrobials. *S.* Enteritidis, one of the most prevalent *S. enterica* serotypes, is relatively more susceptible to antimicrobial agents than are other serotypes. A much higher rate of resistance is found in *S.* Typhimurium, a globally prevalent serotype (Su *et al*., 2004).

1.4.1. Resistance to tetracycline

Tetracycline is clinically known as broad spectrum antibiotic and is effective against many Gram-positive and -negative bacteria (Chopra & Roberts, 2001). Tetracycline mostly act by stopping the binding of tRNA to the A site of the 30S rRNA, causing misreading of the mRNA code or inhibition of the initiation step of protein synthesis (Chopra & Roberts, 2001). Although more than 40 different tetracycline

resistance genes have been described, only five of them – *tet*(A), *tet*(B), *tet*(C), *tet*(D), and *tet*(G) – have been described in *Salmonella* spp. (Michael *et al*., 2013). All these *tet* genes encode an efflux protein, and *tet*(G) has exclusively been detected as a component of the Genomic Island 1 (SGI-1) or 2 (SGI-2)-associated multi-resistance gene clusters (Chopra & Roberts, 2001; Hall, 2010). The *tet*(A) and *tet*(B) genes are associated with transposons Tn*1721* and Tn*10*, respectively. Tn*10* is widespread among different *S. enterica* serotypes, and truncated copies of both transposons have been identified on large multi-resistance plasmids, and plasmids conferring tetracycline resistance alone, or with others antimicrobial agents (Michael *et al*., 2013). In rare cases, more than one *tet* gene is harboured by the same isolate (Michael *et al*., 2006).

1.4.2. Resistance to β-Lactams

This family group comprise penicillin derivatives, cephalosporins, carbapenems and monobactams. Their mechanism of action is by blocking transpeptidation of peptidoglycan strands by covalently binding to one or more of the cell wall transpeptidases, named penicillin-binding-proteins (PBPs) (Guardabassi & Courvalin, 2006). β-lactams are considered bactericidal. In *S. enterica*, resistance to β-lactams is mainly mediated by β-lactamase enzymes (*bla*). There are described more than 340 β-lactamases resistance genes, such as bla_{TEM}, bla_{OXA}, bla_{PER}, bla_{PSE}, bla_{SHV}, $bla_{\text{CTX-M}}$, and bla_{CMY} (Armand-Lefèvre *et al.*, 2003; Michael *et al*., 2013). These enzymes act by hydrolysing the structural rings of the β-lactam, inactivating it. Ambler classification of β-lactamases divides β-lactamases into four classes (A, B, C and D) based upon their amino acid sequences. In *Salmonella* sp., the class A β-lactamases are most commonly found. They provide a range of resistance against penicillins, cephalosporins, and carbapenems. There are many more different gene families encoding for enzymes in this

class, being TEM-type being the most common among *S. enterica* (Michael *et al*., 2013). The genes *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{TEM-52}}$ have been found in many *S. enterica* serotypes including Enteritidis, Dublin, Haadrt, Muenchen, Panama, and Typhimurium (Chen *et al.*, 2004, Gebreyes & Thakur, 2005). The *bla*$_{\text{KPC-2}}$ is a class A β-lactamase gene, which is more active against carbapenems, and was reported in *S.* Cubana (Miriagou *et al*., 2003). The emergence of another class A β-lactamases, known as cefotaximases (*bla*$_{\text{CTX-M}}$), which confer resistance to ampicillin and cephalosporins, including the broad-spectrum cephalosporins, are of clinical importance (Batchelor *et al*., 2005). A wide range of *bla*$_{\text{CTX-M}}$, all of which are extended-spectrum β-lactamases (ESBLs) has been reported in *S. enterica*. ESBLs are β-lactamases conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by β-lactamase inhibitors such as clavulanic acid (Michael *et al*., 2013). This limits the choice of β-lactam use, and carbapenems become the treatment of choice for serious infections due to ESBL-producing organisms. In *S. enterica*, the genes coding for *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-3}}$, *bla*$_{\text{CTX-M-4}}$, *bla*$_{\text{CTX-M-5}}$, *bla*$_{\text{CTX-M-6}}$, *bla*$_{\text{CTX-M-7}}$, *bla*$_{\text{CTX-M-9}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-17}}$, or *bla*$_{\text{CTX-M-18}}$, *bla*$_{\text{CTX-M-27}}$, *bla*$_{\text{CTX-M-28}}$, *bla*$_{\text{CTX-M-32}}$, *bla*$_{\text{CTX-M-53}}$ (Batchelor *et al*., 2005; Doublet *et al*., 2009; Antunes *et al*., 2013) and *bla*$_{\text{CTX-M-83}}$, *bla*$_{\text{CTX-M-86}}$ (Cui *et al*., 2009), has been reported.

The class C β-lactamases, is the second most common class found in *Salmonella*, and provides resistance against all β-lactams except carbapenems, and are encoded by chromosomal *ampC* genes. AmpC β-lactamases, such as *bla*$_{\text{CMY}}$, *bla*$_{\text{AAC}}$ and *bla*$_{\text{DHA}}$, have been reported in *Salmonella*. *bla*$_{\text{CMY-2,}}$ *bla*$_{\text{CMY-4,}}$ and *bla*$_{\text{CMY-7}}$ genes have been described in *S. enterica*, with *bla*$_{\text{CMY-2}}$ being the most common (Michael *et al*., 2013). Only a few

allelic variants of *bla*$_{ACC-1}$, *bla*$_{SCO-1}$, and *bla*$_{DHA-1}$ genes are reported in *S. enterica* (Ktari *et al*., 2009; Michael *et al*., 2013).

The Class B β-lactams like metallo-β-lactamases, provides resistance to all β-lactam agents, including carbapenems (e.g. imipenem) and are usually chromosomally encoded. The class B β-lactamases are not commonly found in *Salmonella*.

Class D β-lactamases are uncommon among *Salmonella*. This class of enzymes provides resistance to β-lactams closely related to oxacillin, such as cloxacillin and methicillin. The *bla*$_{OXA-1}$ gene has been found in serotype Paratyphi (Cabrera *et al*., 2004) and *bla*$_{OXA-30}$ has been found in *S.* Muenchen and Typhimurium (Hanson *et al.*, 2002; Antunes *et al.*, 2004).

1.4.3. Resistance to fluoroquinolones

There are many generations of quinolones, and their mode of action is by targeting mainly DNA gyrase A and B and DNA topoisomerase IV (Michael *et al.*, 2013). *Salmonella* resistance to quinolone has been classified into two mechanisms. The first is the targeting mutations in the quinolone resistance-determining region (QRDR) of two *gyrA* and *gyrB* genes, which encode for the subunits of DNA gyrase, and/or in *parC* and/or *parE* subunit of topoisomerase IV (Casin *et al*., 2003; Baucheron *et al*., 2004; Levy *et al*., 2004). Single or double mutations resulting in amino acid substitution occurs at Ser83 (to Phe or Tyr) and Asp87 (to Asn, Tyr, or Gly) in *gyrA* and at Ser80 (to Arg) and Thr57 (to Ser) in *parC*. A single QRDR mutation, usually at Ser83, confers resistance to nalidixic acid and decreases susceptibility to fluoroquinolones, whereas alterations in ParE are rarely observed in *Salmonella* spp. (McDermott, 2006; Michael *et al*., 2013; Abgottspon *et al*., 2014). A second mode of resistance to quinolones involves changes in expression of the AcrAB-TolC efflux system, as a result of mutations in the regulator

genes of this system, that leads to over expression of this efflux system (Koutsolioutsou *et al*., 2001; Levy *et al*., 2004; Olliver *et al*., 2005), which decreases quinolone susceptibility. However, it is an accumulation of all these mutations that results in resistance, as not one mutation alone will confer high-level resistance to quinolones (Heisig, 1993). In some bacterial organism, such as *E. coli* and *Klebsiella pneumoniae* (Wang *et al*., 2004), the expression of a plasmid mediated gene called *qnr* has also been linked to quinolone resistance (Li, 2005). Several *qnr* genes, such as *qnrA1*, *qnrA2*, *qnrB4*, *qnrB5*, *qnrB6*, *qnrB12*, *qnrB19*, *qnrS1*, *qnrS4*, and *qnrD* have been described in *S. enterica* (Torpdahl *et al*., 2009; Sjölund-Karlsson *et al*., 2010; Veldman *et al*., 2011). The studies conducted on plasmids harbouring *qnr* showed that it could be transferred via conjugation from other bacterial species to *S. enterica* (Martinez *et al*., 2005).

1.4.4. Resistance to aminoglycosides

Aminoglycosides are active and effective against Gram-negative bacteria. They are bactericidal drugs targeting the ribosome, binding irreversibly to 16S rRNA subunit (Michael *et al.*, 2013), leading to protein synthesis inhibition. There are three mechanisms by which bacteria become resistant to aminoglycosides: by reducing antibiotic uptake or decreased permeability; by alteration of the ribosomal binding sites; and by antibiotic modification (Schwarz *et al*., 2006).

According to Shaw *et al*. (1993), there are three groups which are named on the reactions they perform; this includes acetyltransferases, phosphotransferases, and nucleotidyltransferases. Aminoglycoside acetyltransferases (AAC) catalyze acetyl CoA-dependent acetylation of an amino group (Schwarz *et al*., 2006). Many *aac* genes have been found in several *S. enterica* serotypes, including Agona, Typhimurium, Newport, Typhimurium var. Copenhagen, Kentucky, and 4,5,12:i:- (Chen *et al*., 2004; Doublet *et*

al., 2004; Levings *et al.*, 2005). The *aac* genes have been found as part of *Salmonella* genomic islands (Doublet *et al.*, 2004), integrons (Pai *et al*., 2003; Levings *et al.*, 2005), and plasmids (Guerra *et al.*, 2001). AACs provide resistance to tobramycin, gentamicin, and kanamycin (Schwarz *et al*., 2006; Michael *et al*., 2013). Aminoglycoside phosphotransferases, catalyses ATP-dependent phosphorylation of a hydroxyl group (Michael *et al*., 2013). This allows classification into groups depending on the specific sites of phosphorylation. Groups APH (3") and APH (6), provide resistance to streptomycin (Michael *et al*., 2013) and have been found encoded on plasmids harboured by *S. enterica* (Gebreyes & Altier, 2002). Genes *aph* (3")-*Ib* and *aph* (6)-*Id*, are commonly known as *strA* and *strB*, respectively (Shaw *et al*., 1993; Madsen *et al*., 2000). *S. enterica* serotypes Blockely, Bredeney, Agona, Anatum, Derby, Give, London, Saintpaul, Hadar, Heidelberg, and Typhimurium have been found to possess genes from both families (Madsen *et al*., 2000; Pezzella *et al*., 2004). Genes encoding enzymes of the APH (3') subgroup provide resistance to kanamycin and neomycin (Michael *et al*., 2013), and have been found in several *S. enterica* serotypes such as Enteritidis, Haardt, Derby (Chen *et al*., 2004), Typhimurium (Gebreyes & Altier, 2002), and Typhimurium var. Copenhagen (Frech *et al*., 2003). The nucleotidyl transferase is the final group of enzymes providing aminoglycoside resistance (Shaw *et al*., 1993; Michael *et al*., 2013). These enzymes are divided into several groups based on the site of modification and also target the hydroxyl groups. Genes encoding these enzymes are usually designated *aad* (Vanhoof *et al.*, 1998) and some as *ant*. The gene *aadA* is referred to as *ant* (3") (Shaw *et al*., 1993), is found in *Salmonella*, providing resistance for streptomycin (Michael *et al*., 2013). Also, many of these variants genes have been found in serotypes Bredeney, Derby, Agona, Anatum, Enteritidis, Give, Heidelberg, Saint Paul, and Typhimurium (Madsen *et al*., 2000; Chen *et al*., 2004; Pezzella *et al*., 2004). The *aadB* gene, also known as *ant*(2')-*Ia*,

confers resistance to tobramycin and gentamicin (Shaw *et al*., 1993). It has been found in serotypes Typhimurium and Typhimurium var Copenhagen (Frech *et al*., 2003; Antunes *et al*., 2005). Both *aadA* and *aadB* have been found in integron-borne gene cassettes (Winokur *et al*., 2001; Pezzella *et al*., 2004).

1.4.5. Resistance to phenicols

Chloramphenicol is a potent inhibitor of protein synthesis; it binds to the peptidyltransferase centre of the 50s ribosomal unit, preventing formation of peptide bonds. As a result of the binding to enzymes, the drugs will prevent elongation of the peptides. Chloramphenicol is a broad-spectrum antibiotic against both Gram negative and Gram positive bacteria and its effectiveness and ability to cross the blood-brain barrier made it a drug of choice for systematic infections therapy. Chloramphenicol was used in human and veterinary medicine for the treatment of salmonellosis for a long time. In Europe and USA, the chloramphenicol use is prohibited due to the risks associated with the presence of drug residues in food. The most serious adverse effect of the drug is the damage in bone marrow and aplastic anaemia. There are two mechanisms in *S. enterica* conferring resistance to chloramphenicol: (i) by plasmid-located enzymes called chloramphenicol acetyltransferases (CAT) and (ii) by efflux pump encoded by *cmlA* and *floR* genes through which the antibiotic is removed (Schwarz *et al*., 2004). Two *cat* genes, such as *catA1* and *catA2*, have also been found in *S.* Derby, Haardy, Enteritidis, and Typhimurium (Chen *et al.*, 2004). They are located in gene cassettes mainly in class 1 integrons. The *cmlA* and *floR* are closely related genes that have been reported in *S. enterica*, encoding chloramphenicol efflux pumps (White *et al*., 2001; Cabrera *et al*., 2004). Also, *floR* gene appear to be very widespread in *S.* Agona, Kiambo, Albany, Newport, Typhimurium, Typhimurium var Copenhagen (Meunier *et al*., 2003; Cabrera *et*

al., 2004; Doublet *et al*., 2004; Alcaine *et al*., 2005), whereas *cmlA* is less widely distributed. The *floR* gene has been found in *Salmonella* genomic islands and is closely associated with multi-drug resistance, most likely due to its presence on plasmids carrying multiple resistance genes (Meunier *et al*., 2003; Doublet *et al*., 2004; Alcaine *et al*., 2005; Weill *et al*., 2005).

1.4.6. Resistance to inhibitors of folate synthesis

This class of antimicrobial agents inhibit enzymes involved in the synthesis of folic acid. Folic acid is an essential precursor in nucleic acid synthesis, and bacteria cannot utilize exogenous folic acid. Sulphonamides and trimethoprim act on two successive biochemical steps in the synthesis of folic acid and are usually used in combination. Individually they have a bacteriostatic effect but when employed together they show a bactericidal action. Sulphonamides inhibit dihyrdropteroate synthetase (DHPS), while trimethoprim inhibits dihydrofolate reductase (DHFR) (Neu & Gootz, 1996). The resistance of *S. enterica* to sulphonamide has been attributed to the presence of an extra *sul* gene which expresses an insensitive form of DHPS (Antunes *et al*., 2005; Michael *et al*., 2013). The *sul1*, *sul2*, and *sul3* genes are the three main genes that have been identified. Gene *sul1* is harboured by a wide range of *S. enterica* serotypes such as Enteritidis, Hadar, Heidelberg, Orion, Rissen, Agona, Albany, Derby, Djugu, and Typhimurium (Chen *et al*., 2004; Doublet *et al*., 2004; Antunes *et al*., 2005). The class I integrons that contain other resistance genes, have often been associated with all three *sul* genes (Sandvang *et al*., 1998; Guerra *et al*., 2002). These integron gene cassettes have been located on transferable plasmids (Guerra *et al*., 2002) and as part of *Salmonella* genomic island variants (Boyd *et al*., 2002; Doublet *et al*., 2004). Many *S. enterica* serotypes Enteritidis, Agona, and Typhimurium isolates have been found harbouring *sul2*

(Chen *et al*., 2004). Also, *sul3* is known to be associated with plasmids and class I integrons in *Salmonella* (Guerra *et al*., 2002; Antunes *et al*., 2005). *sul3* has been found in serotypes 1,4,[5],12:i:-, Anatum, Bradenburg, Heidelberg, Rissen, Agona, and Typhimurium (Guerra *et al*., 2002; Antunes *et al*., 2005).

Trimethoprim resistance is attributed to the activity of DHFR (Michael *et al*., 2013). Serotypes known to have trimethoprim resistance genes are 4,5,12:i:-, Agona, Djugu, Hadar, Newport, Rissen Albany, Derby, and Typhimurium (Doublet *et al*., 2004; Antunes *et al*., 2005; Martinez *et al*., 2005). Likewise, these genes have been found as part of integron-borne gene cassettes also associated with *sul1* and *sul3* (Antunes *et al*., 2005), on transferable plasmids carrying other resistance genes (Villa & Carattoli, 2005), and *Salmonella* genomic islands (Doublet *et al*., 2004).

1.4.7. *Salmonella* Genomic Island

Multidrug-resistant *S*. Typhimurium definitive phage type (DT) 104 has emerged as a global health problem in the last three decades because of its involvement in disease among both animals and humans. Multidrug-resistant strains of this phage type were first detected in the United Kingdom in cattle and humans in the late 1980s but have since become common in other animal species such as poultry, swine, and sheep (Threlfall *et al.*, 1993). Human infections with multidrug-resistant DT 104 isolates have been associated with the consumption of chicken, beef, swine, sausages, and meat paste (Fone & Barker, 1994; Evans & Davies, 1996). *S*. Typhimurium DT 104 is now distributed worldwide, including Portugal (Antunes *et al*., 2011; Gomes-Neves *et al*., 2013). Multidrug resistance in *S*. Typhimurium DT 104 is mainly due to presence of SGI1. Within SGI-1, genes conferring the penta-resistance phenotype (*i.e.* resistances to tetracycline, ampicillin, chloramphenicol, streptomycin, and sulfonamides) are clustered

in the multi-drug resistance region (Hensel, 2004). SGI1 consists of a backbone of 27.4 kb with a 15 kb complex class 1 integron inserted close to one end, named In104 (Hall, 2010). Streptomycin and spectinomycin resistance is conferred by *aadA2* gene cassette and the *bla*$_{PSE-1}$ cassette confers resistance to ampicillin. A complete *sul1* is located in the longer 3´-CS. The two remaining resistance determinants both encode efflux proteins. They are *tet*(G) conferring resistance to tetracycline, and *floR*, a *cmlA1* homologue that confers resistance to both chloramphenicol and florfenicol (Hall, 2010). There are many variants to SGI1 with different gene cassettes.

1.4.8. Heavy metals resistance

Heavy metals are metals with a density above 5 g/cm^3. Heavy metals occur naturally in the soil, and can increase concentrations with anthropogenic use of heavy metals include mining, manufacturing, electroplating, and use as pesticides), fertilizers, as well as biosolids in agriculture, sludge dumping, industrial discharge, and atmospheric deposition (Ali *et al*., 2013). Some heavy metals examples are arsenic (As), cadmium (Cd), cobalt (Co), silver (Ag), lead (Pb), mercury (Hg), copper (Cu), and zinc (Zn). Heavy metals at low concentrations are necessary in bacteria metabolism, however, at higher concentrations heavy-metal ions form unspecific complex compounds in the cell, which leads to toxic effects. Some heavy-metal cations, *e.g.* Hg^{2+}, Cd^{2+} and Ag^{+}, form strong toxic complexes, which makes them too dangerous for any physiological function. Even highly trace elements like Zn^{2+} or Ni^{2+} and especially Cu^{2+} are toxic at higher concentrations. Thus, the intracellular concentration of heavy-metal ions has to be tightly controlled and heavy-metal resistance is just a specific case of the general demand of every living cell for some heavy-metal homoeostasis system (Nies, 1999). Heavy metals have their toxic effect only inside bacteria. Because some heavy metals are necessary for

enzymatic functions and bacterial growth, uptake mechanisms exist that allow for the entrance of metal ions into the cell. There are two general uptake systems: one is fast and unspecific, driven by chemiostatic gradient across the cell membrane and thus requiring no ATP; the other is slower and more substrate-specific, driven by energy from ATP hydrolysis. The first mechanism is more energy efficient, it results in an influx of a wider variety of heavy metals, and when these metals are present in high concentrations, they are more likely to have toxic effects once inside the cell (Nies & Silver, 1995). To survive under metal-stressed conditions, bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state (Nies, 1999). Products such as disinfectants and heavy metals used in industry and in household products are, along with antimicrobial agents, creating a selective pressure in the environment that is leading mutations in microorganisms that will allow them to survive and multiply in this environment. Co-selection of heavy-metal-tolerant and antimicrobial-resistant phenotypes often occurs when the genes are co-located on the same genetic element such as a plasmid, transposon, or integron (Chapman, 2003). In *S.* Abortus resistance to arsenic, chromium, cadmium and mercury, were identified in transferable plasmids of 0.1, 84, and 85 kb (Ghosh *et al*., 2000). Periplasmic copper binding protein CueP confers copper resistance in *S.* Typhimurium, and has been implicated in the scavenging activity of Reactive Oxygen Species (ROS) transferring copper ion to periplasmic Cu/Zn-superoxide dismutase or by directly reducing the copper ion (Pontel & Soncini, 2009; Yoon *et al*., 2014). In *S.* Typhimurium both *czcD* and *pcoA* genes confer resistance to Zn^{2+} and Cu^{2+} heavy metals ions (Medardus *et al*., 2014). Silver resistance has been described to be associated with plasmid genes, encoding two metal ion efflux pumps (a

P-type ATPase and a three-component cation/proton antiporter) and a periplasmic metal-binding protein (SilE). The SilE protein has been shown experimentally to be specific in its binding to silver (Gupta *et al*., 1999). Many mercury resistance genes were found associated with large resistance transposons that are related to the transposon Tn*1696* and carry a *dfrA5* trimethoprim resistance gene cassette and the *sul1* gene in an In4-type class 1 integron (Cain & Hall, 2012). Resistance to cobalt in Gram-negative bacteria is based on trans-envelope efflux, proton gradient, driven by a resistance, nodulation, cell division transporter (Nies, 1999).

1.4.9. Dissemination of resistance

The widespread of antimicrobial resistance among different bacteria, and the finding of the same or closely related resistance genes in bacteria of different species or genera, suggest the exchange of resistance genes by horizontal gene transfer. In many cases, when resistance genes are integrated into mobile genetic elements, such as plasmids or transposons, they are spread by horizontal gene transfer under the selective pressure imposed by the use of antimicrobial agents (Schwartz *et al.*, 2006). Antimicrobial resistance determinants that confer resistance to many different classes of antibiotics are found in the form of particular genes or as gene cassettes like SGI-1.

1.4.9.1. Plasmids

A common vector for the transmission of antimicrobial resistance genes is an extra-chromosomal DNA, a circular molecule called plasmid. Plasmids can replicate within a cell independently of the chromosomal DNA. Additionally, they can contain genes which are not essential for survival, but which can be evolutionarily beneficial for the bacteria that harbour the plasmid. They can acquire mobile genetic elements such as transposons and Insertion Sequences (IS), which can lead to mobilization of the

antimicrobial resistance genes (Alekshun & Levy, 2007). Plasmids may carry genes that are responsible for conferring resistance to the major classes of antibiotics including β-lactams, quinolones, aminoglycosides, tetracyclines, sulfonamides, trimethoprim, macrolides and chloramphenicol (Carattoli, 2009), as well as heavy metal resistance genes (Ghosh *et al*., 2000).

1.4.9.2. Transposons

Transposons do not possess replication systems, so for stable maintenance they must exist within plasmids or integrate into other transposons or the host chromosome. They vary in their size and structures. These DNA pieces contain terminal regions that facilitate incorporation using specific proteins (e.g. transposase or recombinase) into and from specific genomic regions (Alekshun & Levy, 2007). IS-elements consist of terminal inverted repeats of variable length and transposase gene (Mahillon & Chandler, 1998). Transposons Tn*21* and Tn*1696* have been associated with class 1 integrons in *S.* Typhimurium (Villa & Carattoli, 2005). In *S.* Kentucky, it was found fragments of Tn*1721* contained the tetracycline resistance gene *tet*(A); part of Tn5393 contained the streptomycin resistance gene *strAB*, and a Tn*3*-like region contained $bla_{\text{TEM-1}}$ gene flanked by two IS26 elements in opposite orientations (Doublet *et al*., 2008).

