UNIVERSIDADE DE LISBOA
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CHARACTERIZATION OF ANTIBIOTIC RESISTANCE MECHANISMS IN GRAM NEGATIVE BACTERIA ISOLATED FROM ANIMALS AND FOOD PRODUCTS OF ANIMAL ORIGIN

MARIA DE LURDES TAVARES CLEMENTE

Orientadores

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias
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ABSTRACT

Antibiotics were a truly innovative option in medical therapy for the treatment of diseases caused by microbial agents, having largely contributed for the decrease levels of human and animal morbidity and mortality. Therefore, the overuse and misuse of these drugs in human clinical therapy and in the veterinary medicine, including animal production, contributed for the emergence and dissemination of antibiotic resistant microorganisms, which are a serious threat to human and animal health, and to the ecosystem.

The aim of the present thesis was to search the main acquired antibiotic resistance mechanisms to β-lactams, fluoroquinolones and polymixins in Gram negative bacteria recovered from different animal species and matrices, and to investigate the most important mobile genetic elements involved in the dissemination. Thus, the studies concerning antibiotic susceptibility and molecular characterization were performed in collections of bacterial isolates belonging to *Enterobacteriaceae* family (mainly *Escherichia coli* and *Salmonella enterica*).

Both bacterial species were associated to antibiotic resistant determinants of clinical relevance in human and veterinary medicine, namely, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}32}$, $bla_{CMY\text{-}2}$, *qnrS1*, *aac(6')-Ib-cr*, *mcr-1*. The diversity of detected mobile genetic elements, e.g., IncI1, IncF and IncX4 plasmids, insertion sequences IS*Ecp1*, as well as integrons of class 1 and 2, suggest their involvement in the dissemination of resistance genes interspecies, and movement within the bacterial cell.

Genomic analysis of two isolates (*Morganella morganii* and *Salmonella* Enteritidis), highlighted the potencial of *omic* technologies, as an additional tool to the phenotypic and genotypic characterization of antibiotic resistance.

The results obtained throughout this thesis highlight the importance of the different animal species as reservoirs of antibiotic resistant bacteria. In addition, it was reinforced the need of a permanent research and monitoring of antibiotic resistance in the different ecological niches, and the use of genomic approaches, which had an important role in the understanding of the complex problem represented by the dynamic of antibiotic resistance.

Keywords: Antibiotic resistance; *Enterobacteriaceae*; mobile genetic elements; dissemination; animals; products of animal origin.

RESUMO

Os antibióticos constituíram uma opção verdadeiramente inovadora na terapêutica medicamentosa para o tratamento de doenças provocadas por agentes microbianos, tendo contribuído largamente para a diminuição das taxas de morbilidade e mortalidade humana e animal. Porém, a utilização abusiva e inadequada destes fármacos na prática clínica humana e na medicina veterinária, incluindo a produção animal, contribuiu para a emergência e disseminação de microrganismos resistentes, os quais constituem uma grave ameaça à saúde humana e animal, e para o ecossistema.

A presente dissertação teve como objetivo central investigar os principais mecanismos de resistência adquirida aos antibióticos β-lactâmicos, fluoroquinolonas e polimixinas em bactérias de Gram negativo isoladas de diferentes espécies animais e matrizes, bem como os principais elementos genéticos móveis responsáveis pela sua disseminação. Assim, os estudos de suscetibilidade aos antibióticos e caracterização molecular foram realizados em coleções de estirpes bacterianas pertencentes à família *Enterobacteriaceae* (maioritariamente *Escherichia coli* e *Salmonella enterica*).

Ambas as espécies bacterianas estavam associadas a determinantes de resistência de relevância clínica humana e veterinária, nomeadamente $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-32}}$, $bla_{\text{CMY-2}}$, *qnrS1*, *aac(6')-Ib-cr*, *mcr-1*. A diversidade de elementos genéticos móveis detetados, e.g. plasmídeos IncI1, IncF e IncX4, sequências de inserção IS*Ecp1*, bem como integrões de classes 1 e 2, sugere o seu envolvimento na disseminação de genes de resistência aos antibióticos entre espécies, tal como a sua movimentação dentro da própria bactéria.

A análise do genoma de duas estirpes (*Morganella morganii* e *Salmonella* Enteritidis) realçou o potencial das tecnologias ómicas, como ferramenta adicional na caracterização fenotípica e genotípica da resistência aos antibióticos.

Os resultados obtidos salientam a importância que as várias espécies animais representam como reservatórios de bactérias resistentes aos antibióticos. Foi igualmente reforçada a necessidade de uma permanente investigação e monitorização da resistência aos antibióticos nos vários nichos ecológicos, e do uso de abordagens genómicas, as quais tiveram um papel importante na compreensão do complexo problema que representa a dinâmica da resistência aos antibióticos.

Palavras chave: Resistência aos antibióticos; *Enterobacteraceae*; elementos genéticos móveis; disseminação; animais; produtos de origem animal.

TABLE OF CONTENTS

FIGURES INDEX

TABLES INDEX

ACRONYMS AND ABBREVIATIONS

Aac(6')-Ib-cr	Aminoglicosyde acetyltransferase variant cr
AAC	Aminoglicosyde acetyltransferase
AAD, **ANT**	Aminoglicosyde adenyltransferase
ACT	AmpC Type
ALA	Alanine
APH	Aminoglicosyde phosphotransferase
attI	Attachment site of the integron
attC	Attachment site of the integrons cassette
bla	β-Lactamase coding genes
CAT	Chloramphenicol Acetyltransferases
cat	Chloramphenicol acetyltransferases coding genes
CMY	β-Lactamases active on Cephamycins
CTX-M	β-Lactamases active on Cefotaxim, isolated for the first time in Munich
dfrA	DHFR coding gene
DHFR	Dihydrofolate redutase
DHPS	Dihydropteroate synthase
DNA	Desoxiribonucleic acid
EMA	European Medical Agency
ECDC	European Center for Prevention and Disease Control
ECOFF	Epidemiological cut-off values
EFSA	European Food Safety Authority
ESAC	Extended-spectrum AmpC β-Lactamase
ESBL	Extended-spectrum β-Lactamase
ESVAC	European Surveillance Veterinary Antimicrobial Consumption
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
gyr	Gyrase coding gene
HGT	Horizontal Gene Transfer
ICE	Integrative and Conjugative Element
IMP	β-Lactamase active on Iminipem
Inc	Plasmid Incompatibility group
IntI	Integron integrase
IS	Insertion Sequence
ISCR	Insertion Sequence Common Region
KPC	*Klebsiella pneumoniae* Carbapenemase
MBL	Metallo-β-Lactamase
mcr	Plasmid-mediated colistin resistance gene
MGE	Mobile Genetic Element
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequence Typing
mRNA	Messenger RNA
MS	Mass-Spectrometry
NDM	New Delhi Metalo-β-lactamase
NGS	Next Generation Sequencing
OIE	World Animal Health Organization
OMP	Outer Membrane Protein
oqxAB	Gene coding olanquidox resistance
otr	Oxytetracycline resistance coding gene
ORF	Open Reading Frame
OriIS	Origin Insertion Sequence
OXA	β-Lactamase active on Oxacillin

PBP	Penicillin Binding Protein
PBRT	PCR-Based Replicon Typing
P*intI*	Promoter of Integrase Integron gene
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PMAβ	Plasmid-Mediated AmpC β-lactamase
Pc	Promoter of gene cassette
pMLST	Plasmid Multilocus Sequence Typing
PMQR	Plasmid-Mediated Quinolone Resistance
PMCR	Plasmid-Mediated Colistin Resistance
***qepA*,**	Quinolone efflux pump A coding gene
qnr,	Quinolone resistance coding genes
QRDR	Quinolone-Resistance Determining Region
RAST	Rapid Annotation using Subsystem Technology
rRNA	Ribossomal RNA
sat	Streptothricin resistance coding gene
SHV	β-Lactamase Sulphydryl Reagent Variable
ST	Sequence Type
str	Aminoglycoside resistance coding gene
sul	Sulphamethoxazole resistance coding gene
TEM	β-Lactamase Named after patient Temoniera
TerIS	Termination Insertion Sequence
tet	Tetracycline resistance coding gene
tRNA	Transport RNA
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VAL	Valine
VIM	Verona Integron-encoded Metallo- β-lactamase
WGS	Whole Genome Sequencing

THESIS STRUCTURE

Antibiotic resistance is a worldwide problem with serious repercussions on human health, animal health and the economy, and it should be integrated into a global perspective, as bacteria do not respect geographic and species barriers. The selective pressure exerted by the abusive and inadequate use of antibiotics in human and animal clinical therapy, animal and agricultural production, as well as the environmental impact resulting from these activities, are the main cause for the development of antibiotic resistance. In addition to the investigation through the characterization of resistance mechanisms and the mobile genetic elements involved in the emergence and mobilization of antibiotic resistance genes among the different ecosystems and monitoring through the implementation of surveillance programs on antibiotic resistance and consumption, are of crucial importance, in order to obtain the actual picture of the occurrence and trends on antibiotic resistance.

This thesis is based on twelve papers organized in three distinct chapters (3 to 5), eleven of which have already been published and one submitted for publication, in international peer-reviewed journals. Each research article consists of an introduction, materials and methods, results and discussion related to the scope of the study and preceded by a title page describing the reference of the publication and the contributions of each author.

In addition to the articles, this thesis includes a global overview of antibiotic resistance (Chapter 1, General Introduction), followed by the objectives (Chapter 2, Objectives), a general discussion on the results (Chapter 6, General Discussion) and the main conclusions (Chapter 7, Conclusions).

Considering the different types of manuscripts presented in this thesis followed the recommendations of each scientific journal where they were published or submitted for publication, chapters 3, 4 and 5 were formatted in the same style, with all references gathered in a single section (Chapter 8, References).

The numbering of the figures and tables is presented according to the numbers of the chapter and its article.

The specific content of each of the chapters that integrate this PhD thesis consists of:

Chapter 1 consists of a general introduction, where it is intended to reveal the state of the art in the area of antibiotic resistance focused on Gram negative bacteria. Antibiotic targets and mechanisms of intrinsic and acquired resistance to the main groups of antibiotics, the diversity of antibiotic resistance reservoirs and the main routes of dissemination, are approached. Technological advances and the use of *omics* in research of antibiotic resistance, is also discussed.

Chapter 2 includes the scope and description of the objectives of this thesis.

Chapter 3 consists of a brief review on the dynamic and complex process of antibiotic resistance, and the pathways between the different reservoirs, humans, animals and the environment.

Chapter 4 includes nine publications (***4.1*** to ***4.9***), in which the phenotypic susceptibility and molecular characterization of *Enterobacteriaceae* strains, regarding, the mechanisms of acquired resistance to β-lactam antibiotics [Extended-Spectrum β-Lactamases (ESBL), Plasmid-Mediated AmpC β-lactamases (PMAβ), Extended-Spectrum AmpC β-lactamases (ESAC]), fluoroquinolones, Plasmid-Mediated Quinolone Resistance (PMQR) and to colistin [Plasmid-Mediated Colistin Resistance (PMCR)], and the presence of mobile genetic elements, were evaluated.

Sub-Chapter 4.1, the phenotypic antibiotic susceptibility patterns of 333 strains of *S. enterica* isolated from breeding and broiler flocks, during the period 2009-2011, was evaluated.

Sub-Chapter 4.2, the phenotypic antibiotic susceptibility patterns of 1120 strains of *S. enterica* isolated from poultry, swine and food products of animal origin, and the molecular characterization of ESBL and PMAβ producer strains, mobile genetic elements and genetic environment, were investigated.

Sub-Chapter 4.3, the phenotypic antibiotic susceptibility of 562 *S. enterica* strains isolated from food-producing animals, food products and animal feed, and 598 *E. coli* strains isolated from several animal species, was evaluated. Molecular characterization of acquired resistance mechanisms to β-lactam antibiotics (ESBL and PMAβ) and fluoroquinolones (PMQR), and detection of mobile genetic elements in strains with reduced susceptibility to 3rd generation cephalosporins and/or cephamycins, were performed.

Sub-Chapter 4.4, the zoonotic potential of an *E. coli* strain isolated from a captive dolphin and a set of human clinical strains with similar phenotypic and genotypic characteristics, was assessed.

Sub-Chapter 4.5, the phenotypic antibiotic susceptibility results of 387 strains of *E. coli* isolated from broilers and turkeys at slaughter, and the molecular characterization of 15 strains with reduced susceptibility to 3rd generation cephalosporins and or cephamycins, regarding the detection of ESBL-, ESAC-, PMAβ-, PMQR- and PMCR-encoding genes, were performed. In addition, sequencing of AmpC-encoding gene in three strains in which only the *ampC* gene was detected, and genetic relationship between animal and human strains carrying ESBL-encoding genes from the CTX-M, SHV-12 and TEM-52 family by pulsed-field gel electrophoresis technique (PFGE), were performed. The new CTX-M-166 enzyme-producer strain was also analyzed through New Genome Sequencing (NGS) for further characterization regarding to serotype of *E. coli*, genetic environment of $bla_{\text{CTX-M-166}}$, and identification and typing of the plasmid carrying this new $bla_{\text{CTX-M-166}}$ gene.

Sub-Chapters 4.6 and ***4.7***, biochemical characterization of the new CTX-M-166 enzyme, regarding kinetic parameters, and additional characterization by NGS, concerning antibiotic resistance, virulence and MultiLocus Sequence Typing (MLST), were performed.

Sub-Chapter 4.8, antibiotic susceptibility was determined on 89 strains of *S. enterica* and 91 *E. coli* strains isolated from food-producing, companion and zoo animals. Molecular characterization regarding the detection of PMQR, and genomic analysis and comparison of four strains, two carrying *qnrS1* gene and two carrying *aac(6 ')-Ib-cr* gene, was performed through NGS.

Sub-Chapter 4.9, characterization of a multidrug resistant *Morganella morganii* strain isolated from broilers through NGS, regarding antibiotic resistance and virulence genes, and genomic analysis of the plasmid carrying *qnrD1* gene.

Chapter 5, includes two manuscripts (***5.1*** and ***5.2***), concerning antibiotic resistance mechanisms other than those included in Chapter 4.

Sub-Chapter 5.1, genomic characterization of a *S*. Enteritidis strain isolated from one day-old chicks, regarding antibiotic and heavy metals resistance determinants, virulence factors and mobile genetic elements was performed, through NGS.

Sub-Chapter 5.2, phenotypic susceptibility towards colistin and 3rd generation cephalosporins and/or cephamycins of 1840 *Enterobacteriacaeae* strains (1206 *E. coli* and 634 *S. enterica*), was evaluated. In 138 isolates resistant to colistin, *mcr-1* and *mcr-1.9* genes were detected; in those strains co-resistant to 3rd generation cephalosporins and/or cephamycins, genotypic characterization with respect to the detection of ESBL- and/or PMAβ-encoding genes, was performed. In addition, genotypic characterization of a strain bearing the new variant of the *mcr-1*, *mcr-1.9 gene*, regarding the presence of other antibiotic resistance genes, virulence factors, plasmid's identification and typing and genetic environment, was performed by using Whole Genome Sequencing (WGS).

Chapter 6 includes a global discussion on the results obtained on the studies performed.

Chapter 7, includes the main conclusions of the studies carried out.

Chapters 3, ***4*** and ***5***, that can be read in separate, transcribe the contents of the following publications:

Chapter 3

Manuela Caniça, Vera Manageiro, Daniela Jones-Dias, Lurdes Clemente, Eduarda Gomes-Neves, Patrícia Poeta, Elsa Dias, Eugénia Ferreira. Current perspectives on the dynamic of antibiotic resistance in different reservoirs. *Research in Microbiology*, 2015, 166(7):594-600.

Chapter 4

4.1. Lurdes Clemente, Ivone Correia, Patrícia Themudo, Isabel Neto, Manuela Caniça, Fernando Bernardo, 2014. Antimicrobial susceptibility *of Salmonella enterica* isolates from healthy breeder and broiler flocks in Portugal. *The Veterinary Journal*, 2014, 200(2):276-81.

4.2. Lurdes Clemente, Vera Manageiro, Eugénia Ferreira, Daniela Jones-Dias, Ivone Correia, Patrícia Themudo, Teresa Albuquerque, Manuela Caniça. Occurrence of extended-spectrum β-lactamases among isolates of *Salmonella enterica* subsp. *enterica* from food-producing animals and food products, in Portugal. *International Journal of Food Microbiology*, 2013, 167(2):221-8.

4.3. Lurdes Clemente, Vera Manageiro, Daniela Jones-Dias, Ivone Correia, Patrícia Themudo, Teresa Albuquerque, Margarida Geraldes, Filipa Matos, Cláudia Almendra, Eugénia Ferreira, Manuela Caniça. Antimicrobial susceptibility and oxymino-β-lactam resistance mechanisms in *Salmonella enterica* and *Escherichia coli* isolates from different animal sources. *Research in Microbiology,* 2015*,* 166(7):574-83.

4.4. Vera Manageiro, Lurdes Clemente, Daniela Jones-Dias, Teresa Albuquerque, Eugénia Ferreira, Manuela Caniça. Zoonotic potential of multidrug resistant CTX-M-15-producing *Escherichia coli isolate* of a marine dolphin, in Portugal. *Emerging Infectious Diseases,* 2015, 21:2249-51.

4.5. Vera Manageiro, Lurdes Clemente, Ivone Correia, Teresa Albuquerque, Patrícia Themudo, Eugénia Ferreira, Manuela Caniça. New insights into resistance to colistin and third-generation cephalosporins of *Escherichia coli* in poultry, Portugal: novel $bla_{\text{CTX-M-166}}$ and bla_{ESAC} genes. *International Journal of Food Microbiology*, 2017, 263:67-73.

4.6. Vera Manageiro, Lurdes Clemente, Sílvia Duarte, Luís Vieira, Manuela Caniça. Draft genome sequence of an *Escherichia coli* isolated from a Gallus gallus producing the novel CTX-M-166 variant. *Genome Announcement*, 2016, 4(5), e0102916.

4.7. Vera Manageiro, Rafael Graça, Eugénia Ferreira, Lurdes Clemente, Richard Bonnet e Manuela Caniça. 2017. Biochemical characterization of CTX-M-166, a new CTX-M β-lactamase produced by a commensal *Escherichia coli* isolate. *Journal of Antibiotics*, 2017, 70(6):809-810.

4.8. Daniela Jones-Dias, Vera Manageiro, Rafael Graça, Daniel Sampaio, Teresa Albuquerque, Patrícia Themudo, Luís Vieira, Eugénia Ferreira, Lurdes Clemente, Manuela Caniça. QnrS1- and Aac(6')-Ib-cr-producing *Escherichia coli* among isolates from animals of different sources: susceptibility and genomic characterization. *Frontiers in Microbiology,* 2016, 7:671.

4.9. Daniela Jones-Dias, Lurdes Clemente, Inês Barata Moura, Daniel Sampaio, Teresa Albuquerque, Luís Vieira, Vera Manageiro, Manuela Caniça. Draft genomic analysis of an

avian multidrug resistant *Morganella morganii* isolate carrying qnrD1. *Frontiers in Microbiology,* 2016**,** *7:*1660.

Capítulo 5

5.1. Daniela Jones-Dias, Lurdes Clemente, Conceição Egas, Hugo Froufe, Daniel Sampaio, Luís Vieira, Maria Fookes, Nicholas Thompson, Vera Manageiro, Manuela Caniça. *Salmonella* Enteritidis isolate harboring multiple efflux pumps and pathogenicity factors, shows absence of O antigen polymerase gene. *Frontiers in Microbiology,* 2016, 7:1130*.*

5.2. Lurdes Clemente, Vera Manageiro, Raquel Romão, Catarina Silva, Luís Vieira, Ana Amaro, Ivone Correia, Teresa Albuquerque, Patrícia Themudo, Eugénia Ferreira, Manuela Caniça. 2017. The novel MCR-1.9 variant within colistin-resistant *Enterobacteriaceae* isolates from food-producing animals and meat. Submitted to *International Journal of Food Microbiology (December 2017)*.

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CHAPTER 1

GENERAL INTRODUCTION

1. THE ANTIBIOTICS

"One sometimes finds what one is not looking for"

Sir Alexander Fleming

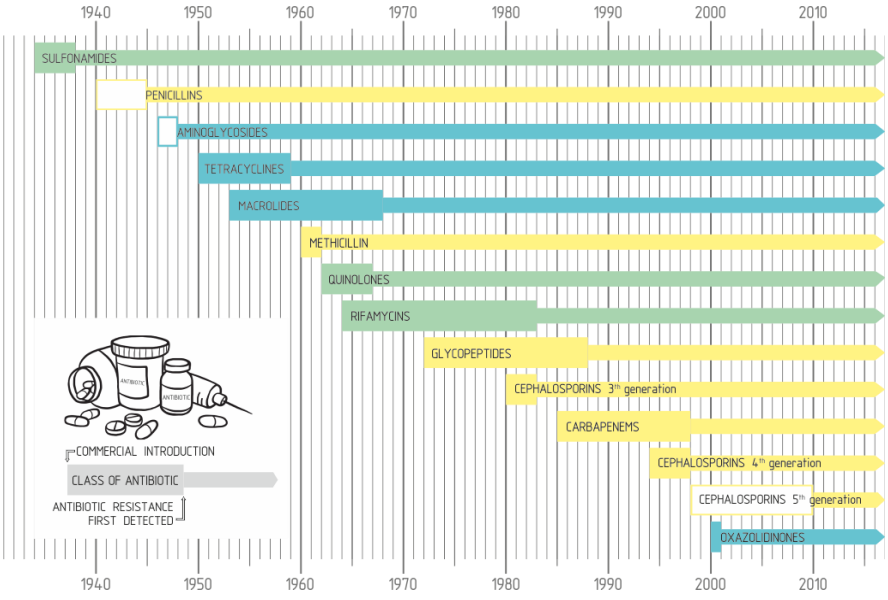
1.1. History of the antibiotics

The discovery of penicillin by Alexander Fleming in 1929 provided treatment for infectious diseases, becoming an indispensable drug in the therapeutic arsenal after the beginning of its industrial production in 1946 (Dantas & Sommer, 2014).

During the "golden age" of antibiotic development (1940-1960), the discovery of new natural, synthetic and semi-synthetic antibiotics deeply changed human medicine in the field of infectious and oncological chemotherapy, organ transplants, and other invasive surgeries, which could fail without the use of these compounds (Wright, 2010). Later on, the use of antibiotics has also been extended to veterinary medicine.

With the introduction of new antibiotics in the clinical practice, the appearance of resistant bacteria was inevitable. However, has always been counter balanced by the development of new substances by the pharmaceutical industry, which since the 1970s focused mainly on the chemical modification of the existing compounds, instead of the development of new molecules (Dantas & Sommer, 2014) (Figure 1.1).

Figure 1.1. The development of new antibiotics is followed by the emergence of resistance (Adapetd from Harbarth *et al.,* 2015).



1.2. Targets of antibiotics

Antibiotics are classified accordingly to their chemical structure, spectrum of activity and mechanism of action (Table 1.1). Bacterial targets may be associated with metabolic processes or structures essential for growth and survival. Antibiotics inducing cell death are bactericidal, and those inhibiting their growth are bacteriostatic (Bernatova *et al.,* 2013).

1.2.1. Inhibition of nucleic acid synthesis

Quinolones and fluoroquinolones are bactericidal antibiotics, which target topoisomerases II and IV, causing inhibition of the deoxyribonucleic acid (DNA) synthesis and consequently, bacterial growth (Kohanski *et al.,* 2010).

1.2.2. Inhibition of cell wall synthesis

Peptidoglycan is an essential compound of the bacterial cell wall, responsible for maintaining the cellular structure. Synthesis of this compound includes three stages: in the first two stages the peptidoglycan percussors are synthesized and incorporated into the lipid molecule (lipid II); in the third stage, the molecules are integrated into the existing peptidoglycan. β-lactam antibiotics are bactericidal and act by blocking peptidoglycan synthesis (Lovering *et al.,* 2012).

1.2.3. Inhibition of protein synthesis

Several antibiotics cause inhibition of protein synthesis, by acting on bacterial ribosomes at different stages of translation (initiation, elongation and termination) (Wright, 2010). Aminoglycosides are bactericidal antibiotics, acting through binding to the 30S subunit of the bacterial ribosome, altering the structure of the complex formed by the aminoacyl-RNA transport (tRNA) and RNA messenger (mRNA) (Kohanski *et al.,* 2010).

Chloramphenicol and florfenicol are bacteriostatic antibiotics and act by preventing the elongation of the peptide chain (Schwarz *et al.,* 2004). Tetracyclines are also bacteriostatic and act by inhibiting the attachment of the aminoacyl-RNA transport complex (tRNA) to the ribosomal receptor A (Chopra & Roberts, 2001).

1.2.4. Inhibitors of folic acid synthesis

Sulfonamides and trimethoprim are bacteriostatic antibiotics acting by competitive inhibition in the synthesis of folic acid. Sulfonamides inhibit the dihydropteroate synthetase enzyme (DHPS), which catalyzes the formation of the dihydrofolate parabenzoic acid, and trimethoprim acts in the next step by inhibiting the enzyme dihydrofolate reductase (DHFR), which catalyzes the synthesis of tetradihydrofolate from dihydrofolate (Huovinen, 2001).

Since these two steps follow each other in the synthesis process, the use of both drugs in combination is advantageous (Huovinen, 2001).

1.2.5. Inhibitors of cell membrane synthesis

Polymyxins are a group of cationic, bactericidal polypeptide antibiotics that act by binding to the phospholipids of the anionic outer bacterial cell membrane, causing a breakdown in the integrity and permeability of the cell wall (Landman *et al.,* 2008).

2. THE USE OF ANTIBIOTICS IN VETERINARY MEDICINE AND ANIMAL PRODUCTION

Antibiotics are used in veterinary medicine for treatment and prevention of infectious diseases in animals. In livestock production they are also used to improve animal growth and feed efficiency (Marshall & Levy, 2011, Aarestrup, 2015, Economou and Gousia, 2015). Antibiotics can also be administered as a metaphylactic treatment, being applied to entire groups of animals, even if only a few show clinical symptoms of a specific disease. This type of treatment is administered in high doses and for a short period of time, eliminating or minimizing the spread of the disease. In constrast, the prophylactic treatment is administered in a subtherapeutic dosage for a longer period, when there is imminent risk of disease emergence. Prophylactic treatment is usually associated with poor facility management and permanent stress, predisposing animals to infection. This type of treatment shows similar effects to the administration of growth promoters (Aarestrup, 2015; Economou & Gousia, 2015) (Table 1.1). Although growth promoters were abolished in the European Union (EU) in 2006, the consumption of antibiotics did not decrease; in contrast, there was an increase in its use for metaphylactic and prophylactic purposes (Woolhouse *et al.,* 2015).

The values for administration of antibiotics at veterinary level in 29 European Community (EC) and non-European Community countries show that tetracyclines rank first in total sales (33.4%), followed by penicillins (25.5%), sulphonamides (11%) and macrolides (7.5%). Polymyxins, fluoroquinolones and cephalosporins, considered critical important antibiotics for humans and animals represented 6.6%, 1.9% and 0.2% of total sales, respectively. It should be noted that colistin represents more than 99% of the total sales of polymyxins (EMA/ESVAC, 2016).