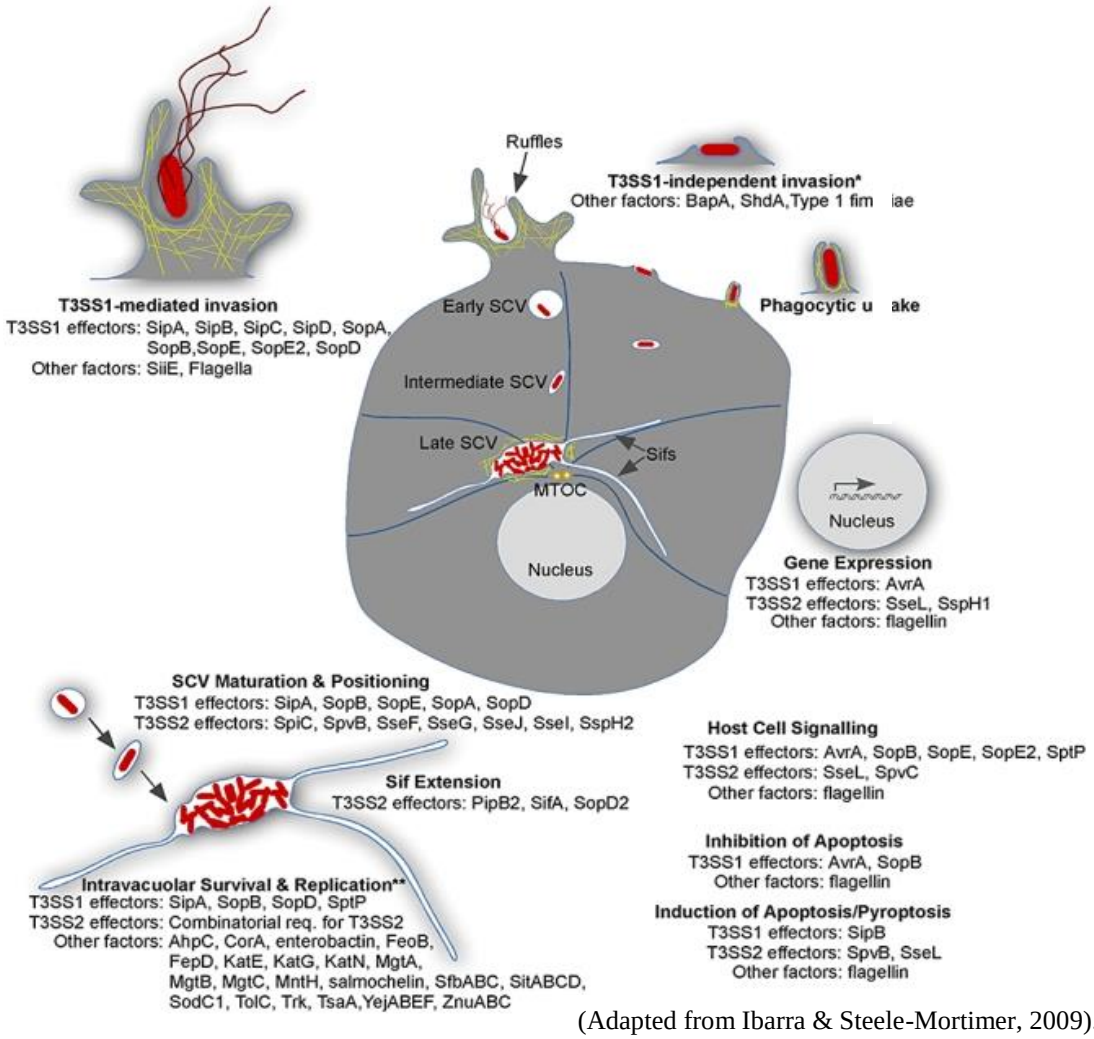
1.4.9.3. Integrons

Integrons are naturally occurring gene capture platforms with a strong promoter that drives gene expression elements that were discovered in the late 1980s (Stokes & Hall, 1989). They are defined as specialized genetic structures that are responsible for the acquisition of resistance genes. Integrons are distinct from all other genetic elements in that they are able to utilize site-specific recombination to acquire and integrate gene cassettes (Collis & Hall, 1992). Several classes of integrons have been described based

on sequence differences in integrase genes (*intI*) (Nield *et al.*, 2001; Rowe-Magnus & Mazel, 1999). The most prominent and widely spread integron among *S. enterica* and also Gram-negative bacteria belongs to the class 1 integron (Guerra *et al*., 2000, Lindstedt *et al*., 2003; Randall *et al*., 2004). Class 1 integrons are largely associated with Tn*21*-related transposons (Rodríguez *et al*., 2006).

1.5. *Salmonella* Pathogenesis

S. enterica entering in host via the fecal-oral route can survive the low pH environment of the stomach are able to colonize multiple sites including the small intestine, colon, and cecum. After entering the intestinal tract, crossing of *S. enterica* through the intestinal barrier constitutes a crucial step for establishment of infection (Figure 1.1).



(Adapted from Ibarra & Steele-Mortimer, 2009).

Figure 1.1. Schematic representation of the major stages underlying *Salmonella* infection.

Internalization of *S. enterica* into host cells can occur via at least two distinct processes. Phagocytes such as macrophages utilize phagocytic uptake to efficiently recognize and internalize bacterial pathogens. *S. enterica* can also actively invade both phagocytic and non-phagocytic cells using a Type III Secretion System (T3SS). *S. enterica* can induce their own entry into enterocytes, but M cells and CD18-expressing phagocytes also facilitate their translocation through the intestinal epithelium (Watson & Holden, 2010). Upon internalisation into non-phagocytic cells, *S. enterica* becomes

enclosed within an intracellular phagosomal compartment termed the *Salmonella*-containing vacuole (SCV), within which they will survive and replicate, a stage characterised by formation of tubulovesicular SCV structures called *Salmonella*-induced filaments (Sifs) (Figure 1.1.) (McGhie *et al*., 2009). During gastroenteritis infection, host colonization is restricted to the intestinal tract. However, *Salmonella* also have the ability to disseminate to extra-intestinal sites at least via CD18-expressing phagocytes, leading to deep organ infection (Vazquez-Torres *et al.*, 1999). Virulence factors specific to each serotype including the presence of surface cell structures, *Salmonella* pathogenicity islands (SPIs), prophages, and plasmid virulence genes (*spv*), are involved in the severity of salmonellosis (Andino & Hanning, 2015). Some virulence factors are present in Table 1.2.

Table 1.2. Some virulence factors of *S. enterica*.

Gene	Function	General function	Reference
agfA	Major curlin subunit precursor	Adhesion	Kröger *et al*. (2012)
fimA	Type-1 fimbrial protein		Kröger *et al*. (2012)
lpfD	Long fimbrial protein		Kröger *et al*. (2012)
pefA	Plasmid-encoded major fimbrial subunit		Haneda *et al*. (2001)
avrA	T3SS effector protein-regulator	SPI-1	Kröger *et al*. (2012)
hilA	Invasion genes transcription activator		McClelland *et al*. (2001)
invA	Secretory apparatus of T3SS		Kröger *et al*. (2012)
sipA	SPI-1 effector protein-involved in actin bundling and polymerisation leading to epithelial cell invasion and formation of the SCV		Kröger *et al*. (2012)
sifA	Lysosomal glycoprotein-containing structures, SifA protein	SPI-2	McClelland *et al*. (2001)
siiE	Large repetitive protein	SPI-3	Kröger *et al*. (2012)
sopB	Type III secretion system effector protein	SPI-5	Kröger *et al*. (2012)
pagN	Outer membrane adhesion	SPI-6	Kröger *et al*. (2012)
cdtB	Cytolethal distending toxin B	Toxin	McClelland *et al*. (2001)
spvR	*Salmonella* plasmid virulence lysR family regulator	Virulence plasmid	Grépinet *et al*. (2012)
shdA	Host colonisation factor	CS54 island	Kröger *et al*. (2012)
phoP	Transcriptional regulatory protein PhoP, regulator of virulence determinants	Regulation	Kröger *et al*. (2012)
phoQ	Sensor protein PhoQ, regulator of virulence determinants		Kröger *et al*. (2012)
rcK	Resistance to complement killing	Serum resistance	Patterson *et al*. (2012)

1.5.1. Adhesion

Crucial to initiating invasion is the attachment of *S. enterica* to host cell. *Salmonella* have various strategies to begin adhesion. Type 1 fimbriae (Fim) are peritrichous structures. The fimbrial subunits are typically encoded by a gene cluster,

together with export, assembly and regulatory proteins (Guo *et al*., 2007). The *fimAICDFHWYZ* operon encodes type 1 fimbriae required for *Salmonella* biofilm formation. This type of fimbriae has been shown to confer *S*. Typhimurium adhesion to a variety of mammalian gut epithelial cells (Bäumler *et al*., 1996a; Boddicker *et al*., 2002). *S*. Typhimurium colonization of the murine intestine by biofilm formation was shown to require type 1 fimbriae carrying an adhesive allelic FimH (Boddicker *et al*., 2002). Other fimbriae, called thin aggregative fimbriae, are thin and extremely aggregative, that lead *Salmonella* to auto-aggregate, a phenomenon which is proposed to allow the survival of bacteria to pass through hostile barrier such as stomach acid (Collinson *et al*., 1993). *S*. Enteritidis *agfBAC* gene sequences are necessary for expression of AgfA, the major structural fimbrin component of an unusual, thin aggregative fimbriae (Tafi), SEF17 (Collinson *et al*., 1996). *S. enterica* Tafi are encoded by two operons, *agfDEFG* and *agfBAC*. SPI-6 have genes that encode a T6SS related with adhesion, and the *safABCD* fimbrial operon that encodes fimbrial subunit, periplasmic chaperone, outer membrane usher protein and alternative fimbrial subunit (Folkesson *et al*., 1999). Mutation in the outer membrane protein PagN encoded by SPI-6, displays a reduction in adhesion and invasion of *S.* Typhimurium into epithelial cells (Lambert & Smith, 2008).

Long-polar fimbriae (Lpf) are long appendages at the cell poles. Bäumler *et al.* (1996b) provided evidence that fimbrial operon of *S*. Typhimurium *lpfABCDE* is involved in adhesion of this pathogen to murine small intestine. Lpf-mediated adhesion seems to result in binding only to certain areas of the alimentary tract, thus targeting the pathogen to its preferred port of entry, the Peyer's patches of the ileum (Bäumler *et al*., 1996b; van der Velden *et al*., 1998). It had been demonstrated that the *lpf*-fimbrial operon oscillates between phases ON and OFF expression states, thereby generating heterogeneity within *S*. Typhimurium populations with regard to expression of long polar fimbrial antigens, a

phase variation mechanism that helps to evade the host immune system (Kingsley *et al*., 2002). The receptors involved in recognition remain unknown.

As described above, several serotypes of *Salmonella* contain virulence plasmids. The plasmid-encoded fimbriae (Pef) contain the *pef* locus, which is composed by four genes, and six additional open reading frames (ORFs). *pefABCD* operon incorporates the genes for *Salmonella* adhesion to the murine small intestine. Mutational inactivation of *pefC* resulted in a moderate decrease in adhesion to the small intestine of mice. Adhesion mediated by Pef is different from that induced by the chromosome-encoded *lpf*, which promote preferentially the adhesion of *S. enterica* to Peyer's patches (Bäumler *et al*., 1996b). However, Pef do not mediate adhesion to various cell lines of human origin, such as HEp-2 and HeLa cells (Bäumler *et al*., 1996a). Pef binds to Lewis X [Le(x)] blood group antigen (Chessa *et al.*, 2008).

BapA and SiiE are two proteins associated to non-fimbrial adhesion, and are secreted by a T1SS, while ShdA, MisL, and SadA are auto-transporters also known as T5SS. BapA and SiiE are the largest proteins in *S. enterica* and share the characteristics of having numerous bacterial immunoglobulin-like domains. The genes encoding these two proteins are highly conserved among *S. enterica* serotypes, like Greenside, Dublin, Grumpensis, Oranienberg, Enteritidis, Newport, Kentucky, Paratyphi B, Typhimurium, Enteritidis, Kentucky, Budapest, Montevideo, Derby, and Gallinarum (Biswas *et al.*, 2011; Suez *et al.*, 2013). The ShdA adhesin is a monomeric fibronectin and collagen-I binding protein that is encoded by *shdA* gene carried on the centisome 54 (CS54 island) (Kingsley *et al.*, 2004). Little is known about the trimeric SadA adhesin (Wiedemann *et al.*, 2015).

Other tool that *S. enterica* have to attach to host cells is the lipopolysaccharide (LPS). Several studies demonstrated that the entire LPS molecule is required for

colonisation of host cell (Nevola *et al*., 1985; Hoare *et al*., 2006; Murray *et al*., 2006; Zenk *et al*., 2009). LPS is a major constituent of the outer membrane of Gram-negative bacteria, including *Salmonella*. Structurally LPS is composed of central hydrophobic glycolipids (lipid A) and a hydrophilic core polysaccharide and a variable O-antigen side chains. Lipid A is the biologically active centre of the LPS molecule and is responsible for the toxic and other biological effects of LPS (Freudenberg *et al.,* 2001). Several SPIs, as described below, encode enzymes associated with LPS.

1.5.2. Regulatory genes

Salmonella sense host signals to regulate properties important for bacterial survival and replication within host tissues. The two-component system PhoP/PhoQ controls a large number of genes responsible for a variety of physiological and virulence functions in *S.* Typhimurium. In *S. enterica*, the sensor PhoQ responds to low Mg^{2+} (García Véscovi *et al*., 1996), mildly acidic pH (Prost *et al*., 2007), and to low concentration of LL-37 and C18G, antimicrobial peptides encounter within macrophages (Bader *et al*., 2005), one important components of innate immunity. PhoQ in the bacterial periplasm promote the active (*i.e.*, phosphorylated) state of the regulator PhoP (*i.e*., PhoP-P). PhoP-P binds to its target promoters and recruits RNA polymerase, thereby advancing transcription of PhoP-activated genes (Shin & Groisman, 2005; Shin *et al*., 2006). *S. enterica* integrate environmental signals from a variety of global regulatory systems to precisely induce transcription of SPIs. SPI-1 is the most studied, and one of the most important in regulation. The regulatory circuit converges on expression of HilA, which directly regulates transcription of the SPI-1 apparatus genes, and then others SPIs. HilA is the central regulator in the overall scheme of SPI-1 regulation and is known to directly bind to promoters and activate expression of the *prg/org*, *inv/spa*, and *sic*/*sip* operons.

The *prg/org* and *inv/spa* operons encode the needle complex *per se*, whereas the *sic/sip* operon encodes the translocon that embeds in the host cell membrane, as well as the primary effectors (Ellermeier & Slauch, 2007). Expression of *hilA* is controlled by the combined action of three transcriptional activators: HilC, HilD and RtsA (Schechter & Lee, 2001; Olekhnovich & Kadner, 2002; Ellermeier & Slauch, 2003).

1.5.3. *Salmonella* Pathogenicity Islands

Salmonella spp. has many clusters of chromosomal virulence genes, termed *Salmonella* Pathogenicity Islands or SPIs. Most SPIs are adjacent to tRNA genes and have a G+C composition different from that of the rest of the chromosome, suggesting acquisition by horizontal transfer (McClelland *et al*., 2001; Porwollik & McClelland, 2003). It has been described that there are at least 21 SPIs, with a wide range of functions, encoding proteins involved in adhesion, invasion, and survival of *Salmonella* in the host cell. SPI-1 through to SPI-5 are common among all *S. enterica* serotypes, while others like SPI-7, -15, -17, -18 are specific for Typhi (Sabbagh *et al*., 2010). The most studied SPI are SPI-1 and SPI-2, associated with adhesion/invasion and host cell survival, respectively. Over thirty SPI-1 and SPI-2 T3SS effectors have been shown to manipulate a succession of key host cellular functions, including signal transduction, membrane trafficking and pro-inflammatory immune responses (McGhie *et al*., 2009). *S. enterica* encodes two T3SS, which are regulated by different environmental signals, such as oxygen concentration, osmolarity and bacterial growth state, and translocate unique sets of effector proteins (Ellermeier & Slauch, 2007).

After bacteria adhere to the apical epithelial surface, profound cytoskeleton rearrangements occur in the host cell. T3SS-1, encoded by SPI-1, is required for SPI-1-dependant internalisation of non-pathogenic cells, such as M cells and epithelial cells,

and mediates the translocation of effector proteins into eukaryotic cells (Mills *et al*., 1995; Lostroh & Lee, 2001; Ehrbar *et al*., 2002). At least 15 effectors can be translocated by T3SS-1. One subset of SPI-1 delivered effector proteins (SipA, SipC, SopB/SigD, SopA, SopD, SopE2 and SptP) mediates the invasion of non-phagocytic cells by *S. enterica*, through the modification of the actin cytoskeleton, resulting in massive localized membrane ruffles and rapid internalization of the bacteria, forming the SCV. SipC and SipA are two actin-binding proteins, which together efficiently nucleate actin polymerization and stabilize the resulting filament architecture (McGhie *et al*., 2001; Hayward & Koronakis, 2002). SipC localizes to the plasma membrane, where it aids in the translocation of other *S. enterica* effectors and initiates actin nucleation. The injected SipA acts in synergy with SipC, as SipA binds to and stabilizes the F-actin filaments (McGhie *et al*., 2004). However, unlike SipC, SipA is not essential for entry but enhances its efficiency (McGhie *et al*., 2004). SopE1, SopE2 and SopB regulate actin polymerization. SopE1 and SopE2 do not bind actin by contrast to SipA and SipC (Patel & Galán, 2005), and SopB regulates indirectly by interfering with inositol phosphate metabolism (McGhie *et al*., 2009). SopA promotes bacterial escape from the SCV in HeLa cells (Diao *et al.*, 2008). It is one of several effectors that is structurally and functionally similar to HECT E3 ubiquitin ligases and is thought to have a role in disrupting SCV membrane integrity (Zhang *et al*., 2006; Steele-Mortimer, 2008). Many of the genes involved in T3SS-1-mediated invasion are highly conserved among the genus *Salmonella*.

The majority of SPI-2 genes are expressed during bacterial growth inside the host (Ochman *et al*., 1996; Shea *et al*., 1996; Waterman & Holden, 2003). Known effectors of T3SS-2, include SpiC, which inhibits fusion of SCV with the lysosome, as well as SseF and SseG; whereas SifA, SifB, PipB, PipB2, SseI, SseJ, SseL, SspH2, GogB are other

effectors of T3SS-2 which are encoded on a different loci (Waterman & Holden, 2003; Lavigne & Blanc-Potard, 2008). To date, the effectors of T3SS-2 remain less understood than effectors of T3SS-1, although it is widely established that T3SS-2 is required for survival within host cells (Hensel *et al*., 1998). A study in SseJ null mutant mice has revealed that SseJ is required for full virulence of *S.* Typhimurium (Ruiz-Albert *et al*., 2002). More specifically, an *in vitro* study has revealed deacylase activity of SseJ, while showing its role in esterification of cholesterol, which is enriched in SCV (Brumell *et al*., 2001; Catron *et al.*, 2002; Garner *et al*., 2002; Ohlson *et al.*, 2005; Kolodziejek & Miller, 2015). Several studies have also highlighted the importance of SPI-2 in systemic infection, while noting its contribution in intestinal disease and inflammation (Bispham *et al*., 2001; Coburn *et al*., 2005; Coombes *et al*., 2005; Hapfelmeier *et al*., 2005).

SPI-3 encodes virulence factors that are important in intestinal colonization and intracellular survival. MgtC is an inner membrane protein, common to several intracellular pathogens, required for intramacrophage survival (Blanc-Potard & Groisman, 1997; Alix & Blanc-Potard, 2007). SPI-3 also encodes MisL, a T5SS (Blanc-Potard *et al*., 1999; Henderson *et al*., 2004). Although not much is known about MisL, a recent study of *S.* Typhimurium demonstrates that MisL is important for intestinal colonization promoting *S.* Typhimurium binding to fibronectin (Dorsey *et al.*, 2005).

SPI-4 encodes a T1SS and SiiE, a large surface-associated non-fimbrial adhesion, translocated by T1SS, that contributes to adhesion and invasion of eukaryotic cells (Gerlach *et al*., 2007). Although encoded by SPI-4, SiiE is also co-regulated by HilA, the master regulator of invasion genes encoded on SPI-1 (Main-Hester *et al*., 2008). A study in both calf and mouse models have revealed a role of SiiE in colonization and intestinal inflammation (Morgan *et al.*, 2007).

Many additional effectors of T3SS-1, including SopA, SopB, SopD, SopE, SopE2, SspH1 and SlrP, are not encoded on SPI-1; rather they are encoded by other horizontally acquired elements, such as on SPI-5 or prophages (Wood *et al.*, 1998; Lavigne & Blanc-Potard, 2008).

Others SPIs encode several genes associated with toxins or extra-intestinal infection. SPI-11 encodes genes related to a toxin, and was reported in serotypes that cause systemic infection like Choleraesuis and Typhi (Sabbagh *et al*., 2010). One of genes of SPI-11, the *cdtB* gene has been described in some isolates of non-Typhi serotypes such as, Bredeney, 9,12:I,v:-, Montevideo, Schwarzengrund, Javiana, and Brandenburg, from human clinical and poultry origin (Mezal *et al.*, 2013; Skyberg *et al*., 2006; Suez *et al.*, 2013). The *cdtB* gene encodes CdtB a homolog of the active subunit of the tripartite cytolethal distending toxin (CDT) (Haghjoo & Galán, 2004). This toxin, which is present in several bacterial pathogens, causes cell-cycle arrest in G2/M transition phase and cellular distension because it inflicts DNA damage on the intoxicated cells (Lara-Tejero & Galán, 2001). SPI-11 encodes also two proteins, PltA and PltB, that exhibit amino acid sequence similarity to the pertussis toxin ‘‘A’’ subunit and one of the components of its heteropentameric ‘‘B’’ subunit, respectively. Like *cdtB*, expression of *pltA* and *pltB* is induced intracellularly. *S.* Typhi was shown to form an intracellular tripartite toxin that is transported to the cell surface via a vesicular mechanism (Spanó *et al*., 2008). However, *S.* Typhi CdtB can induce DNA damage when it is transiently expressed or microinjected into the cytosol of mammalian cells (Haghjoo & Galán, 2004). SPI-11 also encodes the *phoP*-activated genes *pagD, pagC,* and *msgA* involved in intramacrophage survival (Gunn *et al*., 1995; Sabbagh *et al*., 2010).

SPI-12 of *S.* Typhimurium contains 11 ORFs, which include some putative and phage-associated genes as well as *oafA*, encoding a *Salmonella*-specific gene for LPS O-

antigen acetylase (Hansen-Wester & Hensel, 2002; Sabbagh *et al*., 2010). SPI-16 is found in *S.* Typhimurium encoding five ORFs, three of these ORFs show a high level of identity with P22 prophage genes involved in seroconversion (Vernikos & Parkhill, 2006), and were suggested to mediate LPS O-antigen glycosylation (Guan *et al*., 1999) and cell surface variation (Bogomolnaya *et al*., 2008). These ORFs (genes *yfdH, rfbI* and STM0557) were shown to be required for intestinal persistence of *S.* Typhimurium in mice (Bogomolnaya *et al*., 2008).

1.5.4. Prophages

Many bacteriophages integrate into the host genome to form prophages. They encode proteins that can be involved in the virulence of the bacterium. All salmonellae contain prophage genomes and/or prophage remnants in their chromosome. Several *S.* Typhimurium prophages encode T3SS effector proteins (Brüssow *et al*., 2004). *S.* Typhimurium strain LT2 harbours four prophages, including Gifsy-1, Gifsy-2, Fels-1, and Fels-2 (McClelland *et al*., 2001; Brüssow *et al*., 2004). The prophages found in *S. enterica* differ between serotypes. Some encodes TTSS effector proteins like *sopE*, a GDP/GTP nucleotide exchange in Rho GTPases (Stanley *et al*., 2000), and *sodCI*, a CuZn periplasmatic superoxide dismutase that protects the host against oxidative stress (Hardt *et al*., 1998). Other effector proteins, SspH2, the *phoPQ*-activated genes *pagKMO*, and various LPS modification genes are surrounded by prophage gene remnants (Figueroa-Bossi *et al.*, 2001). Prophage regions within bacterial genomes can often be recognized by certain characteristic sequences such as presence of disrupted or interrupted genes. Their proximity to bacterial tRNA genes, unique sequence patterns (such as prophage attachment sites), and long stretches of atypical DNA sequence content also characterize prophages (Switt *et al*., 2015). However, prophage regions do not always exhibit atypical

nucleotide content, nor do prophages always integrate into the same coding regions, nor do they always use tRNAs as their target site for integration (Switt *et al*., 2015).

1.5.5. *Salmonella* Virulence Plasmids

Other *Salmonella* virulence genes are encoded on plasmids. In several *S. enterica* serotypes, especially in Typhimurium, Enteritidis, Choleraesuis, Dublin Gallinarum, and Typhi plasmids have been identified which harbour virulence genes important for systemic infections (Rotger & Casadesús, 1999). These so called *Salmonella* virulence plasmids can vary in size between 30 and 100 kb for different serotypes (Gulig *et al*., 1993; Rotger & Casadesús, 1999). The factors encoded are associated with enhanced virulence such as the *spv* cluster which is essential for infection in laboratory rodents. However, the role of virulence plasmids in gastroenteritis and invasive disease in humans is still unclear. Some reports suggest that a highly conserved horizontally acquired operon *spvABCD* (*Salmonella* plasmid virulence) promotes dissemination of *Salmonella* to extra-intestinal infection (Gulig *et al*., 1993; Fierer, 2001; Raupach *et al*., 2003).

1.5.5.1. Serum resistance

After penetrating the epithelial lining, *S. enterica* serotypes become exposed to host defences encountered in the interstitial fluid, including complement. The complement system is a part of the innate immune system that helps or complements the ability of antibodies and phagocytic cells to clear pathogens from an organism. The complement system can be activated through various ways. However, all pathways rely on the fact that complement component 3 (C3) is activated by conserved microbial structures, such as the LPS of Gram-negative bacteria (Winter *et al*., 2010). This event results in deposition of a C3 cleavage product on the host cell surface, a process known

as C3 fixation. A complement-mediated killing by triggering the formation of a membrane attack complex constituted by several proteins is started on host cell (Müller-Eberhard, 1986). To further reduce the membrane attack complexes *S. enterica* produce accessory components that allows survival. One of these factors is Rck, an outer membrane protein encoded on *Salmonella* virulence plasmid (Heffernan *et al*., 1992). Rck is a 17-kDa outer membrane protein encoded on the virulence plasmid of *S. enterica* serotypes Typhimurium and Enteritidis. When expressed Rck confers serum resistance independent of LPS length. Rck binds complement protein factor H (fH), a protein preventing complement deposition on host cells (Ho *et al*., 2010). The second consequence of C3 fixation is the opsonisation of bacteria, which promotes phagocytosis through complement receptor. While phagocytosis by neutrophils results in killing, *S. enterica* serotypes can survive when they become internalized by macrophages through complement receptor phagocytosis (Santos & Bäumler, 2004).

1.6. *Salmonella* Genome

Several approaches have been done to compare *Salmonella* serotypes genomes. Comparisons of *S.* Typhimurium LT2, *S.* Enteritidis and *S*. Gallinarum showed that genes conserved between serotypes show approximately 99% identity at the nucleotide level, whereas if this broadened to comparisons of *S. bongori* or *E. coli* those figures fall to 91% and 80%, respectively (Porwollik *et al*., 2004; Fookes *et al*., 2011; Anjum & Thomson, 2013). With this high related nucleotide identity, is not easy to define the shared, from core gene set, and determine the number of unique genes present in each strain. The variable genome is considered the acquisition and loss of mobile elements through horizontal gene transfer results in the elasticity of bacterial genomes, which is a major

driving force for their evolution, pathogenicity and adaptation to new and different environments (Kudva *et al*., 2002; Jiang *et al.,* 2004).

In the past few decades an explosive growth in field of molecular biology and genomic technologies allowed advances in biological information. Typing *Salmonella* is crucial to track changes in epidemiology and to trace sources of food-borne infections. Several typing schemes have been used for *Salmonella*. Some phenotypic typing is done by serotyping, phage type, antimicrobial resistance (R-Type), and MALDI-Tof. Serotyping consists in agglutination of somatic "O" and flagella "H" antigens, it can be used to screen *Salmonella* and represents a known method. Antimicrobial susceptibility consist in test of *Salmonella* to different antimicrobial agents, is useful to discriminate between outbreaks isolates. Molecular typing is represented by methods like Random Amplification of Polymorphic DNA (RAPD), PCR for specific genes or islands, plasmid profile, Pulsed Field Gel Electrophoresis (PFGE), Fragment Length Polymorphism (AFLP), MultiLocus Variable Number Tandem Repeat Analysis (MVLA), Multilocus Sequence Typing (MLST), microarrays and new generation of genome sequencing (Wain & Olsen, 2013).

It is possible to assess the genomic diversity using DNA microarray technology and whole genome sequencing. Microarray analysis enables the screening of a large set of targets simultaneously. It comprises DNA-DNA hybridization of the whole genome against an array of known sequences that are pre-amplified and present on the array, and can allow knowing presence or absence of genes of several isolates. DNA microarrays based on the genome sequence of a small number of representatives member of genus *Salmonella* has been used to rapidly screen genetic variability within bacterial population. Microarrays have been used so far in virulence but also in genetic determination of antimicrobial resistance (Majtan *et al.,* 2007; Anjum *et al*, 2011; Huehn *et al*., 2010;

Litrup *et al*., 2010). Comparative genome hybridization DNA based microarrays studies involve genomic DNA from one or more reference strains and a test strain that is labelled and hybridizes to a microarray slide containing probes representatives of all genes present in the reference strain (Anjum *et al*., 2005; Suez *et al*., 2013). Chromosomal DNA hybridization studies have demonstrated that *S. enterica* strains share between 70 and 100% genetic relatedness (Crosa *et al*., 1973), which falls to about 55% when *S. bongori* strains are compared to *S. enterica* strains (Le Minor *et al*., 1986). Genome sequence comparison of two sequenced *S. enterica* strains *S.* Typhi CT18 and *S.* Typhimurium LT2, has suggested the genome to be 89% conserved, interspersed with regions of genomic variation (McClelland *et al*., 2001; Parkhill *et al.,* 2001). Next generation genome sequencing allows high coverage and depth know of genome, which is good for looking at conserved features of genomes. Whole genome sequencing evaluates genomic differences in bacterial strains using indices of genome organization that vary both between species and within isolates of a specie, e.g. gene content (Anjum *et al*., 2003), C+G content (Thomson *et al*., 2004), the occurrence and location of genes duplications, rearrangements, insertions (Sueoka, 1999) and horizontal gene transfer (Szpirer *et al*., 1999; Brown *et al*., 2003; Bischoff *et al*., 2004).

1.7. Aims of the thesis

S. enterica is a foodborne pathogen with capacity to acquire antimicrobial resistance, and have the ability to disperse globally. Monitoring for drug resistance and potential virulence is of extreme importance currently, because helps promote and protect public health by providing information about emerging bacterial resistance, how resistant infections differ from susceptible infections, and the impact of interventions designed to limit the spread of resistance. In Portugal few studies have reported on antimicrobial and heavy metal resistance, as well scarce virulence studies were performed on *Salmonella* strains isolated from food producing animals and processed food. This work aimed to perform phenotypic and genotypic characterisation of numerous antimicrobials and several heavy metals resistances of *Salmonella* spp. isolated from food producing animals and processed food. In addition a new virulence microarray was designed and validated to evaluate virulence determinants present in *S. enterica*. Once known virulence factors content in particular strains missing some SPI-1 genes and containing Typhi-related genes, *in vitro* and *in vivo* assays were performed to evaluated virulence potential in human cells and an *in vivo* model. By gathering information on the antimicrobial resistance, heavy metal resistance, and virulence potential it will to better understand the potential for *Salmonella* isolated from animals producing animals and processed food, to pose a public health threat.

1.8. References

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Chapter 2

Resistência a antibióticos em isolados de *Salmonella enterica* em alimentos de origem animal

Antimicrobial resistance in *Salmonella enterica* from food of animal origin

Rui Figueiredo, Ana Henriques, Rui Sereno, Nuno Mendonça, Gabriela Jorge da Silva

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2.1. Abstract

Antibiotic resistance is an issue of great importance for public health. The use of antibiotic in animal production may lead to the emergence of resistant microorganisms, namely *Salmonella enterica*, an invasive pathogen that usually colonizes animal digestive tract, being necessary to establish several control measures to avoid the entrance of this pathogen in food chain. In this study, 163 isolates of *S. enterica* were recovered from alimentary and production samples of poultry (n = 94), swine (n = 60) and cattle (n = 9), from June 2011 to March 2012. The identification was performed according ISO 6579/A1 guidelines. Antimicrobial susceptibility was tested by microdilution method. Ten serotypes were identified, being *S.* Typhimurium the most frequent (poultry, swine, processed food), followed by *S.* Enteritidis, though not found in swine. Eighty seven percent of the isolates were resistant to at least one antibiotic. The highest frequency of resistance was found for tetracycline (49%) and amoxicillin (37%). Accordingly to animal samples origin, 82% and 65% swine isolates were resistant to tetracycline and amoxicillin, respectively. While in poultry isolates the frequency of resistance was 31% and 21%, and in cattle 22% and 11%, were resistant to tetracycline and amoxicillin, respectively. Poultry isolates showed also resistance to colistin (22%) and moxifloxacin (13%), while swine isolates presented also resistance to chloramphenicol (25%). Processed food isolates showed major resistance to tetracycline (68%), ampicillin (54%) and mezlocillin (52%). Overall, the isolates from swine showed the highest frequency of resistance to the antimicrobials tested. These results, in comparison with previous studies performed in Portugal, suggest that measures to control the spread of *Salmonella* in poultry instituted in the country are effective, but must have to be extended to swine, where *S.* Typhimurium is prevalent, and also more resistant to antimicrobials.

2.2. Introduction

According to the Veterinary Directorate General, in Portugal, the use of antimicrobial agents in animal therapy amounted approximately 180 tonnes in 2010 (DGV, 2010). Antimicrobial resistance is currently considered a public health problem, including microorganisms spread through the food chain. Antimicrobial resistance acquisition of pathogens whose reservoirs are animals for consumption, such as *Salmonella* spp., decreases efficacy of antibiotherapy, with relevant clinical implications.

Salmonella genus is divided into two species, *S. bongori* and *S. enterica* (Brenner *et al.*, 2000). *S. enterica* is divided into six subspecies, one of which, *S. enterica* subs. *enterica* has more than 2500 serotypes, colonizing mainly poultry, swine, and midsize ruminants. *S. enterica* is a Gram-negative bacterium ability to invade the intestinal mucosa, and cause gastroenteritis in humans, called salmonellosis, characterized by diarrhoea, nausea, abdominal pain and sometimes fever (McGhie *et al*., 2009).

In Europe, salmonellosis is the second most common zoonosis in humans, affecting approximately 100 000 individuals per year (EFSA, 2012a). In Portugal was the most common intestinal infectious disease in 2008 (DGS, 2010). Usually, the infection occurs through ingestion of food or water contaminated with intestinal animal waste. Although salmonellosis is usually a self- limiting infectious disease, it is sometimes necessary to resort to antimicrobials due to the invasive nature of the bacteria, especially in children, the elderly or immunosuppressed patients. The treatment is often done with the use of β-lactams and fluoroquinolones (Foley & Lynne, 2008). The use of antimicrobials in the treatment of eating animal can lead to increased

resistance of zoonotic pathogens (Mølbak, 2005), and compromise the clinical efficacy of these drugs, both animal and human.

In the present study, we attempted to identify the serotypes present in animals and food products of animal origin, and to determine the susceptibility to different classes of antimicrobial agents used in human and veterinary clinical practice.

2.3. Methods

2.3.1. Isolation and bacteria identification

Between June 2011 and March 2012, in the central region of Portugal, were collected 163 isolates of *Salmonella* spp. poultry samples (n = 94), swine (n = 60) and cattle (n = 9). The isolates were collected from biological samples (n = 93) as blood, neck, skin, liver, carcasses and faeces, as well as processed foods (n = 70) as minced meat, sausages and hamburgers.

The isolation was done according to standard methods (ISO 6579/A1, 2007). Briefly, 10 g of sample were suspended in buffered peptone water (BPW Merck, Darmstadt, Germany) (1:10). The suspension was homogenized and incubated at 37 °C. After 18h and 0.1 mL were inoculated in 1 mL Rappaport - Vassiliadis broth containing soy peptone (RVS broth, Oxoid, Cambridge, UK) and Muller- Kauffmann tetrathionate broth/novobiocin (MKTTn Broth, Merck, Darmstadt, Germany) respectively. The suspensions were incubated at SVR 41.5 °C ± 1 °C for 24 h ± 3 h, and the MKTTn incubated at 37 ± 1 °C for 24 h ± 3 h. In a second stage a loop of the culture broth was inoculated in selective media Hektoen and xylose-lysine-deoxycholate (XLD) agar (Oxoid, Cambridge, UK). The presumptively identified as *Salmonella* colonies were confirmed by biochemical methods, and the particular serotype in accordance with the

Kauffmann-White scheme for investigation of O and H antigens (Grimont & Weill, 2007).

2.3.2. Antimicrobial susceptibility

The minimal inhibitory concentrations (MICs) were evaluated using a commercial microdilution broth method with pre-prepared microplates with a concentration range to each antibiotic (Microscan Panel, Siemens, West Sacramento, CA, USA), according to the Clinical and Laboratory Standards Institute guidelines (CLSI) (CLSI, 2013). The microplates used included 33 antimicrobial agents ampicillin, piperacillin, mezlocillin, cefazolin, ceftazidime, cefotaxime, cefpodoxime, cefepime, cefuroxime, cefoxitin, ampicillin/sulbactam, amoxicillin/clavulanate, cefotaxime/clavulanate, ceftazidime/clavulanate, piperacillin/tazobactam, imipenem, meropenem, ertapenem, aztreonam, norfloxacin, ciprofloxacin, moxifloxacin, levofloxacin, gentamicin, amikacin, tobramycin tetracycline, colistin, chloramphenicol, nitrofurantoin, tigecycline, fosfomycin, and trimethoprim/sulfamethoxazole. The susceptibility breakpoints were interpreted accordingly to CLSI guidelines (CLSI, 2013), while breakpoints of colistin and moxifloxacin were analysed by the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2011).

2.4. Results and discussion

Results presented in this section were an outcome of the first strains analysed during the work development. The largest number of isolates of *S. enterica* was collected from poultry, followed by swine and processed foods (Table 2.1.). Ten serotypes were identified, being Typhimurium the most frequent, mostly isolated in samples of poultry and swine, followed by serotype Enteritidis. The latter has not been identified in swine (Table 2.1.). Both represent 46% of the identified serotypes. These serotypes are the most frequently associated with human infection (Humphrey, 2000; EFSA, 2012b). In poultry isolates *S.* Typhimurium and *S.* Enteritidis accounted for 42%, while a study of isolates from animal products collected in 2003/04, these two serotypes amounted to 59% of cases in Portugal (Carneiro, 2005; Da Silva & Carneiro, 2006). Being *S.* Enteritidis represented in 44% of poultry isolates in 1999 and 78% in 2003/04 (Antunes *et al.*, 2003; Carneiro, 2005; Da Silva & Carneiro, 2006). In this study, there was a decrease in the prevalence of serotype Enteritidis in poultry in relation to previous studies (Antunes *et al.*, 2003; Carneiro, 2005; Da Silva & Carneiro, 2006), which could be related to the implementation Portugal's National Plan of *Salmonella* Control (PNCS, 2012).

In swine isolates, serotype Typhimurium represented the 46% of cases, compared with 34.5% of 113 isolates of a previous study (DGV, 2007). This study also found up the appearance of serotypes Infantis and Virchow, not reported in 2007 by the Veterinary Directorate General (DGV, 2007). Of note, serotype Infantis was mostly found in swine products, some of which may be eaten raw or undercooked. Is the fourth most frequent serotype in this study, further studies can reveal the importance of the emergence of serotype, as has recently been associated with cases of gastroenteritis in humans (Najjar *et al.*, 2012). Note that, some of the serotypes considered less common,

as Infantis, Mbandaka, Virchow, Derby and Agona, are referenced at Europe, and associated to gastroenteritis in various animal (EFSA, 2012b).

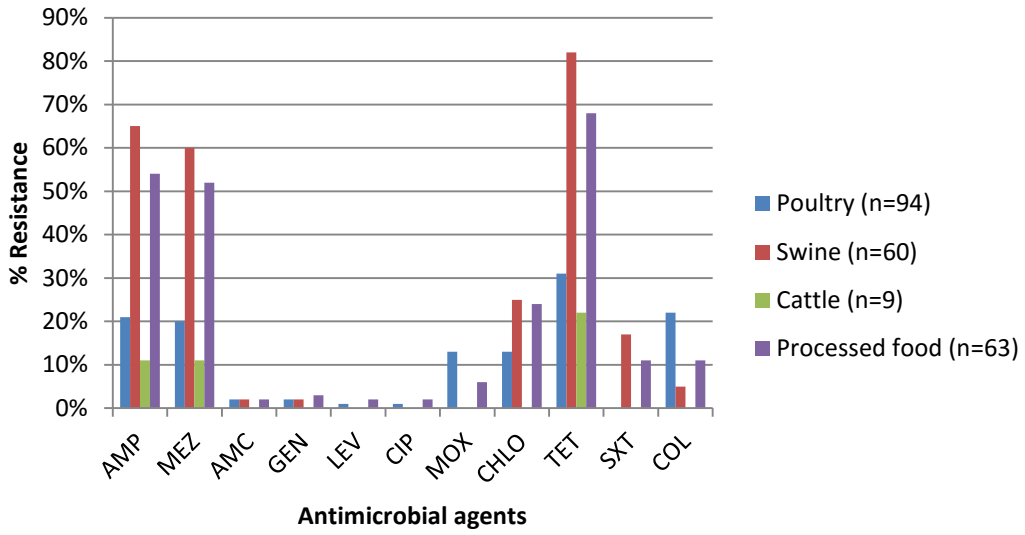
Table 2.1. Prevalence of *Salmonella* serotypes in different types of samples.

Serotype	Poultry Nr (%)	Swine Nr (%)	Cattle Nr (%)	Processed food[a] Nr (%)	Total[b] Nr (%)
Typhimurium	23 (24)	35 (62)	1 (11)	32 (51)	59 (36)
Enteritidis	17 (18)	-	-	3 (5)	17 (10)
Havana	13 (14)	-	1 (11)	-	14 (8)
Infantis	1 (1)	7 (12)	2 (22)	6 (10)	10 (6)
Mbandaka	9 (10)	-	-	-	9 (5)
Derby	2 (1)	-	-	-	2 (1)
Virchow	2 (1)	-	-	-	2 (1)
London	1 (1)	-	-	-	1 (1)
Agona	1 (1)	-	-	-	1 (1)
Patel	1 (1)	-	-	-	1 (1)
Not determined	24 (28)	18 (26)	5 (56)	22 (34)	47 (30)
Total	94	60	9	63	163

[a]Isolates collected samples processed poultry, swine and cattle.

[b]Sum of poultry, swine and cattle isolates.

The resistance to at least one antibiotic was recorded in 87% of the isolates. The isolates showed greater resistance to tetracycline (49%) and ampicillin (37%) (Figure 2.1.), which can be related to be the two most widely used antimicrobial agents in animal's therapy in Portugal (DGV, 2010).



AMP – Ampicillin; MEZ – Mezlocilin; AMC – Amoxicillin/clavulanate; GEN – Gentamicin; LEV – Levofloxacin; CIP – Ciprofloxacin; MOX – Moxifloxacin; CHLO – Chloramphenicol; TET – Tetracycline; SXT – Trimethoprim/Sulfamethoxazole; COL – Colistin.

Figure 2.1. Antibiotic resistance of isolates from poultry, swine, cattle, and processed foods.

Poultry isolates (n = 94) showed high frequency of resistance to tetracycline (31%), colistin (22%), and ampicillin (21%), and 13% were resistant to moxifloxacin, and only 1% were resistant to ciprofloxacin and levofloxacin. The high frequency of resistance to tetracycline into poultry isolates has also been documented in previous studies, which found values of resistance of 36% (Antunes *et al*., 2003). In isolates from swine (n = 60), there was a higher frequency of resistance to tetracycline (82%), ampicillin (65%) and chloramphenicol (25%). These percentages resistance are slightly lower than those reported in swine isolates collected in 2006/07, in which it has been documented 100% resistance to tetracycline, 93% to ampicillin and 37% resistant to chloramphenicol (Antunes *et al.*, 2011). Although the use of chloramphenicol has been banned from veterinary practice for animal production, continues to be observed

resistance to this antimicrobial agent in some isolates. The abolition of antimicrobials in animal production did not translate in a total reverse of resistance to antimicrobial agents, and some strains may continue to remain in certain ecological niches, which emphasizes the need to control the use of antimicrobial agents. Cattle's isolates (n = 9) showed only resistance to tetracycline (22%), ampicillin (11%) and mezlocillin (11%). Importantly, the group which was isolated as few *S. enterica*.

S. enterica isolated from processed foods for consumption (n = 63) showed a higher frequency of resistance to tetracycline (68%), ampicillin (54%) and mezlocillin (52%), and resistance to ciprofloxacin and levofloxacin was low (2%). Within the processed products were collected 14 isolates of products ready for use, in particular filled greaves bars, food usually not subject to cooking, and therefore a possible source of infection to final consumer. Sixty-four percent of isolates from processed food were resistant to tetracycline and fifty percent were resistant to ampicillin. Importantly, the high resistance to tetracycline, and ampicillin also reported previously in Portugal (Antunes *et al*., 2011; Caleja *et al*., 2011). Once isolates presented low levels of fluoroquinolones resistance, in case of infection and need for antibiotherapy, fluoroquinolones may be an option.

Resistance patterns of antimicrobial agents in serotype function are shown in Table 2.2. *S.* Typhimurium, in addition to being the most common serotype in samples of poultry and swine, is also the one with higher frequency of resistance, particularly to tetracycline (83%) and ampicillin (76%), as well as the higher number of multidrug-resistant isolates (36%). Multidrug resistance is an emerging problem. The acquisition of resistance to antimicrobial agents of various classes (at least three) to ensure a high survival bacterial strain capacity in relation to different therapies, enabling the spread of these strains between animals, and animals to humans. Several consequences can

come from multidrug resistance. The clinical therapy of choice is highly reduced and the clinical efficacy of antimicrobials is compromised, leading to increased morbidity host and eventual death (Angulo *et al.*, 2004). It was also observed that swine are a major reservoir of isolates of *S. enterica* resistant, especially Typhimurium. In general, the majority of non-Typhimurium serotypes found presented no resistance to the antibiotic tested. However, the number of isolates is much lower than serotype Typhimurium. These data are consistent with the literature, which describes *S.* Typhimurium as one of the most frequent serotype and antibiotic resistant (EFSA, 2012b). Importantly, tetracycline resistance, and the fact that it was mostly isolated in swine and processed foods, many derivatives of swine, which can be eaten raw or not fully cooked (Table 2.2.).

Table 2.2. Antimicrobial resistance by *Salmonella* serotype.