In Portugal, the total sales of tetracyclines also rank first (38.2%), followed by penicillins (17%) and macrolides (12.2%). The total sales of polymyxins, fluoroquinolones and cephalosporins were 8.7%, 5.6% and 0.2%, respectively (EMA/ESVAC, 2016). Considering the period between 2011 and 2014, there was a decrease in total sales of polymyxins and a marked increase in fluoroquinolones, with a peak in 2014; the sales of 3rd and 4th generation cephalosporins remained stable over the same period (EMA/ESVAC, 2016).

Over the past decade, the World Organization for Animal Health (OIE) has been developing relevant work in the field of antibiotic resistance, through the implementation of international standards applicable to the various antibiotics, being its prudent and responsible use the

main goals. Considering the large number of animal species, it was established by OIE a classification based on the level of importance of antibiotics and according to the following criteria: i) specificity of the infection and lack of alternative therapies; ii:) opinion of the various member countries concerning the antimicrobials of veterinary importance (OIE, 2015).

Table 1.1 lists the classification of the main groups of antibiotics according to the two criteria mentioned above: veterinary critical important antibiotics (VCIA) follow both criteria, veterinary highly important antibiotics (VHIA) follow only one of the criteria, and the veterinary important antibiotics (VIA) are considered if none of the criteria is applicable (OIE, 2015).

Some antibiotics included in the VCIA category, namely 3rd and ^{4th} generation cephalosporins and fluoroquinolones, are also considered by the World Health Organization (WHO) as critical important in humans. For this reason, i) should not be administered to animals, in food or drinking water, in the absence of clinical signs of disease, ii) should not be used as a first line treatment, unless justified, and after antibiotic susceptibility testing, and iii) should be reserved for use, extra label/off-label, when no alternatives are available (OIE, 201

Table 1.1. Main antibiotics of veterinary importance in Gram negative bacteria
(adapted Vaarten, 2012; OIE, 2015).

Group Mechanism of action	Category[1]	Antibiotics	Spectrum	Animal species	Treatment	Primary target	Affected pathways	Resistance mechanisms
Quinolones and Fluoroquinolones								
Inibitors of DNA synthesis	Veterinary antibiotics of high importance Veterinary antibiotics of critical importance	Nalidixic acid Oxolinic acid Flumequine [2] Ciprofloxacin Enrofloxacin [2] Marbofloxacin [2] Danofloxacin [2] Difloxacin Norfloxacin Ofloxacin Orbifloxacin [2] Sarafloxacin [2]	Gram positive aerobes Gram negative aerobes and some anaerobes	Large and small ruminants Poultry Swine Equine Rabbits Fish Bees	Septicemias and respiratory and enteric infections	DNA girase Topoisomerase IV	DNA replication Bactericide	Target protection Enzymatic inactivation Efflux
Sulfonamides and Diaminopirimidins								
Inibitors of DNA synthesis	Veterinary antibiotics of critical importance	Sulfametoxazole [3] Sulfamerazine Sulfaguanidine Sulfadiazine	Gram positive Gram negative Protozoa	Large and small ruminants Poultry Swine Equine Rabbits Fish	Bacterial infections, coccidiosis, infections caused by protozoa	Inibitors of tetrahidrofolic acid synthesis	Nucleotide biosynthesis and DNA replication Bacteriostatic	Target protection Decreased permeability
Inibitors of DNA synthesis	Veterinary antibiotics of critical importance	Trimethoprim [3]	Gram negative	Large and small ruminants Poultry Swine Equine Rabbits	Bacterial infections	Inibitors of tetrahidrofolic acid synthesis	Nucleotide biosynthesis and DNA replication Bacteriostatic	Target protection Decreased permeability

Group Mechanism of action	Category [1]	Antibiotics	Spectrum	Animal species	Treatment	Primary target	Affected pathways	Resistance mechanisms
β-Lactams								
Inhibitors of cell wall synthesis	Veterinary antibiotics of high importance	Cefalexin Cefalotin Cefazolin Cefuroxime	Gram positive aerobes Gram negative aerobes and anaerobes	Large and small ruminants Poultry Swine Equine Rabbits Fish	Septicemia Respiratory infections Urinary infections Mastitis	Penicillin-binding proteins	Biosynthesis of peptidoglican Bactericide	Enzymatic inactivation Efflux Decreased permeability Target protection
	Veterinary antibiotics of critical importance	Cefoperazone Ceftiofur [2] Ceftriaxone Cefquinoma [2]						
	Veterinary antibiotics of critical importance	Penicilin Ampicilin Amoxicilin Meciliname Amoxicilin/Clavulanate Ampicilin/Sulbactam Cloxacilin Dicloxacilin Oxacilin						
Polipeptides								
Inhibitors of cell wall synthesis	Veterinary antibiotics of high importance	Bacitracin Polimixin Colistin	Gram negative	Large and small ruminants Poultry Swine Equine Rabbits Fish	Septicemia Respiratory infections Urinary infections Infections of digestive system Colibacilosis Salmonelosis	Cell membrane	Synthesis of cell wall Bactericide	Efflux Decreased permeability MCR-1 and variants; MCR-2; MCR-3
Aminoglicosides								
Inhibitors of protein synthesis	Veterinary antibiotics of critical importance	Gentamicin Streptomycin Kanamycin; Amikacin Tobramycin Neomycin Apramicin [2] Spectinomycin [2]	Gram positive Gram negative	Large and small ruminants Poultry Swine Equine Rabbits; Fish;	Septicemia Respiratory and Urinary infections Infections of digestive system	30S ribossomal subunit	Inhibition of protein synthesis Bactericide	Enzymatic inactivation Efflux Decreased permeability

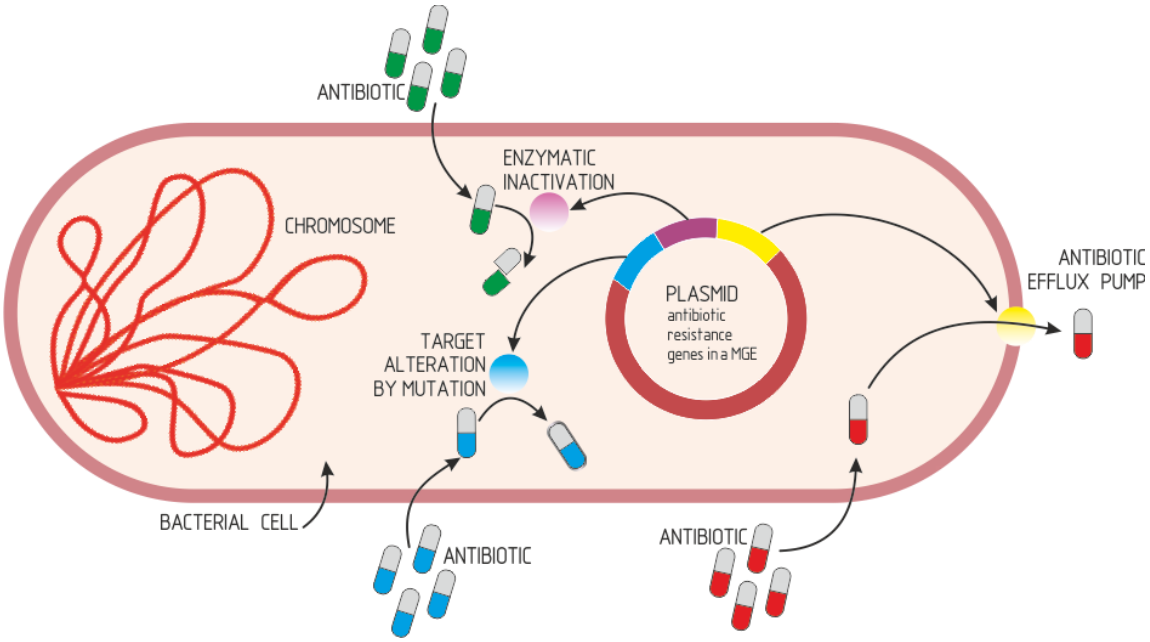
Group Mechanism of action	Category [1]	Antibiotics	Spectrum	Animal species	Treatment	Primary target	Affected pathways	Resistance mechanisms
Tetracyclines								
Inhibitors of protein synthesis	Veterinary antibiotics of critical importance	Clortetraciclina Doxiciclina Oxitetraciclina Tetraciclina	Gram positive Gram negative	Large and small ruminants Poultry Swine Equine Rabbits Fish; Bees	Septicemia Respiratory infections Urinary infections Infections of digestive system	30S ribossomal subunit	Inhibition of protein synthesis Bacteriostatic	Enzymatic inactivation Efflux Decreased permeability Target protection
Fenicols								
Inhibitors of protein synthesis	Veterinary antibiotics of critical importance	Chloramphenicol Florfenicol [2] Tianfenicol	Some Gram positive Gram negative	Large and small ruminants Poultry Swine Equine Rabbits Fish Bees	Respiratory infections in bovine, swine and poultry Infections in fish	30S ribossomal subunit	Inhibition of protein synthesis Bacteriostatic	Enzymatic inactivation Efflux Decreased permeability Target protection

[1]**VHIA**, *veterinary highly important antibiotics*, accomplish one of the criteria established by OIE;
VCIA, *veterinary critical important antibiotics*, accomplish both criteria established by OIE; [1. Specificity of the infection; 2. Opinion of member countries]
[2]Antibiotics of veterinary use;
[3]Can be applied in combination and are bacteriostati

3. MECHANISMS OF ANTIBIOTIC RESISTANCE IN GRAM NEGATIVE BACTERIA

There are four main mechanisms of resistance to antibiotics: i) changes in cell membrane permeability, ii) active efflux, iii) enzymatic modification or inactivation, and iv) target alteration through mutation (Wright, 2010; Blair *et al.,* 2015) (Figure 1.2).

Figura 1.2. Main antibiotic resistance mechanisms. MGE, mobile genetic element. (Adapetd from Levy & Marshall, 2004).



3.1. Decreased cell membrane permeability

The entry of antibiotic molecules in the bacterial cell, including hydrophilic antibiotics (aminoglycosides, β-lactams and colistin), is achieved by diffusion through the outer membrane proteins (Omps), depending on its electrical charge, shape and size. Thus, the reduction of the permeability of the outer membrane by decreasing the number of functional porins, or by replacing for more selective pore channels, are limiting factors to the antibiotics entrance into the bacterium (Figure 1.2) (Blair *et al.,* 2015).

OmpF is the most important porin in *Escherichia coli* and a decrease on its expression contributes to resistance to various antibiotics, including quinolones, aminoglycosides, β-lactams. Karczmarczyk *et al.* (2011) identified it as one of the main mechanisms of resistance to quinolones in strains of *E. coli* isolated from food-producing animals (Karczmarczyk *et al.,* 2011).

3.2. Active efflux

Efflux pumps are one of the main mechanisms of resistance to antibiotics in Gram-negative bacteria, being responsible for the active transport of antibiotics to the outside of the cell (Figure 1.2) (Blair *et al.,* 2015).

This mechanism occurs frequently in strains of *Salmonella enterica* and *E. coli* of animal origin, showing reduced susceptibility to fluoroquinolones, which is frequently associated to mutations in topoisomerase-encoding genes (Randall *et al.,* 2005; Karczmarczyk *et al.,* 2011; Yang *et al.,* 2014).

Active efflux is also the main mechanism of resistance to tetracycline; 30 genetic determinants associated with this resistance mechanism have been identified (Roberts & Schwarz, 2016), of which *tetA*, *tetB*, *tetC* and *tetG* are the most frequent in strains of *S. enterica* and *E. coli* isolated from animals and animal products (Glenn *et al.,* 2013, Gomes-Neves *et al.,* 2014, Chang *et al.,* 2015, Jackson *et al.,* 2015, Shin *et al.,* 2015).

Some efflux pumps act on a specific substrate (Tet tetracycline pumps) (Roberts & Schwarz, 2016), while others, called multidrug resistant (MDR) pumps, act on a larger number of structurally unrelated substrates, such as the OqxAB pump, which is plasmid-mediated and act on quinolones, chloramphenicol and trimethoprim, occurring more frequently in strains of *E. coli* isolated from animals treated with olanquidox (Jacoby *et al.,* 2014; Yang *et al.,* 2014).

3.3. Modification by enzymatic inactivation

The enzymatic inactivation is the most common mechanism of resistance to β-lactam antibiotics, through which β-lactamases cause the cleavage of β-lactam ring (Blair *et al.,* 2015). In *Enterobacteriaceae* strains of animal origin, β-lactamases belonging to different families and different hydrolytic profiles are described (Rubin & Pitout, 2014).

Also in *E. coli* and *S. enterica* strains of animal origin, enzymatic inactivation is the most important mechanism of resistance to other classes of antibiotics, such as phenicols, through the action of acetyltransferases and phosphotransferases (Schwarz *et al.,* 2004), and aminoglycosides, through acetyltransferases, adenyltransferases and phosphotransferases (Ramirez & Tomalsky, 2010; van Hoek *et al.,* 2011; Frye & Jackson, 2013).

The AAC(6 ')-Ib variant, AAC(6')-Ib-cr, encoded by the *aac(6') - Ib-cr* gene, is responsible for co-resistance towards aminoglycosides and fluoroquinolones (Ramirez *et al.,* 2013), occurring frequently in strains of animal origin (Veldman *et al.,* 2011; Jones-Dias *et al.,* 2013).

3.4. Alteration of the target by mutation

Target alteration by the acquisition of mutations at the level of *gyr* (*A* and *B*) and *par* (*C* and *E*) genes is the main mechanism of resistance to quinolones and fluoroquinolones, causing protein alteration and avoiding binding of the antibiotic to its target (Figure 1.2) (Wright *et al.,*

2010). This mechanism is responsible for a high level of resistance to these compounds, and occurs with high frequency in strains of *S. enterica* and *E. coli* of animal origin (Tamang *et al.,* 2012a; Jones-Dias *et al.,* 2013, Wasyl, 2014a, Wasyl *et al.,* 2014b).

4. GENETIC SUPPORT OF ANTIBIOTIC RESISTANCE IN DIFFERENT CLASSES OF ANTIBIOTICS

4.1. Intrinsic and acquired resistance

Bacteria can be naturally resistant to some antibiotics, due to the presence of genetic, structural, and functional characteristics, which inactivate the antibiotic, according to the four resistance mechanisms referred in item 3 and Table 1.1 (Cox & Wright, 2013). Some examples are highlighted in Table 1.2.

Acquired resistance includes genetic mutations in structural or regulatory housekeeping genes conferring resistance, and the horizontal acquisition of mobile antibiotic resistance genes (van Hoek *et al.,* 2011). Some examples of acquired resistance to the antibiotics directly related to the present study are described.

Table 1.2. Some examples of intrinsic resistance and mechanisms

Organisms	Antibiotic	Mechanism
Gram negative bacteria	Vancomicin Daptomicin	Decreased permeability of cell membrane
Klebsiella spp	Ampicilin	Enzymatic inactivation (chromossome-encoded β-lactamase which inactivates the antibiotic)
Escherichia coli	Penicillin Cephalosporins Cephamicins and/ or Aztreonam	Enzymatic inactivation (chromossome-encoded β-lactamase AmpC which inactivates the antibiotic)
Pseudomonas aeruginosa	Sulfamidas Trimetoprim Tetraciclina Cloranfenicol	Decreased permeability of cell membrane
Stenotrophomonas maltophilia	Imipenem	Enzymatic inactivation (chromossome-encoded metalo-β-lactamase which inactivates the antibiotic)

4.2. Mechanisms of resistance (acquired or intrinsic) in different classes of antibiotics

4.2.1. β-lactam antibiotics

In *Enterobacteriaceae*, the production of β-lactamases is the most frequent and important mechanism of resistance, with more than 1000 β-lactamases identified (www.lahey.org) (Seiffert *et al.,* 2013, Rubin & Pitout, 2014).

These periplasmic enzymes are grouped into four molecular classes (A to D), according to the Ambler classification, which is based on the amino acid sequence homology (Ambler, 1980). Ambler classes A, C and D include β-lactamases with serine at the active site, and class B include β-lactamases with Zn^{2+} ion at the active site, that is required as a cofactor in its catalytic activity (Table 1.3) (Ambler, 1980); these are also called metallo-β-lactamases MBL). According to Bush and Jacoby classification, functional groups 1, 2 and 3, and their subgroups, are designated according to the substrate and inhibition profiles; groups 1 and 2 include serine-β-lactamases, and those of group 3 include the metallo-β-lactamases (Table 1.3) (Bush & Jacoby, 2010).

Tabela 1.3. Classification of β-lactamases produced by Gram negative bacteria, with clinical relevance in human and veterinary practice (Bush & Jacoby, 2010)

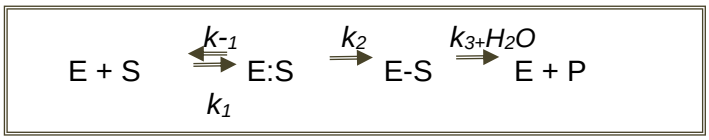
Class Ambler	Functional group or subgroup[1]	Enzyme families	Substrates and inhibition profile	Representative enzymes
A	2b	TEM	Penicillins Early cephalosporins (inhibited by β-lactamase inhibitors)	TEM-1, -2, -13
		SHV		SHV-1,-11, -89
	2be ESBL	TEM	Broad-spectrum cephalosporins (inhibited by β-lactamase inhibitors)	TEM-10, -24, -52
		SHV		SHV-12
		CTX-M		CTX-M-1 to CTX-M-172
		PER		PER-1 to PER-8
		VEB		VEB-1 to VEB-16
	2br IRT/IRS	TEM	Penicillins (resistant β-lactamase inhibitors)	TEM-30, TEM-31
		SHV		SHV-72, -84, -107
	2ber (CMT)	TEM	Broad-spectrum cephalosporins Monobactams (resistant β-lactamase inhibitors)	TEM-50, -158
	2f (Carbapenemases)	GES	Carbapenems Broad-spectrum cephalosporins Cephamycins (variable resistance to β-lactamase inhibitors)	GES-2 a GES-27
		KPC		KPC-2 a KPC-24
		SME		SME-1 a SME-5

Class Ambler	Functional group or subgroup1	Enzyme families	Substrates and inhibition profile	Representative enzymes
B	3a (MBL)	IMP	Carbapenems Broad-spectrum cephalosporins (resistance to β-lactamase inhibitors)	IMP-1 a IMP-53
		VIM		VIM-1 a VIM-46
		NDM		NDM-1 a NDM-16
C	1 (PMAβ)	CMY	Cephalosporins Cephamycins (resistance to β-lactamase inhibitors)	CMY-1 a CMY-136
		DHA		DHA-1 a DHA-23
	1e (ESAC)	CMY	Cephalosporins Cephamycins Increased resistance to ceftazidime (resistance to β-lactamase inhibitors)	CMY-10, -19, -37
D	2d	OXA	Cloxacillin (variable resistance to β-lactamase inhibitors)	OXA-1, -2, -10
	2de (ESBL)	OXA	Cloxacillin Broad-spectrum cephalosporins (variable resistance to β-lactamase inhibitors)	OXA-11, -14, -15
	2df (CHDL)	OXA	Cloxacillin Carbapenems (variable resistance to β-lactamase inhibitors)	OXA-23, -24, -48, -51, -58

[1] **ESBL**, Extended-Spectrum β-Lactamases; **IRT**, Inhibitor Resistant TEM; **IRS**, Inibitor Resistant SHV; **CMT**, Complex Mutant TEM; **MBL**, Metalo-β-Lactamases; **PMAβ**, Plasmid-Mediated AmpC β-lactamases; **ESAC**, Extended-Spectrum AmpC β-lactamases; **CHDL**, Carbapenem-Hydrolysing class-D β-Lactamases.

In a general way, β-lactamases promote the cleavage of the β-lactam ring and may act by: 1) using zinc ions in the case of metallo-β-lactamases, and 2) using the ester-serine pathway in the other classes of β-lactamases. In the latter (Figure 1.3), the hydrolysis is triggered in two distinct steps: i) non-covalent bonding of the enzyme (E) to the antibiotic (substrate, S), resulting in a non-covalent Michaelis complex (E:S), and ii) attack to the β-lactam ring, through the hydroxyl group of the serine residue, resulting in a covalent acyl ester (ES) bond. The ester hydrolysis will finally release the inactive antibiotic (P) before it reaches the PBPs, and the regenerated enzyme is active and available to hydrolyze other antibiotic molecules (Bush & Sykes, 1986).

Figure 1.3. Hidrolysis of a β-lactam antibiotic by an enzyme with serine on its active site (Adapted from Page, 2008).



Due to the successive identification of new variants of β-lactamases, a characterization of their kinetic parameters is fundamental. Thus, the catalysis coefficient, k_{cat}, is the best parameter to describe the hydrolytic activity of β-lactamases and depends on the rates of formation and hydrolysis of the acyl-enzyme complex. This kinetic parameter allows the identification of any mutations affecting the activity of the free enzyme and the hydrolysis step. However, it is not useful to identify mutations affecting the recognition of substrates by the enzyme (Nordmann & Mammeri, 2007; Page, 2008).

Michaelis constant, K_m, is the affinity or semi-saturation constant, that corresponds to an equilibrium constant equal to the concentration of the substrate to be hydrolyzed at a rate equal to 0.5 of the maximum velocity (V_{max}). K_m includes all the rate constants describing the catalytic steps of β-lactams hydrolysis ($K_m = k_3K_S/k_2+k_3$). Thus, it is the most adequate kinetic parameter for the identification of β-lactamases with mutations associated to extended spectruns.

The k_{cat}/K_m constant does not contribute to any of the steps involved in the hydrolysis and depends only on the rate constants involved in the formation of the intermediate acyl-enzyme. Although this parameter is said to measure the "catalytic efficiency," it is independent of the enzyme hydrolytic activity; only mutations affecting the recognition of enzyme substrate, will affect k_{cat}/K_m (Nordmann & Mammeri, 2007; Page, 2008).

From the epidemiological point of view, the most important β-lactamases in *Enterobacteriaceae* are: i) extended-spectrum β-lactamases (ESBL), of which the TEM, SHV and CTX-M families are highlighted, ii) carbapenemases of class A (e.g. KPC), class B (metallo-β-lactamases, e.g. VIM, IMP and NDM), iii) plasmid-mediated AmpC β-lactamases (PMAβ), and iv) extended-spectrum AmpC β-lactamases (ESAC) (Table 1.3) (Kim *et al.,* 2006; Rubin & Pitout, 2014).

4.2.1.1. ESBL

These enzymes confer resistance to β-lactam antibiotics, including penicillins, 1st, 2nd, 3rd and 4th generation cephalosporins and monobactams; generally, carbapenems and cephamycins are not hydrolyzed and are inhibited by β-lactamase inhibitors, such as clavulanic acid, sulbactam, tazobactam and avibactam (Paterson & Bonomo, 2005). There are several families of ESBLs, being the CTX-M family and some variants of the SHV and TEM families

(Table 1.3) the most frequent in the various animal species and products of animal origin (EFSA, 2011a).

Of the ESBL mentioned, it is highlighted the detection of the FEC-1 enzyme (Fujisawa *E. coli*-1), a transferable ESBL, CTX-M type, identified for the first time in Japan, by Matsumoto *et al*. (1988), in a strain of *E. coli* isolated from a canid in captivity (Matsumoto *et al.,* 1988).

- **CTX-M family**

The CTX-M family belongs to class A of the active serine β-lactamases and are divided into six groups, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M -9, CTX-M-25 and KLUC (Table 1.3). Between the different groups, the level of deviation on the amino acid sequence ranges from 9.3 to 32%. Each group includes allelic variants differing only by one or a reduced number (≤5%) of amino acid substitutions (Rossolini *et al.,* 2008; D'Andrea *et al.,* 2013). KLUC-1 and -2 are two chromosomal cefotaximases of *Kluyvera cryocrescens*, differing from each other in an amino acid substitution and sharing 87.6% identity with CTX-M-3. Because KLUC-2 has also been identified in a plasmid, it is suggested that it will be a new cluster or a new member of the CTX-M family (Zhao & Hu, 2013).

CTX-M enzymes have a strong cefotaximase activity. Cefepime is also a good substrate, unlike ceftazidime (Bush & Jacoby, 2010). However, in recent years, the emergence of enzymes with a greater hydrolytic capacity on ceftazidime has been observed, due to the increased use of this antibiotic in clinical practice (Bush & Jacoby, 2010).

The rapid and successful dissemination of enzymes belonging to the CTX-M family in the various epidemiological niches, is due to the dissemination of $bla_{\text{CTX-M}}$ genes through mobile genetic elements, the clonal expansion of certain strains, and the selective pressure caused by the massive use of broad-spectrum cephalosporins and fluoroquinolones in human and veterinary therapy (D'Andrea *et al.,* 2013).

There are currently 172 enzyme variants of the CTX-M family (http://www.lahey.org/Studies) (accessed 09/09/2017). In the CTX-M family, variants -1, -9, -14, -15, -32 are the most frequent in strains of *Enterobacteriaceae* isolated from food-producing animals (Cortés *et al.,* 2010; Geser *et al.,* 2012; Silva *et al.,* 2012; Olsen *et al.,* 2014; Stefani *et al.,* 2014; Michael *et al.,* 2016), companion animals (Costa *et al.,* 2004; Dierikx *et al.,* 2012), and wild animals (Poeta *et al.,* 2009; Pinto *et al.,* 2010; Gonçalves *et al.,* 2012; Jamborova *et al.,* 2015).

CTX-M-15 is the most frequent and widely disseminated enzyme in humans (Nicolas-Chanoine *et al.,* 2014), in food-producing and companion animals, after CTX-M-1 (O'Keefe *et al.,* 2010, Geser *et al.,* 2012, Valentin *et al.,* 2014; Michael *et al.,* 2016).

Other enzyme variants (-24, -27, -55, -98, -102, -104) less common in European countries, occur frequently in eastern countries, in food-producing and companion animals (Tamang *et al.,* 2012b; Zhang *et al.,* 2016).

- **TEM and SHV families**

In the TEM family the variants -20, -52, -106, -126, and in the SHV family the variants -2, -5 and -12 are ESBLs (Table 1.3) and they have also been identified in strains of *Enterobacteriaceae* isolated from food-producing animals (Hasman *et al.,* 2005; Smet *et al.,* 2008; Rodriguez *et al.,* 2009; Dierikx *et al.,* 2010; Geser *et al.,* 2012; Gonçalves *et al.,* 2012; Stefani *et al.,* 2014; Noda *et al.,* 2015; Solà-Ginés *et al.,* 2015; Michael *et al.,* 2016), companion animals (Costa *et al.,* 2004; Carattoli *et al.,* 2005; O'Keefe *et al.,* 2010) and wild animals (Pinto *et al.,* 2010).

4.2.1.2. PMAβ

Plasmid AmpC β-lactamases comprise an important group of β-lactamases with a hydrolytic profile identical to ESBLs, in addition to the hydrolysis of cephamycins, namely cefoxitin and cefotetan (Bajaj *et al.,* 2016) (Table 1.3); they do not confer resistance to cefepime and are inhibited by cloxacillin, but not by β-lactamase inhibitors, namely clavulanic acid and tazobactan (Jacoby, 2009).