Serotype (Nr)	Nr (%) antimicrobial resistance phenotype[a]											
	AMP	**MEZ**	**AMC**	**GEN**	**LEV**	**CIP**	**MOX**	**CHLO**	**TET**	**SXT**	**COL**	**MR[c]**
Typhimurium (59)	45 (76)	44 (76)	2 (3)	1 (2)	0 (0)	0 (0)	5 (9)	19 (32)	49 (83)	3 (5)	5 (8)	21 (36)
Enteritidis (17)	3 (18)	2 (12)	0 (0)	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)	1 (6)	0 (0)	16 (94)	1 (6)
Havana (13)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (31)	1 (8)	0 (0)	0 (0)	0 (0)	0 (0)
Infantis (10)	1 (10)	2 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	4 (40)	2 (20)	0 (0)	1 (10)
Mbandaka (9)	1 (11)	1 (11)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (11)	0 (0)	1 (11)	0 (0)
Virchow (2)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Derby (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	0 (0)
Agona (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Patel (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
London (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ND (48)	10 (21)	6 (13)	1 (2)	1 (2)	1 (2)	1 (2)	2 (4)	6 (13)	24 (50)	5 (10)	2 (4)	6 (13)

[a]Antimicrobial agents: AMP, ampicillin; MEZ, Mezocillin; AMC, amoxicillin/clavulanate; GEN, Gentamicin; LEV, Levofloxacin; CIP, Ciprofloxacin; MOX, Moxifloxacin; CHLO, Chloramphenicol; TET, Tetracycline; SXT, trimethoprim/sulfamethoxazole; COL, colistin.

[b]Not Determined.

[c]Multidrug resistence

In conclusion, in this study there was a larger diversity of serotypes in poultry isolates compared with previous records (PNCS, 2012), including the appearance of Infantis serotype identified in all samples groups, such as Typhimurium (the most prevalent). Isoaltes presented high resistance to tetracycline and ampicillin and a low rate of resistance to fluoroquinolones. There was a decrease in the incidence of resistance to fluoroquinolones compared to the resistance observed in the late twentieth century after the legalization of the use of antimicrobial agents in veterinary use (Threlfall *et al.*, 1997; Malorny *et al.*, 1999). However, it appears that, even after the ban on the use of antimicrobials in animal feed for human consumption in 2006 in the countries of the European Union (Guardabassi & Courvalin, 2006), continue to emerge resistant *Salmonella* to antimicrobial agents. This fact shows that the control measures imposed in the European Union at the level of the use of antimicrobials in feed may be insufficient to minimize the emergence of resistant strains, and like in human therapy, there should be a careful assessment and choice of the need for antimicrobial agents in animals. So, it is important to know the susceptibility profiles of the strains circulating in Portugal. The National Programme for Control of *Salmonella* in poultry appears to be effective, and seems to help reduce the incidence of serotype Enteritidis, usually reported as the most common in this group of animals. However, there was an increase of *S.* Typhimurium in poultry in relation to previous studies, a serotype usually associated with swine, suggesting that this ubiquitous and responsible for most of *Salmonella* serotype may be occupying a new biological niche. To point out that the serotype Typhimurium was most prevalent and presented resistance to different antimicrobials, as demonstrated in this study, which is worrying as it is a serotype with adaptability to various hosts, and the acquisition of antibiotic resistance. The results obtained in this study, and compared with previous data suggest that the spread of

Salmonella control measures imposed in poultry in Portugal are effective, but they have to be extended to swine, where the Typhimurium serotype is prevalent, and also the most resistant to antimicrobial agents.

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Chapter 3

Antimicrobial resistance and extended-spectrum beta-lactamases of *Salmonella enterica* serotypes isolated from livestock and processed food in Portugal: an update

Rui Figueiredo, Ana Henriques, Rui Sereno, Nuno Mendonça, Gabriela Jorge da Silva

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3.1. Abstract

As *Salmonella* is a common foodborne pathogen, the presented study aimed to determine the distribution of *Salmonella enterica* serotypes isolated during 2011-2012 from poultry, swine, cattle and processed food in Portugal, and to characterize the antimicrobial susceptibility and the extended-spectrum β-lactamases (ESBLs). Results were also compared with data obtained before the implementation of the National Control Program in Poultry and the ban of antimicrobial agents in animal feed in European Union (EU). A total of 14 serotypes were identified, from 258 isolates recovered, being *Salmonella* Typhimurium (32.6%, n= 84) and *Salmonella* Enteritidis (10.1%, n = 26) the most common. *S.* Enteritidis in poultry was less frequent than in previous studies, which might be associated with the implementation of the National Control Program for *Salmonella* in poultry. Nevertheless, other serotypes seem to occupy this biological niche, and may be more common in human salmonellosis in the future. The majority of isolates (70.2%, n = 181) were resistant at least to one class of antimicrobial agent and exhibited higher frequency of resistance to tetracycline (47.7%, n = 123) and ampicillin (36.0%, n = 93), being *S.* Typhimurium the more resistant serotype. Resistance to fluoroquinolones was shown in 8% (n = 21) of isolates, a lower value compared to data obtained before 2004. ESBLs-producers *S.* Typhimurium $bla_{\text{CTX-M-1}}$ and *S.* Enteritidis $bla_{\text{SHV-12}}$ were isolated from swine and poultry, respectively. The $bla_{\text{CTX-M-1}}$ and $bla_{\text{SHV-12}}$ genes were carried on conjugative plasmids of IncHI2 replicon types and IncI1, respectively. This was the first report of a $bla_{\text{CTX-M-1}}$ in *S.* Typhimurium in Portugal. Overall, the results revealed changes in animal origin *Salmonella* serotypes, mainly emerging serotypes, in frequency of resistance, and in occurrence of ESBLs-producing *Salmonella.* The control measures taken by EU seem to have some impact in the resistance rate of some antimicrobials like quinolones. The

emergence of ESBLs and its potential spread among animal reservoirs and food chain highlight the continuous antimicrobial surveillance at animal level.

3.2. Introduction

Foodborne diseases caused by non-typhoidal *Salmonella* (NTS) represent an emerging public health problem and an economic burden worldwide. Different serotypes of NTS can be responsible for gastroenteritis in humans, but *S.* Typhimurium and *S.* Enteritidis are the most reported (EFSA & ECDC, 2014). They colonize a wide variety of domestic animals such as poultry, swine and cattle (Sanderson & Nair, 2013; EFSA & ECDC, 2014). Usually, gastroenteritis caused by NTS is a self-limiting infectious disease, and antimicrobial treatment may be not required except in elderly or young children and immuno-compromised patients. However, it was reported that 3-10% of individuals with gastrointestinal illness caused by NTS will develop bacteraemia (Okeke *et al*., 2005), a serious and potentially fatal problem that requires antimicrobial treatment, usually with fluoroquinolones or extended-spectrum cephalosporins (Hohmann, 2001). Nevertheless, the extensive use of many antimicrobials in human and veterinary medicine, and livestock, has led to the emergence of resistant *Salmonella*, which is a serious and rising concern. In fact, *S. enterica* strains belonging to different serotypes showing resistance to three or more antimicrobial agents are now widespread worldwide (Parry & Threlfall, 2008; Barton, 2014; EFSA & ECDC, 2014). The use of antimicrobial agents in animals has been associated to a selection of resistant strains with potential of human infection. In 2006, European Union (EU) banned antimicrobials use as growth promoters in animal food (Guardabassi & Courvalin, 2006). Moreover, many of the resistance determinants are carried in mobile genetic elements that can disseminate through the food chain and to the commensal microbiota. Production of ESBLs is a major mechanism of resistance to β-lactams and has emerged among salmonellae worldwide (Barton, 2014; EFSA & ECDC, 2014). In Portugal the epidemiology of ESBLs in salmonellae isolated from

food-production animals' samples is barely known, though a few studies indicate that these enzymes are disseminating in the country (Clemente *et al*., 2013).

This study aimed to assess the distribution and antimicrobial susceptibility of *S. enterica* serotypes isolated from livestock samples and processed food of animal origin, and to characterize ESBLs. Results were also compared with data obtained before the ban of antimicrobial agents in animal feed in EU, and the implementation of a National Control Program in poultry (NPCS, 2012).

3.3. Material and methods

3.3.1. Strain collection and bacterial identification

Between June 2011 and December 2012, 258 isolates of *Salmonella* spp. were recovered from poultry (n = 144), swine (n = 98) and cattle (n = 16), in centre of Portugal. The isolation was performed in a wide range of samples from biologic samples as blood, fur neck, liver, faeces and carcasses, and processed food like meat cuts, hamburgers or sausages. The isolation was made according to standard methods (ISO 6579/A1, 2007), and is described on section 2.3.1. Serotyping was performed accordingly Kauffmann-White scheme for investigation of O and H antigens (Grimont & Weill, 2007).

3.3.2. Susceptibility testing and ESBLs production

The minimal inhibitory concentrations (MICs) were evaluated using a commercial microdilution broth method with pre-prepared microplates with a concentration range to each antibiotic (Microscan Panel, Siemens, West Sacramento, CA, USA), according to the Clinical and Laboratory Standards Institute guidelines

(CLSI) (CLSI, 2013), and is described in section 2.3.2. Multidrug resistance was defined as the resistance shown at least to three classes of antimicrobial agents. The double-disc synergy test was additionally used in the isolates that showed resistance to the 3rd generation cephalosporins and susceptibility to the of β-lactamases inhibitors, suggesting ESBL production (Giriyapur *et al*., 2011).

3.3.3. PCR and gene sequencing

Presence of TEM-type, CTX-M-type, and SHV-type β-lactamase genes was tested by PCR in isolates with ESBL phenotype (Mendonça *et al*., 2007), as well as the insertion sequence IS*Ecp1* in CTX-M-positive strains, using a forward primer in IS*Ecp1* gene and reverse primer in the $bla_{\text{CTX-M}}$ gene. Presence of mobile genetic elements was assessed by amplification of conserved segments in integrons of classes 1, 2, and 3 (Lévesque *et al*., 1995). Nucleotide sequencing positive amplicons was performed by Macrogen (Amsterdam, The Netherlands). Sequences were edited by BioEdit Software (Ibis Biosciences, Ann Abbot, CA, USA) and identified using BLASTn (http://www.ncbi.nlm.nih.gov/).

3.3.4. Typing of plasmids

Plasmids were recovered using the QIAGEN Plasmid Mini Kit (Quiagen, Hilden, Germany). The PCR-based Inc/rep typing method was used for the identification of the replicon type of plasmids. It consists of five multiplex-PCRs recognizing three different replicon types and three simplex PCRs for K, F, and B/O replicon types (Carattoli *et al.*, 2005).

3.3.5. Conjugation experiments

Conjugation assays were used to assess the transfer of resistance in ESBL-positive strains, using *E. coli* strain J53 as recipient (resistant to azide sodium Azi^R). Briefly, conjugation experiments were performed in trypticase-soy (TS) broth at a ratio donor/recipient of 1:10 with 100 rpm at 37 ºC overnight. Selection was performed on MacConkey agar plates containing 1 mg/L of cefotaxime. Lactose-positive colonies were cultured in TS agar containing cefotaxime (1 mg/L) and sodium azide (10 mg/L). The antimicrobial susceptibility of donor strains, recipient *E. coli* J53, and transconjugants was performed by standard disc diffusion method on Mueller-Hinton agar (Oxoid, Wesel, Germany) following the CLSI recommendations. The MICs for transconjugants, donor strains and recipient strain were determined by E-test (Biomérieux, Marcy l'Étoile, France).

3.4. Results

3.4.1. Diversity of serotypes according their origin

Among the 258 *Salmonella* isolates recovered, a total of 14 serotypes were identified, being *S.* Typhimurium the most prevalent serotype (32.6%, n = 84) followed by *S.* Enteritidis (10.1%, n = 26) (Table 3.1.).

Table 3.1. Frequency of non-typhoidal *Salmonella* serotypes according origin.

***Salmonella* serotype**	**Total (n = 258) Nr (%)**	**Poultry (n= 111) Nr (%)**	**Swine (n= 29) Nr (%)**	**Cattle (n = 6) Nr (%)**	**Processed food Nr (%)**		
					Poultry (n= 33)	**Swine (n= 69)**	**Cattle (n= 10)**
Typhimurium	84 (32.6)	11 (9.9)	17 (58.6)	2 (33.3)	16 (48.5)	35 (50.7)	3 (30)
Enteritidis	26 (10.1)	23 (20.7)	-	-	3 (9.1)	-	-
Infantis	20 (7.8)	3 (2.7)	2 (6.9)	1 (16.7)	-	13 (18.8)	1 (10)
Havana	20 (7.8)	20 (18.1)	-	-	-	-	-
Mbandaka	9 (3.5)	9 (8.1)	-	-	-	-	-
Newport	3 (1.2)	3 (2.7)	-	-	-	-	-
Virchow	2 (0.8)	2 (1.8)	-	-	-	-	-
Derby	2 (0.8)	2 (1.8)	-	-	-	-	-
Agona	1 (0.4)	1 (0.9)	-	-	-	-	-
Patel	1 (0.4)	1 (0.9)	-	-	-	-	-
Altona	1 (0.4)	1 (0.9)	-	-	-	-	-
Brandenburg	1 (0.4)	1 (0.9)	-	-	-	-	-
London	1 (0.4)	1 (0.9)	-	-	-	-	-
Heidelberg	1 (0.4)	-	-	-	1 (3.0)	-	-
ND[a]	86 (33.3)	33 (29.7)	10 (34.5)	3 (50)	13 (39.4)	21 (30.4)	6 (60)

[a]Non determined serotype

S. Typhimurium was mainly isolated from swine samples (58.6%, n = 17) and from swine origin processed food (50.7%, n = 35), but was also present in samples from poultry (9.9%, n = 11) and cattle (33.3%, n = 2). *S.* Typhimurium represented 48.5% of poultry-food products. Isolates from poultry (n = 111) presented 14 different serotypes, being *S.* Enteritidis (20.7%, n = 23) the most prevalent followed by *S.* Havana (18.1%). In cattle isolates (n = 6) were identified 33.3% (n = 2) of *S.* Typhimurium and 16.7% (n = 1) *S.* Infantis. In processed food isolates (n = 112) *S.* Typhimurium represent 48.2%

(n = 54) of isolates, *S.* Infantis 12.5% (n = 14) and *S.* Enteritidis 2.7% (n = 3) (Table 3.1.).

3.4.2. Antimicrobial resistance of *Salmonella* spp. isolates

Antimicrobial susceptibility patterns among the isolates according their origin were show in Table 3.2. One hundred eighty-one (70.2%) isolates were resistant to at least one antimicrobial agent tested. Almost half of the isolates (47.7%, n = 123) were resistant to tetracycline and 36.0% (n = 93) showed resistance to ampicillin.

The highest frequency levels of resistance were observed for isolates of swine (n = 29), namely to tetracycline (89.7%, n = 26), ampicillin (65.5%, n = 19), and chloramphenicol (13.7%, n = 4), only one isolate was pansusceptible. Fifty-three (47.7%) isolates of poultry (n = 111) were pansusceptible, and two (33.3%) isolates of cattle (n = 6) revealed pansusceptibility.

From a total of 112 isolates of processed food, twenty-eight (25%, n = 112) showed pansusceptibility. Resistance levels were higher to tetracycline (72.7%, n = 24) in isolates of poultry-derivate food and lower in isolates of swine-food products (68.1%, n = 47), while resistance to ampicillin was similar in the two types of processed food [56.5% (n = 39) in swine-food and 54.5% (n = 18) in poultry products]. Resistance to chloramphenicol was observed in 20 isolates (30.0%) of products of swine origin. Multidrug-resistance was observed in 6.3% (n = 7), 24.1% (n = 7), 16.6% (n = 1), and 29.5% (n = 33) of poultry, swine, cattle, and processed food isolates, respectively.

Antimicrobial resistance patterns of *Salmonella* serotypes were presented in Table 3.3. *S.* Typhimurium (n = 84) was the serotype more resistant, being 39.3% (n = 33) isolates multidrug-resistant, showing resistance to tetracycline (88.1%, n = 74) and ampicillin (79.8%, n = 67). *S.* Enteritidis (n = 26) presented more frequency of

resistant to colistin (88.5%, n = 23), and ampicillin (15.4%, n = 4), being 11.5% (n = 3) of isolates multidrug-resistant. *S.* Infantis (n = 20) presented more frequency of resistance to tetracycline (35%, n = 7), and to trimethoprim /sulfamethoxazole (15%, n = 3), being multidrug-resistance reported in 10% (n = 2) of isolates. NTS serotypes not determined (n = 86) presented high resistance levels to tetracycline (47.7%, n = 41), and ampicillin (22.1%, n = 19), being 11.6% (n = 10) of isolates multidrug-resistant. Strains of *S.* Newport, *S.* Agona, *S.* Patel, *S.* Altona, *S.* Brandenburg, *S.* London, and *S.* Heidelberg were pansusceptible.

Table 3.2. Antimicrobial resistance pattern of *Salmonella* spp. isolates according their origin.

Antimicrobial agent	Total (n = 258) Nr (%)	Poultry (n = 111) Nr (%)	Swine (n= 29) Nr (%)	Cattle (n = 6) Nr (%)	Processed food (n = 112) Nr (%)		
					Poultry (n = 33)	Swine (n= 69)	Cattle (n = 10)
Tetracycline	123 (47.7)	18 (16.2)	26 (89.7)	4 (66.7)	24 (72.7)	47 (68.1)	4 (40)
Ampicillin	93 (36.0)	11 (9.9)	19 (65.5)	3 (50)	18 (54.5)	39 (56.5)	3 (30)
Amoxicillin/clavulanate	5 (1.9)	2 (1.8)	1 (3.4)	-	2 (6)	-	-
Ampicillin/sulbactam	48 (18.6)	7 (6.3)	8 (27.6)	-	11 (33.3)	20 (28.9)	2 (20)
Mezlocillin	87 (33.7)	9 (8.1)	17 (58.6)	3 (50)	17 (51.5)	38 (55.1)	3 (30)
Piperacillin	87 (33.7)	9 (8.1)	17 (58.6)	2 (33.3)	18 (54.5)	38 (55.1)	3 (30)
Piperacillin/tazobactam	5 (1.9)	-	1 (3.4)	-	3 (9.1)	1 (1.5)	-
Cefazolin	7 (2.7)	3 (2.7)	1 (3.4)	-	1 (3.0)	2 (2.9)	-
Cefuroxime	5 (1.9)	4 (3.6)	-	-	-	1 (1.5)	-
Cefoxitin	2 (0.7)	1 (0.9)	1 (3.4)	-	-	-	-
Ceftazidime	3 (1.1)	2 (1.8)	-	-	-	1 (1.5)	-
Cefotaxime	3 (1.1)	2 (1.8)	-	-	-	1 (1.5)	-
Cefpodoxime	4 (1.6)	3 (2.7)	-	-	-	1 (1.5)	-
Cefepime	1 (0.3)	-	-	-	-	1 (1.5)	-

(Cont.)

Antimicrobial agent	Total (n = 258) Nr (%)	Poultry (n = 111) Nr (%)	Swine (n= 29) Nr (%)	Cattle (n = 6) Nr (%)	Processed food (n = 112) Nr (%)		
					Poultry (n = 33)	Swine (n= 69)	Cattle (n = 10)
Ciprofloxacin	2 (0.8)	-	-	-	2 (6.1)	-	-
Levofloxacin	2 (0.7)	-	-	-	2 (6.1)	-	-
Norfloxacin	2 (0.7)	-	-	-	2 (6.1)	-	-
Moxifloxacin	21 (8.1)	12 (10.8)	-	-	8 (24.2)	1 (1.5)	-
Tobramycin	4 (1.6)	1 (0.9)	-	-	-	3 (4.3)	-
Gentamicin	8 (3.1)	2 (1.8)	-	-	3 (9.1)	3 (4.3)	-
Colistin	37 (14.3)	23 (20.7)	2 (6.9)	-	6 (18.2)	5 (7.2)	1 (10)
Chloramphenicol	43 (16.7)	12 (10.8)	4 (13.7)	1 (16.7)	6 (18.2)	20 (30)	-
Trimethoprim/ Sulfamethoxazole	18 (7.0)	1 (0.9)	3 (10.3)	-	1 (3.0)	13 (18.8)	-
Nitrofurantoin	3 (1.1)	2 (1.8)	1 (3.6)	-	-	-	-
Susceptible to all	77 (29.8)	53 (47.7)	1 (3.6)	2 (33.3)	5 (15.2)	17 (24.6)	6 (60)

All strains were susceptible to ertapenem, imipenem, meropenem, fosfomycin, tigecycline and amikacin.

Table 3.3. Antimicrobial resistance pattern of *Salmonella* spp. isolates according their serotypes.

Antimicrobial agent	Serotypes								
	Typhimurium	**Enteritidis**	**Havana**	**Infantis**	**Mbandaka**	**Virchow**	**Derby**	**Others[a]**	**ND[b]**
	(n= 84)	**(n= 26)**	**(n = 20)**	**(n = 20)**	**(n = 9)**	**(n = 2)**	**(n = 2)**	**(n = 9)**	**(n = 86)**
	Nr (%)	**Nr (%)**	**Nr (%)**	**Nr (%)**	**Nr (%)**	**Nr (%)**	**Nr (%)**	**Nr (%)**	**Nr (%)**
Tetracycline	74 (88.1)	3 (11.5)	-	7 (35.0)	1 (11.1)	-	1 (50.0)	-	41 (47.7)
Ampicillin	67 (79.8)	4 (15.4)	-	2 (10.0)	1 (11.1)	-	-	-	19 (22.1)
Ampicillin/sulbactam	35 (41.7)	1 (3.8)	-	-	-	-	-	-	12 (14.0)
Amoxicillin/clavulanate	2 (2.3)	-	-	-	-	-	-	-	3 (3.5)
Piperacillin	67 (79.8)	3 (11.5)	-	2 (10.0)	1 (11.1)	-	-	-	14 (16.3)
Piperacillin/tazobactam	3 (3.6)	-	-	-	-	-	-	-	2 (2.3)
Mezlocillin	66 (78.6)	3 (11.5)	-	3 (15.0)	1 (11.1)	-	-	-	14 (16.3)
Cefazolin	3 (3.6)	1 (3.8)	-	-	-	-	-	-	3 (3.5)
Cefuroxime	1 (1.1)	1 (3.8)	1 (5.0)	-	-	-	-	-	2 (2.3)
Cefoxitin	1 (1.1)	-	-	-	-	-	-	-	1 (1.2)
Ceftazidime	1 (1.1)	1 (3.8)	-	-	-	-	-	-	1 (1.2)
Cefotaxime	1 (1.1)	1 (3.8)	-	-	-	-	-	-	1 (1.2)
Cefpodoxime	1 (1.1)	1 (3.8)	-	-	-	-	-	-	2 (2.3)
Aztreonam	1 (1.1)	1 (3.8)	-	-	-	-	-	-	-
Ciprofloxacin	-	-	-	-	-	-	-	-	2 (2.3)
Levofloxacin	-	-	-	-	-	-	-	-	2 (2.3)
Norfloxacin	-	-	-	-	-	-	-	-	2 (2.3)
Moxifloxacin	6 (7.1)	1 (3.8)	5 (25.0)	2 (10.0)	-	-	-	-	7 (8.1)

(Cont.)	Serotypes								
Antimicrobial agent	**Typhimurium (n= 84) Nr (%)**	**Enteritidis (n= 26) Nr (%)**	**Havana (n = 20) Nr (%)**	**Infantis (n = 20) Nr (%)**	**Mbandaka (n = 9) Nr (%)**	**Virchow (n = 2) Nr (%)**	**Derby (n = 2) Nr (%)**	**Others[a] (n = 9) Nr (%)**	**ND[b] (n = 86) Nr (%)**
Tobramycin	4 (4.8)	-	-	-	-	-	-	-	-
Gentamicin	5 (6.0)	-	-	-	-	1 (50.0)	-	-	2 (2.3)
Colistin	10 (11.9)	23 (88.5)	-	1 (5.0)	1 (11.1)	-	-	-	2 (2.3)
Chloramphenicol	27 (32.1)	1 (3.8)	1 (5.0)	2 (10.0)	-	-	-	-	12 (14.0)
Trimethoprim/ Sulfamethoxazole	7 (8.3)	1 (3.8)	-	3 (15.0)	-	-	-	-	7 (8.1)
Nitrofurantoin	1 (1.1)	-	-	-	-	2 (100.0)	-	-	-
Susceptible to all[c]	2 (2.4)	1 (3.8)	14 (70.0)	11 (55.0)	7 (77.8)	-	1 (50.0)	9 (100.0)	32 (37.2)

[a]Serotypes Newport, Agona, Altona, London. Branderburg, Patel and Heidelberg.

[b]Serotypes Not Determined

All strains were susceptible to ertapenem, imipenem, meropenem, fosfomycin, tigecycline and amikacin.