In *E. coli* (wild-type), and due to the presence of a weak promoter and a repressor, the expression of chromosomal AmpC enzyme does not confer resistance to β-lactams. However, mutations in the promoter and repressor genes may lead to resistance towards penicillins, cephalosporins, cephamycins and/or aztreonam (Jacoby, 2009). *Salmonella* spp., *Klebsiella* spp. and *Proteus mirabilis* do not have chromosomal β-lactamases of the AmpC type, and resistance to cephalosporins and cephamycins is plasmid-encoded. These enzymes derive from chromosomal AmpC cephalosporinases and encompass families: CMY, DHA, ACT, FOX, MIR, ACC, LAT, CFE and MOX (Table 1.3) (Jacoby, 2009).

There are currently 136 variants in the CMY family, 23 variants in the DHA family, 38 variants in the ACT family, 5 variants in the ACC family, 12 variants in the FOX family, 18 variants in the MIR family, 11 variants in the MOX family, and a variant in the CFE family (http://www.lahey.org/Studies) (accessed 9/9/2017).

CMY-2 (CMY family) is the enzyme that is most frequently detected in *Enterobacteriaceae* strains isolated from animals of different species and products of animal origin (Ewers *et al.,* 2012; Tamier *et al.,* 2012b; Dierikx *et al.,* 2012; Liebana *et al.,* 2013), as well as from human isolates recovered from nosocomial and community-acquired infections (Nicolas-Chanoine *et al.,* 2014, Alonso *et al.,* 2016). Although rarely, DHA-1 (DHA family). ACC-1 (ACC family) variants were also identified in *E. coli* strains isolated from food-producing animals (Hasman *et al.,* 2005; Rayamajhi *et al.,* 2008; Dierikx *et al*., 2010), and in *Klebsiella pneumoniae* strains isolated from canine (Hidalgo *et al.,* 2015).

Most ESBL and PMAβ producing strains also carry resistance determinants to other antibiotics, namely sulfamides, tetracyclines, fluoroquinolones, antibiotics commonly used in

veterinary medicine and animal production. The persistence and dissemination of these strains in production animals may be due, not only to the use of 3rd generation cephalosporins but also due to other antibiotics by co-selection (D'Andrea *et al.,* 2013, Liebana *et al.,* 2013).

4.2.1.3. ESAC

Genomic events such as insertions, delections and amino acid substitutions have been described in chromossome and plasmid-encoded AmpC β-lactamases, responsible for increasing the catalytic efficiency over oxymino-cephalosporins, as in ESBLs (Nordmann & Mammeri, 2007). Broad-spectrum AmpC β-lactamases are responsible for the hydrolysis of 3rd generation cephalosporins, including cefepime and cefpiroma. Its hydrolytic activity on cefoxitin is variable; some chromosome-encoded ESACs produced by *E. coli* isolates have a hydrolytic activity on cefoxitin lower than that produced by wild-type cephalosporinases, and may express a susceptibility phenotype to that antibiotic (Nordmann & Mammeri, 2007). This resistance pattern may also be associated with ESBLs, which may lead to an incorrect phenotype interpretation (Nordmann & Mammeri, 2007).

The target of these cephalosporinases comprises of two regions: R1 surrounded by the Ω-loop, and R2 surrounded by R2 loop, containing the H-10 and H-11 helices (Nordmann & Mammeri, 2007; Jacoby, 2009). These changes generally increase the catalytic efficiency for ceftazidime compared with the wild-type enzyme that imply the expression of high MIC values, whereas MIC values for cefotaxime and cefepime reflect only a reduced susceptibility (Jacoby, 2009).

This resistance mechanism is emerging in human clinical isolates (Mammeri *et al.,* 2008; Jørgensen *et al.,* 2010) and may be found in animals; it has been described for the first time in strains of *E. coli* isolated from cattle in France (Haenni *et al.,* 2014).

4.2.1.4. Metalo-β-lactamases and other Carbapenemases

The use of carbapenems in veterinary practice is restricted to severe multidrug resistant post-surgery and urinary tract infections caused by *E. coli* in companion animals. Due to this fact, the occurrence of carbapenemases in strains of animal origin is low (Abraham *et al.*, 2014, Woodford *et al.,* 2014).

In *Enterobacteriaceae*, resistance to carbapenems arises mainly through two mechanisms: i) acquisition of enzyme-encoding genes responsible for the hydrolysis of carbapenems, or ii) a decrease in antibiotic uptake due to deficient qualitative or quantitative porine expression (OmpF and OmpC), in association with an overexpression of β-lactamases with weak affinity for carbapenems (Nordmann *et al.,* 2012).

Some studies detected carbapenemases in strains of *E. coli* and *S. enterica* subsp *enterica* (e.g. both producing VIM-1, in Germany), *Acinetobacter baumannii* (OXA-23, in France and

NDM-1 in China) and *Acinetobacter lwoffii* (NDM-1, in China), isolated from food-producing animals (Fischer *et al.,* 2012, Poirel *et al.,* 2012a Wang *et al.,* 2012b; Fischer *et al.,* 2013a; Fischer *et al.,* 2013b; Zhang *et al.,* 2013).

The detection of carbapenemase-producing bacteria is probably underestimated, since carbapenems are not routinely included in the antibiotic susceptibility tests in veterinary laboratories (Abraham *et al.,* 2014). Existing data comes from scientific studies carried out in several countries, in isolates from different animal matrices, using different methodologies and interpretation criteria (Guerra *et al.,* 2014).

Since carbapenems are considered as one of the last resources to treat human infections caused by ESBLs and so do not have the adverse effects of colistin, preservation of its efficacy is essential, in order to have clinical success against severe infections caused by Gram negative organisms (Evans *et al.,* 2014).

The production of carbapenemases in strains of *E. coli* (NDM-1 and OXA-48, United States), *K. pneumoniae* (OXA-48) and *Acinetobacter* spp. (OXA-23, Belgium and Germany), isolated from companion animals (Smet *et al.,* 2012, Stolle *et al.,* 2013, Sun *et al.,* 2015, Liu *et al.,* 2016, Ewers *et al.,* 2017), and *Salmonella* Corvallis (NDM-1, Germany) isolated from wild birds (Fischer *et al.,* 2013b), has been detected.

There are currently 53 variants in the IMP family, 46 in the VIM family, 24 in the KPC family, 16 in the NDM family and 27 in the GES family (http://www.lahey.org/Studies) (accessed 09/09/2017).

4.2.2. Quinolones and fluoroquinolones

In *Enterobacteriaceae*, the major resistance mechanisms to fluoroquinolones are chromosome-encoded, and associated to nucleotide modifications in genes encoding the DNA subunits gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) (Jacoby et al. al., 2014). Mutations occur in the Quinolone Resistance Determinant Region (QRDR), resulting in amino acid substitutions, altering the target protein structure and subsequently the fluoroquinolone-binding affinity of the enzyme (Jacoby *et al.,* 2014). The other two mechanisms of resistance, also chromosome-encoded, are the decrease in cell membrane permeability with loss of porins, and the occurrence of efflux pump (Jacoby *et al.,* 2014).

The emergence of plasmid-mediated quinolone resistance (PMQR) emerged in 1998, constituting a new threat due to its rapid expansion in different reservoirs. The genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS* and *qnrVC* encode proteins that protect the topoisomerases II and IV subunits from fluoroquinolones (Jacoby *et al.,* 2006; Poirel *et al.,* 2012d). The second mechanism, *aac(6')-Ib-cr,* encodes an acetyltransferase responsible for co-resistance to fluoroquinolones and aminoglycosides (kanamycin, tobramycin and amikacin). The third mechanism includes efflux transporters, encoded by the *qepA*, and *oqxAB* genes,

responsible for the active export of fluoroquinolone molecules outside the cell (Poirel *et al.,* 2012d).

Various animal species constitute the reservoirs of PMQR and their occurrence has been increasing (Veldman *et al.,* 2011, Tamang *et al.,* 2012a; Wang *et al.,* 2012a; Jones-Dias *et al.,* 2013). In European countries, QnrB, QnrS, and variants, and also AAC(6 ')-Ib-cr, are the most frequent mechanisms in strains of *E. coli* and *S. enterica* (Veldman *et al.,* 2011; *et al.,* 2013, Wasyl, 2014a, Wasyl *et al.,* 2014b, Jamborova *et al.,* 2015). Efflux pumps QepA and OqxAB occur more frequently in strains of *E. coli* isolated from olanquidox-treated animals, used as growth promoters in some Asian countries (Jacoby *et al.,* 2014; Yang *et al.,* 2014).

Although the presence of PMQR does not confer high level of resistance to quinolones, it favours the selection of bacteria with a high level of resistance, increasing the effect of other chromosome-encoded resistance mechanisms even in the absence of pressure antibiotics (Guan *et al.,* 2013; Redgrave *et al.,* 2014).

4.2.3. Polymyxins

Intrinsic resistance to polymyxins includes adaptive and mutational mechanisms, acting on lipopolysaccharides of the cell membrane (LPS). In Gram negative bacteria, polymyxins act on two components, PhoP/Q and PmrA/B, corresponding to cationic regulation systems of antimicrobial response. Overall, resistance involves the modification of 4'-phosphoethanolamine (PEA). This chemical modification results in a reduction of the negative charge by breaking the outer membrane covalent bonds, and a decrease in the antibiotic binding to the outer membrane fraction A of the LPS. Other mechanisms may also occur, such as active efflux, porine depletion, capsule formation and hypervesiculation (Olaitan *et al.,* 2014; Gao *et al.,* 2016; Trimble *et al.,* 2016).

Recently in China, Liu *et al.* (2016) identified, for the first time, in *E. coli* strains isolated from humans, food-producing animals and meat, a new mechanism of resistance to colistin called MCR-1, which belongs to the phosphoethanolamine-transferase family. This plasmid-mediated mechanism encoded by the *mcr-1* gene is thought to catalyze the modification of the lipid A fraction (Liu *et al.,* 2016). This chemical modification represents a unique mechanism, unlike that occurring in chromosome resistance, triggered by two operons, *pmrAB* and *phoPQ* and a *mgrB* regulator (Gao *et al.,* 2016).

Lately, several studies from different countries including Portugal, reported the detection of the *mcr-1* gene in strains of *E. coli* and *S. enterica* from humans, food-producing animals, meat and in the environment (Hasman *et al.,* 2015; Campos *et al.,* 2016; Figueiredo *et al.,* 2016; Kluytmans-van den Bergh *et al.,* 2016; Malhotra-Kumar *et al.,* 2016; Perrin-Guyomard *et al.,* 2016; Quesada *et al.,* 2016; Zhang *et al.,* 2016; Zurfuh *et al.,* 2016).

More recently, in Belgium, a new gene, *mcr-2*, in *E. coli* pathogenic strains from cattle and swine (Xavier *et al.,* 2016), and in Italy, a new variant of the *mcr-1*, *mcr-1.2*, in a human

strain of *K. pneumoniae*, were detected (DiPilato *et al.,* 2016). The phylogenetic analysis of MCR-2 seems to point that this protein can be originated in *Moraxella catarrhalis*, evidencing 76.75% homology with MCR-1 (Xavier *et al.,* 2016).

Some authors report the co-occurrence of *mcr-1* gene in strains producing ESBLs and also resistant to tetracyclines and sulfonamides, antibiotics widely used in animal production, stressing the importance that the selective pressure exerted by other antibiotics can also contribute for the emergency and dissemination of this gene (Haenni *et al.,* 2016). MCR determinants and known variants of *mcr-1* gene are given in Table 1.4.

5. DISSEMINATION OF ANTIBIOTIC RESISTANCE GENES

Antibiotic resistance can be transmitted:

- vertically, by accumulation of genetic changes during the natural process of genome replication, and transfer to subsequent generations;
- horizontally, by exchange of resistance determinants between bacteria, through three mechanisms: conjugation, transformation and transduction (Figure 1.4) (Dantas & Sommer, 2014).

Conjugation is an important mechanism involved in the horizontal gene dissemination, in which DNA transfer is performed through *pili* or adhesins. Transformation and transduction are less frequent mechanisms; in the transformation occurs the uptake and integration of extracellular DNA fragments after bacterial lysis, and in transduction the transfer of resistance determinants is mediated by bacteriophages (von Wintersdorff *et al.,* 2016) (Figure 1.4).

Figure 1.4. Mechanisms involved in horizontal gene transfer (adapetd from Levy & Marshall, 2004).

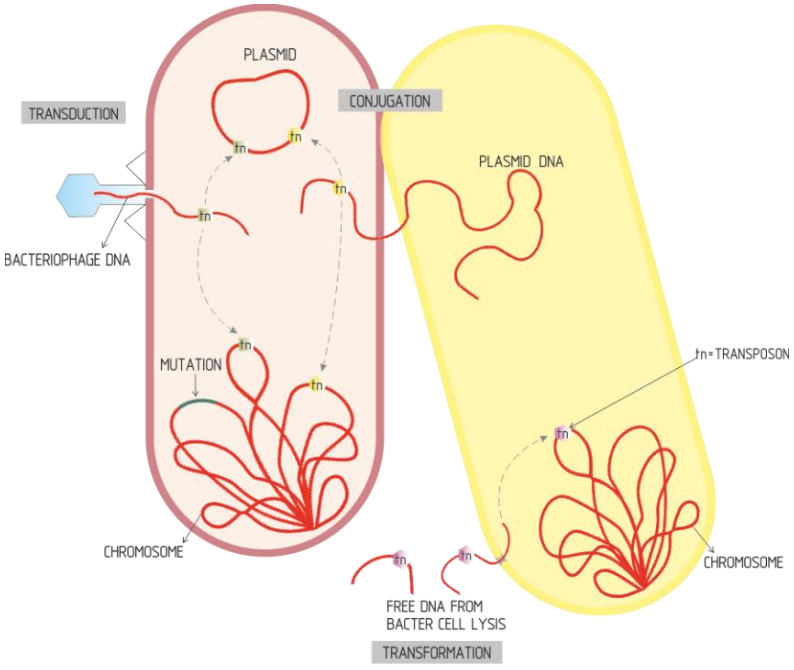


Table 1.4. Comparison of aminoacid substitutions, and the occurrence of the first cases of MCR-producing isolates.

MCR variant	Aminoacid substitutions	Bacterial species	Host	Country	GenBank acess nb.
MCR-1	-	*E. coli, K. pneumoniae*	Human; Swine; Meat	China	KP347127
MCR-1.2	Gln3Leu	*K. pneumoniae*	Human	Itália	KX236309
MCR-1.3	Ile38Val	*E. coli*	Poultry	China	NG_052861
MCR-1.4	Asp440Asn	*E. coli*	Sewage	China	KY041856
MCR-1.5	His452Tyr	*E. coli*	Human	Argentina	KY283125
MCR-1.6	Arg536His	*Salmonella* Typhimurium	Healthy human	China	NG_052893
MCR-1.7	Ala215Thr	*E. coli*	Sewage	China	KY488488
MCR-1.8	Gln3Arg	*E. coli*	Poultry	Brunei	KY683842
MCR-1.9*	Val413Arg	*E. coli*	Swine	Portugal	KY780959
MCR-2	80.7% identity to MCR-1	*E. coli*	Sick calves and piglets	Bélgica	NG_051171
MCR-3	32.5% and 31.7% identity to MCR-1 and MCR-2, respectively	*E. coli*	Swine	China	KY924928

* This study: **Chapter 5.2**: The novel MCR-1.9 variant within colistin-resistant *Enterobacteriaceae* isolates from food-producing animals and meat

In *Enterobacteriaceae*, the spread of acquired antibiotic resistance is mainly carried out through mobile genetic elements, such as plasmids, insertion sequences, transposons and some integrons (Table 1.4). These mobile elements allow the transfer of antibiotic resistance genes between DNA molecules inside the cell, i.e., from the chromosome to the plasmid or between plasmids; the plasmids are responsible for the dissemination of resistance genes between bacteria of the same species, or from different species (Partridge, 2015).

Table 1.5. Main mobile genetic elements and molecular features

Mobile genetic element (MGE)	Molecular features
Insertion sequences (ISs)	The simplest MGE, consisting of an open reading frame (*orf*) codifying the enzyme transposase, flanked by a repeated and inverted sequence of 10-40bp in each extremity (Gyles & Boerlin, 2015).
Insertion sequence common region (ISCR)	Insertion sequences like those of *IS*91 family, inserted by a mechanism of transposition designated by *rolling circle.* ISCRs have terminal sequences designated by origin (*ori*S) and ending sequences (*ter*IS) (Bennett, 2008; Zhao & Hu, 2013).
Integrative conjugative elements (ICEs)	MGE sharing features inherent to transposons, bacteriophages and plasmids, non-replicable and capable of integration or excision from the chromossome, and transfer from one bacterial cell to another, through conjugation (Gyles & Boerlin, 2015).
Transposons	MGE capable of moving inside the same DNA molecule, from one plasmid to another, or from the chromosome to a plasmid and vice-versa (Bennett, 2008).
Plasmids	MGE composed by extrachromosome linear or circular double-strand DNA, with autonomous replication (Leplae *et al.,* 2004). Some conjugative plasmids carry genetic determinants allowing transfer through conjugation; non-conjugative plasmids use mechanisms from other transferable plasmids to be transfered (Gyles & Boerlin, 2015).
Integrons	MGE able to capture gene cassetes and integrate by recombination (Hall & Collis, 1995). These elements consist of three regions, being two regions, 5' (5'-CS) e 3' (3'-CS), highly conserved and flanking a third region, variable, and where gene cassetes are located. 5'-CS region includes the transposase encoding gene (*intI*), the recombination site (*attI*), and a promoter (*Pc*), ensuring expression of the operon (Partridge, 2015). Integrons are grouped in three classes (1, 2 and 3), according to the nucleotide sequence of the integrase (*intI*) (Di Gonza & Gutkind, 2010).
Bacteriophages	Viruses, obligate intracelular and able to mediate the transfer of resistance genes. They multiply inside the bacterial cell by using host biosynthetic machinery (Balcazar, 2014).

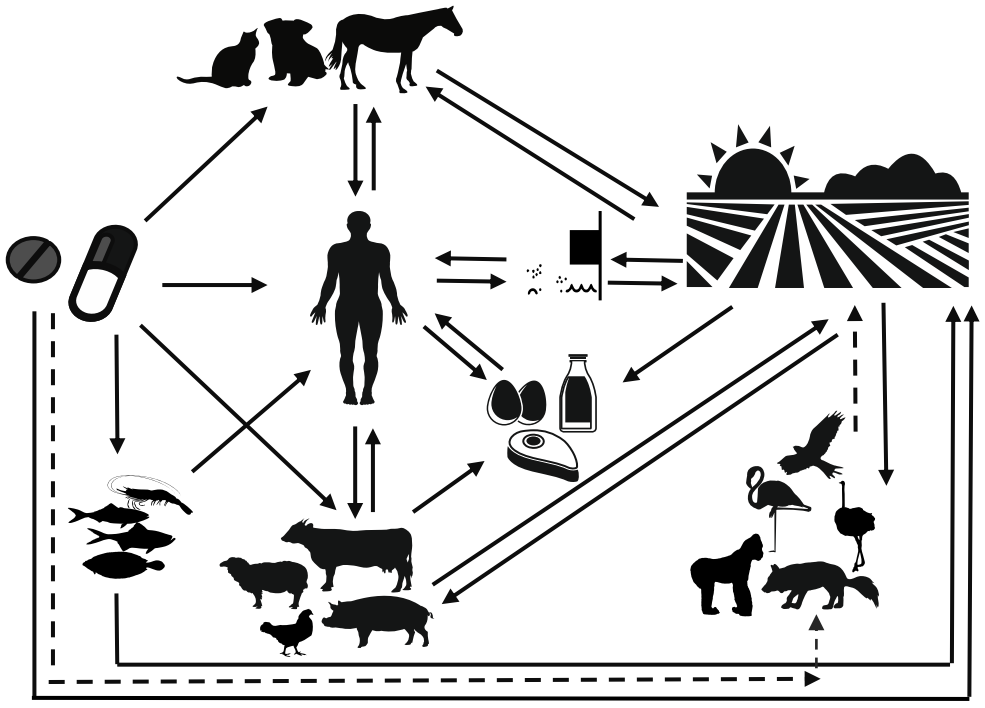
6. DIVERSITY OF ANTIBIOTIC RESISTANCE RESERVOIRS

Nowadays, the globalized world favors the spread of antibiotic resistance, for example through trade of foodstuffs, live animals and free movement of people. This may occur through direct or indirect contact, the food chain, water and manure applied to agricultural fields, among others (Marshall & Levy, 2011; Cares *et al.,* 2013; Aarestrup, 2015).

Thus, antibiotic resistance emerges as a dynamic, complex, multifactorial process involving humans, animals and the environment. Multiple connections are established between the various reservoirs, through which resistance genes and mobile genetic elements exchange information by horizontal gene transfer. Water (water courses, urban and residual effluents) and sludge are primordial sites of occurrence of these genetic events (Cantas *et al.,* 2013, Butaye *et al.,* 2014, Roca *et al.,* 2015, Woolhouse *et al*. 2015) (Figure 1.5). Horizontal gene transfer and genetic recombination among microbial communities of the various reservoirs, contribute to the diversity and adaptability of microorganisms to various environments (Woolhouse *et al.,* 2015).

Antibiotic resistance from human pathogenic strains is a major concern for health professionals and authorities, although they represent a limited number of species, compared with commensals and opportunistic pathogens (Djordjevic *et al.,* 2013). Some pathogenic and commensal bacteria are zoonotic, which allows them to infect/colonize food-producing animals, companion and wild animals, and the environment (Lewis *et al.,* 2008; Garcia-Alvarez *et al.,* 2012; Overdevest *et al.,* 2011; Harrison *et al.,* 2013; Pomba *et al.,* 2013; Franco *et al.,* 2015).

Figure 1.5. Some transmission pathways of antibiotic resistance, between different reservoirs: humans, animals and the environment (adapted from Woolhouse *et al.,* 2015).



6.1. Animals as reservoirs of antibiotic resistance

6.1.1. Food-producing animals

The use of antibiotics in animal production seem to have some impact on the emergence of resistant and multidrug resistant human pathogens, particularly *E. coli*, *Campylobacter* spp. and *S. enterica*, which are zoonotic agents transmitted through the food chain.

The high level of resistance to fluoroquinolones and tetracyclines observed in strains of *S. enterica* and *Campylobacter jejuni* isolated from humans, poultry and poultry meat in several European countries is consistent with the high consumption of fluoroquinolones and tetracyclines by humans and animals (Carreira *et al.,* 2012; Jones-Dias *et al.,* 2013; EFSA/ECDC, 2015; EMA/ESVAC, 2015; EMA/ESVAC, 2016). Food-producing animals are potential reservoirs of antibiotic resistant strains such as *Salmonella* spp. Thus, factors such as the environment of slaughterhouses and food processing plants, as well as operations along the slaughter and processing line may contribute for cross-contamination and for the emergence of human infections caused by strains with genotypic profiles identical to those detected in animal strains (Gomes-Neves *et al.,* 2012; Gomes-Neves *et al.,* 2014; Leverstein-van Hall *et al.,* 2011). The increase in the incidence of ESBL and PMAβ-producing *Enterobacteriaceae* infections in livestock suggests that animals, food and the environment are potential reservoirs of these microorganisms (Seiffert *et al.,* 2013). Studies performed elsewhere detected a high frequency of ESBL-encoding genes in *E. coli* strains isolated from poultry (75-80%), with a genotypic profile identical to strains isolated in man (Leverstein-van Hall *et al.,* 2011; Overdevest *et al.,* 2011). Also, in Italy, Franco *et al.,* (2015) detected a clone of *Salmonella* Infantis MDR isolated in poultry responsible for gastrointestinal infections in man (Franco *et al.,* 2015). Although carbapenems are not registered for use in the veterinary practice of food-producing animals (Poirel *et al.,* 2014), recent studies have reported the detection of carbapenemases in the following: VIM-1-producing *E. coli* and *S. enterica* isolated from pigs in Germany (Fischer *et al.,* 2012; Fischer *et al.,* 2013a), OXA-23-producing *A. baumannii* in cattle in France (Poirel *et al.,* 2012a), NDM-1-producing *A. baumannii* and *A. lwoffii* isolated from pigs and poultry in China (Wang *et al.,* 2012b; Zhang *et al.,* 2013) and more recently in poultry, OXA-48b-producing *Shewanella xiamenensis* isolated from broilers and *Shewanella aneidensis* isolated from cattle, and OXA-252-producing *S. xiamenensis* isolated from pigs at slaughter (Ceccarelli *et al.,* 2017). In recent years, severe human infections have been caused by multidrug resistant strains of *Pseudomonas aeruginosa* and *A. baumannii*, in which colistin is the last therapeutic resource (Catry *et al.,* 2015).

This antibiotic has been used in the veterinary practice and animal production since the 1950s for the treatment and prevention of infections caused by *Enterobacteriaceae* in different animal species, namely pigs, large and small ruminants, poultry, rabbits and aquaculture fish (EMA, 2015; Paterson & Harris, 2016). Currently, colistin is the fifth most commonly used antibiotic in the EU (EMA/ESVAC, 2015, EMA/ESVAC, 2016). Recently, the identification of *mcr* and variant *mcr-1* genes in strains of *Enterobacteriaceae* isolated from human, animal, food and environmental samples poses a serious threat to public health due to the fast dissemination through plasmids and other MGE (Hasman *et al.,* 2006; Campos *et al.,* 2016; Figueiredo *et al.,* 2016; Jones-Dias *et al.,* 2016b; Liu *et al.,* 2016; Malhotra-Kumar *et al.,* 2016; Perrin-Guyomard *et al.,* 2016; Quesada *et al.,* 2016; Xavier *et al.,* 2016; Zhang *et al.,* 2016; Zurfuh *et al.,* 2016; Yin *et al.,* 2017). Although it has not been demonstrated that the use of colistin in the veterinary practice has resulted in transfer of resistance to humans, this hypothesis should not be excluded, whereby the European Medicines Agency (EMA) advert for the restrict use of colistin only for the treatment of infected animals or cohabitants, discouraging its use as a preventive treatment (EMA, 2015).

6.1.2. Companion animals

Since antibiotics are essential for the treatment of infectious diseases of both humans and companion animals, very often an overlap between the antibiotic classes used in these two medical practices occurs (OIE, 2014). In companion animals, the use of critical important antibiotics in human clinical practice, should be strictly assessed due the emergence of multidrug resistant strains and the fact it may constitute the last therapeutic resource for humans (Table 1.1) (OIE, 2014; WHO, 2014).

The occurrence of ESBL and PMAβ-type β-lactamase-producing bacteria in canine, feline and equine species is a growing concern (Ewers *et al.,* 2012; Hordijk *et al.,* 2013). The detection of ESBL-producing *E. coli* strains belonging to the ST131 lineage in different species, like companion (Pomba *et al.,* 2009; Bogaerts *et al.,* 2015) and food-producing animals (Solà-Ginés *et al.,* 2015) suggest the occurrence of transmission from humans to animals, due to the higher frequency of these strains in humans (Nicolas-Chanoine *et al.,* 2014; Rubin & Pitout, 2014).

Being carbepenems antibiotics of critical importance for the treatment of human infections, its use in companion animals is restricted to cases of severe infections caused by multidrug resistant strains of *Enterobacteriaceae* (Poirel *et al.,* 2014). Recently, NDM-1 and OXA-48 carbapenemases were detected in strains of *E. coli* and *K. pneumoniae* isolated from companion animals, (Stolle *et al.,* 2013; Liu *et al.,* 2016) and OXA-23 in *Acinetobacter* spp isolated from horses in Belgium (Smet *et al.,* 2012).

6.1.3. Wild animals

In their natural habitat, wild animals are rarely treated with antibiotics, so, a low level of resistance may be expected. However, the increasing proximity of wildlife to urban habitats, as well as to other animals, contributed for the transfer of antibiotic resistant bacteria. Contaminated surface water (wastewater, rainwater, urban and industrial effluents from agricultural and animal farms) is one of the main vehicles for the dissemination of resistance determinants and mobile genetic elements among bacteria of different origins (Baquero et al. 2008).

Thus, wild life constitutes a reservoir of resistant bacteria, namely ESBL, PMAβ and carbapenemase-producing bacteria (Fisher *et al.,* 2013b, Radhouani *et al.,* 2013, Veldman *et al.,* 2013, Guerra *et al.,* 2014). In Portugal, ESBL and/or PMAβ producing bacteria were detected in the ecosystem of the Iberian lynx and red fox, and their presence is attributed to feeding habits based on wild rabbits, rodents and birds (Gonçalves *et al.,* 2011; Radhouani *et al.,* 2013; Radhouani *et al.,* 2014).

6.1.4. Aquaculture

Aquaculture is a new sector of food production, which has been experiencing a significant increment over the last two decades, due to the world growing demand for a healthy protein source (Done & Halden, 2015). Although disease control in aquaculture is essentially based on the application of vaccines, antibiotherapy with fluoroquinolones, florfenicol, tetracycline, sulfonamides, amoxicillin and colistin is also frequently used.

Administration of antibiotics via medicated feed or in water, may lead to contamination of the surrounding environment with residues of antibiotics, favoring the selection pressure and the dissemination of antibiotic resistance genes through mobile genetic elements (Vieira *et al.,* 2010; Marshall & Levy, 2011; Done & Halden, 2015).

Although scarce, studies carried out in Portugal in strains of *E. coli* isolated from aquaculture gilt (*Sparus aurata*), revealed the detection of resistance genes to several groups of antibiotics (chloramphenicol, tetracycline, aminoglycosides, β-lactams and sulfonamides), of which ESBL-encoding genes ($bla_{\text{TEM-52}}$ and $bla_{\text{SHV-12}}$) were highlighted (Sousa *et al.,* 2011). Indeed, the presence of carbapenemase-producing *E. coli* strains in oysters, shrimps and fish pond water was detected in Brazil (Vieira *et al.,* 2010). Carbapenemases of the OXA family (OXA-48) were identified in strains of *Stenotrophomonas* spp, *Myroides* spp. and *Pseudomonas* spp. isolated from frozen seafood, and the variants OXA-181 and OXA-515 detected in strains of *Shewanella* spp. isolated from ornamental fish imported from Asian countries. The detection of carbapenemases in bacterial species not usually searched in surveillance programs suggest that these bacteria may also act as reservoirs of transmissible resistance genes (Morrison & Rubin, 2015; Ceccarelli *et al.,* 2017).

6.2. The role of the environment

Environmental bacteria act as a source of antibiotic resistance genes that may be spread among human and animal pathogenic bacteria. This phenomenon is exacerbated by the influx of resistance genes from animals (including food-producing), humans (including the hospital environment and health care units), and from the great amounts of antibiotic waste disposed to the environment (pharmaceutical industry, elimination of metabolites by humans and animals) (Robinson *et al.,* 2016) (Figure 1.5).

In fact, exposure to intensive agricultural practices is also a risk factor for antibiotic resistance due to direct selective pressure resulting from the application of antibiotics in crops or indirect exposure due to use of manure and other biofertilizers (Cantas *et al.,* 2013, Berendonk *et al.,* 2015). Different agricultural practices influence the composition of the soil, with regard to its microbiome, presence of antibiotic resistance genes (resistome), and presence of mobile genetic elements (mobilome) (Jones-Dias *et al.,* 2016b).

7. THE IMPORTANCE OF RESEARCH AND MONITORING ANTIBIOTIC RESISTANCE

Since antibiotic resistance is a serious problem for human and animal health, some initiatives were taken to control it, in particular those relating to the strengthening of antibiotic resistance monitoring programmes. Some networks have been developed in different countries, for example Denmark (DANMAP), Netherlands (MARAN), Sweden (SVARM), Norway (NARMS), among others.

Recently, the European Commission, through Decision 652/2013 established a surveillance programme to monitor antibiotic susceptibility of commensal and zoonotic agents from livestock and meat for a period of seven years (2014-2020), using standardized and validated techniques, and harmonized interpretation criteria. The implementation of this program allows the collection of important information on the emergence and spread of antibiotic resistance, warning in advance changes and future trends in antibiotic resistance patterns within the different animal populations (CD652/2013).

In Portugal, a recent study by Marinho *et al*. (2016), included a large number of strains collected from several animal species and also from humans, highlighting the importance of monitoring antibiotic resistance due to the declining antibiotic efficacy and the lack of new antibiotics in the near future (Marinho *et al.,* 2016).

Recently, the European Food Safety Authority (EFSA), the European Center for Disease Prevention and Control (ECDC) and the European Medical Agency (EMA) have joined forces and released the first integrated report data analysis on antibiotics, and the occurrence of antibiotic resistance in bacteria isolated from humans, food-producing animals and food products (EFSA/ECDC/EMA, 2015).

7.1. The current threat

The emergence and spread of multidrug resistant pathogenic bacteria in humans has been increasing at an alarming rate. Annually, in the European Union, about 400,000 patients suffer from infections caused by multidrug resistant bacteria, of which 25,000 die (WHO, 2014). It is estimated that progressively more people worldwide will die due to multidrug resistant bacterial infections, reaching 10,000,000 deaths in the year 2050, associated with an approximate cost of $100 trillion dollars (O'Neill, 2016).

Veterinary data concerning medical and therapeutic costs involved with the use of antibiotics, as well as direct consequences affecting animal welfare (decline in growth, production and food efficiency, increased morbidity and mortality) are limited, but nonetheless constitute a cause of concern, requiring immediate action (Vaarten, 2012; Weese *et al.,* 2015).

7.2. Technological advances in diagnosis

Conventional microbiological techniques encompassing culture and phenotypic characterization of antibiotic susceptibility, although important and informative, are time consuming and in most cases, treatment is applied before the laboratory result is obtained. Likewise, the clinician obtains information about the therapeutic option taken and the presence of multidrug resistant strains, but mechanisms of resistance and mobile genetic elements are not identified (Anjum, 2015).

The genotypic characterization of the strains can be carried out using molecular biology techniques, namely Polymerase Chain Reaction (PCR), including conventional PCR, real-time multiplex PCR, and microarrays (Anjum, 2015). Microbiology has undergone a significant transformation over the last decades. Specifically, new technologies have been developed, such as New Generation Sequencing (NGS) and Mass Spectrometry (MS), which consistitute valuable tools for the rapid and complete analysis of genomes and proteomes, respectively, both in research and in diagnosis (Franzosa *et al.,* 2015).

7.2.1. Microarrays

The microarray also known as a DNA chip or biochip, is one of the most powerful genomic technologies, used to characterize the genotype of multiple genome regions, or simultaneously measure expression levels of a large number of genes (Bumgarner, 2013; Silva *et al.,* 2014).

As far as antibiotic resistance is concerned, studies carried out by some authors (Jacobsen *et al.,* 2011; Mendonça *et al.,* 2016) emphasize the importance of using this technology in the detection of several resistance and virulence determinants in strains of human and animal origin.

7.2.2. “Omic" technologies

The new "omic" technologies include the detection of genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) in a biological sample, to fully characterize its phenotype (Franzosa *et al.,* 2015). Genomics and transcriptomics progressed due to advances in the microarray technology, and mass spectophotometry was the most widely used method for the detection of analytes, by proteomics and metabolomics. The amount of data generated is enormous and its analysis is complex, requiring specialized statistical and bioinformatic treatment.

7.2.2.1. Genomics

The New Genome Sequencing (NGS) allows to determine the complete DNA sequence of the bacterial genome, both chromosomal and plasmid DNA.

Regarding antibiotic resistance, and depending on the type of information intended, this technique allows the sequencing of nucleic acids, DNA and RNA (in the form of cDNA); when applied to DNA, reveals the presence of antibiotic resistance genes, and when applied to RNA, allows the detection of resistance genes expression (Chan, 2016). All the information obtained is analyzed with the support of bioinformatics tools, and the identification of genes may be confirmed by detection of homology with known reference databases, such as ResFinder and the Comprehensive Antibiotic Resistance Database (Anjum, 2015, Chan, 2016).

The equipments used, such as HiSeq and MiSeq series by Illumina Inc. (CA, USA), may allow the simultaneously sequencing of many bacterial genomes, as well as the detection of new resistance determinants carried by multiple plasmids. Metagenomic analysis of clinical or environmental samples (blood, faeces, soils, waste water) through the sequencing of the 16SrRNA gene of bacteria allows the study of all the members of a specific bacterial community contained in the sample and evaluate the influence of the environmental conditions through the detection of specific genes, such as antibiotic resistance encoding genes (Franzosa *et al.,* 2015, Chan, 2016).

7.2.2.2. Proteomics and transcriptomics

Proteomics is defined as the global analysis of all cellular proteins that make up the proteome; it is more complex than the genome, since the number of encoding genes can be predicted in the genome, whereas the number of different proteins that an organism can synthesize is not deduced directly of the proteome analysis (Fouhy *et al.,* 2015).

Transcriptomics identifies the transcripts present in the cell, which is the mRNA transcribed from the DNA, while proteomics identifies the proteins resulting from the translation of the transcripts into protein.

Mass spectophotometry, based on the Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight (MALDI-TOF) technology, can be used in the study of antibiotic resistance in both human and animal strains, through the analysis of the total protein content, which is measured after the contact of isogenic susceptible and resistant strains with previously selected antibiotics.

The response is specific for each antibiotic, and the proteins secreted at high levels under selective pressure correspond to the expressed genes (Fouhy *et al.,* 2015; Pérez-Llarena & Bou, 2016).

7.2.2.3. The current and future perspectives: veterinomics

Veterinomics represents the future vision of the veterinary science, with the use of "omic" technologies in the fields of research and clinical practice, with the aim of improving animal health, and thereafter protection of public health (Katsafadou, 2016).

As the costs involved in the NGS techniques continue to decline, this technology is progressively being used alone, or in combination with conventional phenotypic methods, for the assessment of susceptibility to various antibiotics, whether in the field of surveillance or clinical diagnosis (Zankari *et al.,* 2013).

Studies made by some authors (Zankari *et al.,* 2013) on bacteria of animal origin (*Salmonella* spp., *E. coli* and *Enterococcus* spp.), revealed that results obtained by the phenotypic methods match with those predicted through NGS. However, its use as a single technique should be carefully evaluated in terms of standardization, to allow comparison of results between different laboratories (Zankari *et al.,* 2013, Anjum, 2015).

Proteomics may have a broad application in the study of various diseases and pathological processes, detecting new target proteins. These proteins will allow the detection of the disease in a sub-clinical phase and may contribute for the development of new diagnostic methods, therapeutic approaches, and the development of vaccines (Katsafadou, 2016).

Although promising, MALDI-TOF (MS) technique still requires validation, simplification and automation, namely in the analysis of results. One of the most important challenges concerns an in-depth study on the correlation between the results obtained in proteomics and the minimum inhibitory concentration (MIC) values, in order to get an adequate therapeutics and monitoring of infection (Pérez-Llarena & Blou, 2016).

The food industry is another branch where proteomics is being developed and applied in terms of composition, quality and certification of food, safety assessment of genetically modified feedstuffs and identification of allergens and toxins (Piras *et al.,* 2016).

8. **ONE HEALTH**

Antibiotic resistance is a global and transversal serious problem, involving human and veterinary medicine, agriculture and the environment. International organizations responsible for each sector, such as the World Health Organization (WHO), the World Organization for Animal Health (OIE), the Food and Agriculture Organization of the United Nations (FAO) and EFSA, have a key role to play.

Their main concern and goal is to recognize the importance of antibiotic resistance and the impact of the use of antibiotics in animals, and to promote the necessary collaboration between local, national and global authorities, namely from the most important sectors, veterinary, environmental and human sectors. Strict measures must be adopted, to maximize animal health, by minimizing the likelihood of antibiotic resistance, and safeguard the future effectiveness of antibiotics, in the interest of human and animal health and welfare (Vaarten, 2012; Weese *et al.,* 2015, Robinson *et al.,* 2016).

Veterinary authorities should also promote strict compliance of the veterinary prescription, giving priority to biosafety and vaccination. Research in infectious diseases and antibiotic resistance should be encouraged by developing and enforcing standards and guidelines, and promote the responsible use of antibiotics in animals.

Chapter 2

OBJECTIVES

Escherichia coli is one of the most prevalent bacteria in the gastrointestinal tract of mammals, constituting one of the most frequent causes of infection in humans and animals (Alocatti *et al.,* 2013). The flexibility and genetic ability of this pathogen to adapt to different hosts stands out as an important feature in the spread of resistance, and that is why it is one of the target bacteria in many research studies and antibiotic resistance surveillance programs (EFSA, 2017). *Salmonella enterica* is a zoonotic agent and the second cause of human food-borne infection in the European Union, mainly associated with consumption of foods, such as poultry meat, eggs, milk, shellfish and other fresh products (Pui *et al.,* 2011; EFSA, 2017). Just like *E. coli*, it is also considered as a target bacterium in research studies and antibiotic resistance surveillance programs (EFSA, 2017).

According to recent data, and among the 29 European countries, Portugal has one of the highest frequencies of antibiotic resistant *E. coli*, *Campylobacter* spp and *S*. *enterica* strains isolated from food-producing animals and food products. These strains are frequently resistant to antibiotics which are considered of critical importance for the treatment of human infections, namely fluoroquinolones, polymyxins and 3rd generation cephalosporins (EFSA/ECDC, 2017).

However, information on the main antibiotic resistance determinants and mobile genetic elements involved in the spread of the above-mentioned resistance mechanisms is still scarce.

Thus, the main objectives of this thesis were:

i) Phenotypic characterization of *E. coli* and *S*. *enterica* strains isolated from different animal species and products, with regards to susceptibility to antibiotics of several classes;

ii) Genotypic characterization of strains resistant to critically important antibiotics in human and veterinary medicine, namely, 3rd and 4th generation cephalosporins, fluoroquinolones and polymyxins;

iii) Investigation and identification of mobile genetic elements associated with antibiotic resistance determinants and involved in their dissemination;

iv) Genomic characterization of clinically relevant multidrug resistant isolates, with regards to determinants associated to antibiotic and biocide resistance, virulence, mobilization, and DNA transfer.

The studies developed in this thesis will contribute to increase the knowledge about molecular mechanisms of acquired resistance to critically important antibiotics in human and veterinary medicine, particularly in *E. coli* and *S*. *enterica* strains. It will be of added value to discuss the results obtained in a European and worldwide context, considering the ease of dissemination of the mechanisms involved.

CHAPTER 3

DYNAMICS OF ANTIBIOTIC RESISTANCE

Current perspectives on the dynamic of antibiotic resistance in different reservoirs

This research paper was submitted as:

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Contributions of the authors for the manuscript:

Manuela Caniça: conception and design of study, drafting of the article and final approval of manuscript;
Vera Manageiro: final revision and contribution in writting the manuscript and final approval of manuscript;
Daniela Jones-Dias: final revision and contribution in writting the manuscript and final approval of manuscript;
Lurdes Clemente: final revision and contribution in writting the manuscript and final approval of manuscript;
Eduarda Gomes-Neves: final revision, and final approval of manuscript;
Patrícia Poeta: final revision, and final approval of manuscript;
Elsa Dias: final revision, and final approval of manuscript;
Eugénia Ferreira: final revision, and final approval of manuscript;
.

ABSTRACT

Antibiotic resistance consists of a dynamic web. In this review we describe the path by which different antibiotic residues and antibiotic resistance genes disseminate among relevant reservoirs (human, animal, and environmental settings), evaluating how these events contribute to the current scenario of antibiotic resistance. The relationship between the spread of resistance and the contribution of different genetic elements and events is revisited, exploring examples of the processes by which successful mobile resistance genes spread across different niches. The importance of classic and next generation molecular approaches, as well as action plans and policies are also reviewed, which might aid in the fight against antibiotic resistance.

Keywords:
Antibiotics, Resistome, Mobilome, Reservoirs, Dissemination

3.1. Introduction

Many classes of antibiotics are not only clinically valuable for human medicine, but also in other fields such as, veterinary medicine and food animal production, including aquaculture (Marshall & Levy, 2011; Garcia-Alvarez *et al.,* 2012). The agricultural setting also plays an important role in the spread of antibiotic residues in the environment, due to their use as additives and biocides in crops. Consequently, all the adjacent natural environments consisting of water, soil and plants are also environmental niches to be considered for the dynamic of antimicrobial resistance (Baquero *et al.,* 2008; Heuer *et al.,* 2011).

The use of antibiotics might have dangerous and long-term effects that extend beyond the selection of specific resistance mechanisms (Gillings, 2013). The selection pressure applied to bacterial communities, through the widespread discharge of antibiotic residues in the environment, highly contributes to the exposure of several niches to antibiotic resistant bacteria (commensal and/or pathogenic) (Perron *et al.,* 2008). For example, applying animal manure may enhance viable antibiotic resistant coliform bacteria in soil, increasing the frequency of detection of some antibiotic resistance genes (Heuer *et al.,* 2011). These bacteria may reach the food chain considering that vegetables are grown in soil (Marti *et al.,* 2013). Acquired antibiotic resistance is also frequent among isolates from wild animals, which represents a niche of concern. Indeed, many reports refer wild animals as reservoirs of resistant determinants that commonly appear in other habitats, namely in human settings (Sousa *et al.,* 2014).

It should also be noted that the bacterial resistance to antibiotics is related to soil and aquatic native microorganisms, which may be producers of antimicrobial compounds (DeLorenzo *et*

al., 2014 ; Zhang *et al.,* 2014). Several other factors contribute to antibiotic resistance. Indeed, the existence of major anthropogenic actions such as international travel, and global trade of foodstuffs highly contribute to its amplification (Barbosa & Levy, 2000; Martinez *et al.,* 2002; Allen *et al.,* 2010; Gaze *et al.,* 2011) (Table 3.1).

Table 3.1. Antibiotic resistance is generated by several factors
Adapted from (Barbosa & Levy, 2000; Martinez *et al.,* 2002; Allen *et al.,* 2010).

Factors depending on biological and physical influences:
Human activities Animals (namely insects, birds, wildlife) Water Environmental changes Wind Changes in geographic localization of bacteria
Factors dependent on humans and their management of antibiotics:
Preservation of ecosystems (eventually bioremediation) Intensive farming Sanitation and hygiene measures Runoff and leak Manure Demographic changes (increasing number of elderly people) Anthropogenic contacts Socioeconomic factors Bioterrorism (biological war) Travel of people and foodstuffs Patient movement within and between medical institutions Infection control measures (prevention of infection) Appropriateness of use
Factors related to the antibiotic itself
Antibiotic use Novel antibiotics Dose of treatment Duration of treatment Antibiotic residues Food additives Selection of antibiotic resistant bacteria
Factors related to microorganisms
Wide spread of commensal bacteria Extensive spread of old or new pathogens Higher number of infections (opportunistic) Increased number of host-pathogen contacts Modification of microbial diversity
Factors related to the genetic basis of resistance
Cross selection Non-antibiotic selection Gene transfer Clonal spread

A classic example of a vehicle for transmission of antibiotic resistant bacteria are the human hands, which can became easily contaminated by environmental surfaces near patients in hospitals, or animals in husbandry settings (Gomes-Neves *et al.,* 2012; Park *et al.,* 2014). Consequently, World Health Organization is strongly committed to make people aware of the problem of antibiotic resistance, especially care takers, namely through campaigns of hand hygiene aiming to fight antibiotic resistance (Park *et al.,* 2014 ; WHO, 2014).

There is an urgent need to learn about possible connections between antibiotics, environmental organisms, and associated bacterial communities, as they may threaten diverse ecosystems, and consequently, human health (Martinez, 2009). In this review we emphasize that these settings are linked and may constitute reservoirs of antibiotic resistance determinants, playing important roles in this dynamic.

3.2. Antibiotic residues *versus* resistome in the environment

Before the antibiotic era, environmental antibiotic resistant bacteria already existed, carrying genes that became critically important in medicine (D'Costa *et al.,* 2011). Indeed, for many years the environment consisted of an under-recognized reservoir of resistance genes that have the potential to be transferred and emerge in clinically important bacteria (D'Costa *et al.,* 2006; Allen *et al.,* 2009). Groh et al. (2006) showed that homologues of multidrug resistance genes present in bacterial pathogens are essential for the sediment fitness in nonpathogenic bacteria, by conferring an ecological advantage on these microorganisms (Groh *et al.,* 2007).

Several reports have demonstrated the existence of antibacterial activity in extracts from different microorganism genera/species against distinct bacteria. A recent study showed that some antibiotic resistance Gram-negative strains recovered from an industrial alpine location highly polluted with oil hydrocarbons, had the ability to produce antimicrobial compounds active against Actinobacteria and Gammaproteobacteria. Thus, the selection pressure present in this environment could lead not only to high antibiotic resistance, but also to the capacity of this population to produce antimicrobial compounds (Hemala *et al.,* 2014). LeBel *et al*. (2013) have also demonstrated that the heat-stable bacteriocin nisin (naturally produced by *Lactococcus lactis*), displayed antimicrobial activity against the emerging zoonotic agent *Streptococcus suis* (Lebel *et al.,* 2013).

In fact, several studies have demonstrated that freshwater/marine bacteria are also able to produce antibacterial compounds that exhibit antimicrobial activity similar to standard drugs, which is the case of cyanobacteria (Singh *et al.,* 2011). Besides, it was also demonstrated that extracts from *Anabaena* spp. were effective against vancomycin-resistant *Staphylococcus aureus* (Bhateja *et al.,* 2006). Some authors have considered that cyanobacteria antibacterial activity is more effective against Gram-positive bacteria (Kreitlow *et al.,* 1999; Martins *et al.,* 2008) than Gram-negative, which was attributed to the protection conferred by the

lipopolysaccharides barrier of Gram-negative cell wall (Martins *et al.,* 2008); however, some Gram-negative bacteria, including pathogenic species, were also affected by cyanobacterial compounds. The potential application of bacterial compounds to the development of new antimicrobials seems, therefore, a promising research area.

The relation between bacteria and antibiotics may be approached in a variety of ways. In the case of cyanobacteria, considering their ubiquity and importance in the ecosystems (Vasconcelos *et al.,* 2001), increasing concern has been attributed to the effects of environmental stressors in these bacteria. In fact, although cyanobacteria can easily adapt to different environmental conditions, they can also be severely affected by environmental changes and water contaminants (López-Rodas *et al.,* 2006; González-Pleiter *et al.,* 2013). On the other hand, and considering that cyanobacteria are exposed to antibiotics and to resistant bacteria in their natural habitats (Martinez, 2009), we can hypothesize that they are able to develop antibiotic resistance mechanisms. Their ability to produce a variety of bioactive antibacterial compounds with potential pharmaceutical interest (Singh *et al.,* 2011), suggests that they might have developed defense mechanisms against their own toxicity (Waksman, 1941).

Overall, the relation of antibacterial compounds-producing bacteria might challenge the scientific community because these new molecules may constitute a promising future source of antimicrobials. Thus, further research will be needed to understand the role of those genera/species on the resistome.

3.3. Mobilome associated to antibiotic resistance genes

Genomic events constitute a central process in the mobilization of genetic elements and associated mobile antibiotic resistance-encoding genes between different settings (Burrus & Waldor, 2004). The movement of bacteria from the environment to animals and humans (and vice-versa) contributes to the increase of the mobilome (mobile gene pool) (Brown *et al.,* 2012). These genetic exchanges are significantly reported among the human and animal gut (Devirgiliis *et al.,* 2011). Indeed, lateral gene transfer and recombination of genetic material within bacterial populations highly contributes to the diversity and adaptability of strains to different environments (Fig. 3.1).

The wide divergence within specific functional genes, the creation of mosaic-structured genomic regions, as well as the high prevalence of mobile genetic elements contribute to the success of different gene pools, producing new worldwide dispersed hotspots (Burrus & Waldor, 2004; Wright, 2007; Gaze *et al.,* 2011; Gillings, 2013). For instance, the acquisition of a single plasmidic but pleiotropic gene that encodes resistance to two structurally and functionally different classes of antibiotics also highlights the remarkable adaptive nature of Gram negative bacteria (Robicsek *et al.,* 2006).

In general, mobile genetic elements, such as plasmids, insertion sequences (IS), transposons, genomic islands, and phages, constitute the arsenal of bacterial genomes in what concerns genetic transfer, contributing to the emergence of novel genotypic and phenotypic variants (Leavis *et al.,* 2007; Brown *et al.,* 2012; Gomes-Neves *et al.,* 2015). Frequently, these genetic structures may be organized in cascade-like arrangements, contributing to the amplification of mobilization events.

Several reports revealed that mutations in the promoter region and acquisition of functional promoters can turn on a silent gene (Salyers & Shoemaker, 2006). For example, IS may disrupt open reading frames and activate gene expression through transposition. Functional promoter may be created through the -35 promoter-like sequence existing in the terminal of some IS elements (Salyers & Shoemaker, 2006).

The mobilome and the resistome usually follow parallel paths. The environment is broadening the origin of antibiotic resistance genes. This was the case of *Kluyvera* spp., commensal bacteria of both humans and animals, which went ahead to the mobilization of their chromosomal CTX-M-type β-lactamase-encoding genes into the plasmids of other bacteria. Thus, CTX-M extended-spectrum β-lactamase (ESBL) has its origin in *Kluyvera* spp., which possibly shares other genes with enteric bacteria (Bonnet, 2004). The understanding that genes from non-related species might be expressed in new hosts is now evident (Perron *et al.,* 2008; Sousa *et al.,* 2014; Zhang *et al.,* 2014). Other example is OXA-type-β-lactamases. Interestingly, the genes encoding these enzymes have already been described in plasmids prior to the human use of antibiotics, since they have moved through horizontal gene transfer between bacterial phyla for millions of years (Barlow & Hall, 2002). Recently, an OXA carbapenemase (from Ambler class D) that supposedly had its origin in Turkey (OXA-48) has been described in Europe and in the United States of America (USA) among *Klebsiella pneumoniae* isolates, causing considerable morbidity and mortality (Poirel *et al*., 2012b; Manageiro *et al*., 2014). The first report of *Shewanella xiamenensis* carrying a $bla_{\text{OXA-48-like}}$ gene, suggested that the emergence of different genes from this group had their origin in different *S. xiamenensis* strains (Tacão *et al*., 2013). The contribution of different mobile genetic elements and mechanisms for the dissemination of these carbapenemase-encoding genes ($bla_{\text{OXA-48-like}}$) from *Shewanella* spp. to *Enterobacteriaceae,* and to other Gram-negative bacteria has been reported (Poirel *et al*., 2012c). These antibiotic resistance genes constitute one of the greatest threats in terms of public health because of their ability to resist carbapenems (Nordmann *et al.,* 2012; Poirel *et al.,* 2012c).

The idea that some resistance mechanisms, such as carbapenemase production, are only linked to infections and human healthcare facilities is no longer valid (Patel & Bonomo, 2013). The efficacy of $bla_{\text{OXA-48-like}}$ gene transfer between bacterial species in human cases (Manageiro *et al.,* 2014), have also been reported in food-producing, companion and wild

animals, as well as in natural environments (Stolle *et al.,* 2013; Tacão *et al.,* 2013; Woodford *et al.,* 2014), highlighting its importance in the dissemination of antibiotic resistance among different reservoirs.