3.4.3. PCR and DNA sequencing of ESBL producers

S. Typhimurium Sal25 and *S.* Enteritidis Sal353 were resistant to several β-lactams, including extended-spectrum cephalosporins, and Sal25 showed also resistance to aminoglycosides. Sal25 and Sal353 strains were resistant to cefotaxime (MIC ≥ 256 mg/L and MIC 12 mg/L, respectively) and ceftazidime (MIC 8 mg/L and MIC ≥ 256 mg/L, respectively). Both strains showed a typical double-disc synergy that suggested ESBL production. Nucleotide sequencing of the amplicons identified the $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-1}}$ resistance genes in *S.* Typhimurium Sal25 and the $bla_{\text{SHV-12}}$ resistance gene in *S.* Enteritidis Sal353.

Class 1 integrons were detected in both strains Sal25 and Sal353. The nucleotide sequencing of class 1 integron from the aminoglycoside resistant Sal25 strain showed only the *aadA1* gene, which confers resistance to streptomycin and spectinomycin.

3.4.4. Conjugation assays

Resistance to β-lactams was transferred from Sal25 and Sal353 to *E. coli* J53 recipient strain by conjugation. Transconjugants and the parental strain Sal25 were positive for IS*Ecp1* sequence, located upstream $bla_{\text{CTX-M-1}}$ gene. The incompatibility group of ESBL's plasmid was identified as IncHI2 in Sal25 strain and IncI1 in Sal353 strain. Class 1 integrons were also transferred, confirmed by PCR.

3.5. Discussion

In Europe, more than 100,000 salmonellosis human cases are reported each year (EFSA & ECDC, 2014). In Portugal, little information is available on antimicrobial resistance of *Salmonella*. The main objective of this study was to assess the distribution and antimicrobial susceptibility of *S. enterica* serotypes isolated from animals and

processed food, and to characterize the ESBLs. Moreover, analysis of the results allowed us to compare with data obtained before the antimicrobial agents were banned in animal feed in EU in 2006, and the implementation of the National Control Program for *Salmonella* in poultry.

S. Typhimurium and *S.* Enteritidis are reported as the main sources of salmonellosis (EFSA & ECDC, 2014). Here, they represented 42.7% (n = 110) of the isolates (Table 3.1.), suggesting that other serotypes, referred usually as less common, might becoming important as a cause of human salmonellosis.

S. Typhimurium was the major serotype isolated in swine (58.6%, n = 17), which is in concordance with other reports (EFSA & ECDC, 2014). However, we found higher frequency when compared with previous Portuguese studies (Da Silva & Carneiro, 2006; DGV, 2007; Clemente *et al*., 2013). *S.* Enteritidis was the prevalent serotype in poultry, (20.7%, n = 23), while it was present in 44%, 78% in isolates collected in 1999 and 2003/2004, respectively (Antunes *et al.*, 2003; Da Silva & Carneiro, 2006). A recent study showed a reduction of *S.* Enteritidis in poultry as well (32.8%) (Clemente *et al.*, 2013). The administration of *Salmonella* vaccine as part of the National Program for Control for *Salmonella* implemented after 2008, might be associated with the reduction in this host and the emergence of others serotypes, including Typhimurium. Our data show an increase of *S.* Typhimurium in poultry comparing with previous reports (Antunes *et al*., 2003; Da Silva & Carneiro, 2006; Clemente *et al.*, 2013), suggesting an occupation of a new biological niche. *S.* Typhimurium was also the serotype with the greatest frequency and diversity for antimicrobial resistance, which is a public health concern. The results obtained, and compared with previous data, indicated that the control measures to decrease the *Salmonella* dissemination in poultry have been effective, but they should be extended to swine (Rasschaert *et al*., 2012). *Salmonella* serotypes Infantis, Mbandaka,

Newport, Virchow, Derby and Agona have been consistently in the top 10 most frequently reported serotypes in Europe, although not so common as Typhimurium and Enteritidis (EFSA & ECDC, 2014). All are known to induce gastroenteritis in a broad range of unrelated host species (Eurosurveillance editorial team, 2012), and our results revealed an emergence of these serotypes, especially in poultry, which might be more common in a near future (Clemente *et al*., 2013).

In Portugal, the use of antimicrobial agents in animal therapeutic ascended to 180 tonne in 2010 (DGV, 2010). A high rate of resistance to ampicillin and tetracycline was observed, especially in isolates from processed food (Table 3.2.), which were the main antimicrobial agents used in animal therapeutic in Portugal (DGV, 2010). The high frequency of resistance to ampicillin and tetracycline had been described in uncooked food and ready for consumption (Da Silva & Carneiro, 2006; Clemente *et al*., 2013; EFSA & ECDC, 2014), which may compromise effective treatment with these antimicrobials in case of infection (Hohmann, 2001). A decrease in the frequency of fluoroquinolone resistance was observed when compared to the resistance reported previously (Antunes *et al.,* 2003; Da Silva & Carneiro, 2006), after the legalization of the use of this antimicrobial class in veterinary use (Threlfall *et al*., 1997). This finding might be associated with the ban of antimicrobial agents as growth promoters in animal feed in 2006 in the European Union countries (Guardabassi & Courvalin, 2006). However, resistant *Salmonella* strains continued to be isolated demonstrating that the control measures instituted at feed level may be insufficient to minimize the emergence of resistant strains. Like in human therapy, there must be a continuous surveillance and an evaluation of the need and careful choice of antimicrobial agents in livestock.

In this study, the prevalence of ESBLs was low. In Portugal, ESBL-producing *E. coli* is relatively common in animals (Ramos *et al*., 2013), but scarce in *Salmonella*

isolates (Clemente *et al*., 2013). A *S.* Typhimurium strain produced *bla*$_{\text{TEM-1}}$ and the *bla*$_{\text{CTX-M-1}}$ ESBL, associated with IncHI2 type plasmid, while the *bla*$_{\text{SHV-12}}$ was identified in a *S*. Enteritidis strain. Recently, *bla*$_{\text{CTX-M-1}}$ gene was found in *Salmonella* isolated from animal and human samples in France, but in an IncI1 conjugative plasmid (Cloeckaert *et al*., 2010). In Portugal, ESBLs in strains of animal origin, like *bla*$_{\text{CTX-M-9}}$ and *bla*$_{\text{CTX-M-15}}$ enzymes, were identified in *E. coli* clinical strains and were associated to IncHI2 and IncFII plasmid groups, respectively (Novais *et al*., 2006; Coque *et al*., 2008). The European spread of the *bla*$_{\text{CTX-M-9}}$ in clinical *E. coli* and *S. enterica* strains is largely due to dissemination of plasmids of the IncHI2 group (Guardabassi & Courvalin, 2006; Novais *et al*., 2006). Plasmids of the IncHI2 group were also associated with the dissemination of *bla*$_{\text{CTX-M-2}}$ gene in animal reservoirs (Fernández *et al*., 2007). An IncHI2 replicon associated to *bla*$_{\text{CTX-M-9}}$ in a clinical isolate of *S.* Bovismorbificans was reported in Portugal (Antunes *et al*., 2013). To the best of our knowledge, *S.* Typhimurium with IncHI2 plasmids carrying *bla*$_{\text{CTX-M-1}}$ have not been described so far in Portugal. Its spread by conjugation was demonstrated, which is a public health concern. Overall, IncHI2 replicon type plasmids seem to have a role on the dissemination of CTX-M-type ESBLs among animals' reservoirs.

We also reported the first *bla*$_{\text{SHV-12}}$ in *S.* Enteritidis, one of the most common sources of human gastroenteritis (EFSA & ECDC, 2014). This ESBL has been reported worldwide in other *Salmonella* serotypes (Miriagou *et al*., 2004; Morosini *et al*., 2010; Clemente *et al*., 2013). Despite the low number of ESBL-producing *Salmonella* encountered, this study confirmed the importance of food-producing animals and food products as important reservoirs of *Salmonella* isolates carrying ESBLs-encoding genes, which can easily be transferred to humans through food chain or direct contact with animals infected, or even transferred to others human pathogens (Liebana *et al*., 2013).

In conclusion, our data revealed changes in animal origin *Salmonella* serotypes and antimicrobial resistance rate, and occurrence of ESBLs-producing *Salmonella*. Through the years a decrease of resistance to some antimicrobial agents like fluoroquinolones was observed, suggesting that fluoroquinolones may be considered a good alternative against *Salmonella* human infection. In contrast, there is an emergence of ESBLs-producing strains that can compromise foodborne *Salmonella* infection treatment. *Salmonella* control program reduced Enteritidis serotype in poultry, but Typhimurium seems to adapt to these hosts, being the serotype more resistant to antimicrobial agents, and should be extended to other animals. The control measures taken by EU seem to have some impact in the frequency of resistance. However, data from a continuous monitoring of food production is of critical importance, and will contribute to the evaluation of strategies for prevention and control of antimicrobial resistance.

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Chapter 4

Multidrug and heavy metal resistance in *Salmonella enterica* isolated from food-producing animals and food products

Rui Figueiredo, Roderick Card, Javier Nunez, Nuno Mendonça, Gabriela Jorge da Silva, Muna F. Anjum

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4.1. Abstract

To determine antimicrobial resistance profile in *Salmonella* isolated from food and food-producing animals in Portugal; in selected isolates detect heavy metal (HM) resistance, the associated genetic elements and their transferability. Perform antimicrobial testing phenotypically and antimicrobial resistance (AMR) gene detection using microarrays for 106 *Salmonella*. Perform whole genome sequencing (WGS), HM susceptibilities and conjugations for selected strains. Phenotypic and genotypic AMR testing detected high levels of resistance to tetracycline (65.1%) and ampicillin (58.8%), mainly due to presence of *tet*(B), $bla_{\text{TEM-type}}$ and $bla_{\text{PSE-type}}$. *Salmonella* Genomic Island-1 was detected in seventeen strains which harboured the *hld*_DT104 gene marker. WGS of *S.* Typhimurium Sal199 harbouring cytolethal distending toxin confirmed resistance to tetracycline but detected no HM resistance genes despite showing phenotypic resistance to a number of HMs. WGS of multi-drug resistant *S.* Typhimurium Sal25, showing HM resistance, confirmed $bla_{\text{CTX-M-1}}$ gene on a conjugative IncHI2 plasmid which also harboured a plethora of genes encoding resistance to tellurite, zinc, copper, cobalt, arsenic, mercury, silver and cadmium. WGS of tetracycline and ampicillin resistant *S.* Typhimurium Sal368, showed for the first time in *Salmonella*, presence of a *tet*(M) gene inserted within Tn*3* transposase in an IncF plasmid that also harboured $bla_{\text{TEM-1}}$ gene and resistance genes to arsenic, mercury, and silver, to which it expressed high levels of resistance. Both plasmids easily transferred by conjugation to *Escherichia coli*. When monitoring AMR genes dissemination it is important also to consider HM resistance and their involvement in maintenance/selection of enteric pathogens in the food chain.

4.2. Introduction

Salmonella spp. are a common source of foodborne diseases that cause morbidity and mortality worldwide (Majowicz *et al*., 2010). In the European Union (EU), salmonellosis is the second most commonly reported gastrointestinal infection, with a confirmed case rate of 20.4 cases per 100,000 individuals in 2011 (EFSA, 2013). Gastroenteritis infections caused by non-typhoidal *Salmonella* (NTS) are mostly self-limiting and antimicrobial treatment is usually not required. However, 3-10% of individuals with gastrointestinal illness caused by NTS will develop bacteraemia (Okeke *et al*., 2005). Numerous serotypes of isolated NTS have been found to overlap between farm animals and humans. Antimicrobial resistance is an important cause of treatment failure in humans and reduces significantly the therapeutic options available (EFSA, 2013).

There has been a rapid emergence in recent years of multidrug-resistant (MDR) isolates of NTS, showing resistance to three or more antimicrobial classes tested (Whichard *et al*., 2010; Magiorakos *et al*., 2012). Antimicrobial resistance genes located within mobile genetic elements, such as plasmids and chromosomal islands, can lead to the spread of resistance both within *S. enterica* strains and among other bacterial species, including anaerobes (Anjum *et al*., 2011; Szmolka *et al*., 2012; Kirchner *et al*., 2013). Moreover, *Salmonella* are zoonotic pathogens that can pass between animals and humans through the food chain, causing infection in both; with *Salmonella* Typhimurium being one of the main serovars associated with enteric disease. *S*. Typhimurium DT 104 a virulent pathogen for animals and humans, including in Portugal is an epidemic strain that has added to the problem of treating *Salmonella* infections worldwide (Hall, 2010; Antunes *et al*., 2011). Multidrug resistant (MDR) *S*. Typhimurium DT 104 isolated in animals is typically characterized by resistance to five antimicrobials which are present

on a *Salmonella* Genomic Island (SGI-1) (Hensel, 2004); mobilisation of SGI-1 from Typhimurium DT 104 has resulted in its dissemination to other *Salmonella* serotypes (Frana *et al.*, 2001; Anjum *et al*., 2011). Emerging MDR *S.* Typhimurium 4,[5],12:i:− has been identified in several European countries, and has also been identified with heavy metal (HM) tolerance determinants (Mourão *et al*., 2015).

Selective pressure due to substantive use of antibiotics in food-producing animals has been implicated in the selection and maintenance of antimicrobial resistance determinants in *Salmonella*; but the possibility of co-selection of these successful MDR *Salmonella* spp. by other compounds widely used in animal husbandry (e.g. biocides, metals) remains less explored. HMs with antimicrobial activity are currently used in animal farming management due to the antibiotic restriction directives and/or to limit transfer of bacterial foodborne zoonotic pathogens. Silver and copper are appropriate for use as disinfectants, antiseptics or preservatives; copper and zinc are suitable to use as feed additives for growth promotion of farm animals; and mercury can be found as a contaminant in animal feed (Seiler & Berendonk, 2012). Zinc is also known to inhibit some of the bacterial populations in the intestinal tract and thereby improve the health or feed conversion ratio of the food animals (Medardus *et al.,* 2014). So, HMs can remain in the animal production environment and accumulate to toxic concentrations, which can represent a long-term selective pressure driving the co-selection of antibiotic-resistant bacteria including pathogens such as *Salmonella* spp.

The aim of the present study was to assess the antimicrobial resistance profile for a panel of *Salmonella* isolates from food-producing animals from farms, slaughter houses and retail meat whose virulence profile we have previously described (Figueiredo *et al*., 2015a). We also aimed to characterise the genetic elements associated with HMs resistance in selected *S.* Typhimurium isolates showing AMR, including MDR, by whole

genome sequencing; and determine the expression of these genes to a selection of HMs, and their transferability to a non-*Salmonella spp.* such as *E. coli*, which is ubiquitous in the environment.

4.3. Methods

4.3.1. Bacterial isolation and phenotypic resistance typing

A total of 106 *S. enterica* non-Typhi strains from a previous study were used (Figueiredo *et al*., 2015a). Strains were isolated from poultry (n = 39), swine (n = 14), and cattle (n = 1), and processed food (n = 52). The serotypes were: Typhimurium (n = 59), Enteritidis (n = 21), Havana (n = 6), Infantis (n = 6), Mbandaka (n = 2), Virchow (n = 2), Derby (n = 1), and non-determined serotype (n = 9). Antimicrobial susceptibilities were tested to 21 antimicrobials or antimicrobial combinations (Table S4.1.). The Minimal Inhibitory Concentrations (MICs) were determined by the microdilution broth method (Microscan Panel, Siemens, West Sacramento, CA, USA), according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2013). All susceptibility breakpoints were interpreted according to CLSI, except for moxifloxacin for which the European Committee on Antimicrobial Susceptibility Testing breakpoint was used (EUCAST, 2014).

4.3.2. Microarray Hybridization

Genomic DNA was isolated as previously described (Anjum *et al*., 2007), and used for a linear multiplex amplification reaction that included a labelling step with biotin-dUTP and all AMR primers (Card *et al*., 2013). A total of 128 antimicrobial resistance determinants target to aminoglycosides (n = 19); β-lactamics (n = 64); chloramphenicol (n = 5); integrases I and II (n = 2); quinolones (n = 9); sulphonamides (n = 3); tetracycline (n = 10); trimethoprim (n = 9) and to others antimicrobials agents (n

= 7) not used in *Salmonella* treatment (Kirchner *et al*., 2013; Card *et al*., 2013). Labelled DNA was hybridised to immobilized probes present on the microarray using the HybPlus Kit buffers (Alere Technologies, Jena, Germany). Microarray signals were detected with the ArrayMate device (Alere Technologies) using IconoClust software (Standard version; Alere Technologies). Mean signal intensities of two replicate spots per probe were used for analysis and values ≥ 0.5 were considered positive.

4.3.3. Plasmid profiles

Plasmid profile was performed on selected isolates, which were chosen based on their microarray results. It included MDR *S.* Typhimurium strains Sal25, Sal199 with CdtB toxin, and Sal368 with *tet*(M) gene. They were grown in Luria broth (LB) at 37ºC, with shaking at 300 rpm. Cells were harvested at 13000rpm for 5 minutes, the pellets were lysed, plasmid extracted and plasmid profile determined by gel electrophoresis as previously described (Kado & Liu, 1981). DNA was separated on 0.8% agarose gels and visualized after staining with ethidium bromide, using a Ultraviolet (UV)-transiluminator. The approximate size of each plasmid was determined after comparison with an *E. coli* 39R861 (Threlfall *et al*., 1986); containing 4 plasmids of known size and a supercoiled DNA ladder (Sigma).

4.3.4. Whole genome sequencing of selected isolates

The genomic DNA of isolates Sal25, Sal199, and Sal368 was purified and pair-end sequenced using an Illumina Miseq Sequencer. Raw sequences were filtered and trimmed to minimized sequencing errors (Trimmomatic software) (Bolger *et al*., 2014). The raw data was mapped onto the reference *S.* Typhimurium LT2 (BWA software) using SAMTOOLS software (Li & Durbin, 2009; Li *et al*., 2009). Between 85% and 91% of

the raw data was mapped to LT2 with a mean coverage parameter between 17 and 33. Between 89% and 97% of the reference genome LT2 was shared with the strains used in this study. In house software was used to identify the core genome (genomic regions shared by the strains in the study), SNPs, possible deletions (uncovered regions of the reference genome LT2) and insertions (*de novo* assembly of unmapped raw data) with respect to LT2 strain. Genome was also annotated using RAST (http://rast.nmpdr.org), which helped to identify HM resistance genes. The sequences of the *S.* Typhimurium Sal25, *S.* Typhimurium Sal199, *S.* Typhimurium Sal368 are deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB9808, which is available from http://www.ebi.ac.uk/ena/data/view/PRJEB9808.

4.3.5. Heavy metal resistance

MICs for sequenced strains were determined by microdilution method using Mueller-Hinton broth (BioMérieux, Marcy-l'Étoile, France) supplemented separately with cobalt ($CoSO_4$), cadmium ($CdSO_4$), mercury ($HgCl_2$), arsenic ($NaAsO_2$), lead ($[CH_3COO]_2Pb$), silver ($AgNO_3$), and tellurite (K_2TeO_3) at doubling dilutions from 8 to 0.001 mM (final concentrations), adjusted to pH 7.2. MICs for cupper ($CuSO_4$) and zinc ($ZnSO_4$) were determined at doubling dilutions from 36 to 0.25 mM (final concentrations). Approximately 0.2 µL of a 10^7 CFU/mL suspension of each isolate was added to each well of a 96-well plate containing Muller-Hinton broth supplemented with the afore mentioned HMs in the concentration ranges given, the plates were incubated at 37 ºC for 24h.

Conjugation assays for sequenced strains were performed to assess plasmid mediated transfer of HM resistance, using *E. coli* J53 strain as recipient (resistant to azide sodium, Azi^R). Briefly, conjugation experiments were performed in trypticase-soy (TS)

broth at a ratio of donor: recipient of 1:10 with shaking at 100 rpm at 37ºC for 16h. Selection was performed on MacConkey agar plates separately supplemented with of $CoSO_4$, $CdSO_4$, $HgCl_2$, $NaAsO_2$, $(CH_3COO)_2Pb$, $AgNO_3$, or K_2TeO_3 (10 µM), and $CuSO_4$ and $ZnSO_4$ (2 mM). Lactose-positive colonies were cultured in TS agar supplemented with the correspondent HM compound at the appropriate concentration and sodium azide (10 mg/L). The susceptibility of recipient *E. coli* J53 and transconjugants to the HMs included in this study was performed as described above. The efficiency of conjugation calculated, which is estimated as the number of transconjugants per donor cell.

4.4. Results

4.4.1. Antimicrobial resistance microarray

Phenotypic resistance to one or more antimicrobials was found in 82% of isolates (Table S4.1.), with 27% being MDR. The majority of resistance phenotypes correlated with the microarray genotype data. Resistance to tetracycline was shown in 65.1% of isolates which harboured: *tet*(B) (52.2%), *tet*(G) (23.2%), *tet*(A) (21.7%), *tet*(E) (20.2%) and *tet*(M) (1.5%). The *tet*(E) and *tet*(G) genes were both present in some isolates. Only one isolate was positive for *tet*(M), which has previously not been reported in *Salmonella*. Phenotypic resistance to the β-lactams tested (Table S4.1.) was observed in 58.5% of all the isolates, and was genotypically associated with bla_{TEM} (70.3%), bla_{PSE} (26.6%), $bla_{\text{OXA-1}}$ (2.1%), bla_{SHV} (1%), and $bla_{\text{CTX-M-1}}$ (1%). MDR *S.* Typhimurium Sal25 showed by microarray the simultaneous presence of $bla_{\text{CTX-M-group-1}}$ and bla_{TEM} genes; further sequencing indicated presence of $bla_{\text{TEM-1}}$ and the Extended Spectrum Beta-Lactamase (ESBL) determinant $bla_{\text{CTX-M-1}}$. This strain also harboured genes coding for resistance to gentamicin (*aac3-IV*, *aadA1*-like genes), chloramphenicol (*catA1*) and tetracycline [*tet*(B)].

Resistance to the aminoglycosides tested was reported in 3.8% of *Salmonella* isolates, they all harboured *aac3*-IVa or *aac6'-aph2'* genes. Presence of *aadA1*-like (11.3%) and *aadA2*-like (30.2%) genes were also found in isolates that did not express aminoglycoside resistance phenotypically. Aminoglycosides resistance genes were usually associated with presence of the class 1 integron (32.1%). Fourteen percent of *Salmonella* isolates were resistant to quinolones, of which 80% harboured the *qnrB* gene. Resistance to trimethoprim/sulfamethoxazole was observed in 11.3% of isolates, encoded in most by *dfrA01* gene (8.3%) associated with *sul1* or *sul2*. Resistance to chloramphenicol was observed in 26.4% of *Salmonella* isolates, of which 67.9% was conferred by *floR1*, 17.9% by *cmlA1*-like gene and 7.1% by *catA1* gene (Table S4.1.).

4.4.2. Whole genome sequencing of selected isolates

We performed whole genome sequencing on 3 *S.* Typhimurium isolates which were selected based on their microarray genotypes: MDR isolate Sal25, Sal199 strain which harbours a CdtB toxin (Figueiredo *et al.*, 2015a) and *tet*(E), and AMR Sal368 which showed presence of a *tet*(M) gene, that is mainly associated with Gram-positive bacteria. The draft genomes from each isolate were mapped to the reference *S.* Typhimurium LT2 genome and annotated using the RAST server (http://rast.nmpdr.org). From genome sequencing and annotation of the isolates we verified presence of all AMR genes that were detected by microarray (Table S4.1.).

In addition, we detected genes which may enable selection in presence of HMs. These included several efflux systems which are known to pump out a wide range of toxins including antibiotics and HMs from the bacterial cells. For Sal199, which harboured the CdtB tripartite toxin, no HM resistance genes were detected from genome annotation. MDR Sal25 strain carried a tellurite resistance operon (*terABCDEFXWZ*),

arsenic resistance operon (*arsRDABC*) and mercury resistance operon (*merACFDPTR*) (Table 4.1.). Genes *cusC* and *cusF*, and a RND transporter of the CzcB family related to cobalt, zinc, and cadmium resistance were present; as well as zinc resistance mediated by *zitB* gene, and *cutA*, *cop*, *cusR*, and *pcoE* genes which are associated with copper resistance were also present. An IncHI2 conjugative plasmid harboured a *bla*$_{\text{CTX-M-1}}$ gene with the IntIPac and Tn*21* transposase downstream and the insertion sequences, ISE*cp1* and IS*5*-like element, upstream (Figure 4.1.).