International travel and medical tourism have rapidly driven the resistance mechanisms into an alarming public health warning (Cantón *et al.,* 2012; Leonard *et al.,* 2013). Apart from the enzymes belonging to Ambler class D, this also has been happening with other carbapenemases, such as KPC (β-lactamases from Ambler class A) and NDM (metalo-β-lactamase from Ambler class B) (Cantón *et al.,* 2012).

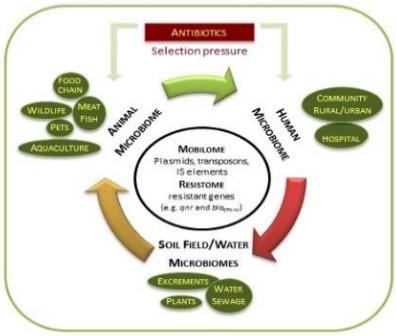
3.4. Investigating antibiotic resistance

Routine investigation of the most relevant antibiotic resistance mechanisms (such as ESBLs, carbapenemases, methicillin-resistant *S. aureus*/MRSA, and vancomycin-resistant enterococci/VRE) is becoming common in the human sector; however, it is still scarce in the area of food, animals, and environment. The detection of ESBL- and carbapenemase-producing bacteria in the environment, farms, food, and companion animals is an urgent matter, in order to contain these resistance mechanisms. This is a matter of concern as carbapenems are currently one of the last resources to treat human complicated infections caused by multidrug resistant Gram-negative bacteria (Patel & Bonomo, 2013).

The colonization of healthy animals can represent a silent source of these resistance mechanisms, being a possible transmission pathway to humans via food chain. On the other hand, the carriage of such resistance mechanisms by healthy humans can also represent a pitfall for their transmission to non-human sources. Moreover, infection and colonization is higher among animal owners, farmers and veterinarians. They can be carriers and transmit bacterial antibiotic resistance genes to animals, namely due to prior selection pressure caused by hospitalization, antibiotic consumption, contact with healthcare settings and/or international voyages (Figure 3.1) (Woodford *et al.,* 2014).

Data collection and analysis on antibiotic resistance will improve detection of outbreaks (in a micro level), and support decision-making (in a macro level), highlighting the importance of the One-Health approach to combat its global rise in human infectious diseases (Mills, 2014; WHO, 2014).

Figure 3.1. Crosswalk between resistome and mobiloma among different environment



Our capability to contain the increase and dissemination of resistance mechanisms such as carbapenemase-producing bacteria will eventually indicate the efficiency of the antibiotic use and antibiotic resistance containment policies (Woodford *et al.,* 2014; WHO, 2014). In 2006, the European Union (EU) interdicted the antibiotics as growth promoters in animal feed, which was an important step in the use of antibiotics for non-medical purposes. However, the antibiotic resistance of foodborne pathogens is still a matter of concern. The notification rates of zoonoses in confirmed human cases in EU for the year of 2013 showed that *Campylobacter*, *Salmonella*, and *Escherichia coli* were the most frequent bacteria causing human foodborne zoonoses. Despite all efforts, these pathogens still present high levels of resistance to antibiotics used in humans (EFSA/ECDC, 2015). About 30% of human *Salmonella* spp. isolates exhibited multidrug resistance (EFSA/ECDC, 2015).

Emerging antibiotic resistant foodborne pathogens from animal origin are appealing to food and veterinary microbiology laboratories to be increasingly alert due to the impact that these bacteria can have on public health. Next-generation sequencing technologies have greatly accelerated the rate and reduced the cost of genomic data acquisition.

The whole-genome sequencing (WGS) and molecular epidemiology studies constitute important assets to explore the bacterial genomes (Leavis *et al.,* 2007; Allen *et al.,* 2009; Ho *et al.,* 2011). The possibility of performing comparative genomic analysis in short periods of time can allow the rapid detection of resistance genes that, in turn, can be helpful to distinguish different bacterial subpopulations (Allen *et al.,* 2009; Ho *et al.,* 2011; Harrison *et al.,* 2013).

These methodologies are very helpful to monitor the mobilome, as mobile antibiotic resistance genes are frequently clustered in complex genetic arrays. For instance, hitchhiking genes, such as antibiotic resistance and heavy metal or disinfectant resistance genes are commonly genetically associated. Moreover, there are genomic regions that allow the gathering of antibiotic resistance genes, such as the integrons, and those responsible for their transfer, like plasmids or the conjugative transposons (Salyers & Shoemaker, 2006). The plasmidome sequencing is also an interesting approach to evaluate antibiotic resistance genes that may spread among different settings (Brown *et al.,* 2012). In the same way, a complete proteome might be obtained through the matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS). Correia *et al.,* (2014) reported the confirmation of several proteins in *S.* Typhimurium Phage Type 104 clinical strain through this method, emphasizing the presence of the Aac(6')-Ib-cr enzyme responsible for both plasmid-mediated aminoglycoside and quinolone resistance (Robicsek *et al.,* 2006; Correia *et al.,* 2014).

Likewise, the high-throughput sequencing of nine genomes was used for the rapid identification of data in an outbreak caused by enteroaggregative verocytotoxin-producing *E. coli* O104:H4 (STEC) associated with the consumption of raw vegetables (EFSA, 2011; Ho *et al.,* 2011). This outbreak was responsible for a hemolytic uremic syndrome in patients due to TEM- and CTX-M-15-producing STEC isolates. The exposure path involved in this outbreak

could have been more than one (EFSA, 2011; Ho *et al.,* 2011): firstly, a primary human infection could have arisen from consumption of contaminated food or direct contact with an animal carrying STEC strains and then, a subsequent infection might have occurred by the fecal-oral way, through manipulation of contaminated vegetables (EFSA, 2011; Ho *et al.,* 2011).

Using the WGS approach, two independent farm human cases of *mecC*-MRSA infection, directly linked to a livestock (cow and sheep) reservoir, were identified in Denmark, supporting zoonotic spread. In these cases, it was demonstrated that the CC130 MRSA lineage was transmitted between animals and humans, and that livestock may be a reservoir for MRSA (Harrison *et al.,* 2013).

Thus, new generation approaches allow a better understanding of specific resistance mechanisms, enabling an effective control of complex epidemiological situations. Overall, the use of many types of "omic" approaches is already providing more advanced hypotheses, mechanisms and models of antibiotic resistance evolution (Franzosa *et al.,* 2015).

3.5. Strengthening the combat against antibiotic resistance

Actions should be taken to diminish the selection pressure imposed by antibiotics in the human communities. Other priority actions should also include risk management to minimize antibiotics and antibiotic resistant bacteria in animals (companion and food-producing) and in the environment, namely in fresh and wastewater.

Several measures are necessary to fight antibiotic resistant bacteria, but the main concern involves putting them into practice in all countries, regions and settings (European Council, 2001; Review on Antimicrobial Resistance, 2014; WHO, 2014; Mills, 2014; EFSA/ECDC, 2015).

The Council Recommendation/2002/77/EC on the prudent use of antimicrobials agents in human medicine highlights the need to the "*relationship between the occurrence of antimicrobial resistance in certain human pathogens and their occurrence in animals and the environment*" (European Council, 2001). In addition, we are made aware to the fact that "*coordination between human, veterinary and environment sectors should be ensured and the magnitude of the relationship between the occurrence of antimicrobial resistant pathogens in humans, animals and the environment should be further clarified and therefore this Recommendation does not preclude further initiatives in other areas*." (European Council, 2001). Recommendations from other entities also reinforce the need to fight against the rising threats of antibiotic resistance through the publication of detailed action plans and some ideas underlining important pressure points (Bronzwaer *et al.*, 2004; WHO, 2014). We also highlight that the Transatlantic Taskforce on Antimicrobial Resistance of 2014 released recommendations for future collaboration between the USA and EU. In this document the strategies to improve the pipeline of new antibacterial drugs was emphasized (Livermore,

2011). The lack of new antibiotics is the major drawback in the field of antibiotic resistance (Livermore, 2011).

The implementation of DC 613/2013 by the EU countries concerning antimicrobial resistance monitoring in zoonotic and commensal agents in 2014 in food-producing animals and meat, for a period of seven years (2014-2020), using standardized and validated antimicrobial susceptibility testing methods and harmonized interpretive criteria, will complement and provide early warning of changes in resistance patterns in animal populations and monitor future trends in the occurrence of antimicrobial resistance (Commission Implementing Decision 613/2013).

Many European programs have also been leading the development of policies for diminishing antibiotic use and antibiotic resistance genes in different sources, namely water (Fatta-Kassinos *et al.,* 2015). In the water reservoir should be highlighted the recent COST Action that takes into account the problematic of antibiotics, and their consequences, which main objective is to make possible the wastewater reuse [http://www.nereus-cost.eu/].

Meanwhile, national strategies for combating antibiotic resistant bacteria, identifying priorities and coordinating investments were also recently reviewed in the USA (The Whitehouse, 2014).

The evaluation of antibacterial products used as feed additives, and the impact of antimicrobial resistance on the antibiotics of human and veterinary importance has also been revised (EFSA, 2012d).

3.6. Conclusions

Antibiotic resistance constitutes a health crisis that has consequences all over the world, striking several settings simultaneously: humans, animals, and the natural environments. Until the decade of the 90's the majority of drugs, including antibiotics, were derived from natural products; since then, a substantial increase in the use of synthetic and semi-synthetic substances as therapeutic agents, was noticed (Li *et al.,* 2009). However, the major approach of the pharmaceutical industry to overcome this problem was the improvement of the pre-existing antibiotics in detriment of the research on new molecules (Li *et al.,* 2009). In the future, special attention should be paid to the potential of new natural antimicrobial products as effective and less toxic alternatives (Singh & Barrett, 2006).

Overall, actions must be taken to diminish the selection pressures imposed by antibiotics, in order to reduce the exposure of humans to high rates of antibiotic resistant strains. Priority actions include risk management to access the use of antibiotics and presence of resistant bacteria in the different environments. In the end, a higher economic and human investment in the field is necessary.

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CHAPTER 4

SUSCEPTIBILITY AND MOLECULAR CHARACTERIZATION OF ENTEROBACTERIACEAE STRAINS - ACQUIRED RESISTANCE MECHANISMS TO: β-LACTAMS (*ESBL, PMAβ, ESAC*) ***AND FLUOROQUINOLONES*** (*PMQR*)

4.1. Antimicrobial susceptibility of Salmonella enterica isolates from healthy breeder and broiler flocks in Portugal

This research paper was submitted as:

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Contributions of the authors for the manuscript:

Lurdes Clemente: conception and design of study, acquisition of laboratory data, analysis of data, drafting of article and critical revision of the manuscript, final approval of manuscript;
Ivone Correia: acquisition of laboratory data, final approval of manuscript;
Patrícia Themudo: acquisition of laboratory data, final approval of manuscript;
Isabel Neto: orientation on statistical data, final approval of manuscript;
Manuela Caniça: critical revision of the manuscript, final approval of manuscript;
Fernando Bernardo: critical revision of the manuscript, final approval of manuscript;

ABSTRACT

Three hundred and thirty three isolates representing 40 different serotypes of *Salmonella enterica,* recovered from environmental and faecal samples of breeder and broiler flocks from 2009 to 2011, were studied. Antimicrobial susceptibility was determined by measuring the minimal inhibitory concentration of 11 antimicrobials using the agar dilution method. *Salmonella* Havana*, S.* Enteritidis and *S.* Mbandaka were the most common serotypes isolated from broiler flocks, while *S.* Enteritidis was the common isolate from breeder flocks. The frequency of non-wild-type *Salmonella* isolates (isolates with decreased susceptibility) to the different antimicrobials varied according to serotype. *S.* Mbandaka in broilers and *S.* Enteritidis in both breeders and broilers showed higher frequencies of reduced susceptibility to quinolones, but clinical resistance towards ciprofloxacin was not observed. Reduced susceptibility to sulfamethoxazole, tetracycline, ampicillin and streptomycin were common in *Salmonella* Typhimurium isolates*.* Two isolates of *S.* Havana from broilers were resistant to cefotaxime and phenotypically categorised as extended-spectrum β-lactamase producers (ESBL). Results presented in this study provide useful data on the antimicrobial susceptibility of different *Salmonella* serotypes and highlight the high diversity of multidrug resistance patterns present.

Keywords**:** *Salmonella enterica*; Breeders; Broilers; Antimicrobial susceptibility

4.1.1. Introduction

Salmonella enterica is the second most important cause of food-borne disease in the European Union with a total of 99020 confirmed cases in humans in 2010 (EFSA, 2012b). Raw eggs are still the most frequent source of outbreaks, followed by fresh poultry meat, pork, fruit and vegetables. The continued increase in consumption of poultry products per capita also increases the potential for human exposure to *Salmonella* via the food chain.

Salmonella enterica infection in humans usually results in a self-limiting gastroenteritis; however, young children, elderly and immune-compromised people may experience enteric fever or an invasive form of the disease requiring antimicrobial treatment (Pui *et al.,* 2011). In poultry, the clinical signs vary considerably depending on age of birds and/or infecting serotype. Infections caused by serotypes Enteritidis and Typhimurium are rarely responsible for severe illness and animals frequently become asymptomatic carriers; except in young chicks and poults where acute outbreaks exhibiting clinical disease accompanied by high mortality rates may occur (Padron, 1990; Foley *et al.,* 2008).

Salmonella can be introduced at all stages of the production cycle, though breeding flocks and hatcheries are critical sources and responsible for the quick spread of the infection (Foley *et al.,* 2008). Several factors may affect the susceptibility of poultry to colonization, such as age, serotype, initial dose level, environmental stress, antimicrobial or anti-inflammatory treatments and competition with the enteric microbiota (Foley *et al.,* 2008). Besides causing illnesses or death in both humans and poultry, there is a worldwide concern that the persistence of *Salmonella* serotypes that are resistant or show decreased susceptibility to several antimicrobials may reduce treatment options and, more importantly, lead to treatment failure (Newell *et al.,* 2010). Fluoroquinolones such as ciprofloxacin are critically important antimicrobials in human and veterinary medicine. In animal isolates, the highest occurrence of decreased susceptibility to ciprofloxacin has been recorded in *Salmonella* spp. recovered from live chickens (*Gallus gallus*) and broiler meat (EFSA, 2012b). It was hypothesised that the withdrawal of growth promoters in Europe in 2006 would lead to decreased antimicrobial resistance in pathogenic microorganisms, but trade has resulted in the importation of poultry products from regions where the use of antimicrobials and growth promoters is not as well regulated as it is in the EU, resulting in the introduction of resistant organisms (Barrow *et al.,* 2012).

This paper reports the results of a monitoring programme examining the antimicrobial susceptibility patterns of *Salmonella* serotypes isolated from breeder and broiler flocks in Portugal during 2009-2011. The ultimate aim of this programme is to contribute to a better understanding of the zoonotic potential of the circulating strains of *Salmonella* in a country where consumption of poultry meat is significant.

4.1.2. Material and Methods

Bacterial isolates

The National Veterinary Reference Laboratory (INIAV) received *Salmonella* isolates from the *Salmonella* National Control Programmes in food producing animals and were serotyped and susceptibility tested according to the guidelines of Commission Decision (CD) 2007/407/CE, concerning harmonised monitoring of antimicrobial resistance in *Salmonella* in poultry and pigs. This analysis includes data from a total of 333 *Salmonella* isolates, from both breeders (*n*=58) and broilers (*n*=275). All samples were, collected in the period of 2009-2011. The breeder and broiler farms were sampled and selected by the official authorities and were distributed throughout the country. The parent stock (breeders) were imported from other European countries as day old chicks while broilers were born in Portugal. Birds were raised and managed in industrial units designed for a temperate climate.

Faecal and environmental samples using sterile boots/sock swabs were collected in broiler flocks 3 weeks prior to slaughtering and, from breeder flocks three times during the production cycle.

All samples were examined according to ISO norm 6579: 2002 applied to *Salmonella* detection in food and animal feeding stuffs (Anonymous, 2002). Suspected colonies were further characterized by means of biochemical tests, using triple sugar iron agar slopes and API 20E strips (BioMérieux).

Salmonella serotyping

Salmonella isolates were biochemically confirmed and serotyped (Table 4.1.1), using the Kauffmann-White scheme (Grimond & Weill, 2007).

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) for 11 antimicrobials were determined by the agar dilution method (Clinical Laboratory and Standards Institute, 2008). Antimicrobials were tested in two fold concentration series over a range which was specific to each antibiotic: Ampicillin (0.5-64mg/L); cefotaxime (0.06-8mg/L); chloramphenicol (2-256mg/L); ciprofloxacin (0.008-8mg/L); florfenicol (1-128mg/L); gentamicin (0.25-32mg/L); nalidixic acid (2-512mg/L); streptomycin (2-512mg/L); sulfamethoxazole (8-1024mg/L); tetracycline (0.5-64mg/L); and trimethoprim (0.25-32mg/L). *E. coli* ATCC25922 strain was used as a control for MICs.

In order to assess decreased susceptibility of the isolates, epidemiological cut-off values from the European Committee for Antimicrobial Susceptibility Testing (EUCAST) were used (Table 4.1.1) allowing the detection of any deviation in the susceptibility of the wild type population (EFSA, 2012b). MIC_{50} and MIC_{90} values, as well as rates of decreased susceptibility and resistance to critically important antimicrobials for humans (cefotaxime and ciprofloxacin) were calculated according to clinical breakpoints established by EUCAST for *Enterobacteriaceae* (Table 4.1.1). Isolates were considered to be multi-drug resistant (MDR) if they presented reduced susceptibility to three or more structurally unrelated antimicrobials.

Phenotypic screening of extended-spectrum β-lactamases (ESBL)

Isolates exhibiting a non-wild-type MIC for cefotaxime (> 0.5 mg/L) were tested phenotypically for the presence of ESBL by testing for synergy through disk combination (Mast Laboratories) including cefotaxime (30µg), ceftazidime (30µg) and cefpodoxime (10µg), as single drugs and in combination with clavulanic acid (10µg).

Statistical analysis

All statistical analyse were undertaken using SPSS v19.0 (IBM). The chi-square test was used to assess the association between *Salmonella* serotypes and antimicrobial susceptibility profiles. When the assumptions of the asymptotic method were not met, the exact significance was calculated by applying the Fisher exact test. Pairwise comparisons of different susceptibilities were undertaken using the Bonferroni correction.

4.1.3. Results

Antimicrobial susceptibility

Of the 333 *Salmonella* isolates selected and tested for antimicrobial susceptibility, 11 serotypes of *Salmonella enterica* were identified in breeders and 29 in broilers (Table 4.1.1). Of the serotypes recovered from broilers, *S.* Enteritidis and *S.* Mbandaka showed a higher frequency of reduced susceptibility to quinolones when compared with *S.* Havana and *S.* Typhimurium; the same comparison with other serotypes was observed in *S.* Enteritidis isolates recovered from breeders. Although no clinically-apparent resistance against ciprofloxacin was detected, 53.4% and 60.5% of the isolates recovered from breeders and broilers, respectively, exhibited a reduced susceptibility to this antimicrobial. *S.* Typhimurium and *S.* Enteritidis showed higher frequencies of decreased susceptibility to sulfamethoxazole and tetracycline. Decreased susceptibility to ampicillin, chloramphenicol, florfenicol, streptomycin and gentamicin was either absent, or very low, in serovars Havana, Enteritidis and Mbandaka. Although few isolates of *S.* Virchow were tested, a high level of resistance to gentamicin and quinolones was detected.

Multiple resistance patterns

Thirty different patterns of decreased susceptibility were observed, of which half were classified as MDR (Table 4.1.2). In isolates recovered from broilers, MDR was most evident in isolates of *S.* Typhimurium (45.5%), followed by *S.* Enteritidis (31%), *S.* Havana (14.3%), *S.* Mbandaka (4.5%) and 13.7% in isolates in the group of other serotypes (Table 4.1.2). In breeders, MDR was only detected in isolates of *S.* Enteritidis (5.6%). Two *S.* Havana isolates recovered from broilers, with MICs for cefotaxime ≥ 8 mg/L and an ESBL phenotype, were also MDR.

Table 4.1.1. Summary of antimicrobial susceptibility of *Salmonella enterica* isolated from breeder and broiler flocks (n=333).

	Broilers							Breeders				
Antibiotics	***S.* Havana**	***S.* Enteritidis**	***S.* Mbandaka**	***S.* Typhimurium**	***S.* Virchow**	**Other serotypes***	**Total**	***S.* Enteritidis**	***S.* Havana**	**Other serotypes****	**Total**	**Breakpoints (mg/L)**
	n = 77	*n* = 71	*n* = 66	*n* = 11	*n* = 6	*n* = 44	***n* = 275**	*n* = 36	*n* = 8	*n* = 14	*n* = 58	
Ampicillin												
MIC50	2	2	4	8	1	2	2	1	8	4	2	
MIC90	8	4	8	>64	4	>64	8	8	8	8	8	
% DS	11.7^{A,B,C}	2.8^{B,C}	0^{C}	36.4^{A}	0^{A,B,C}	15.9^{A,B}	7.9	0^{A}	0^{A}	0^{A}	0	> 8^{a}
Cefotaxime												
MIC50	0.125	0.125	0.25	0.125	≤0.06	0.125	0.125	0.125	≤0.06	0.125	0.125	
MIC90	0.25	0.125	0.25	0.125	≤0.06	0.125	0.25	0.25	0.125	0.125	0.125	
% DS	2.6^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0.7	0^{A}	0^{A}	0^{A}	0	> 0,5^{a}
% R	2.6^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0^{A}	0.7	0^{A}	0^{A}	0^{A}	0	> 2^{b}
Nalidixic acid												
MIC50	8	512	256	4	4	4	32	>512	4	4	256	
MIC90	32	>512	512	8	256	128	> 512	>512	4	4	>512	
% DS	15.6^{A}	95.8^{B}	92.4^{B}	9.1^{A}	33.3^{A}	11.4^{A}	54	83.3^{B}	0^{A}	0^{A}	51.7	> 16^{a}
Ciprofloxacin												
MIC50	0.03	0.25	0.25	0.03	0.015	0.015	0.125	0.25	0.015	0.015	0.125	
MIC90	0.5	1	0.5	0.03	0.25	0.5	0.5	0.25	0.03	0.015	0.25	
% DS	35.1^{A}	95.8^{B}	97^{B}	9.1^{A}	33.3^{A}	11.4^{A}	60.5	86.1^{B}	0^{A}	0^{A}	53.4	> 0,06^{a}
% R	0	0	0	0	0	0	0	0	0	0	0	> 1^{b}
Chloramphenicol												
MIC50	8	4	16	8	4	4	8	8	8	4	8	
MIC90	16	8	16	64	8	8	16	16	8	8	8	
% DS	1.3^{A,B,C,D}	0^{C,D}	0^{B,D}	18.2^{A}	0^{A,B,C,D}	0^{A,B,C,D}	1.1	0^{A}	0^{A}	0^{A}	0	>16^{a}
Florfenicol												
MIC50	8	4	16	8	4	4	8	8	2	2	4	
MIC90	16	8	16	16	8	8	16	8	4	8	8	
% DS	1.3^{A}	0^{A}	0^{A}	9.1^{A}	0^{A}	0^{A}	1.1	0^{A}	0^{a}	0^{a}	0	>16^{a}

Antibiotics	Broilers							Breeders				
	***S.* Havana**	***S.* Enteritidis**	***S.* Mbandaka**	***S.* Typhimurium**	***S.* Virchow**	**Other serotypes***	**Total**	***S.* Enteritidis**	***S.* Havana**	**Other serotypes****	**Total**	**Breakpoints (mg/L)**
	n = 77	*n* = 71	*n* = 66	*n* = 11	*n* = 6	*n* = 44	***n* = 275**	*n* = 36	*n* = 8	*n* = 14	*n* = 58	
Streptomycin												
MIC50	≤2	≤2	4	8	4	4	4	2	≤2	16	2	
MIC90	8	8	8	256	4	128	8	8	4	32	16	
% DS	$6.5^{C,D,G,H}$	$1.4^{E,F,G,H}$	$1.5^{B,D,F,H}$	36.4^{A}	$0^{A,B,C,D,E,F,G,H,}$	$11.4^{A,B,C,D,E,F,G,H}$	5.8	0^{A}	0^{A}	7.1^{A}	1.7	>16[a]
Gentamicin												
MIC50	≤0.25	≤0.25	0.5	0.5	0.5	0.5	0.5	≤0.25	≤0.25	0.5	0.25	
MIC90	0.25	0.5	0.5	0.5	32	1	0.5	0.5	0.5	0.5	0.5	
% DS	1.3^{B}	0^{B}	1.5^{B}	$0^{A,B}$	33.3^{A}	$2.3^{A,B}$	1.8	0^{A}	0^{A}	0^{A}	0	>2[a]
Sulfamethoxazole												
MIC50	32	64	64	64	32	64	32	128	32	32	64	
MIC90	>1024	>1024	64	>1024	32	>1024	>1024	256	64	256	128	
% DS	$16.9^{A,B,C}$	31^{C}	4.5^{B}	$45.5^{A,C}$	$0^{A,B,C}$	$18.2^{A,B,C}$	18.5	5.6^{A}	0^{A}	0^{A}	3.4	>256[a]
Tetracycline												
MIC50	2	2	2	2	2	2	2	2	1	2	2	
MIC90	4	>64	4	>64	2	>64	>64	4	2	>64	2	
% DS	1.3^{B}	32.4^{A}	1.5^{B}	36.4^{A}	$0^{A,B}$	18.2^{A}	13.4	5.6^{B}	$0^{A,B}$	35.7^{A}	0	>8[a]
Trimethoprim												
MIC50	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	
MIC90	>32	>32	0.5	≤0.25	≤0.25	0.5	>32	0.5	≤0.25	0.5	0.5	
% DS	$13^{A,B}$	28.2^{B}	3^{A}	$9.1^{A,B}$	$0^{A,B}$	2.3^{A}	12.3	5.6^{A}	0^{A}	0^{A}	3.4	>2[a]

DS, decreased susceptibility; [a] EUCAST epidemiological breakpoints; **R**, resistance; [b] EUCAST clinical breakpoints.
Superscripts within the same line indicate significant differences between serotypes.
Serotypes mentioned in bold are those with higher frequencies of decreased susceptibility to the antimicrobials tested.
*, Serotypes: Altona, Agona, Anatum, Bradenberg, Cerro**, Derby,** Heidelberg, Indiana, Infantis, Kedougou, Kentucky, Kottbus, Lexington, London, Newport, **Rissen, Seftenberg,** Spartel, Taksony, Tennessee, **I 4,[5],12:i:-,** II 42,b,e,n,x,z15; II 48:z10:[1,5]; IIIb 61:l,v:1,5,7; **Serotypes: Give, Mbandaka, Nima, Seftenberg**, Tennessee,** Virchow, I 1,3,19:-:-:z27, II 4,12:b:-, IIIb 61:k:1,5, (7)

Table 4.1.2. Antimicrobial patterns of decreased susceptibility in *Salmonella enterica* isolates recovered from breeder and broiler flocks.

Antibiotics	Broilers						Breeders	
	***S.* Havana**	***S.* Enteritidis**	***S.* Mbandaka**	***S.* Typhimurium**	***S.* Virchow**	**Other serotypes ***	***S.* Enteritidis**	**Other serotypes****
	%	%	%	%	%	%	%	%
NAL	1.3							
CIP	16.8		3				2.8	
TE						2.3		30.8
NAL, CIP	9.1	62	87.9			11.3	77.8	
CIP, TRI			1.5					
SMX, AMP						2.3		
SMX; TRI	2.6		1.5					
SMX, TE						2.3		
TE, STR								7.7
NAL, CIP, AMP	1.3							
NAL, CIP, CLO	1.3							
NAL, CIP, GEN	1.3				33.3			
NAL, CIP, STR	1.3							
NAL, CIP, TE		1.4						
NAL, CIP, TRI		1.4						
CIP, SMX, STR	3.9							
SMX, TRI, AMP	6.5			9.1				
SMX, STR, AMP						2.3		
SMX, TE, CLO				9.1				
NAL, CIP, GE, TRI			1.5					
NAL, CIP, SMX, TE		4.2	1.5					
SMX, TE, GE, AMP						2.3		
SMX, TRI, AMP, CTX	1.3							
SMX, TE, STR, AMP				18.2		6.8		
NAL, CIP, SMX, TRI, TE		25.4	1.5					
CIP, TRI, SMX, AMP, CTX	1.3							
SMX, TRI, STR, TE, AMP	1.3					2.3		
NAL, CIP, SMX, TRI, STR							5.6	
NAL, CIP, SMX, TRI, STR, TE		1.4						
NAL, CIP, STR, TE, SMX, CLO, FLO, AMP				9.1				

AMP, ampicillin; **CTX**, cefotaxime; **NAL**, nalidixic acid; **CIP**, ciprofloxacin; **CLO**, chloramphenicol; **FLO**, florfenicol; **STR**, streptomycin; **GE**, gentamicin; **SMX**, sulphamethoxazole; **TRI**, trimethoprim; **TE**, tetracycline

Serotypes mentioned in bold are those with higher frequencies of MDR phenotypes.

***,** Serotypes: Altona, Agona, Anatum, Bradenberg, Cerro, **Derby**, Heidelberg, Indiana, Infantis, Kedougou, Kentucky, Kottbus, Lexington, London, Newport, **Rissen**, **Seftenberg**, Spartel, Taksony, Tennessee, **I 4,[5],12:i:-**, II 42,b,e,n,x,z15; II 48:z10:[1,5]; IIIb 61:l,v:1,5,7; **, Serotypes: Give, Mbandaka, Nima, Seftenberg, **Tennessee**, Virchow, I 1,3,19:-:-:z27, II 4,12:b:-, IIIb 61:k:

4.1.4. Discussion

This study of *Salmonella* serotypes in Portugal supports previous studies (Papadopoulou *et al.,* 2009), that infected breeding flocks and hatcheries, contaminated feed, environment and rearing sites are important potential sources for broiler contamination and, subsequently, human food poisoning.

S. Enteritidis, *S.* Havana and *S.* Mbandaka are all considered zoonotic or potentially pathogenic serovars for humans (Schiff & Saphra, 1941; Menon *et al.,* 1994; Scheil et al.1998; Backer *et al.,* 2000; Boisrame-Gastrin, *et al.,* 2011), and were among the most prevalent serotypes recovered from Portuguese broiler flocks. It is likely that, at least for Serovars Havana and Mbandaka, poultry feed containing cereal grain imported from non-European countries is one of the main sources for these serotypes in live birds.

The antimicrobial susceptibility profiles of *Salmonella* isolates in this study was is serotype dependent, as has been reported in other studies (Musgrove *et al.,* 2006; Newell *et al.,* 2010; EFSA, 2012b). In strains from broilers, important differences between MIC_{50} and MIC_{90} (3 - to ≥ 7-fold dilutions) were observed for ampicillin, chloramphenicol, sulfamethoxazole, streptomycin and tetracycline in *S.* Typhimurium, for sulfamethoxazole and trimethoprim in *S.* Havana, for sulfamethoxazole, tetracycline and trimethoprim in *S.* Enteritidis and for ampicillin, nalidixic acid, streptomycin, sulfamethoxazole and tetracycline in other serotypes. In breeders, significant differences between MIC_{50} and MIC_{90} were observed for sulfamethoxazole and tetracycline in isolates grouped as 'other' serotypes (Table 4.1.1). Similarly, de Jong et. al. (2009) observed differences for sulfisoxazole, streptomycin and tetracycline, showing the heterogeneity of *S. enterica* isolates, as far as antimicrobial susceptibility is concerned.

The reduced susceptibility of *Salmonella* isolates to fluoroquinolones, particularly in serovars of *S.* Enteritidis and *S.* Mbandaka, may be attributed to the widespread use of enrofloxacin in Portugal (EMA, 2012), particularly in the broiler and turkey sectors. In fact, Portugal is the European country with the highest frequency of isolates with MICs for ciprofloxacin greater than that seen in wild-type isolates (72%) and is the sixth highest user of fluoroquinolones in Europe (EFSA, 2012b); however, no clinical resistance was observed presumably due to the prohibitive fitness costs of the resistant organisms (O'Reagan *et al.,* 2010). Despite the successes of *Salmonella* National Control Plans in European countries, *S.* Enteritidis still remains one of the most important serovars in poultry production and is strongly associated with MDR phenotypes (Barrow *et al.,* 2012). In our study, 31% of Enteritidis isolates were MDR and resistant to quinolones. Although third generation cephalosporins are not authorised for treatment of chickens in Portugal, two isolates of *S.* Havana, from two different poultry farms (one from Northern Portugal and one from the central region), had an MIC for cefotaxime that was >8mg/L (the MIC for the wild-type isolate); both were characterized as ESBL producers. Polymerase chain reaction and sequencing of the amplicons in those isolates, confirmed the

presence of *bla*$_{CTX-M-1}$ (Clemente *et al.,* 2013). Resistance against third generation cephalosporins in *Salmonella* isolates due to ESBL has been recently identified worldwide (Newell *et al.,* 2010). Factors which have been linked to the recent emergence of ESBL include: (1) The use of third generation cephalosporins (ceftiofur) in combination with Marek's disease vaccine in young chicks in some sectors of the poultry industry; (2) the use of ceftiofur as a spray or by subcutaneous injection in hatcheries, particularly alongside in ovo vaccines (Liebana *et al.,* 2012; MARAN, 2010); (3) importation of day-old grandparent chickens from UK and USA (EFSA, 2011); and (4) the rise in the use of fluoroquinolones in poultry, cattle and pigs, which selects for bacterial clones carrying ESBLs (Rodriguez-Bano et al. 2010; Wener et al. 2010; Cavaco et al. 2008). The origin of the two ESBL isolates in this study is unclear, but all the parent stock was imported from Europe, as day old chicks, so the resistance may have arisen before arrival in Portugal. In European countries, the occurrence of resistance to cefotaxime in *Salmonella* isolates was low (0.2 % to 3 %) for most countries, except for the Netherlands where it was moderate (12 %) (EFSA, 2012b). However, even low levels of resistance to critically important antimicrobials are of great concern because the spreading of MDR isolates producing ESBLs or AmpC, may also co-select for other resistance determinants through the use of other antimicrobials (Dierick *et al.,* 2010). In our study, MDR phenotypes were found in both ESBL isolates, which may be linked to antimicrobials frequently used in poultry production (EFSA, 2012b). Indeed, the selective pressure exerted by the use of antimicrobials in both human and animal populations can contribute to the spreading of particularly resistant clones of *Salmonella* (Newell *et al.,* 2010). The spread of clones of *Salmonella* can also be influenced by factors independent of antimicrobial usage, such as human foreign travel, the integrated structure of some animal production systems, animal movement and management and hygienic practices on farms (Levy, 2002; Hawkey & Jones, 2009; EFSA, 2012b). The need for the use of antimicrobials in animal production should be reduced through disease prevention, supported by good animal husbandry and management practices, animal welfare and vaccination (Newell *et al.,* 2010; Barrow *et al.,* 2012).

4.1.5. Conclusion

The widespread decreased susceptibility of *Salmonella* isolates to critically important antimicrobials, such as fluoroquinolones, highlighted in the present study, should raise concern about the prudent use of such antibiotics in poultry production. In the framework of ongoing *Salmonella* national surveillance programmes (EFSA, 2012a), monitoring should continue and be enlarged to cover other animal species, food products, and pharmaceutical drugs in order to provide early warning of changes in resistance patterns in animal populations. Monitoring antimicrobial susceptibility in vivo, alongside the use of epidemiological cut-off values to interpret final results, seems to be the most sensitive way to detect such changes.

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4.2. Occurrence of extended-spectrum β-lactamases among isolates of Salmonella enterica subsp*. enterica from food-producing animals and food products, in Portugal*

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Contributions of the authors for the manuscript:

Lurdes Clemente: conception and design of study, acquisition of laboratory data, analysis of data, drafting of article, critical revision and final approval of manuscript;
Vera Manageiro: acquisition of laboratory data, analysis of data, critical revision and final approval of manuscript;
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ABSTRACT

A total of 1120 *Salmonella* spp isolates, recovered from poultry, swine and food products of animal origin (bovine, swine and poultry) over the period of 2009-2011, were investigated in order to determine their serotype, susceptibility to a panel of eleven antimicrobials (A, ampicillin; Ct, cefotaxime; Cp, ciprofloxacin; Tm, trimethoprim; Su, sulfamethoxazole; C, chloramphenicol; S, streptomycine; G, gentamicine; T, tetracycline; NA, nalidixic acid; Fl, florfenicol) and the presence of resistance determinants of extended-spectrum cephalosporins. Overall, *Salmonella* Enteritidis was the most common serotype in all three animal species. In poultry of 618 isolates, 32.8% comprised *S.* Enteritidis, 18.3% *Salmonella* Havana and 16.5% *Salmonella* Mbandaka; in pigs of 101 isolates 21.8% comprised *Salmonella* Rissen and *Salmonella* Typhimurium, 10.9% *Salmonella* Derby and *Salmonella* London. *Salmonella* I 4,[5],12:i:- was the most common serotype recovered from pork and beef food products comprising 32.6% and 30% of isolates respectively, followed by *Salmonella* Rissen (26% and 24%) and *S.* Typhimurium (18.2% and 19%), respectively. In poultry products, *S.* Enteritidis, was the most frequent serotype (62.7%), followed by *Salmonella* Mbandaka (10.2%) and *Salmonella* Derby (8.5%). Susceptibility profiles differed according to the origin of the isolates. Five multidrug resistant isolates (0.45%) were further characterized as extended-spectrum β-lactamase (ESBL) producers. Polymerase chain reaction and sequencing of the amplicons confirmed the presence of $bla_{\text{CTX-M-1}}$ (*n*=2), $bla_{\text{CTX-M-14}}$ (*n*=1), $bla_{\text{CTX-M-15}}$ (*n*=1) and $bla_{\text{CTX-M-32}}$ (*n*=1); $bla_{\text{SHV-12}}$ and $bla_{\text{TEM-1}}$ genes were also detected in two isolates of *S.* I 4,[5],12:i:-. Four isolates, two *S.* Havana and two *S.* I 4,[5],12:i:-, carried class 1 integrons and in three, two *S.* I 4,[5],12:i:- and one *S.* Havana, IS*Ecp1* was identified associated to $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-32}}$ and $bla_{\text{CTX-M-14}}$ genes. Additionally, in one *S.* I 4,[5],12:i:- isolate, *orf477* was identified linked to $bla_{\text{CTX-M-32}}$. No plasmid mediated quinolone resistance-encoding genes were detected. Here, we report for the first time the presence of $bla_{\text{CTX-M}}$ genes in *Salmonella enterica* subsp *enterica* isolates recovered from poultry and food products of swine origin in Portugal.

Keywords: *Salmonella enterica*; food-producing animals; ESBL; CTX-M group; Multidrug resistance

4.2.1. Introduction

Infections with *Salmonella enterica* are one of the most important causes of food-borne diseases worldwide and the great majority are associated with the consumption of products such as meat, poultry, eggs, milk, seafood and other fresh products (Pui *et al.,* 2011). *S. enterica* infection is the second most frequent cause of gastrointestinal infection in humans in the European Union (EU), with a total of 99020 confirmed cases in 2010, although fewer cases had been observed in the previous years (EFSA, 2012c). *Salmonella* Enteritidis and

Salmonella Typhimurium were the most frequently reported serotypes accounting for 45% and 22.4%, of all isolates respectively (EFSA, 2012c).

Serotype I 4,[5],12:i:- is considered a monophasic variant of *S.* Typhimurium and one of the first reports of its occurrence in Europe was in Portugal from a chicken carcass (Machado & Bernardo, 1990). A survey conducted in 2010 in animals and animal products showed that this serovar was the second most common in swine (9.3% of all isolates), and the third most common in pork products (7.4%), cattle (4.7%) and beef products (10%) and was also present in turkey (2.4%) and chicken meat (0.4%) (EFSA, 2012c). This serotype is considered to be a new pandemic strain of *Salmonella* in Europe, typically possessing resistance to four antimicrobials comprising ampicillin, streptomycin, sulphonamides and tetracycline, ASSuT (Hopkins *et al.,* 2010).

Salmonella Havana is a potential pathogenic serotype for humans (Bekal *et al.,* 2012; Boisrame-Gastrin, *et al.,* 2011; Backer *et al.,* 2000; Menon *et al.,* 1994). In animals, it was isolated from different species, such as: broiler flocks (Soerjadi-Liem *et al.,* 1984), cattle (Bernardo *et al.,* 1996), turkeys (Pedersen *et al.,* 2002), wild mammals and birds (Caleja *et al.,* 2011; Reche et. al., 2003) and meat products from different animal species, including chicken, beef, veal and mutton (Mehrabian *et al.,* 2007).

The rapid development of resistance to extended-spectrum cephalosporins in different serovars of *S. enterica* has been observed worldwide and is predominantly linked to plasmid-mediated production of β-lactamases (EFSA, 2011). The most frequently detected β-lactamases in isolates from animal origin are extended-spectrum β-lactamases (ESBL) and plasmid-mediated AmpC β-lactamases (PMAβ); these are important causes of treatment failure in humans, when produced by potential zoonotic bacteria such as non-typhoid *Salmonella* (EFSA, 2011).

The aim of the present study was to evaluate the antimicrobial susceptibility and to characterize the ESBL- and PMAβ-producing *S. enterica* isolates recovered from different parts of the poultry industry, pigs and food products of animal origin in Portugal.

4.2.2. Materials and Methods

Bacterial isolates

This study included 1120 *Salmonella* spp isolates, representing 59 serotypes, recovered from breeders (*n*=58), broilers (*n*=275), layers (*n*=285), pigs (*n*=101) and food products of animal origin (*n*=401), in the period of 2009-2011. Samples were collected under the scope of the *Salmonella* National Control Programme in food-producing animals and food of animal origin, according to the guidelines of the Commission Decision (CD) 2007/407/CE. The isolates collected through the control and surveillance programmes, established in conformity with Regulation CE nº 2160/2003 of the European P arliament and/or Decisions 2006/662/CE and

2006/668/CE, were submitted for antimicrobial susceptibility testing after serotype characterization.

The poultry and swine farms sampled were distributed throughout Portugal and the isolates were recovered from faecal and environmental samples collected using sterile boots/sock swabs; the swabs were placed in sterile bags and transported to the laboratory. Food products consisted of uncooked fresh products like minced meat, hamburgers, meat cuts, sausages and table eggs, randomly collected at various retail stores. The isolates were recovered from bovine (*n*=100), swine (*n*=242) and poultry (*n*=59) products. All samples were examined according to ISO norm 6579: 2002 applied to *Salmonella* detection in food and animal feeding stuffs (Anonymous 2002). Suspected colonies were further characterized by means of biochemical tests, using triple sugar iron agar slopes and API 20E strips (bioMérieux, France). After biochemical confirmation, *Salmonella* isolates were sent to the *Salmonella* National Reference Laboratory (INIAV-Lisbon), in triple sugar iron slopes or SMID (Oxoid) plates, for serotype characterization (Table 4.2.1).

Serotyping of *Salmonella* isolates

Salmonella isolates were serotyped by the slide agglutination method for their O and H antigens, using the method of Kauffmann-White scheme (Grimond & Weill, 2007).

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) were determined by agar dilution, following standard recommendations, on a panel of eleven antimicrobial compounds: ampicillin (A), cefotaxime (Ct), nalidixic acid (NA), ciprofloxacin (Cp), streptomycin (S), gentamicin (G), chloramphenicol (C), florfenicol (Fl), tetracycline (T), sulphamethoxazole (Su) and trimethoprim (Tm) (Table 4.2.2). Antimicrobials were tested in a two-fold concentration series over the following ranges: ampicillin and tetracycline (0.5 - 64µg/mL), gentamicin and trimethoprim (0.25 - 32µg/mL), ciprofloxacin (0.008 - 8µg/mL), cefotaxime (0.06 - 8µg/mL), nalidixic acid and streptomycin (2 - 512µg/mL), chloramphenicol (2 - 256µg/mL), florfenicol (1 - 128µg/mL) and sulphamethoxazole (8 - 1024µg/mL). In order to assess decreased susceptibility of the strains, interpretation of the results was done according to the epidemiological cut-off values recommended by the European Committee on Antimicrobial Susceptibilty Testing (EUCAST), as intended to be for surveillance purposes (http://www.srga.org/eucastwt/wt_eucast.htm). Important parameters like MIC_{50} and MIC_{90} values, rates of decreased susceptibility, as well as resistance to critically important antimicrobials for humans (cefotaxime and ciprofloxacin), were calculated according to breakpoints established by EUCAST for *Enterobacteriaceae* (EUCAST, 2012) (Table 4.2.2). Isolates were considered multidrug resistant (MDR) if they presented diminished susceptibility to three or more structurally unrelated antimicrobials.

Screening and identification of ESBL and PMAβ

Isolates exhibiting MIC > 0.5µg/mL (non-wild type) to cefotaxime were tested for the presence of ESB, by the presence of synergy through disk combination, including cefotaxime, ceftazidime and cefpodoxime, as single drugs, and in combination with clavulanic acid (Mast Laboratories, UK). PMAβ producing isolates were phenotypically identified according to clinical breakpoints (EUCAST, 2012) by presenting inhibition zone diameters for cefoxitin of less than 19mm.

In order to detect the presence of β-lactamase-encoding genes in the isolates selected with decreased susceptibilities to cefotaxime and cefoxitin, different PCR reactions were performed according to the phenotypes found. Briefly, total DNA was prepared as previously described (Féria *et al.,* 2002). The genes *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, *bla*$_{CTX-M}$ and *ampC* were detected by PCR as previously described (Mendonça *et al.,* 2007) using a thermal cycler Biorad C1000 (Bio-Rad Laboratories, Portugal). PCR products were purified with ExoSAP IT (USB Corporation, Cleveland, Ohio, USA), and all amplicons from phenotype-like-ESBL producer strains were further sequenced directly on both strands using the automatic sequencer ABI3100 (Applied Biosystems, Warrington, UK). Results were compared with sequences included in the database found at http:\\www.lahey.org/studies/.

Surrounding of bla$_{CTX-M}$ genes

Genetic arrangement of *bla*$_{CTX-M}$ genes was investigated by PCR mapping technique, using primers that specifically targeted IS*Ecp1*, IS*26*, IS*903* and *orf477* genetic elements, in combination with primers for *bla*$_{CTX-M}$ genes, as previously described (Mendonça *et al.,* 2007; Eckert *et al.,* 2006). Additionally, the isolates were subjected to the detection of class 1 and 2 integrons through amplification of integrase encoding genes, as reported elsewhere (Leverstein-Van Hall *et al.,* 2002).*2.6. Detection of plasmid mediated quinolone resistance* (*PMQR*) *genes*

All CTX-M positive *Salmonella* spp. isolates were screened for the following PMQR-encoding genes: *qnr*A (Martinez-Martinez *et al.,* 1998), *qnr*B (Jacoby *et al.,* 2006), *qnr*C (Wang *et al.,* 2009), *qnr*D (Cavaco *et al.,* 2009), *qnr*S (Hata *et al.,* 2005), and *aac*(*6´*)-*Ib-cr* (Park *et al.,* 2007). A set of primer pairs 5'-GAACCGATGACGAAGCACAG-3' and 5'-CGTCGTTAAAGCATTCTTGTCC-3' were designed in this study to target and amplify *qepA gene.* The amplification conditions were: initial denaturation at 94ºC for seven minutes, followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 53.2ºC for 60 sec and extension at 72ºC for 60 sec, and then a final extension at 72ºC for 10 minutes. Positive and negative controls were used in each PCR reaction.

Statistical analysis

OpenEpi software, version 2.3.1 (Dean *et al.,* 2013), was used for statistical analysis. Fisher exact test was used to assess differences in antibiotic resistance between different groups. Two-sided P values of ≤ 0.05 were considered to be statistically significant.

4.2.3. Results

Serotypes of *Salmonella* spp

Among the 618 *Salmonella* spp. isolates (Table 4.2.1) recovered from live poultry (breeders, broilers and layers), three main serotypes were identified: *S.* Enteritidis (32.8%, 203/618), *S.* Havana (18.3%, 113/618) and *Salmonella* Mbandaka (16.5%, 102/618). Among the 101 isolates recovered from pigs, *Salmonella* Rissen and *S.* Typhimurium (21.8%, 22/101), *Salmonella* Derby and *Salmonella* London (10.9%, 11/101) were the most common serotypes found.

Considering food products *per* animal group (swine, poultry and bovine), *Salmonella* I 4,[5],12:i:- was the most common serotype recovered from food products of swine and bovine origin (32.6% and 30%), followed by *Salmonella* Rissen (26% and 24%) and *S.* Typhimurium (18.2% and 19%), respectively. In poultry products, *S.* Enteritidis, was the most frequently recovered serovar (62.7%), followed by *Salmonella* Mbandaka (10.2%) and *Salmonella* Derby (8.5%).

Antimicrobial susceptibility phenotype

Susceptibility profiles are shown in Table 4.2.2 and differ according to the origin of the isolates. Although resistance to ciprofloxacin was absent or very low, the prevalence of decreased susceptibility of the poultry isolates (non-wild type) to the remaining quinolones was higher when compared with the isolates recovered from pigs and food products derived from bovine and swine. Among swine isolates, decreased susceptibility was higher for tetracycline, sulfamethoxazole, ampicillin and streptomycin; the same was observed in isolates recovered from food products from swine and bovine. In order to compare resistance phenotypes within the same serovar, the seven most frequent serotypes are shown in Table 4.2.3, omitting serotypes with $n < 5$. Overall, and considering MIC_{90} for all samples and antimicrobials tested, no significant differences (more than one step dilution above the lowest value) were observed within the same serotype. Differences considered to be statistically significant ($p \leq 0.05$) are highlighted on Table 4.2.3.

MDR was recorded in two isolates from breeders (3.4%), five isolates from layers (1.8%), 47 isolates from broilers (17.1%) and 56 isolates from pigs (55.4%); from food products, it was

noticed in 147 isolates from swine (60.7%), 57 isolates from bovine (57%) and eight isolates from poultry (13.6%).

Table 4.2.1. Serotypes of 1120 *Salmonella* spp. isolates.

Serotype	Poultry			Pigs	Food of animal origin			Total (*n*=1120)
	Breeders (*n*=58)	Broilers (*n*=275)	Layers (*n*=285)	(*n*=101)	Bovine (*n*=100)	Swine (*n*=242)	Poultry (*n*=59)	
Altona	-	1	-	-	-	-	-	1
Anatum	-	2	2	2	3	4	-	13
Bovismorbificans	-	-	-	2	1	3	-	6
Brandenburg	-	2	1	2	-	1	-	6
Braenderup	-	-	9	-	-	-	-	9
Bredeney	-	-	-	1	4	9	-	14
Cerro	-	1	2	-	-	-	-	3
Corvallis	-	-	1	-	-	-	-	1
Derby	-	4	1	11	6	20	5	47
Dublin	-	-	-	-	2	-	-	2
Enteritidis	36	71	96	-	3	3	37	246
Give	1	-	5	5	-	4	-	15
Gloucester	-	-	-	2	-	-	-	2
Goldcoast	-	-	-	1	-	-	-	1
Hadar	-	-	-	-	2	1	1	4
Havana	8	77	28	-	-	-	-	113
Heidelberg	-	3	7	-	-	1	3	14
Indiana	-	2	-	-	-	-	-	2
Infantis	-	1	3	-	-	-	-	4
Kedougou	-	1	-	-	-	1	-	2
Kentucky	-	1	2	-	5	-	-	8
Kottbus	-	1	-	-	-	-	-	1
Lagos	-	-	-	-	-	1	-	1
Lexington	-	1	7	-	-	-	-	8
London	-	1	-	11	-	4	-	16
Mikawasima	-	-	2	-	-	-	-	2
Mbandaka	1	66	35	2	-	-	6	110
Menston	-	-	-	1	-	-	-	1
Molade	-	-	-	-	1	-	-	1
Montevideo	-	-	2	-	-	-	-	2
Muenchen	-	-	-	3	-	-	-	3
Newport	-	4	1	-	-	-	-	5
Nima	1	-	-	-	-	-	-	1
Ohio	-	-	2	-	-	1	-	3
Ouakam	-	-	1	-	-	-	-	1
Poona	-	-	1	-	-	-	-	1
Reading	-	-	-	-	-	2	-	2
Rissen	-	2	1	22	24	63	-	112
Schwarzengrund	-	-	2	-	-	-	-	2
Senftenberg	3	2	8	1	-	-	1	15
Spartel	-	1	-	-	-	-	-	1
Taksony	-	1	3	-	-	-	-	4
Tennessee	4	1	9	-	-	1	-	15
Typhimurium	-	11	8	22	19	44	2	106
Virchow	1	6	10	-	-	-	1	18
Winslow	-	-	1	-	-	-	-	1
I 1,3,19:-:-:Rz27.	1	-	13	7	-	-	-	21
I 4,[5],12:i:-	-	7	3	5	30	79	3	127
I 6,7, 14:-:1,2	-	-	2	-	-	-	-	2
I 8, 20:r:-	-	-	1	-	-	-	-	1
I 13, 22, i:-	-	-	1	-	-	-	-	1
I 21:d:-	-	-	1	-	-	-	-	1
II 4,12:b:-	1	-	1	-	-	-	-	2
II 42:b:e,n,x,z15	-	2	1	-	-	-	-	3
II 48:z10:[1,5]	-	1	2	-	-	-	-	3
IIIb 61:k:1,5,(7)	1	-	-	-	-	-	-	1
IIIb 61:l,v:1,5,7	-	1	-	-	-	-	-	1

Table 4.2.2. Antimicrobial susceptibility, MIC_{50} and MIC_{90} of 1120 *Salmonella enterica* isolates.