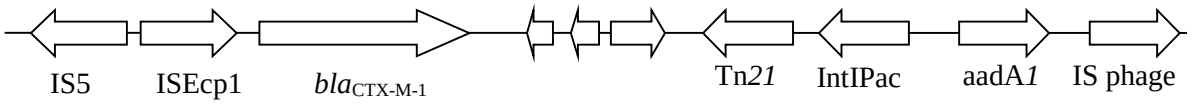

Figure 4.1. Genetic environment surround *bla*$_{\text{CTX-M-1}}$ in *S.* Typhimurium Sal25.

In addition, it carried the chloramphenicol resistance *catA1* gene associated with a *tnpA* transposon, tetracycline resistance gene *tet*(B) was located downstream of the Tn*21* (transposase) and *tnpR* (resolvase) genes, and the streptomycin *strA* and *strB* genes were surrounded by phage replication genes. The silver binding protein (*silE*) gene was carried on Sal25, and in Sal368. Whole genome sequencing confirmed presence of the *tet*(M) in strain Sal368, in addition to *tet*(B), and *bla*$_{\text{TEM-1}}$ genes. An IncF plasmid harboured the *tet*(M) gene which was inserted within a Tn*3* transposase, and *bla*$_{\text{TEM-1}}$ inserted on Tn552, within the intact prophage Gifsy-2 (Figure 4.2.).

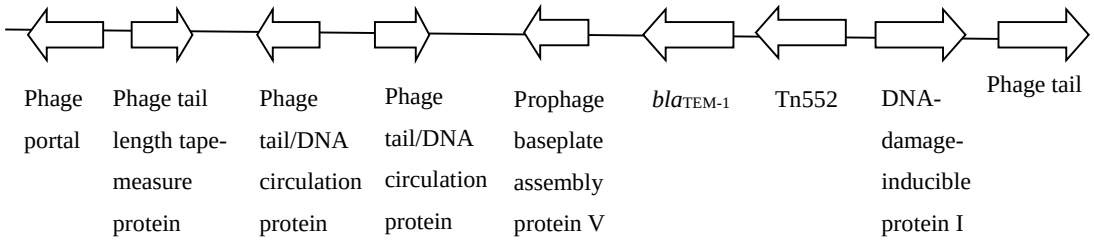

Figure 4.2. Phage genes enclose *bla*$_{\text{TEM-1}}$ in *S.* Typhimurium Sal368.

An *aadA2*-like gene was present within a class I integron. Resistance to arsenic (*arsRDABC*) and the mercury resistance operon (*merACFDPTR*), were also identified in Sal368 (Table 4.1.).

4.4.3. Phenotypic characterization of isolates to HM resistance

We determined whether these isolates, which harboured a number of genes encoding HM resistance, expressed these resistances. Susceptibilities of the 3 *S.* Typhimurium isolates to a variety of HMs were performed. *S.* Typhimurium LT2 was used as a negative control strain to compare MIC values. Our results showed that all 3 isolates had higher MIC to a number of HMs with respect to LT2 (Table 4.1.). Resistance to arsenic was 8-fold higher in Sal25 and Sal368 than either LT2 or Sal199. Resistance to cobalt was 2-fold higher in Sal25 and 4-folds higher in Sal199 and Sal368 in comparison to LT2. Resistance to zinc was similar for all 3 isolates, which was double that for LT2. Only Sal25 and Sal368 showed increased tolerance to copper with respect to LT2.

Table 4.1. MICs to antimicrobial agents and heavy metals of *Salmonella* isolates and transconjugants.

Strain	Antimicrobial agents[a] MIC (µg/mL)				HM MIC (mM)								
	AMP	**CTX**	**TET**	**SM**	**Arsenic**	**Cadmium**	**Cobalt**	**Zinc**	**Copper**	**Lead**	**Mercury**	**Silver**	**Tellurite**
S. Typhimurium LT2	64	0.06	16	2	1	0.5	0.25	8	8	0.008	0.03	0.016	2
S. Typhimurium Sal25	>256	>256	256	>16	>8	1	0.5	16	16	1	0.25	0.125	>8
S. Typhimurium Sal199	2	<0.016	16	0.5	1	0.25	1	16	8	0.016	0.03	0.03	2
S. Typhimurium Sal368	256	0.125	124	16	8	0.5	1	16	16	0.5	0.06	0.125	2
E. coli J53 (recipient)	16	0.03	1	0.125	0.004	0.25	0.004	1	1	0.008	0.004	0.001	0.08
T1 - J53+Sal25	>256	16	1	8	4	0.5	0.25	4	8	0.25	0.125	0.016	4
T2 - J53+Sal368	128	0.03	32	8	4	0.5	0.25	4	4	0.25	0.03	0.016	0.5

[a]AMP – ampicillin; CTX – Cefotaxime; TET – Tetracycline; SM; Streptomycin

Mercury resistance was 15.6-fold higher in Sal25, 3.8-fold higher in Sal368 and 1.9-fold higher in Sal199 compared to LT2. Tellurite resistance was 4-fold higher in Sal25 than LT2. In contrast, lead resistance was 125-fold higher in Sal25 and 62.5-fold higher in Sal199 and Sal368 than LT2. Silver resistance was similarly high, being 125-fold higher in both Sal25 and Sal368 than LT2.

4.4.4. Transfer of heavy metal and antimicrobial resistance

Phenotypic characterisation showed that all 3 isolates which were AMR were also highly resistant to a number of HMs; whole genome sequencing data indicated these were encoded by genes which were possibly plasmid associated. Conjugation studies were performed to determine the transferability of HM and antimicrobial resistance from the donor *S*. Typhimurium to *E. coli* J53 (Table 4.1.). No transconjugants were obtained from conjugation of *E. coli* J53 with Sal199. However, there were a number of transconjugants as a result of conjugation of *E. coli* J53 with Sal25 and Sal68. The frequency of plasmid transfer in the presence of different HM for the two isolates is presented in Table 4.2. The HM plasmid associated with Sal25 (pSal25) consistently showed a higher frequency of transfer than the HM plasmid associated with Sal368 (pSal368). Selection using zinc, copper and tellurite resulted in higher frequency of transfer of the plasmid to the recipient strain from both Sal25 and Sal368, than was observed for lead and silver (Table 4.2.).

Table 4.2. Efficiency of conjugation of pSal25 between Sal25 and *E. coli* J53; and pSal368 between Sal368 and *E. coli* J53.

	Conjugative plasmid	**Transconjugants CFU/ml**	**Frequency of transfer[a]**
Arsenic	pSal25	$1.05x10^{6}$	$6.48x10^{-3}$
	pSal368	$7.50x10^{5}$	$4.34x10^{-3}$
Cadmium	pSal25	$8.50x10^{5}$	$5.24x10^{-3}$
	pSal368	$7.00x10^{5}$	$4.05x10^{-3}$
Cobalt	pSal25	$1.10x10^{6}$	$6.79x10^{-3}$
	pSal368	$9.50x10^{5}$	$5.49x10^{-3}$
Zinc	pSal25	$1.20x10^{6}$	$7.41x10^{-3}$
	pSal368	$1.05x10^{6}$	$6.01x10^{-3}$
Copper	pSal25	$1.15x10^{6}$	$7.10x10^{-3}$
	pSal368	$1.10x10^{6}$	$6.36x10^{-3}$
Lead	pSal25	$6.00x10^{5}$	$3.70x10^{-3}$
	pSal368	$4.50x10^{5}$	$2.60x10^{-3}$
Mercury	pSal25	$1.10x10^{6}$	$6.79x10^{-3}$
	pSal368	$9.00x10^{5}$	$5.20x10^{-3}$
Silver	pSal25	$7.50x10^{5}$	$4.63x10^{-3}$
	pSal368	$4.50x10^{5}$	$2.60x10^{-3}$
Tellurite	pSal25	$1.20x10^{6}$	$7.41x10^{-3}$
	pSal368	$1.05x10^{6}$	$6.07x10^{-3}$

All conjugation experiments were performed using $1.62x10^{8}$, and $1.73x10^{8}$ CFU/ml of donor strains Sal25 and Sal368, respectively. *E. coli* J53 at $1.55x10^{8}$ CFU/ml was used as recipient, in a donor: recipient ratio of 1:10. All values were from three replicates in two independent experiments.

[a]The frequency of transfer is the number of transconjugants per donor cell.

A representative *E. coli* J53 transconjugant from each donor was purified and their sensitivity to a panel of HMs compared with the parent strain *E. coli* J53 and *S.* Typhimurium LT2. Arsenic, cobalt, silver, zinc, lead, and mercury resistance was significantly higher in the transconjugants T1 (J53/pSal25) and T2 (J53/pSal368). Transconjugants T1 and T2 conferred 4-fold and 1000-fold more resistance to arsenic than LT2 and *E. coli* J53 respectively; although the resistance was half that of the donor

strains. Both transconjugants showed 63-fold higher cobalt resistance than recipient strain *E. coli* J53 and equal to LT2, but T1 showed half the MIC to cobalt as Sal25, and T2 was 4-fold less than Sal368. Transconjugants T1 and T2 also showed approximately 31-fold higher resistance to lead than *E. coli* J53 and LT2. Mercury resistance of T1 was 31-fold higher than *E. coli* J53 and 4-fold higher than LT2, while T2 presented 8-fold higher resistance than *E. coli* J53, and equal MIC to LT2. Resistance to silver was transferred to transconjugants T1 and T2; it was similar to LT2, thereby increasing the tolerance of the recipient strain by approximately 10 times. Tellurite resistance present in Sal25 was transferred to T1 increasing resistance by 50-fold in J53and transfer of T2 from Sal368 increased tellurite resistance by 6-fold in J53. Neither transconjugant showed increased resistance to cadmium in comparison to LT2, only double that of J53.

The co-transfer of antimicrobial resistance in T1 and T2 verified that the AMR genes were present on the same plasmid that harboured HM resistances (Table 4.1.). Resistance of ampicillin in T1 was 16-fold, and 8-fold higher in T2 compared to J53. Resistance to cefotaxime was transferred from MDR Sal25 to T1, increasing the tolerance of the recipient strain by approximately 533 times. No resistance to cefotaxime was observed in T2, but resistance to tetracycline was transferred to T2, increasing the tolerance of the recipient strain by approximately 32 times. No resistance to tetracycline was transferred from Sal25. Streptomycin resistance was 64 higher in T1 and T2 when compared to recipient strain J53.

4.5. Discussion

Infections with *S. enterica*, by consumption of contaminated food products, are one of the most important causes of food-borne diseases worldwide. Furthermore, there has been a rapid development of resistance to antimicrobial agents in different *S. enterica*

serotypes including Typhimurium worldwide (EFSA, 2013), which will impact the therapeutic options in particular the use of β-lactams (Hohmann, 2001), sometimes required to clear salmonellosis infection.

In concordance with previous work on AMR present in Portuguese *Salmonella (*Antunes *et al*., 2011), we also detected a high rate of resistance to ampicillin in our panel of isolates, associated mainly with the presence of $bla_{\text{TEM-type}}$ and $bla_{\text{PSE-type}}$, and to tetracycline; both of which are the main antimicrobial agents used in animal therapy in Portugal (DGV, 2010). Two ESBL genes, $bla_{\text{CTX-M-1}}$ and $bla_{\text{SHV-12,}}$ were also detected by the microarray, which have previously been scarce in animals and humans in Portugal (Antunes *et al*., 2013; Clemente *et al*., 2013; Figueiredo *et al*., 2015b). Resistance to tetracycline was mainly associated with *tet*(B) and *tet*(G) genes; *tet*(G) genes have only recently been reported in *S.* Typhimurium in Portugal (Antunes *et al*., 2011). Presence of *tet*(G) in 17 strains which also harboured the *hld*_DT104 gene marker in addition to the class 1 integron, $bla_{\text{PSE-1}}$, *aadA2*, *floR1* and *sul1* genes, suggested the presence of SGI1 (Mulvey *et al*., 2006), responsible for pentaresistance. The *tet*(B) gene was found in both *S.* Typhimurium and *S.* Infantis, serotypes associated with swine, suggesting possible transfer of plasmid between isolates shared in this host. The *tet*(M) gene was detected in a *S.* Typhimurium Sal368 isolated in swine raw meat. This is the first report where *tet*(M) associated with the Tnp*A1* transposase was present in an IncF conjugative plasmid from *S.* Typhimurium. The *tet*(M) gene has been reported in *Streptococci* from swine (Aminov *et al*., 2001), and in Portuguese *Enterococcus* strains, but is not common in Gram-negative bacteria (Araújo *et al*., 2010), and is another example of horizontal gene transfer within the host gut microbiota. The *catA1* gene, not reported before in Portuguese *Salmonella* strains was also present in two strains of *S.* Typhimurium. This could suggest

co-selection of this gene with other resistance markers or the possible illegal use of the chloramphenicol, which is banned for animal husbandry in Portugal.

Whole genome sequencing of 3 *S.* Typhimurium strains, Sal25, Sal199, and Sal368, provided insight into possible HMs resistance genes that may be present in this panel of isolates. Their presence indicated a potential adaptation and proliferation of *Salmonella* in the HM contaminated environments of animal husbandry and the food processing chain. Few studies have previously report on HM resistance in conjunction with AMR resistance. Here we demonstrate that a *S.* Typhimurium strain, Sal25, harbouring the $bla_{\text{CTX-M-1}}$ gene on conjugative IncHI2 plasmid, also harboured a plethora of genes encoding resistance to mercury, arsenic, tellurite, zinc, cobalt, lead and cadmium. The plasmid was easily transferred to an *E. coli* laboratory strain (J53) and both the parent and transconjugant showed high levels of resistance to afore mentioned HMs in comparison to *S.* Typhimurium LT2 or *E. coli* J53. Presence of the *pco* genes conferring resistance to zinc and copper has previously been reported in IncA/C or IncR plasmids in Europe *(*Medardus *et al*., 2014; Mourão *et al.,* 2015), and its presence in an IncHI2 plasmid indicates further dissemination. A conjugative IncF plasmid, harbouring mercury, silver, cobalt, lead and arsenic resistance, was present in *S.* Typhimurium Sal368; this plasmid, harbouring both HM and AMR genes, was also transferable. The association of *mer* and *sil* genes with multiple antibiotic resistance genes on IncA/C and IncHI1 plasmids have recently been described in Europe (Gupta *et al*., 2001; García-Fernández *et al*., 2007; Lindsey *et al*., 2011; Moreno-Switt *et al*., 2012). *S*. Typhimurium Sal199 was of particular interest to us as it harboured the first reported cytolethal distending toxin in *S*. Typhimurium. This tripartite toxin is common in *S.* Typhi and we have shown infection of *Galleria mellonella* with this strain results in increased mortality compared to LT2 (Figueiredo *et al*., 2015a). This strain showed higher sensitivity to

antimicrobial agents, with tetracycline being an exception, and lower levels of HM resistance, although the MIC was higher than that of LT2 for zinc, cobalt and lead. Whole genome sequencing and annotation did not detect known genes conferring specific HM resistances in this strain, and there were no plasmids associated with these resistances, as demonstrated by the lack of any HM resistant transconjugants.

Co-localization of antimicrobial and HMs resistance has previously been reported (Chapman, 2003). In this study, phenotypic tolerance of transconjugants, due to horizontal gene transfer of plasmids to the ubiquitous *E. coli*, to mercury, arsenic, cobalt, zinc, silver, lead, tellurite, and cadmium, as well as a number of antimicrobial resistances, demonstrated the ease with which these plasmids can disseminate under laboratory conditions and possibly also do in the environment. We noted the highest level of tolerance of Sal25 and Sal368 to arsenic was 8 mM, which is 8-fold higher than the levels reported previously (Joerger *et al*., 2010). For silver, Sal25 and Sal368 isolates, like other *Salmonella* studied in Portugal (Mourão *et al*., 2015) showed a much higher tolerance than its recognized antimicrobial activity at 0.06 mM, (Araújo *et al*., 2012) which has attracted interest in the scientific community. Metals such as cobalt, copper, and zinc are applied as nutritional additives in animal feed for livestock farming production in Europe (EC, 2003). A few studies have reported different tolerance levels of *Salmonella* to zinc and copper. This study reported 16 mM as the highest level of zinc tolerance in the 3 *Salmonella* isolates, which is the double of the levels previously reported; but notably the copper tolerance seen in this study was 1.75-fold lower than previously (Aarestrup & Hasman, 2004; Medardus *et al*., 2014).

In conclusion, this study has clearly shown the benefits of using an antimicrobial resistance microarray to screen *Salmonella* isolates for antimicrobial resistance. Furthermore, it has shown the benefits of looking for the presence other selective markers

such as HM resistance to the survival of these pathogens in hostile environments. The presence of plasmid associated HM resistance which also harbour AMR genes, highlights the necessity to monitor the co-dissemination of HM and antimicrobial resistance genes in zoonotic bacteria. HM contamination, which is much less studied than AMR, nevertheless, represents a long-standing, widespread and persistent selection pressure with both environmental and clinical importance that, as demonstrated by this study, potentially contributes to the maintenance and spread of antibiotic resistance. In future studies examining AMR genes disseminating in enteric bacteria should also consider HM resistance and their involvement in maintenance of enteric pathogens in the food chain.

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4.7. Supplementary data

On digital format (CD-Rom)

Table S4.1. - Antimicrobial Resistance Phenotype and Resistance Genotype Associated

Chapter 5

Virulence characterization of *Salmonella enterica* by a new microarray: detection and evaluation of the cytolethal distending toxin gene activity in the unusual host *S.* Typhimurium

Rui Figueiredo, Roderick Card, Carla Nunes, Manal AbuOun, Mary C. Bagnall, Javier Nunez, Nuno Mendonça, Muna F. Anjum, Gabriela Jorge da Silva

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5.1. Abstract

Salmonella enterica is a zoonotic foodborne pathogen that causes acute gastroenteritis in humans. We assessed the virulence potential of one-hundred and six *Salmonella* strains isolated from food animals and products. A high through-put virulence genes microarray demonstrated *Salmonella* Pathogenicity Islands (SPI) and adherence genes were highly conserved, while prophages and virulence plasmid genes were variably present. Isolates were grouped by serotype, and virulence plasmids separated *S.* Typhimurium in two clusters. Atypical microarray results lead to whole genome sequencing (WGS) of *S.* Infantis Sal147, which identified deletion of thirty-five SPI-1 genes. Sal147 was unable to invade HT-29 cells and showed reduced mortality in *Galleria mellonella* infection model, in comparison to a SPI-1 harbouring *S.* Infantis. Microarray and WGS of *S.* Typhimurium Sal199, established for the first time in *S.* Typhimurium presence of *cdtB* and other Typhi-related genes. Characterization of Sal199 showed *cdtB* genes were upstream of transposase *IS*911, and co-expressed with other Typhi-related genes. Cell cycle arrest, cytoplasmic distension, and nuclear enlargement were detected in HeLa cells infected by Sal199, but not with *S.* Typhimurium LT2. Increased mortality of *Galleria* was detected on infection with Sal199 compared to LT2.

Thus, *Salmonella* isolates were rapidly characterized using a high through-put microarray; helping to identify unusual virulence features which were corroborated by further characterisation. This work demonstrates using suitable screening methods for *Salmonella* virulence can help assess the potential risk associated with certain *Salmonella* to humans. Incorporation of such methodology into surveillance could help reduce the risk of emergence of epidemic *Salmonella* strains.

5.2. Introduction

Foodborne diseases caused by non-typhoidal *Salmonella* (NTS) represent an important public health problem and economic burden worldwide (Majowicz *et al.*, 2010). In Europe, there are over 100,000 salmonellosis human cases each year (EFSA, 2013). *Salmonella* serovars associated with gastroenteritis transmit through the faecal-oral route, either directly or via contaminated food or water (Hohmann, 2001). Gastroenteritis infections caused by NTS are mostly self-limiting and antimicrobial treatment is usually not required. However, 3-10% of individuals with gastrointestinal illness caused by NTS will develop bacteraemia (Okeke *et al.*, 2005), a serious and potentially fatal condition that requires antimicrobial treatment (Hohmann, 2001). The increase in bacterial antimicrobial resistance has led to an increased risk of treatment failure in humans and reduces significantly the therapeutic options available (EFSA, 2013). While a large number of *Salmonella* serotypes can cause gastroenteritis, a few serotypes such as *S.* Typhi can cause invasive infection.

The differences in pathogenicity of *Salmonella* serotypes depend upon the virulence potential of the microorganism and the susceptibility of the host. Bacterial virulence factors are necessary to adhere, invade and replicate inside host cells. These are encoded by genes present on a wide range of genetic elements, including the bacterial chromosome, plasmids, prophages and *Salmonella* Pathogenicity Islands (SPIs). Some SPIs are conserved throughout the genus, e.g. SPI-1, which encodes a Type 3 Secretion System 1 (T3SS-1) which is important for infection of the host cell and SPI-2, which encodes T3SS-2 that facilitates intracellular survival and replication (McGhie *et al.*, 2009). Other SPIs are serotype specific *e.g.* SPI-7 in *S.* Typhi (Anjum *et al.*, 2005), and increase the virulence potential of the pathogen. Twenty-percent of the *Salmonella* genome comprises DNA of bacteriophage origin which encodes a variety of virulence

factors (McClelland *et al*., 2001). Plasmids are frequently found in *Salmonella* serotypes associated with infections of humans and animals, including the *Salmonella* virulence plasmid. In addition to the risk presented by NTS serotypes as a potential foodborne hazard, there has been a rapid emergence in recent years of multidrug-resistant isolates of *Salmonella* (Anjum *et al*., 2011; Magiorakos *et al*., 2012). So, it is relevant to investigate the presence of virulence determinants in strains that have been implicated in *Salmonella* disease of food origin to help assess and understand the risk posed by strains from different sources. High through-put DNA microarrays have been developed which enable detection of large numbers of virulence genes, allowing many isolates to be studied simultaneously (Anjum *et al*., 2007; Franklin *et al*., 2011; Braun *et al*., 2012; Yoshida *et al*., 2014). This chip is unique as it is based on detecting virulence determinants and not serotype, unlike the other *Salmonella* high through-put chips (Franklin *et al*., 2011; Braun *et al*., 2012), which makes this chip much more informative to determine the potential risk posed by these isolates. It is also much cheaper and easier to handle unlike other *Salmonella* glass-slide microarray chips which have been used to look at virulence profile previously (Anjum *et al*., 2007; Scaria *et al*., 2008; Huehn *et al*., 2010).

The aim of the present study was to assess the diversity of virulence genes in *S. enterica* in order to better understand the potential risk that they might present. A customised *Salmonella* virulence gene microarray was constructed targeting *Salmonella* pathogenicity islands, virulence regulators, prophages, toxin, and virulence plasmids genes. The array screening informed the selection of atypical isolates for further *in vitro* and i*n vivo* virulence characterisation using whole genome sequencing, adhesion/invasion assays, *Galleria mellonella* infection model, and flow cytometry.

5.3. Methods

5.3.1. *Salmonella* strains

A total of 106 *Salmonella* strains were selected from a previous epidemiological study (Figueiredo *et al*., 2015). Strains had been isolated from poultry (n = 39), swine (n = 14), cattle (n = 1), and processed food (meat cuts, hamburgers or sausages) (n = 52). The serotypes found were Typhimurium (n = 59), Enteritidis (n = 21), Havana (n = 6), Infantis (n = 6), Mbandaka (n = 2), Virchow (n = 2), Derby (n = 1) and non-determined serotype (n = 9) (Table 5.1.).