Antimicrobial	Breeders *n*=58	Broilers *n*=275	Layers *n*=285	Pigs *n*=101	Food (*n*=401) Bovine *n*=100	Swine *n*=242	Poultry *n*=59	Breakpoints (mg/L) DS[a]	R[b]
Ampicillin									
MIC50	2	2	2	2	>64	>64	4		
MIC90	8	8	4	>64	>64	>64	>64		
% DS	1.7	8	3.2	46.5	59	54.1	15.3	>8	
Cefotaxime									
MIC50	0.125	0.125	0.125	0.125	0.125	0.125	0.06		
MIC90	0.125	0.25	0.125	0.125	0.125	0.125	0.125		
% DS	0	0.7	0	0	0	1.2	0	>0.5	
% R	0	0.7	0	0	0	1.2	0		>2
Nalidixic acid									
MIC50	256	32	4	4	4	4	8		
MIC90	>512	> 512	> 512	4	8	8	> 512		
% DS	51.7	54.2	20.7	4	4	5.8	47.5	>16	
Ciprofloxacin									
MIC50	0.125	0.125	0.03	0.015	0.015	0.03	0.125		
MIC90	0.25	0.5	0.25	0.03	0.03	0.125	0.25		
% DS	53.4	60.7	22.1	2	4	6.2	50.8	>0.06	
% R	0	0	0.70	0	0	0	0		>1
Chloramphenicol									
MIC50	8	8	8	8	8	8	4		
MIC90	8	16	8	256	> 256	128	8		
% DS	0	1.1	0	17	26	21.1	5.1	>16	
Florfenicol									
MIC50	4	8	8	8	8	8	4		
MIC90	8	16	8	16	32	16	8		
% DS	0	1.1	0	8.9	10	9.1	0	>16	

Antimicrobial	Breeders *n*=58	Broilers *n*=275	Layers *n*=285	Pigs *n*=101	Food (*n*=401) Bovine *n*=100	Swine *n*=242	Poultry *n*=59	Breakpoints (mg/L)
Streptomycin								
MIC50	2	4	4	32	16	16	4	
MIC90	16	8	16	256	256	256	16	
% DS	5.2	5.8	4.9	45.5	39	46.7	5.1	>16
Gentamicin								
MIC50	0.25	0.5	0.5	0.5	0.5	0.5	0.5	
MIC90	0.5	0.5	0.5	1	0.5	0.5	0.5	
% DS	0	1.8	0.4	1	3	5	0	>2
Sulfamethoxazole								
MIC50	64	32	64	1024	>1024	>1024	128	
MIC90	128	>1024	128	>1024	>1024	>1024	>1024	
% DS	0	18.5	1.4	55.4	59	60.3	16.9	>256[c]
Tetracycline								
MIC50	2	2	2	>64	>64	>64	2	
MIC90	2	>64	4	>64	>64	>64	64	
% DS	0	13.5	4.6	61.4	73	78.5	11.9	>8
Trimethoprim								
MIC50	≤0.25	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	
MIC90	0.5	>32	0.5	>32	>32	>32	0.5	
% DS	3.4	12.7	0.4	24.8	19	19.8	8.5	>2

Resistance (**R**); Decreased susceptibility (**DS**)
[a] EUCAST epidemiological breakpoints
[b] EUCAST, 2012, clinical breakpoints
[c] For sulfonamides

Table 4.2.3. MICs among the most common serotypes of *Salmonella* spp[a].

Antimicrobials	*S.* Derby				*S.* Enteritidis				*S.* Havana			*S.* Mbandaka			*S.* Rissen			*S.* Typhimurium					*S.* 4, [5],12:i:-			
		Food						Food						Food		Food					Food				Food	
	Pigs (*n* =11)	Bovine (*n* = 6)	Swine (*n* = 20)	Poultry (*n* = 5)	Breeders (*n* = 36)	Broilers (*n* = 71)	Layers (*n* = 96)	Poultry (*n* = 37)	Breeders (*n* = 8)	Broilers (*n* = 77)	Layers (*n* = 28)	Broilers (*n* = 66)	Layers (n = 35)	Poultry (*n* = 6)	Pigs (*n* = 22)	Bovine (*n* = 24)	Swine (*n* = 63)	Broilers (*n* = 11)	Layers (*n* = 8)	Pigs (*n* = 22)	Bovine (*n* = 19)	Swine (n = 44)	Broilers (*n* = 7)	Pigs (*n* = 5)	Bovine (*n* = 30)	Swine (*n* = 79)
A																										
MIC_{50}	1	2	2	4	1	2	2	4	8	2	1	4	1	1	>64	>64	4	8	4	>64	>64	>64	>64	>64	>64	>64
MIC_{90}	2	2	2	>64 [b]	8	4	4	4	8	8	2	8	2	8	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
Ct																										
MIC_{50}	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.06	≤0.06	0.125	0.125	0.25	≤0.06	0.125	0.125	0.125	0.125	0.125	0.125	0.125	≤0.06	≤0.06	≤0.06	0.125	≤0.06	≤0.06
MIC_{90}	0.125	0.125	0.125	0.125	0.25	0.125	0.125	0.125	0.125	0.25	0.25	0.25	0.125	0.125	0.25	0.25	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.25
Na																										
MIC_{50}	4	4	4	4	>512	512	4	>512	4	8	4	256	4	≤2	4	4	4	4	4	4	4	4	4	4	4	4
MIC_{90}	4	4	4	>512 [b]	>512	>512	>512	>512	4	32 [b]	8	512 [b]	4	≤2	4	4	4	8	8	8	4	256 [b]	8	4	8	8
Cp																										
MIC_{50}	0.015	0.015	0.015	0.03	0.25	0.25	0.03	0.125	0.015	0.03	0.03	0.25	0.03	0.015	0.015	0.015	0.03	0.03	0.03	0.015	0.015	0.03	0.015	0.03	0.03	0.03
MIC_{90}	0.03	0.015	0.03	0.25 [b]	0.25	1 [b]	0.25	0.25	0.03	0.5 [b]	0.5	0.5 [b]	0.03	0.015	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.25 [b]	0.06	0.03	0.03	0.03
C																										
MIC_{50}	8	8	8	8	8	4	8	4	8	8	8	16	8	4	8	8	8	8	4	128	128	8	8	16	8	8
MIC_{90}	8	8	8	64 [b]	16	8	8	8	8	16	16	16	8	8	16	64 [b]	128 [b]	64 [b]	4	512 [b]	256 [b]	256 [b]	8	128 [b]	128 [b]	128 [b]
Fl																										
MIC_{50}	8	8	8	8	8	4	4	4	2	8	8	16	8	4	8	16	8	8	4	16	8	8	8	16	8	8
MIC_{90}	16	8	8	16	8	8	8	8	4	16	8	16	8	4	16	16	16	16	8	64 [b]	64 [b]	64 [b]	8	16	16	16
S																										

Antimicrobials	*S.* Derby				*S.* Enteritidis				*S.* Havana			*S.* Mbandaka			*S.* Rissen			*S.* Typhimurium					*S.* 4, [5],12:i:-			
		Food						Food						Food		Food			Food				Food			
	Pigs (*n* =11)	Bovine (*n* = 6)	Swine (*n* = 20)	Poultry (*n* = 5)	Breeders (*n* = 36)	Broilers (*n* = 71)	Layers (*n* = 96)	Poultry (*n* = 37)	Breeders (*n* = 8)	Broilers (*n* = 77)	Layers (*n* = 28)	Broilers (*n* = 66)	Layers (n = 35)	Poultry (*n* = 6)	Pigs (*n* = 22)	Bovine (*n* = 24)	Swine (*n* = 63)	Broilers (*n* = 11)	Layers (*n* = 8)	Pigs (*n* = 22)	Bovine (*n* = 19)	Swine (n = 44)	Broilers (*n* = 7)	Pigs (*n* = 5)	Bovine (*n* = 30)	Swine (*n* = 79)
MIC_{50}	128	8	8	4	2	≤2	≤2	≤2	≤2	≤2	4	4	8	8	32	16	8	8	8	128	64	128	128	256	128	128
MIC_{90}	>256 [b]	128 [b]	128 [b]	16	8	8	8	4	4	8	8	8	8	8	128 [b]	16	32	256	256	>256 [b]	128	256	256	512	256	256
G																										
MIC_{50}	0.5	0.5	0.5	0.5	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
MIC_{90}	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.25	0.5	0.5	0.5	0.5	1	0.5	0.5	0.5	1	1	0.5	1	1	0.5	0.5	1
Su																										
MIC_{50}	1024	64	64	64	128	64	128	64	32	32	32	64	64	64	>1024	>1024	64	64	32	>1024	>1024	>1024	>1024	>1024	>1024	>1024
MIC_{90}	>1024	>1024	>1024	>1024	256	>1024 [b]	128	128	64	>1024 [b]	128	64	128	64	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
T																										
MIC_{50}	>64	>64	>64	2	2	2	2	2	1	2	2	2	2	2	>64	>64	>64	2	2	>64	>64	>64	>64	>64	>64	>64
MIC_{90}	>64	>64	>64	>64 [b]	4	>64 [b]	4	4	2	4	4	4	2	2	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
Tm																										
MIC_{50}	0.5	<0.25	<0.25	<0.25	0.5	0.5	<0.25	<0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	>32	>32	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	0.5	≤0.25	≤0.25
MIC_{90}	0.5	<0.25	<0.25	>32 [b]	0.5	>32 [b]	0.5	0.5	≤0.25	>32 [b]	0.5	0.5	≤0.25	≤0.25	>32	>32	>32	≤0.25	≤0.25	0.5	≤0.25	≤0.25	0.5	>32 [b]	0.5	>32

A, ampicillin; **Ct**, cefotaxime; **Cp**, ciprofloxacin; **Tm**, trimethoprim; **Su,** sulfamethoxazole; **C**, chloramphenicol; **S**, streptomycine; **G**, gentamicine; **T**, tetracycline; **Na**, nalidixic acid; **Fl**, florfenicol

[a] Serotypes with ≥ 5 isolates.

[b] Differences statistically significant ($p \leq 0.0$

Characterization of ESBL, PMQR, IS and integrons

Five isolates of *S. enterica* (0.45%, 5/1120), two being of serotype Havana recovered from broilers, and three of serotype I 4,[5],12:i:-, obtained from food products of swine origin, presented non-wild type MICs for cefotaxime (MIC ≥ 8µg/mL) and an ESBL phenotype (Table 4.2.4). In *S.* Havana isolates, (showingACtCpTm and ASuCtTm phenotypes), the *bla*$_{CTX-M-1}$ gene was detected and in *S.* I 4,[5],12:i:- (presentingASSuCtC, ASSuTCtCG and ATCtCTm phenotypes), the *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-32}$ genes were identified,, among which two isolates also contained the *bla*$_{TEM-1}$ and *bla*$_{SHV-12}$ genes. Although only one isolate of *S.* Havana showed a non wild-type MIC for ciprofloxacin (0.25µg/mL), no PMQR-encoding genes were detected in all five isolates.

Class 1 integrons were detected in two isolates of *S.* Havana and two of *S.* I 4,[5],12:i:-, and in two isolates of *S.* I 4,[5],12:i:- and one of *S.* Havana, IS*Ecp1* was identified upstream of the *bla*$_{CTX-M}$ gene. In *S.* I 4,[5],12:i:- one isolate , *orf477* was also detected and associated with *bla*$_{CTX-M-32}$.

4.2.4. Discussion

In Portugal, little information regarding antimicrobial susceptibility in food-borne and commensal bacteria isolated from food-producing animals is available. Moreover, the data available on antimicrobial susceptibility of *Salmonella* spp recovered from animals is frequently obtained from different methods and interpretation criteria, which are not always comparable (Antunes et al. 2006; Caleja *et al.,* 2011).

This study analysed a large number of isolates recovered from a range of different matrices and showed a wide variety of serotypes (*n*=59) revealing that several sources, such as infected breeding flocks and hatcheries, contaminated feed, environment and rearing sites, may be involved in *Salmonella* contamination. Indeed, *Salmonella* spp are widely distributed in nature and its long survival and transmission to vectors, such as flies, rats and birds, may favour its dissemination to those different reservoirs from which animals can be infected orally (Pui et. al., 2011).

Among 1120 *Salmonella* isolates, *S. Enteritidis* was the most common serotype particularly in live poultry (32.8%) and their food products (62.7%) and constitutes the primary cause of human salmonellosis in the EU (EFSA, 2012c).

Our results suggest that *Salmonella* contamination of food products arises mainly from the live animals, but the slaughter line of the abattoir, the contact with meat handlers, the processing plants and equipments and retail stores, can contribute to further cross-contamination (Gomes-Neves *et al.,* 2012; Pui et. al., 2011). Some variation in the frequency.

Table 4.2.4. Phenotypic and genotypic features of ESBL-producing isolates (*n*=5).

Isolate no	Serotype	Origin	MIC Cefotaxime (µg/mL)	β-lactamases	Integrons	Insertion sequence	Resistance phenotype[a]
13933/2011	Havana	Broiler	> 8	CTX-M-1	Class 1	*ISEcp1*	ACtCpTm
16627/2011	Havana	Broiler	> 8	CTX-M-1	none	Negative	ASuCtTm
10029/2010	I 4,[5],12:i:-	Pork	8	CTX-M-15; SHV-12	Class 1	Negative	ASSuCtC
21003/2011	I 4,[5],12:i:-	Pork	> 8	CTX-M-32; TEM-1	Class 1	*ISEcp1* + o*rf477*	ASSuTCtCG
12675/2011	I 4,[5],12:i:-	Pork	> 8	CTX-M-14	Class 1	*ISEcp1*	ATCtCTm

[a] **A**, ampicillin; **Ct**, cefotaxime; **Cp**, ciprofloxacin; **Tm**, trimethoprim; **Su**, sulfamethoxazole; **C**, chloramphenicol; **S**, streptomycine; **G**, gentamicine; **T**, tetracycline

of recovery of some serotypes may occur, as observed in our study, for example with serovar I 4,[5],12:i:- recovered from pigs and pork products and, serotype Havana among broilers, and poultry food products.

In some European countries, the prevalence of ampicillin, streptomycin, sulfonamides and tetracyclines resistant I 4,[5],12:i:-, has been increasing in human foodborne outbreaks and in pigs and pork (EFSA, 2011; Hopkins *et al.,* 2010). In this study, 11.3% of the isolates belonging to this serovar were distributed among broilers, layers and pigs and food products, particularly from pigs and cattle, which may be important reservoirs. Within this serotype, decreased susceptibility to ampicillin, streptomycin, sulphonamides and tetracycline (ASSuT phenotype) was detected in 59.8% isolates. Additionally, 21.3% of these isolates showed co-resistance to gentamicin, trimethoprim, chloramphenicol, florfenicol, quinolones and cefotaxime, contributing to MDR.

Important differences between MIC_{50} and MIC_{90} were noticed (3- to ≥ 8-fold dilutions) regarding ampicillin, chloramphenicol and streptomycin (in pigs)**,** chloramphenicol and streptomycin in food products from bovine and swine, nalidixic acid in broilers, layers and food products from poultry, sulphamethoxazole and tetracycline in broilers and food products from poultry and trimethoprim in broilers, pigs and food products from swine and bovine. This has previously been reported in swine by de Jong et al. (2009) and Ibar et al. (2009) for
ampicillin, chloramphenicol, sulfamethoxazole and tetracycline; indeed, this reveals variability of the isolates as far as antimicrobial susceptibility is concerned.

The higher frequency of poultry-derived quinolone non-wild type isolates might be explained by the widespread use of enrofloxacin in poultry production (Hao-Van et al, 2012; Levy, 2002). In fact, Portugal has the highest frequency of non-wild type isolates towards ciprofloxacin (72%) and the sixth highest user of fluoroquinolones in Europe (EMA, 2012). Although no clinical resistant isolates were detected in our study, the population showing decreased susceptibility (non-wild type) might be an indicator of emerging resistance (de Jong *et al.,* 2009).

In the isolates recovered from both swine and bovine food products, non-wild type resistance to tetracycline, sulfamethoxazole and ampicillin, was observed as in other countries, namely, the Netherlands, Ireland and Greece (EFSA, 2012b), suggesting that the widespread use of these antimicrobials in livestock production is creating resistance problems (EMA, 2012). Within the same serotype, the relevant differences observed in MIC_{90} values for some of the antimicrobials tested should be due to a range of factors, including animal species, type of production, antibiotic pressure exerted during production cycle and circulating strains (wild and non wild-type isolates), (EFSA, 2012a; Hao-Van *et al.,* 2012; Levy, 2002).

In Portugal the use of third-generation cephalosporins, namely ceftiofur, is only allowed in bovine and swine production; in this study, 0.45% of *S. enterica* (3 *S.* I 4,[5],12:i:- and 2 *S.* Havana isolates, recovered from food products of swine origin and broilers, respectively) showed a non-wild type MIC for cefotaxime, due to the production of CTX-M β-lactamases.

In *S.* Havana, recovered from broilers and isolated from two different poultry farms, the *bla*$_{\text{CTX-M-1}}$ gene was detected harbouring relevant mobile genetic elements, which might contribute to the dissemination of this resistance mechanism (Barlow *et al.,* 2008). *S.* Havana has not been frequently isolated from food-producing animals in Europe (EFSA, 2012c). However, recently, the detection of *bla*$_{\text{CTX-M-15}}$ harbouring *S.* Havana isolates in two African children in France was reported (Boisrame-Gastrin *et al.,* 2011). In Portugal, besides poultry, *S.* Havana has been identified in wild rabbits (Vieira-Pinto et al. 2011), wild boars (Caleja *et al.,* 2011) and exotic birds (Gomes *et al.,* 2002). The *bla*$_{\text{CTX-M-1}}$ gene has also been detected all over Europe among *Enterobacteriaceae* of animal origin (Bortolaia *et al.,* 2011; Gándara *et al.,* 2011; Dierikx *et al.,* 2010; Machado *et al.,* 2008a; Kempf *et al.,* 2007).

In the three isolates of *S.* I 4,[5],12:i:- recovered from food products of swine origin, a diversity of *bla*$_{\text{CTX-M}}$ genes was detected. In fact, the presence of CTX-M-9 group enzymes in other serovars has been identified, such as in *Salmonella* Virchow isolated from broilers and a laying hen, and *S.* Enteritidis from broilers (Rião *et al.,* 2006), as well as the presence of CTX-M-15 and CTX-M-14 enzymes in isolates of *S.* Enteritidis and *Salmonella* Essen, recovered from different poultry specimens including meat, faeces and diseased birds (Tamang *et al.,* 2011b).

In our study, CTX-M-15 enzyme was co-produced with the ESBL SHV-12 in an isolate which harboured class 1 integron. SHV-12 enzyme was also previously identified in *S. enterica* isolates from poultry and swine (Chiaretto *et al.,* 2008; Rião *et al.,* 2006), which might indicate the dissemination of this β-lactamase throughout the food chain. Here, one isolate of *S.* I 4,[5],12:i:- carried a class 1 integron, but also IS*Ecp1* and *orf477*, which were associated with *bla*$_{\text{CTX-M-32}}$ gene. Although CTX-M-32 enzyme does not seem to be a common β-lactamase found in animals, Politi et al. (2005) in Greece reported it in isolates of *S.* Virchow from poultry products.

In Portugal, CTX-M-14, CTX-M-15 and CTX-M-32 enzymes were detected in *E. coli* isolates recovered from faecal samples of Iberian lynx, seagulls, dog, food-producing animals and marine water samples (Gonçalves *et al.,* 2011; Simões *et al.,* 2010; Pomba *et al.,* 2009; Machado *et al.,* 2008a; Machado *et al.,* 2008b).

To our knowledge, this was the most comprehensive national study of *Salmonella* isolates recovered from poultry, pigs and food products of animal origin and the first report of ESBL-encoding genes, particularly from *bla*$_{\text{CTX-M}}$ family, in Portugal in *Salmonella*, serotypes Havana and I 4,[5],12:i:-. Serotype Havana is not a common serotype in European countries, either in live broilers or any other animal species (EFSA, 2012c); however, it is the most common

serotype isolated from the Portuguese broiler population (P. Themudo, personal communication).

In our study, *S.* I 4,[5],12:i:- was the most common serotype recovered from food products of swine and bovine origin. In Europe, it is responsible for 1.5% of the human cases of salmonellosis (EFSA, 2012c); however, if the isolates are ESBL producers and MDR as in this study, they may represent a serious threat to human health.

Our results agree with other studies that animals may act as important reservoirs of *Salmonella* isolates carrying ESBL-encoding genes, which might be transmissible to humans through contact or the food chain and, in addition, might be a potential source for human pathogens to acquire these resistance genes (Caratolli, 2008; Liebana *et al.,* 2012).

Within Europe, the occurrence of resistance to cefotaxime in *Salmonella* isolates is low in most countries and, moderately frequently as observed in the Netherlands (EFSA, 2011). However, even low levels of resistance to these critically important antimicrobials are of great concern because the spreading of MDR isolates producing ESBLs or AmpC, may also co-select other resistance determinants through the use of other antimicrobials (Dierick *et al.,* 2010). The inappropriate use of antimicrobials in veterinary medicine or in feed additives has been linked to the emergence and spread of *Salmonella* resistant strains with potentially serious effects in food safety (Hao- Van *et al.,* 2012; Rodriguez *et al.,* 2009).

In conclusion, the growing concern of the emergence of bacterial strains bearing ESBL in food-producing animals highlights the importance of continuous monitoring (EFSA, 2011).

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4.3. Antimicrobial susceptibility and oxymino-β-lactam resistance mechanisms in Salmonella enterica and Escherichia coli isolates from different animal sources

This research paper was submitted as:

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ABSTRACT

The impact of extended-spectrum β-lactamases (ESBL) and plasmid-mediated AmpC β-lactamases (PMAβ) of animal origin has been a public health concern. In this study, 562 *Salmonella enterica* and 598 *Escherichia coli* isolates recovered from different animal species and food products were tested for antimicrobial resistance. Detection of ESBL-, PMAβ-, plasmid-mediated quinolone resistance (PMQR)-encoding genes and integrons was performed in isolates showing non-wild type phenotypes.

Susceptibility profiles of *Salmonella* spp. isolates differed according to serotypes and origin of the isolates. The occurrence of cefotaxime non-wild type isolates was higher in pets than in other groups. In nine *Salmonella* isolates, $bla_{\text{CTX-M}}$ (*n*=4), $bla_{\text{SHV-12}}$ (*n*=1), $bla_{\text{TEM-1}}$ (*n*=2), $bla_{\text{CMY-2}}$ (*n*=2) were identified. No PMQR-encoding genes were found. In 47 *E. coli* isolates, $bla_{\text{CTX-M}}$ (*n*=15), $bla_{\text{SHV-12}}$ (*n*=2), $bla_{\text{CMY-2}}$ (*n*=6), $bla_{\text{TEM-type}}$ (*n*=28) and PMQR-encoding genes, *qnrB* (*n*=2), *qnrS* (*n*=1) and *aac(6')-Ib-cr* (*n*=6) were detected. To the best of our knowledge, this study is the first to describe, the presence of $bla_{\text{CMY-2}}$ (*n*=2) and $bla_{\text{SHV-12}}$ (*n*=1) genes among *S. enterica* from broilers, in Portugal.

This study highlights that animals may act as important reservoirs of isolates carrying ESBL, PMAβ and PMQR encoding genes, which might be transferred to humans through direct contact or the food chain.

Keywords: Antimicrobial resistance; *Salmonella enterica*; *Escherichia coli*; ESBLs; PMAβ; PMQR

4.3.1. Introduction

Salmonella is a widely distributed foodborne pathogen and one of the most common causes of bacterial foodborne illnesses, with tens of millions of human cases occurring worldwide every year (http://www.who.int). In the European Union it is the second most reported zoonotic disease in humans, with a total of 92.916 cases; most infections are caused by serovars Enteritidis, Typhimurium and Typhimurium monophasic 1,4,[5], 12:i:- (EFSA, 2014).

Escherichia coli is the most prevalent commensal of gastrointestinal tract of humans and animals, remaining as one of the most frequent causes of several bacterial infections in both, humans and animals (Allocati *et al.,* 2013).

Antimicrobial resistance in *Enterobacteriaceae*, namely in non-typhoidal *Salmonella* serotypes and *E.* coli, is an expanding problem (EFSA, 2014). The wide and uncontrolled use of antimicrobial compounds in human and veterinary practices, animal production, agriculture, industrial technology, the increase on the movement of people and on the circulation of food and raw materials for food production across the different countries are, all

together, factors responsible for the emergence and dissemination of resistant and multi-resistant bacterial strains, constituting a risk for the human and animal health, due to the increase of morbidity, mortality and the cost associated to the treatment of infections (Marshall & Levy, 2011; EFSA, 2014).

β-lactams and fluoroquinolones are two important classes of antimicrobials used to treat severe infections in humans and in animals (EFSA, 2014). Resistance to third generation cephalosporins is generally due to the production of extended-spectrum β-lactamases (ESBL) and plasmid-mediated AmpC β-lactamases (PMAβ). Various β-lactamase-encoding genes have been detected in diverse serotypes, located in plasmids or in integrons, facilitating its transmission between serotypes and other bacteria (EFSA, 2011).

Animals have the potential to act as reservoirs for a number of zoonotic infections, including those caused by pathogenic and commensal *E. coli* ESBL-producers, which might be transmitted to humans through direct contact or the food chain (EFSA, 2011; Marshall & Levy, 2011).

In Europe, a wide range of ESBL genotypes have been reported from animals (Caratolli, 2008; EFSA, 2011) some of them also found in humans (Leverstein-van Hall *et al.,* 2011). Companion animals, like horses, dogs and cats, also constitute a potential reservoir of ESBL-encoding genes, as often they live in close contact with their owners, making the occurrence of transmission more likely (Sun *et al.,* 2010; Dierikx *et al.,* 2012; Hordijk *et al.,* 2013). Wild animals living in the wilderness or in captivity, may also represent a source of ESBL-producing *E. coli* isolates to the ecosystems (Gonçalves *et al.,* 2011; Veldman *et al.,* 2013), stressing the importance of the environment on the dissemination of resistance genes and the potential zoonotic transmission due to the contact between zoo animals, zoo keepers and visitors (Wang *et al.,* 2012a)**.**

In this study, we present updated data on antimicrobial resistance in *Salmonella* recovered from animals, with particular emphasis in food-producing animals, poultry feed and food of animal origin, as well as in *E. coli* isolates collected from food-producing animals, pets and zoo animals. Evaluation of the presence of antimicrobial resistance mechanisms in isolates with reduced susceptibility to third-generation cephalosporins and/or cephamycins was also performed.

4.3.2. Materials and Methods

Bacterial isolates

This study included 562 *Salmonella* spp. isolates representing 50 different serotypes (Table 4.3.1), recovered from breeders (*n*=23), broilers (*n*=193), layers (*n*=73), turkeys (*n*=17), animal feed (*n*=52), other animal species (*n*=22) and food products of animal origin (*n*=182), over the period of 2012-2013.

In poultry farms, samples were collected from faeces and environment using sterile

boots/sock swabs. Food products consisted of: uncooked fresh products like minced meat, hamburgers, meat cuts, sausages and table eggs, randomly collected at various retail stores. Samples from other animal species (pigeons, partridges, ducks, pets and exotic animals) consisted of hemoculture and organs (lung, liver, spleen, kidneys and intestine), collected during post-mortem examination for bacteriological examination.

All samples were examined according to ISO norm 6579:2002 applied to *Salmonella* detection in food and animal feeding stuffs. After biochemical confirmation, *Salmonella* isolates were sent to the *Salmonella* National Reference Laboratory (INIAV-Lisbon), in triple sugar iron slopes or SMID plates, for serotype characterization.

Also included in this study, 598 *Eschericia coli* isolates (Table 4.3.1) were collected over the period of 2009-2013 from food-producing animals [(bovine, swine and poultry), (*n*=215), pets (dogs, cats, horses and cage birds), (*n*=113) and zoo animals (terrestrial and aquatic mammals, birds and reptiles), (*n*=270)]. Samples consisted of swabs from organic fluids and cavities, faecal samples, urine samples, hemocultures and organs collected during post-mortem examination and submitted for bacteriological analysis. Suspected *E. coli* colonies obtained in MacConkey agar were confirmed by means of API 20E strips.