5.3.2. Design and validation of *Salmonella* virulence gene microarray probes

A total of 114 *Salmonella* virulence determinants (Table S5.1.) present on the chromosome (n = 90; SPIs, n = 52, and other chromosomal genes, n = 38), phage related (n = 12), and plasmid related (n = 12), were selected. The genes span a range of virulence and regulatory associated functions including adhesion (n = 28), toxins (n = 2), regulation (n = 2), and invasion/survival within non-phagocytic enterocytes (n = 82). The hybridisation probes and labelling primer sequences were designed in this study and their specificity tested *in silico* as previously described (Batchelor *et al*., 2008); the sequences are presented in Table S5.1. In brief, sequence specific primers for the labelling reaction and hybridisation probes were designed based on sequence data available in GenBank. First, a sequence entry was retrieved for each target gene in the gene list (Table S5.1.), as a reference. Then BLASTn searches against the whole GenBank (NR database) were performed to find any further entries similar to the reference sequence. This was performed for every target gene. The hits for each target were aligned. The alignments were generated directly from the BLASTn results and used to identify conserved regions. Probes and primers were designed, if possible, within these regions. If no sufficient consensus site was found, pairs of perfect match/mismatch primers or probes were

designed which were specific for the allelic variants. The size of the oligonucleotides was varied so the melting temperatures were nearly identical for all probes and primers. A total of 117 gene probes and 114 labelling primers were designed for these genes, including four probes being designed for one gene to encompass allelic variations. Specificity of all probes and primers were determined by performing BLASTn searches against the NCBI database. Each oligonucleotide probe was spotted in duplicate and the microarrays printed by Alere Technologies (Jena, Germany); each replicate probes were spotted at different positions to circumvent any spatial effect of hybridization. The performance of the probes was assessed using control strains, in which presence of the gene had been verified by PCR and sequencing (see below). The specificity of each probe was estimated by comparing the microarray result with PCR and sequence data for the control panel of strains.

Table 5.1. Frequency of non-typhoidal *Salmonella* serotypes according origin.

***Salmonella* Serotype**	**Total (n = 106) Nr (%)**	**Poultry (n = 39) Nr (%)**			**Swine (n = 14)[a] Nr (%)**		**Cattle (n=1)[b] Nr (%)**	**Processed food of animal origin Nr (%)**	
		Farm	**Slaughter house**	**Distribution**	**Slaughter house**	**Distribution**	**Slaughter house**	**Poultry (n = 21)**	**Swine (n = 31)**
Typhimurium	59 (55.7)	2 (5.1)	6 (15.4)	-	10 (71.4)	1 (7.1)	1 (100)	14 (66.6)	25 (80.6)
Enteritidis	21 (19.8)	9 (23.1)	-	9 (23.1)	-	-	-	3 (14.3)	-
Infantis	6 (5.7)	-	1 (2.6)	-	1 (7.1)	-	-	-	4 (12.9)
Havana	6 (5.7)	6 (15.4)	-	-	-	-	-	-	-
Mbandaka	2 (1.9)	2 (5)	-	-	-	-	-	-	-
Virchow	2 (1.9)	2 (5)	-	-	-	-	-	-	-
Derby	1 (0.9)	1 (2.6)	-	-	-	-	-	-	-
ND[a]	9 (8.4)	1 (2.6)	-	-	2 (14.2)	-	-	4 (19)	2 (6.5)

[a]Any isolates recovered from Farm

[b]Any isolate recovered from farm and distribution.

[c]Not determined

5.3.3. Microarray Hybridization

Genomic DNA was isolated and used for a linear multiplex amplification reaction which was performed at 55 ºC, as previously described (Anjum *et al*., 2007). The PCR mixture for linear DNA amplification and labelling contained DNA (0.5µg) in a total volume of 10 µl, mixed with 1 µl of 10x Therminator amplification buffer, 0.1µl Therminator DNA polymerase (BioLabs), 1 µl of virulence primer-mix (0.135 µM per oligonucleotide in the stock solution), 1 µl of dNTP-mix including the biotin label (1 mM dACGTP; 0.65 mM dTTP) and 0.35 µl Biotin-16-dUTP (1 mM Biotin-16-dUTP, Roche). Hybridization was achieved using the HybridisationPlusKit (Alere Technologies, Jena, Germany) employing a heated mixer (Thermomixer, Eppendorf, Hamburg, Germany). Single-stranded labelled amplified products were hybridised to the arrays as described previously (Card *et al*., 2015) and signals read on an ArrayMate apparatus (Alere Technologies, Jena, Germany) using IconoClust software (standard version; Inverness Technologies, Jena, Germany) (Batchelor *et al*., 2008).

5.3.4. Microarray data analysis and probes validation

Mean signal intensities of two replicate spots per probe were used for analysis. Based on PCR data, for the virulence gene probes intensities ≥ 0.4 were considered positive, while those with value < 0.4 were considered absent. The microarray data across all isolates were converted to a binary format, whereby 1 indicated gene presence and 0 indicated gene absence, and compared in BioNumerics 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). An Unweighted Pair Group Method with Arithmetic mean (UPGMA) dendrogram was used to cluster isolates based on their virulence determinants.

PCR primers for each virulence gene were designed using the Primer3Web software (version 0.4.0) (http://frodo.wi.mit.edu/) using the reference sequences given in

Table S5.1., to give a 200 to 500 bp amplicons. PCR amplifications were performed using standard conditions, as reported previously (Batchelor *et al*., 2008). A control *Salmonella* strain was identified for each virulence gene (Table S5.1.) and presence of the gene confirmed by PCR and sequencing.

5.3.5. Whole Genome Sequencing

The genomic DNA from selected isolates showing atypical microarray results was pair-end sequenced using an Illumina Miseq Sequencer. Raw sequences were filtered and trimmed to minimized sequencing errors (Trimmomatic software) (Bolger *et al*., 2014). The raw data was mapped onto the reference *S.* Typhimurium LT2 (BWA software) (Li & Durbin, 2009) and SAMTOOLS software (Li *et al*., 2009). Between 85% and 91% of the raw data was mapped to LT2 with a mean coverage parameter between 17 and 33. Between 89% and 97% of the reference genome LT2 was shared with the strains used in this study. In house software was used to identify the core genome (genomic regions shared by the strains in the study), SNPs, possible deletions (uncovered regions of the reference genome LT2) and insertions (*de novo* assembly of unmapped raw data) with respect to LT2 strain (Accession number: AE006468). The sequences of the *S.* Typhimurium Sal199, *S.* Infantis Sal147, and *S.* Infantis Sal280 are deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB9808, which is available from http://www.ebi.ac.uk/ena/data/view/PRJEB9808.

5.3.6. Reverse transcriptase PCR of SPI-11 genes and typhoid-associated virulence genes

Total RNA was isolated from *S.* Typhimurium Sal199 and *S.* Typhimurium LT2 strains grown on Mueller-Hinton broth for 2, 16 and 24 h at 37° C by using Tri reagent (Sigma Aldrich, Poole, UK) as described before (Abuoun *et al.*, 2005). Reverse

transcriptase PCR (RT-PCR) was carried out using the OneStep RT-PCR kit (Quiagen, UK), according to the manufacturer's instructions. RT-PCR was performed using primers designed to SPI-11 genes *envF, pagC, pagD, pltA, pltB, cdtB* and three typhoid-related virulence genes *taiA, tcfA* and *hlyE* (Table S5.2.). Genomic DNA and *agfA*, a fimbrial gene, were used as positive controls, in normal PCR and RT-PCR, respectively. A negative control with no DNA was also included in the assays. RT-PCR was performed with an initial incubation step of 50° C for 30 min, during which time cDNA was synthesized from the RNA template. The reverse transcriptase was inactivated and the cDNA denatured by incubation at 95° C for 15 min, followed by an amplification reaction comprising 30 cycles of 94° C for 1 min, 55° C for 0.5 min, and 72° C for 2 min. RT-PCR products were visualized on 1% (w/v) agarose gels.

5.3.7. Cell distending assays and flow cytometry

Cytolethal toxin assays for *S.* Typhimurium Sal199 strain were performed as described previously by Spanó *et al*. (2008) with minor modification using *S.* Typhimurium LT2 as negative control. Briefly, HeLa cells were seeded into 6-well tissue culture plates at a density of 1×10^5 cells per well. The cells were then incubated for 72 h at 37° C with 5 % CO_2. After the incubation, cells were washed three times with Phosphate-Buffered Saline 0.1M, pH 7.2 (PBS), fixed with 3 % paraformaldehyde, and observed under light microscope Axiovert 40 CFL (Carl Zeiss Microscopy, LLC, United States), for demonstration of morphological changes in the cells. To measure the cell cycle arrest flow cytometry was followed as described by Mezal *et al*. (2014). The cells' pellets were resuspended with propidium iodide (PI) staining solution (0.1% Triton X-100, 40 μg of PI mL^{-1}, and 100 μg of RNase A mL^{-1}) and incubated at room temperature in the dark for 15 min. Approximately, 1.0×10^4 cells were examined using a

Fluorescence-activated cell sorting (FACS) analysis with the excitation set at 488 nm and the emission set at 630 nm. The experiment was conducted in triplicate.

5.3.8. Adhesion/invasion assay

Two *S.* Infantis isolates, Sal147 missing 35 SPI-1 genes, and Sal280 with all SPI-1 genes were selected. Adhesion and invasion assays were conducted using HT-29 colon human cells in presence of gentamicin as previously described (Searle *et al.*, 2010). Mean of the counts from each well for the four replicates were $\log_{10}$ transformed to equalise the variances for each strain regardless of the mean. The resulting four means for adhesion and four means for invasion were subjected to a two-ways analysis of variance (ANOVA) using the statistical package GraphPad Prism 5 (GraphPad Software, Inc., California, USA). A *p*-value of < 0.05 was taken to indicate statistical significance.

5.3.9. *Galleria mellonella* Infection model

G. mellonella was used as an *in vivo* virulence model, as previously described (Kirchner *et al*., 2013) to compare the *in vivo* virulence of Sal199, Sal147, and Sal280, in comparison with to *S.* Typhimurium LT2. Briefly, *Salmonella* grown in Mueller-Hinton broth for 2, 16 and 24h at 37°C, was harvested to give a final concentration of 10^8 bacteria in 1 mL 0.1 M PBS (pH 7.2). Ten larvae were injected with a 10 µL 10^4 CFU mL^{-1} in the right fore pro-leg, using a Hamilton syringe (Sigma, UK). All larvae were incubated at 37º C for 24 h before determining rates of survival and macroscopic appearance. Non-injected and 0.1 M PBS-injected animals were included in each experiment as controls. The exact dose was determined after plating the inoculum on LB agar. Representative results were obtained from three independent experiments. Data are expressed as percentage of survival, and analysed using the statistical package GraphPad Prism 5

(GraphPad Software, Inc., California, USA). A *p*-value of < 0.05 was taken to indicate statistical significance.

5.4. Results

5.4.1. Virulence genes detected in isolates

One hundred and nine probes designed in this study for 106 genes were validated with control strains. Eight genes could not be validated as the probes were non-specific and have not been considered any further (data not shown). Microarray was performed on the aforementioned 106 *Salmonella* isolates. The microarray results were transformed to a binary form *i.e.* present or absent (Table S5.3.) and analysed in BioNumerics by an UPGMA cluster analysis (Figure 5.1.). Cluster analysis showed four different clusters: one contained 25 isolates which were mainly from the non-common serotypes; one contained all *S.* Enteritidis isolates and there were two clusters of *S.* Typhimurium, one with 41 isolates without virulence plasmid and prophage associated genes and another comprising 18 isolates with these determinants (Figure 5.1.).

The results indicated that virulence determinants located in SPIs 1-6 were highly conserved (Figure 5.1.). In most isolates all SPI-1 genes selected for the microarray were present. One strain of *S.* Infantis (Sal147) isolated from a food product of swine origin was deficient in many SPI-1 genes. Sixteen isolates of non-common serotypes, lacked *rhuM* (hypothetical DNA-binding protein), and seven isolates lacked *sugR* (hypothetical ATP-binding protein) from SPI-3. Virulence determinants of SPI-4 and SPI-5 were conserved in all isolates. Some islet genes were present in all serotypes (Fig. 5.1.). The islet group was highly conserved genes and included those encoding regulator (*envR*, *fhuA*, *oxyR*, *entF*, *slyA*, *leuO*, *msgA*) or effector proteins (*sseK2*, *sfrJ*, *iroB* and *pagK*).

The prophage genes showed the greatest variation in their occurrence (Fig. 5.1.). Gifsy-1-associated gene *gogB* were present in half of *S.* Typhimurium and *S.* Infantis isolates. The Gifsy-2-associated gene *sodCI* was detected in 100 % of *S.* Enteritidis and 96.6 % strains from *S.* Typhimurium and the non-common serotypes. The Gifsy-3-associated gene *sspH2* was reported in 89.8% strains of *S.* Typhimurium, and 90.5 % of *S.* Enteritidis.

The genes *spvC* and *spvR* are located on the *Salmonella* virulence plasmid. They were present in all isolates of *S.* Enteritidis and in 33.9 % of *S.* Typhimurium. Gene *rck* was detected in all *S.* Enteritidis, and in 32.2 % *S.* Typhimurium isolates (Table S5.3.).

The fimbrial genes *agfA*, *bcfC*, *bcfG*, *stdB*, *stiC*, STM4595 were present in all isolates. The *sefA*/*sefR* was found in 90.5 % *S.* Enteritidis. All *S.* Enteritidis isolates had the plasmid-located genes (*rck*, *pefA*, *pefD*, *pefI*, *spvC*, *spvR*, *srgA*, and *srgC*). Typhi colonization factor (*tcf*) operon was found in 18.9 % of isolates from serotypes Typhimurium, Mbandaka, Havana, Virchow, and Infantis. However, the presence of pilus-fimbrial *pilV* gene was only found in two isolates of non-determined serotype and three of *S.* Enteritidis (Table S5.3.; Figure 5.1.).

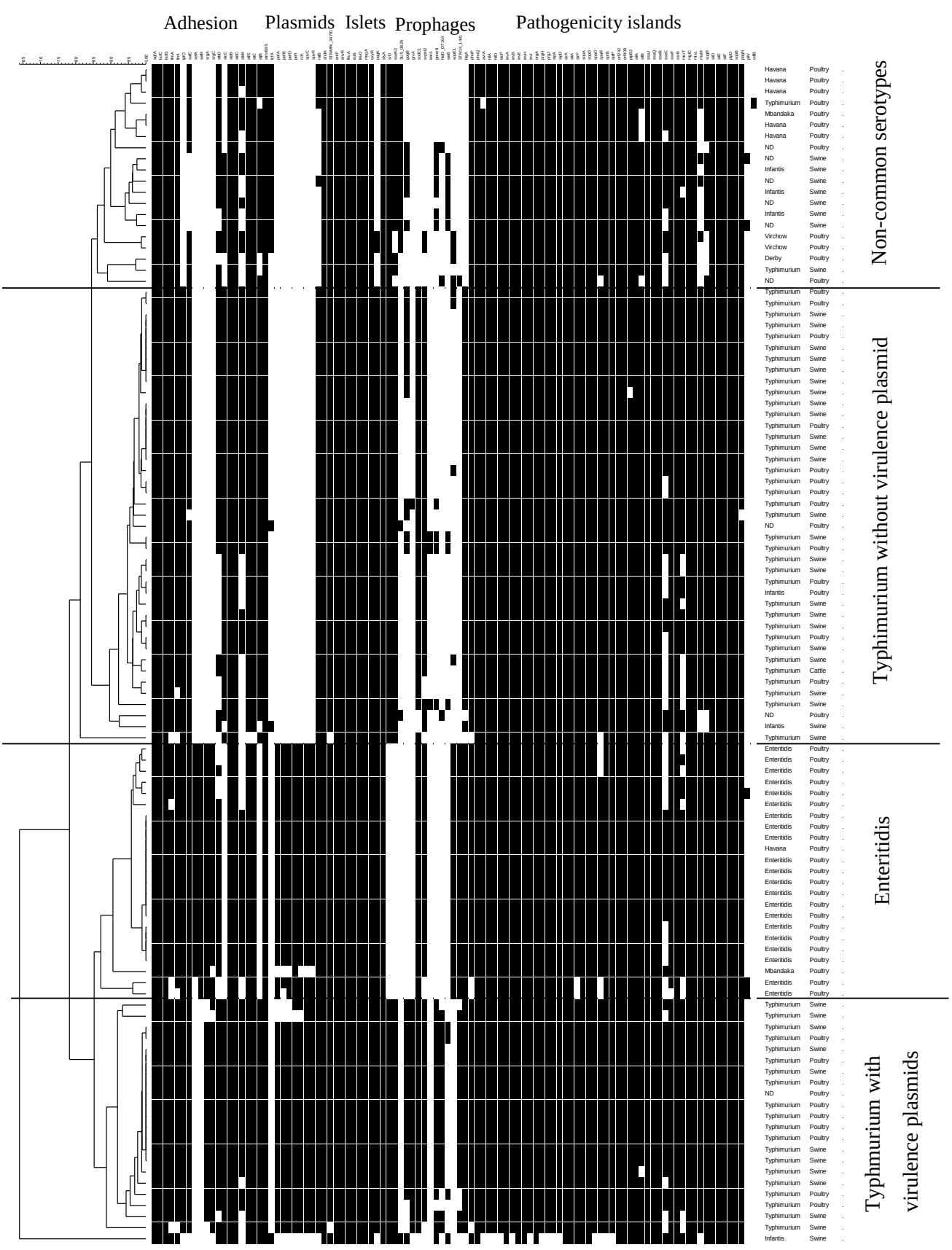

Figure legend – next page.

Figure 5.1. Virulence determinants microarrays data for 106 *Salmonella* strains analysed.
At the top, the analysed genes are grouped according to their particular genomic location or function (fimbrial). The order of strains represents their relatedness according to the UPGMA dendrogram type performed in BioNumerics 5.1. The hybridization result of a distinct strain is shown by row. A white box indicates the absence and a black box indicates the presence of the target sequence in the strain.

5.4.2. Whole Genome Sequencing data

Whole genome sequencing (WGS) was performed on two isolates which showed atypical microarray results; Sal199 which showed presence of the *cdtB* gene by array and Sal147 which showed absence of SPI-1 genes included on the array. The WGS data was analysed by mapping contigs to the *S.* Typhimurium LT2 genome, which was used as reference. In *S.* Typhimurium Sal199, the insertion of *cdtB* toxin together with *pltA* and *pltB*, was positioned upstream of the transposase IS*911*. Analysis of *S.* Infantis Sal147 genome, corroborated arrays results showing deletion of a large number of SPI-1 genes, namely *avrA*, *sprB*, *hilC*, *orgC*, *orgB*, *orgA*, *prgK*, *prgJ*, *prgI*, *prgH*, *hilD*, *hilA*, *iagB*, *sptP*, *sicP*, *iacP*, *sipA*, *sipD*, *sipC*, *sipB*, *sicA*, *spaS*, *spaR*, *spaQ*, *spaP*, *spaO*, *spaM*, *invJ*, *invI*, *invC*, *invB*, *invA*, *invE*, *invG*, *invF*. Overall, both genomes were very elastic with many phages, and mobile elements being inserted or deleted, with respect to LT2.

5.4.3. SPI-11 genes and typhoid-related virulence genes

Arrays and WGS data indicated the unusual presence of *cdtB* gene on *S.* Typhimurium Sal199. The *cdtB* gene is encoded on SPI-11, together with *pltA* and *pltB*; when these genes are expressed together they form a tripartite toxin, which was originally described in *S.* Typhi (Spanó *et al.*, 2008). In order to check the expression of these three genes, and *taiA*, *tcfA*, and *hlyE* which are other typhoid associated genes, RT-PCR was performed. The RT-PCR experiments showed the expression of SPI-11 genes,

as well as the expression of typhoid-related virulence genes (Table S5.2.). *cdtB* gene as well as other SPI-11 genes were detected on *S.* Typhimurium Sal199, and some SPI-11 genes were also present in LT2, but not the *cdtB* toxin genes (Table S5.2.).

5.4.4. Toxin cells assays and flow cytometry

Cytolethal distending intracellular tripartite toxin causes cell arrest in G2 / M transition phase, and consequently a nucleus enlargement and an increase in the amount of DNA can be observed in eukaryotic cells. *S.* Typhimurium Sal199 was examined using flow cytometry for its ability to block cell cycle arrest by analysing the DNA content of HeLa cells. HeLa cells infected with *S.* Typhimurium LT2 were essentially identical to the control (no infected cells), with a large accumulation of cells in the G0 / G1. In contrast, HeLa cells infected with Sal199 become arrested in G2 / M phase after 72 h in comparison to un-infected cells (Figure 5.2a.). The ratio G0 / G1 to G2 / M of cell cycle profiles is shown in Figure 5.2b. The results from profiles of the DNA content suggest that *S.* Typhimurium Sal199 exhibited G2 / M arrest.

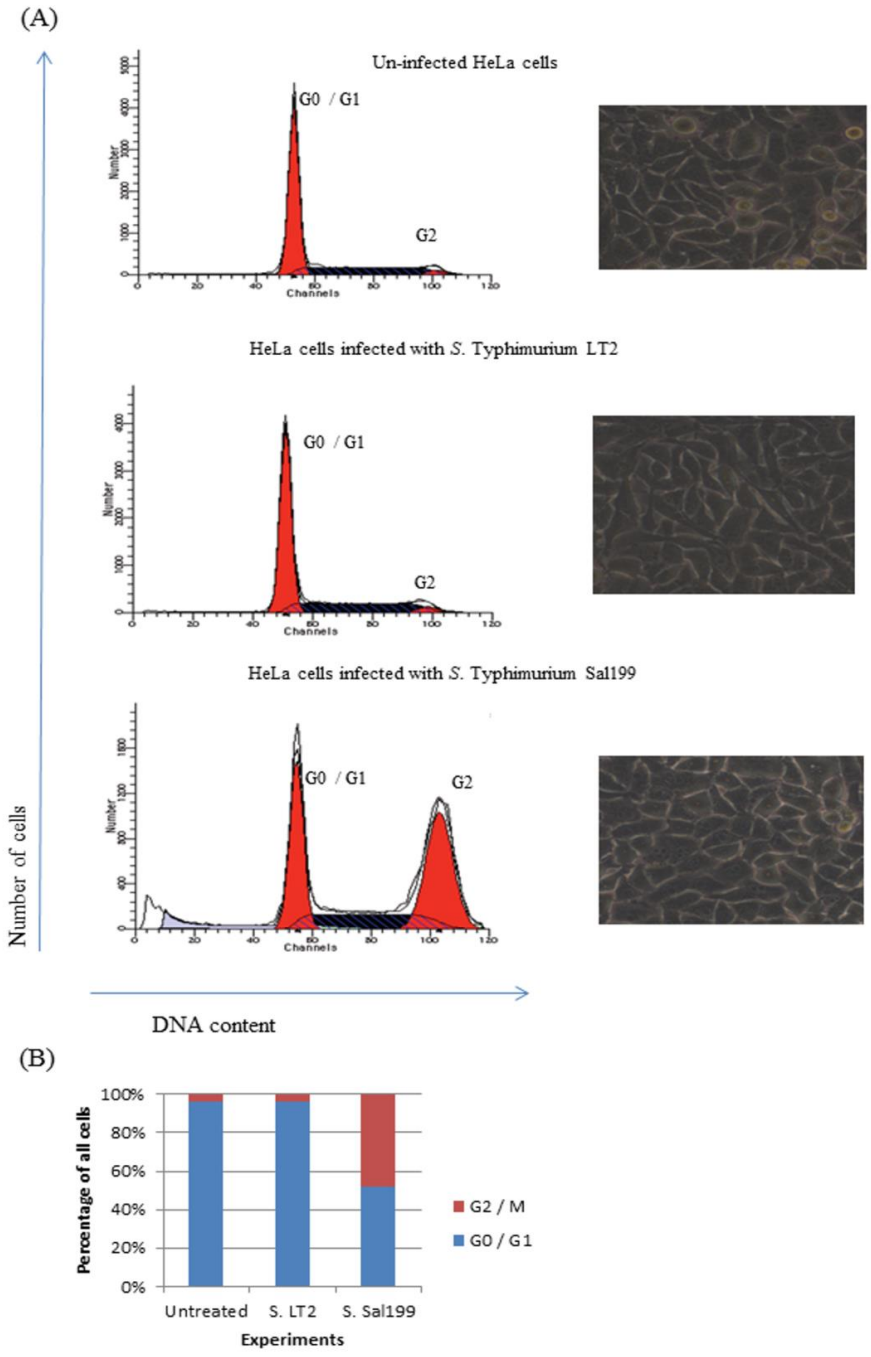

Figure 5.2. Effect of the CdtB toxin in HeLa cells, 72h after *S.* Typhimurium Sal199 infection. (a) On top, untreated HeLa cells, in middle, HeLa cells infected with *S.* Typhimurium LT2, and on bottom, HeLa cells infected with *S.* Typhimurium Sal199. The cells were examined at same magnification (40x) by light microscope, and the cell cycle arrest measured by flow cytometry. The peaks corresponding to cells in G1, S and G2/M are indicated. (b) Ratio G0/G1/G2/M of cell cycle profiles from at least three independent experiments.

5.4.5. Adhesion/invasion assays of selected isolates

The arrays and WGS results predicted a difference in virulence between two *S.* Infantis strains tested, therefore we characterised these strains further by adhesion/invasion assays. From microarray and whole genome sequencing, one strain Sal147, has a large deletion in SPI-1 genes while Sal280 harboured all SPI-1 genes. The results of adhesion and invasion assays are shown in Fig. 5.3. There was a statistically significant difference to the extent that *S.* Typhimurium LT2 control strain was able to adhere compared to both *S.* Infantis strains. In addition, *S.* Typhimurium LT2 was 2.6-fold more invasive in HT-29 cells than *S.* Infantis Sal280 ($p < 0.001$). When comparing the two *S.* Infantis strains no statistically significant difference in adhesion was observed, but *S.* Infantis Sal147, was unable to invade HT-29 cells, unlike Sal280 ($p < 0.001$; Figure 5.3.).

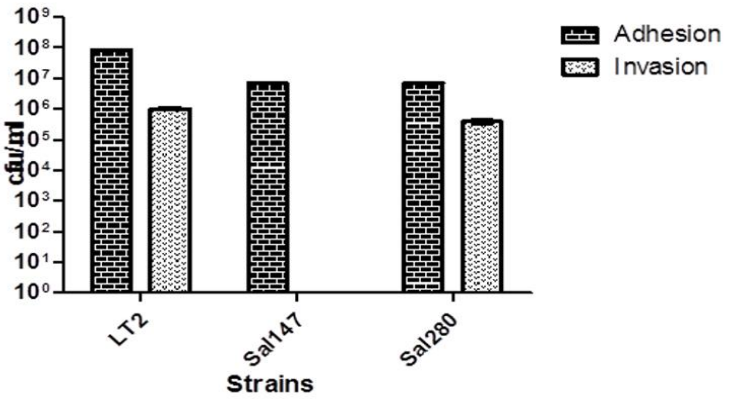

Figure 5.3. Adhesion and invasion of HT-29 cells by control strain *S.* Typhimurium LT2, *S.* Infantis Sal147, and *S.* Infantis Sal280.

Data shown are means ± SEM from four independent experiments.

5.4.6. *In vivo* assays

To further assess the virulence potential of *S.* Typhimurium Sal199 and *S.* Infantis Sal147 strains, *in vivo* assays were performed using the larvae of the Greater wax moth

G. mellonella. The mean rate of survival of *Galleria* at this bacterial dose was strain and growth time dependent. Sal147 strain, grown for 24h, showed a higher percentage of survival, at an average of 50 % (range 40-60 %) than *S.* Typhimurium Sal199 that presented a mean survival of 10 % (range 0-20 %). *S.* Typhimurium LT2 and *S.* Infantis Sal280 presented a survival rate of 16.7 % (range 10-20 %) (Figure 5.4.). Therefore, the mean rate of survival for Sal147 infected larvae was up to 3 times higher when compared to Sal280; while the mortality rate of Sal199 infected larvae was 1.6 times higher compared to LT2. A colour change was observed in some larvae following 24 h incubation. Those killed following infection were black or dark brown while surviving larvae were white and looked identical to the uninfected and PBS controls.

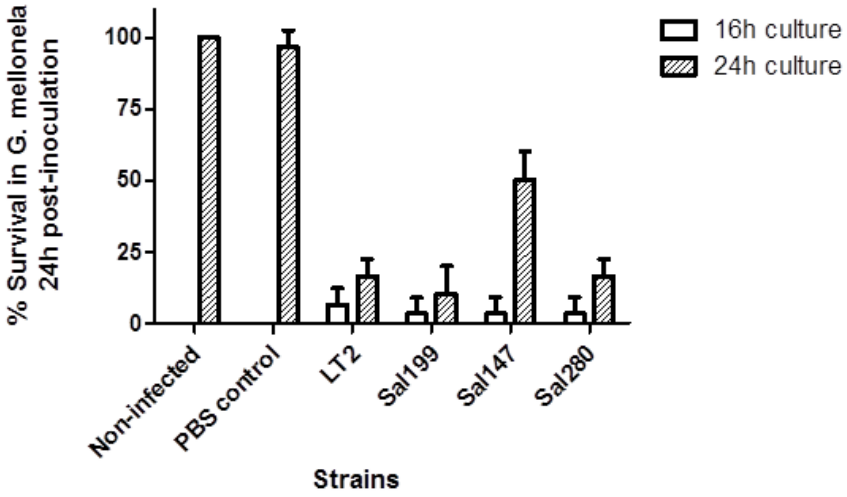

Figure 5.4. Percentage survival in *G. mellonella* 24h post-infection in different strains.

5.5. Discussion

Infection with *S. enterica* is one of the most important causes of food-borne diseases worldwide. In this study we have developed and tested a high through-put microarray chip for *Salmonella* virulence genes using a collection of *Salmonella* isolates recovered from farm animals, slaughterhouses and retail meat, in order to assess the

ability of this array to help identify the potential risk associated with *Salmonella* harbouring different virulence determinants entering the food chain and causing disease in man.

This study showed that adhesion and SPI-located genes were highly conserved in all strains except one *S.* Infantis, while prophages and virulence plasmid were generally variable among the strains and serotypes, as reported previously (Thomson *et al.*, 2008; Huehn *et al*., 2010; Anjum *et al*., 2011). Nonetheless, some phage-associated genes were more frequently detected, e.g. *sodCI* (encoding a periplasmatic superoxide dismutase) and *sspH2* (encoding an ubiquitin ligase protein), suggesting its stable maintenance. A core set of fimbrial genes were detected in all strains which could contribute to the colonization in a broad host range; while presence of particular fimbrial genes in *S.* Enteritidis, probably contribute to host specificity (Huehn *et al.*, 2010; Anjum *et al.*, 2011). All *S.* Infantis strains were swine-associated and most showed presence of SPI-3 *sugR* gene, contrasting with previous reports (Huehn *et al*., 2010). Virulence plasmids are important genetic elements in *Salmonella*. *S.* Typhimurium strains harbouring the *spv* and *rck* genes, which are required for bacterial multiplication in the reticulo-endothelial system are more able to produce systemic disease (Rotger & Casadesús, 1999). These plasmids were mainly present in isolates from the extra-intestinal environment, like liver, muscle, and food products such as raw meat and ready to eat food such as sausages. The *pef* fimbrial operon and *rck* gene that are plasmid borne and play a role in stages of the infection process, were present in all *S.* Enteritidis and in some *S.* Typhimurium isolates allowing their separation into two groups which may correspond to a difference in the potential for these *S.* Typhimurium strains to cause infection and pathogenesis. It also highlights the virulence plasticity of *S. enterica,* and its ability to adapt to others hosts. Therefore having a more extensive knowledge of virulence genes will help understands

the evolution in different serotypes and to infer their relative virulence. Genome variability is common among *S.* Typhimurium isolates (Suez *et al*., 2013) and may be an important factor to consider in relation to virulence genes and consequence of infection. For our panel of isolates ~99% of *S.* Typhimurium strains harboured one or more antibiotic resistance determinant, including to Extended Spectrum β-Lactamases (ESBL), with more than 39 % showing a multidrug resistance (MDR) phenotype (Figueiredo *et al*., 2015).

Moreover, using the virulence arrays we detected a *cdtB* gene, in association with *pltA* and *pltB* genes in *S.* Typhimurium Sal199 strain isolated from raw poultry meat. CdtB forms a tripartite toxin with PltA and PltB, causing cell-cycle arrest in *S.* Typhi, thereby increasing its virulence potential (Mezal *et al.*, 2014). Our findings are in concordance with previous reports showing the CdtB toxin plays a role in cellular distension of host cells (Spanó *et al*., 2008; Mezal *et al.*, 2014). The *cdtB* gene has been described in only six NTS serotypes from human clinical and poultry origin (Skyberg *et al.*, 2006; Mezal *et al*., 2013; Suez *et al.*, 2013), but never in *S.* Typhimurium. This study shows that the HeLa cells infected with *S.* Typhimurium Sal199 became distended with prominent cell cycle arrest compared with the control (un-infected cells and cells infected with *S.* Typhimurium LT2). Presence of *cdtB, pltA* and *pltB* upstream of an IS*911* transposase might have resulted in horizontal transfer of these genes from a *S.* Typhi to Sal199, a *S.* Typhimurium strain. To the best of our knowledge, this is the first report detecting the presence of this typhoid-related toxin in *S.* Typhimurium, which is one of the most common serotypes associated with human gastroenteritis. Furthermore, the increased virulence of Sal199 is corroborated by our *in vivo* findings which showed increased mortality of *G. mellonella* by *S.* Typhimurium Sal199 with respect to *S.* Typhimurium LT2, which might be associated with expression of the CdtB toxin.

Another interesting finding from using the virulence arrays, and confirmed by WGS, was the absence of thirty-five SPI-1 genes in the MDR *S.* Infantis Sal147. Adhesion/invasion assays showed that this strain was able to adhere but not invade a human colon cell line in comparison to controls. These results indicate a possible reduction in the virulence capacity for Sal147 in comparison to another *S.* Infantis isolate and LT2, which was confirmed using the *G. mellonella in vivo* virulence model and suggests a reduced risk to humans.

Therefore, in this study we have clearly shown the benefits of using a virulence genes microarray to assess the risk of infection associated with *Salmonella* strains isolated from farm animals, slaughter houses and food products which can enter the food chain and cause infection in humans. Identification of strains with high virulence potential and infectivity in the food animal breeding section at an early stage may help facilitate interventions that could reduce the risk of dissemination of epidemic strains such as *S.* Enteritidis PT4, *S.* Typhimurium DT104 and monophasic *S.* Typhimurium DT193, which have all entered the food chain to cause human infections. In addition to providing details of the virulence profile of isolates this chip also helped group isolates by serotype, which indicates that isolates of the same serotype usually harbour the same virulence profile, and so this chip can be used as a surrogate for serotyping in future. In addition, it has the benefit of spotting isolates from a serotype with atypical virulence profile which may pose either increased or decreased risk to humans. Currently, these arrays are a cheaper and more accessible option than some of the other methods explored in this study including whole genome sequencing, but this may change in future. Also, they can be used by any laboratory worldwide, as a rapid and useful epidemiological surveillance tool to detect and assess the risk posed to humans by *Salmonella* isolated from the food chain and to help investigate outbreaks.

5.6. References

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5.7. Supplementary data

On digital format (CD-Rom)

Table. S5.1. Details of the *Salmonella* virulence genes for which probes and labelling primers were designed in this study.

Table. S5.2. RT-PCR results to SPI-11 and typhoid-associated genes.

Table. S5.3. Binary form microarrays virulence results.

Chapter 6

General Discussion

Infections with *Salmonella enterica*, usually by consumption of contaminated food products, are one of the most important causes of food-borne disease worldwide. On occasions *Salmonella* infection can require antimicrobial therapy. Antimicrobial compounds are widely used in medicine and agriculture, to treat and prevent infections in food animals. There has been a rapid development of resistance to antimicrobial agents in different *S. enterica* serotypes worldwide, which will impact the success of therapeutic options for salmonellosis. The work presented represents the largest test of antimicrobial agents and heavy metals as well access to diverse genetic resistance in combination to virulence determinants performed in Portugal in *Salmonella* isolated from food and animal-producing food.

Chapter 2 describes the first results of the epidemiology of antimicrobial resistance (AMR) for the first 163 *Salmonella* isolates performed phenotypically. Ten serotypes were identified with *S.* Typhimurium being the most frequent (poultry, swine, processed food), followed by *S.* Enteritidis. The highest frequency of resistance was found for tetracycline (49%) and amoxicillin (37%). Overall, the isolates from swine showed the highest frequency of resistance to the antimicrobials tested. In **Chapter 3** it reports AMR epidemiologic studies and molecular characterization of the extended spectrum β-lactamases (ESBLs) of *Salmonella* isolates. This chapter included all *Salmonella* studied in this work. Results were compared with data obtained before the implementation of the National Control Program in Poultry and the ban of antimicrobial agents in animal feed in European Union. A total of 14 serotypes were identified from the 258 isolates recovered, with *S.* Typhimurium (32.6%) and *S.* Enteritidis (10.1%) being the most common. These two *S. enterica* serotypes were the most frequently reported of all known serotypes in human cases (EFSA & ECDC, 2012). *S.* Enteritidis in poultry was less frequent than in previous studies performed in Portugal, which might be associated with

the implementation of the National Control Program for *Salmonella*, which aim to reduce *S.* Enteritidis in poultry (NPCS, 2012). Nevertheless, other serotypes seem to occupy this biological niche, and may be more common in human salmonellosis in future. The majority of isolates (70.2%) were resistant at least to one class of antimicrobial agent and exhibited higher frequency of resistance to tetracycline (47.7%) and ampicillin (36.0%), which were the main antibiotics used in animal therapeutic in Portugal (DGV, 2010). Resistance to fluoroquinolones was shown in 8% of isolates, a lower value compared to data obtained before 2004. Therefore, fluoroquinolones could be a choice of treatment in case of *Salmonella* infection. The two more common serotypes, also expressed ESBLs. ESBL-producers *S.* Typhimurium $bla_{\text{CTX-M-1}}$ and *S.* Enteritidis $bla_{\text{SHV-12}}$ were isolated from swine and poultry, respectively. The $bla_{\text{CTX-M-1}}$ and $bla_{\text{SHV-12}}$ genes were carried on conjugative plasmids of IncHI2 and IncI1 replicon types, respectively. The two ESBLs genes were able to be transferred to bacteria from a different genus than *Salmonella*, like the ubiquitous commensal *E. coli*. An IncHI2 replicon associated with $bla_{\text{CTX-M-9}}$ in a clinical isolate of *S.* Bovismorbificans was reported in Portugal (Antunes *et al*., 2013). To the best of our knowledge, *S.* Typhimurium with IncHI2 plasmids carrying $bla_{\text{CTX-M-1}}$ have not been described so far in Portugal. Its spread by conjugation was demonstrated, which is a public health concern. We also reported the first $bla_{\text{SHV-12}}$ in *S.* Enteritidis, which is one of the most common sources of human gastroenteritis (EFSA & ECDC, 2014). Despite the low number of ESBL-producing *Salmonella* encountered, this work confirmed the importance of food-producing animals and food products as important reservoirs of *Salmonella* isolates carrying ESBL-encoding genes. The emergence of ESBLs and its potential spread among animal reservoirs and food chain highlight the need for continuous surveillance for antimicrobials at the animal level. Overall, these results revealed changes in the frequency of resistance in *Salmonella* serotypes of animal origin,

especially in emerging serotypes, and in the occurrence of ESBLs-producing *Salmonella.* The control measures taken by EU seem to have some impact in the resistance rate of some antimicrobials like quinolones.

In **Chapter 4** and **5** a subset of 106 *S. enterica* isolates were selected, that included all *S.* Typhimurium, *S.* Enteritidis, and other AMR strains belonging to less common serotypes. In **Chapter 4** the analysis of AMR profile in 106 *Salmonella* strains isolated from food and food-producing animals in Portugal was performed by using an AMR microarray. In selected isolates heavy metal resistance was detected, as well as associated genetic elements, and their transferability confirmed by conjugation. In this subset of isolates, phenotypic and genotypic AMR testing detected high levels of resistance to tetracycline (65.1%) and ampicillin (58.8%), mainly due to presence of *tet*(B), $bla_{\text{TEM-type}}$ and $bla_{\text{PSE-type}}$, which has only recently been reported in *S.* Typhimurium in Portugal (Antunes *et al*., 2011). *Salmonella* Genomic Island-1 was detected in seventeen strains, which harboured the *hld*_DT104 gene marker. *S.* Typhimurium Sal199 harbouring the CdtB toxin confirmed resistance to tetracycline. No heavy metal resistance genes were detected despite showing phenotypic resistance to a number of heavy metals. Multidrug resistant (MDR) *S.* Typhimurium Sal25, showing heavy metal resistance, confirmed $bla_{\text{CTX-M-1}}$ gene on a conjugative IncHI2 plasmid that also harboured numerous genes encoding resistance to tellurite, zinc, copper, cobalt, arsenic, mercury, silver, and cadmium. AMR *S.* Typhimurium Sal368, showed for the first time in *Salmonella*, the presence of a *tet*(M) gene inserted within Tn*3* transposase in an IncF plasmid that also harboured $bla_{\text{TEM-1}}$ gene and resistance genes to arsenic, mercury, and silver, to which it expressed high levels of resistance. The *tet*(M) gene has been reported in *Streptococci* from swine, and in a Portuguese *Enterococcus* strain (Araújo *et al*., 2010). Both plasmids

were transferred by conjugation to *E. coli*. These findings highlight the need for monitoring both AMR and heavy metal resistance across the food chain.

In **Chapter 5,** the study assessed the virulence potential of the same 106 *Salmonella* strains isolated from food-producing animals and food products. A high through-put virulence gene DNA microarray was designed for this study. DNA microarrays comprised probes that target *Salmonella* Pathogenicity Island (SPI), adhesion, prophages, virulence plasmids and regulation genes. The results demonstrated that SPIs and adherence genes were highly conserved, while prophages and virulence plasmid genes were variably present. A core set of fimbrial genes were detected in all strains which could contribute to the colonization in a broad host range; while presence of particular fimbrial genes in *S.* Enteritidis, probably contribute to host specificity (Huehn *et al.*, 2010; Anjum *et al.,* 2011). Isolates grouped by serotype and virulence plasmids separated *S.* Typhimurium isolates in two clusters. As a consequence of some unexpected results from the virulence microarrays further studies were performed to check the potential virulence of the respective strains. Atypical microarray results lead to Whole Genome Sequencing (WGS) of *S.* Infantis Sal147, which identified deletion of thirty-five SPI-1 genes which are involved in host cell invasion. In order to determine the infection capacity of this strain, adhesion/invasion studies were performed in human colon cells. *S.* Infantis Sal147 showed capacity to adhere but not to invade the human cells line HT-29, in comparison with *S.* Infantis Sal280 which harbouring SPI-1 that had capacity of adhesion and invasion of HT-29. Additional studies performed *in vivo* in *G. mellonella* larvae of the Great wax moth, showed reduced mortality in this infection model, in comparison to a SPI-1 harbouring *S.* Infantis. Microarray and WGS of *S.* Typhimurium Sal199 established for the first time in *S.* Typhimurium the presence of *cdtB* and other *S.* Typhi-related genes. Characterization of Sal199 showed *cdtB* genes

were upstream of transposase *IS*911 and co-expressed with other Typhi-related genes. *In vitro* assays using human cell line HeLa cells infected by Sal199, showed cellular distension of eukaryotic cells, but not in HeLa cells infected with *S.* Typhimurium LT2 control. Moreover, studies *in vivo* showed increased mortality of *G. mellonella* larvae with Sal199 infection compared to LT2.

6.1. References

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Chapter 7

General Conclusions

This work not only described the antimicrobial and heavy metal resistance patterns but also the virulence potential of *Salmonella enterica* strains recovered from food-producing animals and processed food. *S enterica* Typhimurium and Enteritidis, the most common serotypes isolated, presented high resistance rates to ampicillin and tetracycline. Resistance to fluoroquinolones was low, and can be used as therapy in case of human infection. *S. enterica* recovered from processed food showed high levels of antimicrobial resistance. This work presented the first report on genes of plasmidic origin, which include $bla_{\text{SHV-12}}$ in *S.* Enteritidis, and $bla_{\text{CTX-M-1}}$ and *tet*(M) in *S.* Typhimurium highlighting the lateral transfer of resistance genes among diverse bacterial populations in the food chain. Bacterial strains harbouring extended spectrum β-lactamase (ESBL) in food producing animals highlight the importance of continuous monitoring to protect public health limiting antimicrobial resistance spread. Work performed on antimicrobial resistance coupled with heavy metal tolerance highlights that heavy metal present in toxic concentrations in food-animal environments can contribute to persistence of genetic platforms carrying metal/antibiotic resistance genes in *S. enterica*. From the work performed on virulence characterization *Salmonella* Pathogenicity Island (SPI) and adhesion genes being highly conserved in different serotypes suggest conferring broad host adaptability. The immense variation not only in plasmid virulence but also in prophage genes shows the importance of transduction in *Salmonella* spp., suggesting the role mobile elements, such as phages, plasmids play in the rapid evolution of *Salmonella* in adaptation to different environmental stresses, enabling the survival and widespread distribution of these *Salmonella* serotypes throughout the food chain. Our data showed that the genes present in SPI-1 in *S.* Infantis are not essential for adherence but are crucial to invade host cells. Furthermore, detection of CdtB toxin genes suggests they might have been horizontally transferred by a prophage that is integrated into the bacterial DNA

chromosome. To our knowledge, this is the first report showing that *S.* Typhimurium strains from food sources contain active CdtB toxin genes similar to that in *S*. Typhi.

So, in case of human infection, the antibiotherapy can be compromised, due antimicrobial resistance, and the *Salmonella* infection can be worst due presence of Typhi-related genes. Identification of strains with high virulence potential and infectivity in food at an early stage may help facilitate interventions, reducing the risk of dissemination of epidemic strains that may affect human health. This work emphasizes both the role and the risk of the selective pressure imposed by overuse and misuse of antimicrobials and heavy metals, and the potential of concomitant selection of virulence factors encoded in the same elements with antimicrobial resistance genes. Microarray and whole genome sequencing are powerful tools to help improve our understanding of these pathogens, providing an insight into the potential risk for consumers along the food chain and to perform epidemiological analysis and trace outbreaks. In future these tools can be used in an efficient, timely manner and at a reasonable cost. Incorporation of such methodology into surveillance could help reduce the risk of emergence of epidemic *Salmonella* strains that poses a public health risk.

Chapter 8

Future perspectives

In future, it will be important to continue to study the epidemiology of antimicrobial resistance in *S. enterica* of animal origin, with special attention for the emergence of extended spectrum β-lactamases. Epidemiologic studies allow the discovery of peculiar and interesting results. On the same way, the understanding of genetic mobilization of antimicrobial and heavy metal resistance determinants between *Salmonella* serotypes, but also between different species is of extreme importance. A more deep and extended study, for understanding the impact of heavy metals and disinfectants use in agriculture and animal production could be good to know their importance in *Salmonella* selection through the food chain. In this way, a microarray of heavy metal resistance genes could be designed, and implemented in food control, and veterinary laboratories. In other hand, in epidemiologic virulence approach futures studies may include *cdtB* and other Typhi-related genes, once these genes appear and maybe are emerging in non-Typhi serotypes. Prophage and virulence plasmids should be included in next studies to monitor and understand the adaptation of *Salmonella* serotypes to new hosts. Some studies could be performed in particular strains, which had curious results in this work, like Sal147 and Sal199. The potential use of *S.* Infantis Sal147 for a vaccine in animals can be explored, once this strain was able to attach but not to invade host cells. In *S.* Typhimurium Sal199, a deeper work could be done, for understand the mobility, expression and effect of CdtB toxin in eukaryotic cells, and little mammals. *In vivo* assays to test CdtB toxin in mice, doing knock-out of the Typhi-related genes in Sal199 and see the virulence associated. The study of AMR, heavy metal resistance and virulence were only performed in selected isolates, and in future the remaining isolates could be studied. Finally, the technology and general idea of study AMR, heavy metals resistance and virulence determinants applied in this study can be applied to other foodborne pathogens.

Chapter 9

Supplementary data

On digital format (CD-Rom)

Chapter 4

Table. S4.1. Antimicrobial Resistance Phenotype and Resistance Genotype Associated

Chapter 5

Table. S5.1. Details of the *Salmonella* virulence genes for which probes and labelling primers were designed in this study.

Table. S5.2. RT-PCR results to SPI-11 and typhoid-associated genes.

Table. S5.3. Binary form microarrays virulence results.