Salmonella spp. and *E. coli* isolates were preserved in cryovials containing tryptose soya broth and glycerol at -70ºC for further antimicrobial susceptibility tests and molecular assays.

Serotyping of *Salmonella* isolates

Salmonella isolates were serotyped by the slide agglutination method for their O and H antigens, using the method of Kauffmann-White scheme (Grimmont & Weill, 2007)**.**

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) were determined by agar dilution, following standard recommendations (CLSI, 2013), with a panel of nine antimicrobial compounds: ampicillin (A), cefotaxime (Ct), nalidixic acid (Na), ciprofloxacin (Cp), gentamicin (G), chloramphenicol (C), tetracycline (T), sulphamethoxazole (Su) and trimethoprim (Tm) (Table 4.3.2). To assess non wild-type strains, interpretation of the results was done according to the epidemiological cut-off values recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://mic.eucast.org/Eucast2/). For *Salmonella* spp., the cut-off value used for sulfamethoxazole was that for sulfonamides from Clinical Standards Laboratory Institute (CLSI, 2013). *E. coli* ATCC 25922 was used as the quality control strain.

Table 4.3.1. *Salmonella* spp. (*n*=562) and *Escherichia coli* (*n*=598) isolates.

Samples	*Salmonella* spp.							*Escherichia coli*	
	Most common reported serotypes in Humans		Emergent reported serotype in Humans	Important serotypes in national poultry population		Other serotypes[a]	Total	Samples	Isolates (*n*)
	S. Enteritidis	*S.* Typhimurium	*S.* 4,[5],12,i:-	*S.* Havana	*S.* Mbandaka				
Animal species								Animal species	
Breeders	1	1	0	0	0	21	23	Pets	113
Broilers	31	4	6	102	5	45	193	Zoo	270
Layers	13	4	1	1	13	41	73	Food producing	215
Turkeys	1	2	5	0	0	9	17		
Animal feed	9	2	0	8	7	26	52		
Other species	2	8	4	0	0	8	22		
Total	57	21	16	111	25	150	380		
Food									
Bovine	0	3	2	0	0	20	25		
Swine	0	12	29	0	0	33	74		
Poultry	10	26	16	2	2	27	83		
Total	10	41	47	2	2	80	182	Total	598

[a] **Serotypes**: Agona, Altona, Anatum, Bardo, Bovismorbificans, Brandenburg, Bredeney, Cerro, Cubana, Derby, Duesseldorf, Give, Heidelberg, Hadar, Indiana, Kentucky, Kingston, Kottbus, Javiana, Landau, Lexington, Llandoff, London, Muenchen, Newport, Reading, Redba, Rissen, Saintpaul, Schwarzengrund, Seftenberg, Stanleyville, Taksony, Tennessee, Tomegbe, Virchow, I 3,19 :-:-z27, I:17:b:?, II 48:z10:[1,5], II 6,7:z6:?, II 42:b:e,n,x,z15, I 9,46:?:?, II 4, [5],12:?, 4,12:eh:?, I 6,7,?:

Rates of resistance to important antimicrobials to humans (ampicillin, cefotaxime, ciprofloxacin and gentamicin), were calculated according to clinical breakpoints established by EUCAST for *Enterobacteriaceae* (EUCAST, 2014). Isolates were considered multidrug resistant (MDR) if they presented diminished susceptibility or non-wild type phenotypes against three or more antimicrobials not structurally related (Tables 4.3.2 and 4.3.3).

Screening and identification of ESBL and PMAβ

Salmonella spp. and *E. coli* isolates exhibiting MIC > 0.5µg/mL and > 0.25µg/mL, respectively, were considered non-wild type to cefotaxime and tested for the production of ESBL, by combined disk test using cefotaxime, ceftazidime and cefpodoxime, as single drugs and in combination with clavulanic acid.

In addition, a cefoxitin disk (30µg) was added to this test to detect presumptive PMAβ producers. All isolates classified as intermediate or resistant using CLSI criteria (≤17mm) to cefoxitin (CLSI, 2013), were suspected to be PMAβ producers and also included in this study.

Molecular characterization of resistance

In order to detect the presence of β-lactamase-encoding genes in the isolates showing non wild-type phenotypes to cefotaxime and/or cefoxitin, different PCR reactions were performed according to the phenotypes found. The bla_{ESBL} (bla_{TEM}, bla_{SHV}, $bla_{OXA\text{-}1\text{-}type}$, $bla_{CTX\text{-}M}$) and $bla_{PMA\beta}$ (bla_{CMY}, bla_{MOX}, bla_{FOX}, bla_{LAT}, bla_{ACT}, bla_{MIR}, bla_{DHA}, bla_{MOR}, bla_{ACC}) genes were detected by PCR, as previously described Positive and negative controls were used in all PCR reactions (Mendonça *et al.,* 2007). PCR products were purified and all amplicons were further sequenced directly on both strands using automatic sequencer ABI3100 (Applied Biosystems).

Additionally, the isolates were subjected to the detection of class 1 and 2 integrons through amplification of integrase encoding genes, as reported elsewhere (Leverstein-van *et al.,* 2002).

Salmonella spp. and *E. coli* isolates evidencing a non-wild phenotype to cefotaxime and simultaneously non-susceptible to quinolones, were screened for the following PMQR encoding genes: *qnr*A, *qnr*B, *qnr*C, *qnr*D, *qnr*S, *aac*(*6´*)-*Ib-cr* and *qepA* (Wang *et al.,* 2003; Park *et al.,* 2007; Cavaco *et al.,* 2009; Wang *et al.,* 2009)**.**

4.3.3. Results

Serotypes of *Salmonella* spp.

A great diversity of serotypes (*n*=50) was identified among the 562 *Salmonella* isolates. Overall, in poultry, particularly in broilers, *S.* Havana and *S.* Enteritidis were the two main serotypes identified; *S.* Mbandaka was the most common serotype in layers (Table 4.3.1). *S.* Enteritidis is one of the most common reported serotypes in Humans and it was found in

poultry food products (100%, 10/10), in broilers (54.4%, 31/57), in layers (22.8%, 13/57) and in poultry feed (15.8%, 9/57). *S.* I 4,[5],12:i:- and *S*. Typhimurium were the most common serotypes recovered from food products of swine and poultry origin. Being serovar I 4,[5],12:i:- considered the emergent serotype in Humans, it was identified in 61.7% (29/47) isolates recovered from swine and 34% (16/47) from poultry products; in live birds, 37.5% (6/16) and 31.3% (5/16) were from broilers and turkeys, respectively (Table 4.3.1).

Antimicrobial susceptibility phenotype of *Salmonella* spp. and *E. coli* isolates

Susceptibility profiles of *Salmonella* spp. differed according to the origin (Table 4.3.2) and the serotypes of the isolates (Table 4.3.3).

Although clinical resistance to ciprofloxacin was absent (Table 4.3.2), the frequency of non-wild type isolates to this antimicrobial was higher in poultry and poultry food products (both 30.1%). Therefore, isolates recovered from bovine and swine food products, showed higher non wild-type phenotypes for tetracycline (64% and 73%, respectively), sulfamethoxazole (60% and 67.6%, respectively) and ampicillin (20% and 50%, respectively) (Table 4.3.2).

Regarding cefotaxime, nine isolates of *S. enterica* (1.6%, 9/562) (being four of serotype Havana and one of serotype Enteritidis recovered from broilers, two of serotype I 4,[5],12:i:-, obtained from a partridge and pork sausage, one of serotype Heidelberg from broiler neck skin and one of serotype London from a pork hamburger) presented non-wild type MICs for cefotaxime (MIC ranging from 1 to ≥ 8µg/mL) (Table 4.3.4).

MDR was observed in five isolates from turkeys (29.4%), 26 isolates from broilers (13.5%), two isolates from layers (2.8%), six isolates from other animal species (27.3%), 44 isolates from food of swine origin (59.5%), 22 isolates from food of poultry origin (26.5%), 5 isolates from food of bovine origin (20.0%), and two isolates from animal feed (3.9%). No MDR isolates were detected in broiler breeders. The higher frequency of MDR isolates was observed in *S.* I 4,[5],12:i:- and *S*. Typhimurium serovars (Table 4.3.3).

With regard to susceptibility of *E. coli* isolates (Table 4.3.2), the frequency of non-wild type isolates to all antimicrobials tested was higher when comparing with *Salmonella* isolates, particularly to ciprofloxacin, where clinical resistance is important, namely in food-producing animals (24.2%) and pets (21.1%) (Table 4.3.2).

Eight isolates of *E. coli* from food-producing animals (3.7%, 8/215), twelve from pets (10.6%, 12/113) and seven from zoo animals (2.6%, 7/270) showed a non-wild type to cefotaxime (MIC ranging 0.5 to ≥8mg/L) (Table 4.3.4).

MDR was recorded in 115 isolates from food-producing animals (53.7%), 39 isolates from pets (34.5%) and 81 isolates from zoo animals (30%) (Table 4.3.2).

Table 4.3.2. MIC_{50} and MIC_{90} for *Salmonella* spp. (*n*=562) and *Escherichia coli* (*n*=598) isolates.

Antibiotics	*Salmonella* spp.									*Escherichia coli*		
	Breeders *n*=23	Broilers *n*=193	Layers *n*=73	Turkeys *n*=17	Animal feed *n*=52	Other species *n*=22	Food Bovine *n*=25	Swine *n*=74	Poultry *n*=83	Food animals *n*=215	Pets *n*=113	Zoo animals *n*=270
A												
MIC_{50}	2	2	1	2	2	2	2	2	2	8	8	8
MIC_{90}	2	>64	2	>64	4	>64	>64	>64	>64	>64	>64	>64
% DS [a]	8.7	16.1	4.2	35.3	3.8	27.3	20	50	30.1	47.4	43.9	38.9
Ct												
MIC_{50}	≤0.06	≤0.06	≤0.06	0.125	≤0.06	≤0.06	0.125	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06
MIC_{90}	0.125	0.25	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	1	0.125
% DS [a]	0	3.1	0	0	0	4.5	0	2.7	1.2	3.7	10.6	3
% R[b]	0	2.6	0	0	0	0	0	1.4	1.2	3.7	8	3
Na												
MIC_{50}	4	8	4	4	4	4	4	4	4	4	4	4
MIC_{90}	4	512	256	256	4	>512	4	8	>512	>512	>512	>512
% DS [a]	8.7	26	11.1	17.6	0	18.2	0	4.1	28.9	38.6	23.7	14.1
Cp												
MIC_{50}	0.015	0.03	0.015	0.03	0.015	0.03	0.03	0.03	0.03	0.03	0.015	0.015
MIC_{90}	0.03	0.5	0.125	0.125	0.03	1	0.03	0.03	0.5	>8	>8	8
% DS [a]	8.7	30.1	9.7	17.6	0	22.7	0	4.1	30.1	41.9	27.2	17.4
% R [b]	0	0	0	0	0	0	0	0	1.2	24.2	21.1	11.1
C												
MIC_{50}	4	8	4	8	8	4	4	8	4	8	4	4
MIC_{90}	8	16	8	8	8	16	256	>256	8	128	8	8
% DS [a]	0	3.1	0	5.9	0	9.1	12	20.3	3.6	21.4	7	5.5
G												
MIC_{50}	0.5	≤0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
MIC_{90}	0.5	0.5	0.5	0.5	0.5	8	1	2	1	8	1	1
% DS [a]	0	1	0	0	0	13.6	0	9.5	2.4	11.6	5.3	5.2
Su												
MIC_{50}	32	64	64	64	64	32	>1024	>1024	64	128	32	32
MIC_{90}	64	>1024	128	>1024	64	>1024	>1024	>1024	>1024	>1024	>1024	>1024
% DS [a]	4.3	27.5	19.4	47.1	5.8	13.6	60	67.6	27.7	47	25.4	24.7
T												
MIC_{50}	1	2	1	16	2	2	64	>64	2	64	2	2
MIC_{90}	2	64	2	>64	4	>64	>64	>64	>64	>64	>64	>64
% DS [a]	0	10.4	1.4	52.9	1.9	27.3	64	73	31.3	62.3	30.7	31.7
Tm												
MIC_{50}	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	0.5	0.5	0.5
MIC_{90}	≤0.25	0.5	0.5	≤0.25	0.5	0.5	≤0.25	>32	0.5	>32	>32	>32
% DS [a]	0	9.8	1.4	0	1.9	45.5	0	14.9	8.4	36.7	22	19.6
MDR [c] % DS	0	13.5	2.8	29.4	3.9	27.3	20	59.5	26.5	53.7	34.5	30

A, Ampicillin; **Ct**, Cefotaxime; **Na**, Nalidixic acid; **Cp**, Ciprofloxacin; **C**, Chloramphenicol; **G**, Gentamicine; **Su**, Sulphamethoxazole; **T**, Tetracycline; **Tm**, Trimethoprim

[a] Decreased susceptibility - EUCAST epidemiological breakpoints

[b] Resistance - EUCAST clinical breakpoints

[c] Multidrug resistance

Table 4.3.3. MIC_{50} and MIC_{90} among the most important *Salmonella* serotypes.

Antimicrobials	*S.* Enteritidis				*S.* Typhimurium		*S.* 4, [5], 12, i:-				*S.* Havana	
	Broilers (*n*=31)	Layers (*n*=13)	Animal feed (*n*=9)	Food Poultry (*n*=10)	Food Swine (*n*=12)	Poultry (*n*=26)	Broilers (*n*=6)	Turkeys (*n*=5)	Food Swine (*n*=29)	Poultry (*n*=16)	Broilers (*n*=102)	Animal feed (*n*=16)
A												
MIC_{50}	2	2	2	2	>64	2	>64	>64	>64	>64	4	2
MIC_{90}	2	2	2	4	>64	4	>64	>64	>64	>64	>64	2
% DS [a]	6.5	0	0	0	66.7	7.7	83.3	100	79.3	56.3	18.6	0
% R [b]	6.5	0	0	0	66.7	7.7	83.3	100	79.3	56.3	18.6	0
Ct												
MIC_{50}	0.125	≤0.06	≤0.06	0.125	0.125	≤0.06	≤0.06	≤0.06	≤0.06	≤0.06	0.25	0.125
MIC_{90}	0.125	0.125	0.125	0.125	0.125	0.125	≤0.06	0.125	0.125	0.125	0.25	0.125
% DS [a]	3.2	0	0	0	0	0	0	0	3.4	0	3.9	0
% R [b]	3.2	0	0	0	0	0	0	0	0	0	3.9	0
Cp												
MIC_{50}	0.25	0.125	0.03	0.25	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
MIC_{90}	0.25	0.25	0.03	0.25	0.03	0.25	0.03	0.03	0.03	0.03	0.5	0.03
% DS [a]	80.6	53.8	0	90	0	15.4	16.7	0	34.5	0	26.5	0
% R [b]	0	0	0	0	0	0	0	0	0	0	0	0
G												
MIC_{50}	≤0.25	0.5	≤0.25	≤0.25	0.5	0.5	≤0.25	0.5	≤0.25	0.5	≤0.25	0.5
MIC_{90}	0.5	0.5	0.5	0.5	0.5	1	0.5	1	1	0.5	0.5	0.5
% DS [a]	0	0	0	0	0	0	0	0	6.9	0	2	0
% R [b]	0	0	0	0	0	0	0	0	6.9	0	2	0
% MDR [c]	3.2	0	0	0	66.7	3.8	83.3	80	82.8	50	13.7	0

A, Ampicillin; **Ct**, Cefotaxime; **Cp**, Ciprofloxacin; **G**, Gentamicine
[a] Decreased susceptibility - EUCAST epidemiological breakpoints
[b] Resistance - EUCAST clinical breakpoints
[c] Multidrug resistance

Characterization of ESBL, PMAβ, PMQR and integrons

ESBL and PMAβ phenotypes were detected in five and two isolates, respectively (Table 4.3.4). In two MDR *S.* Havana (ACtSuT, ACtCpSuTm), one *Salmonella* Heidelberg (ACtSuTm) and one *Salmonella* London (ACt) isolates, *bla*$_{\text{CTX-M-type,}}$ *bla*$_{\text{CTX-M-1}}$, and *bla*$_{\text{CTX-M-14}}$ genes were detected, respectively. Additionally, *bla*$_{\text{CMY-2}}$ gene was encountered in two *S.* Havana isolates showing ACt phenotype. The *bla*$_{\text{SHV-12}}$ gene was detected in one MDR *S. Enteritidis* isolate (showing ACtNaCpCSuT) recovered from a broiler flock. Two *S.* I 4,[5],12:i:- isolates (with ACtSuT and ACtNaCpGT phenotypes), recovered from a pork sausage and a partridge, presented *bla*$_{\text{TEM-1}}$ gene.

Although three isolates, being two *S.* Havana and one *S.* I 4,[5],12:i:-, showed a non wild-type MIC for ciprofloxacin (0.25 and 1µg/mL, respectively), no PMQR-encoding genes were detected (Table 4.3.4).

One isolate of each serotype Havana, Enteritidis and Heidelberg, harboured class 1 integrons and one isolate belonging to serotype I 4,[5],12:i:- carried a class 2 integron.

The ESBL/PMAβ-encoding genes identified in *E. coli* from food-producing animals were *bla*$_{\text{CTX-M-1}}$ (*n*=4), *bla*$_{\text{SHV-12}}$ (*n*=2), *bla*$_{\text{CMY-2}}$ (*n*=2) and *bla*$_{\text{CTX-M-32}}$ (*n*=1); in pets it were *bla*$_{\text{CMY-2}}$ (*n*=4), *bla*$_{\text{CTX-M-15}}$ (*n*=2) and *bla*$_{\text{CTX-M-14}}$ (*n*=1); and in zoo animals were *bla*$_{\text{CTX-M-15}}$ (*n*=5), *bla*$_{\text{CTX-M-1}}$ (*n*=1), and *bla*$_{\text{CTX-M-14}}$ (*n*=1) (Table 4.3.4).

Although the frequency of non-wild type isolates to ciprofloxacine was lower in zoo animals than in pets and food-producing animals (Table 4.3.2), PMQR encoding genes were more frequently detected in isolates recovered from these animals, being *aac(6')-Ib-cr* prevalent (Table 4.3.4).

Six out of nine ESBL/PMAβ isolates from food-producing animals (66.7%), three out of seven isolates from pets (42.9%) and seven out of seven isolates from zoo animals (100%) were MDR. No ESBL/PMAβ-encoding genes were detected in two and three isolates recovered from food-producing animals and pets, respectively, showing resistance to cefoxitin and none of these enzymes were produced by *E. coli* isolates with intermediate susceptibility to this antibiotic.

Most of the *E. coli* isolates harboured class 1 integrons. Nevertheless, class 2 was also found in one isolate recovered from a dog and in one isolate from a turkey.

4.3.4. Discussion

In the last six years, besides *S*. Enteritidis, the serovars Havana and Mbandaka were the most frequently isolated from broilers, layers and poultry feed in Portugal, suggesting that they are well adapted to the poultry population (Clemente *et al.,* 2013; Clemente *et al.,* 2014). It is likely that poultry feed containing cereal grain, imported from some countries, is one of the main sources for the high frequency of occurrence of these serotypes in live birds.

Table 4.3.4. Characteristics of *Salmonella* spp. and *E. coli* isolates displaying non wild-type phenotypes to cefotaxime and or cefoxitin.

Sample	Isolate	Species identification	Animal species	Antimicrobial resistance phenotype	Decreased susceptibility			Genetic profile		Integrons
					Ct[a]	Cp[a]	FOX[b]	*bla*[c] genes	PMQR[d] genes	
Food-producing animals										
	SA12434	*S.* Enteritidis	broiler	A, Ct, Na, Cp, C, Su, T	>8	0.25	S	*bla*$_{SHV-12}$		Class 1
	SA22067	*S.* Havana	broiler	A, Ct, Su, T	>8	0.03	S	*bla* $_{CTX-M-1}$		
	SA34303	*S.* Havana	broiler	A, Ct, Cp, Su, Tm	>8	0.25	S	*bla* $_{CTX-M-type}$		Class 1
	SA6423	*S.* Havana	broiler	A, Ct	8	0.03	R	*bla* $_{CMY-2}$		
	SA6427	*S.* Havana	broiler	A, Ct	8	0.06	R	*bla* $_{CMY-2}$		
	SA18281	*S.* 4,[5],12:i:-	partridge	A, Ct, Na, Cp, G, T	1	1	S	*bla* $_{TEM-1}$		Class 2
	EC32	*E. coli*	turkey	Na, Cp, Su, G, T	≤0.06	8	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1; Class 2
	EC230	*E. coli*	swine	A, Na, Cp, Su, T	≤0.06	0.125	*R*	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	EC235	*E. coli*	turkey	A, Na, Cp, Su, C, T	0.25	>8	R	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	EC241	*E. coli*	swine	A, Su, C, T	0.125	0.015	I	*bla*$_{AMP-C}$, *bla* $_{OXA-type}$		Class 1
	EC261	*E. coli*	broiler	A, Ct, Na, Cp	4	2	R	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$, *bla* $_{CMY-2}$	*qnrS1*	
	EC269	*E. coli*	bovine	A	≤0.06	0.03	I	*bla*$_{AMP-C}$		
	EC276	*E. coli*	swine	A, Ct, Na, Cp, C, Su, T, Tm,	>8	>8	S	*bla*$_{AMP-C}$, *bla* $_{CTX-M-32}$		Class 1
	EC296	*E. coli*	Broiler chicks	A	≤0.06	0.03	R	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$, *bla* $_{CMY-2}$		
	EC350	*E. coli*	turkey	A, Na, Cp, C, Su, T, Tm	0.125	8	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	EC382	*E. coli*	Bovine calf	A, Na, Cp, G, Su, T, Te, C	0.25	>8	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	EC383	*E. coli*	swine	A, Su, T, Tm	≤0.06	≤0.008	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	EC421	*E. coli*	bovine	A, Ct, Na, Cp, C, Su, T, Tm,	>8	>8	S	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$, *bla* $_{CTX-M-1}$		Class 1
	EC427	*E. coli*	chicks	A, Ct, Na, C, Su, T, Tm,	>8	0.06	S	*bla*$_{AMP-C}$, *bla* $_{CTX-M-1}$		Class 1
	EC439	*E. coli*	swine	A, Ct, Su	>8	0.015	S	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$, *bla* $_{CTX-M-1}$		
	LC64	*E. coli*	bovine	A, Ct, Na, Cp, Su, Tm	>8	>8	S	*bla*$_{AMP-C}$, *bla* $_{CTX-M-1}$		Class 1
	LC215	*E. coli*	Broiler chicks	A, Na, Cp, G, T	0.125	8	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	LC217	*E. coli*	Broiler chicks	A, Cp, T	0.125	0.25	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$	*qnrB 19*	
	LC219	*E. coli*	rabbits	A, Na, Cp, Su,	0.125	4	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	EC492	*E. coli*	bovine	A, Ct, Na, Cp, C, Su, T	4	>8	S	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$, *bla*$_{SHV-12}$		Class 1
	EC505	*E. coli*	bovine	A, Ct, Na, Cp, C, Su, T	4	>8	S	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$, *bla*$_{SHV-12}$		Class 1
Pets										
	EC30	*E. coli*	dog	A, Na, Cp, Ct, C, Su, TE, Su, Tm	>8	0.25	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	EC50	*E. coli*	cat	A, Ct	1	0.015	R	*bla*$_{AMP-C}$		
	EC175	*E. coli*	dog	A, Ct	>8	0.015	R	*bla*$_{AMP-C}$, *bla* $_{CMY-2}$		
	EC200	*E. coli*	dog	A, Te	≤0.06	0.015	R	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		
	EC274	*E. coli*	cat	A, Ct, Na, Cp, G, Su, T, Tm	>8	>8	R	*bla*$_{AMP-C}$		Class 1
	EC315	*E. coli*	dog	A, Ct, Su, T, Tm	>8	0.015	S	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$, *bla* $_{CTX-M-14}$		Class 1
	EC321	*E. coli*	cat	A, Ct	0.5	0.015	I	*bla*$_{AMP-C}$		

Sample	Isolate	Species identification	Animal species	Antimicrobial resistance phenotype	Decreased susceptibility			Genetic profile		Integrons
					Ct[a]	Cp[a]	FOX[b]	*bla*[c] genes	PMQR[d] genes	
	EC356	*E. coli*	dog	A, Ct, Na, Cp	>8	>8	S	*bla*$_{AMP-C}$, *CTX-M-15*		
	EC385	*E. coli*	dog	A, Na, Cp, Su, T, Tm	0.25	8	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	EC425	*E. coli*	cat	A, Ct, Na, Cp	8	>8	R	*bla*$_{AMP-C}$, *bla* $_{CMY-2}$		
	EC433	*E. coli*	dog	A, Ct, An, Cp, G, Su, Tm	>8	>8	S	*bla*$_{AMP-C}$, *CTX-M-15;*	*aac(6')-Ib-cr*	Class 1
	EC443	*E. coli*	dog	A, Ct, Na, Cp, Tm	>8	>8	R	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$, *bla* $_{CMY-2}$		Class 2
	EC459	*E. coli*	mandarim	A, Ct	8	0.015	R	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$, *bla* $_{CMY-2}$		
	EC498	*E. coli*	parrot	A, Ct, Na, Cp, Su	2	>8	S	*bla*$_{AMP-C}$		
Zoo animals										
	EC92	*E. coli*	dolphin	A, Ct, An, Cp, G, T	>8	>8	S	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$, *bla* $_{OXA-type}$; *bla* $_{CTX-M-15}$	*aac(6')-Ib-cr*	Class 1
	EC126	*E. coli*	turtledove	A, Na, Cp, C, Su, T, Tm	≤0.06	0.25	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	EC128	*E. coli*	lemur	A, Na, Cp, C, Su, T, Tm	≤0.06	0.25	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	EC163	*E. coli*	frog	A	≤0.06	0.015	I	*bla*$_{AMP-C}$		
	EC212	*E. coli*	dolphin	A, Ct, Na, Cp, G, T	>8	>8	I	*bla*$_{AMP-C}$, *bla* $_{OXA-type}$; *bla* $_{CTX-M-15}$	*aac(6')-Ib-cr*	
	EC248	*E. coli*	tiger	A, Ct, S, T, Tm	>8	0.015	S	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$, *bla* $_{CTX-M-1}$		Class 1
	EC325	*E. coli*	dolphin	A, Ct, Na, Cp, G, T	>8	>8	S	*bla*$_{AMP-C}$, *bla* $_{OXA-type}$; *bla* $_{CTX-M-15}$	*aac(6')-Ib-cr*	
	EC337	*E. coli*	dolphin	A, Na, Cp, C, Su, T, Tm	0.125	>8	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		Class 1
	EC338	*E. coli*	dolphin	A, Ct, Na, Cp, G, T	>8	>8	S	*bla*$_{AMP-C}$, *bla* $_{OXA-type}$; *bla* $_{CTX-M-15}$	*aac(6')-Ib-cr*	
	EC361	*E. coli*	dolphin	A, Ct, Na, Cp, G, T	>8	>8	S	*bla*$_{AMP-C}$, *bla* $_{OXA-type}$; *bla* $_{CTX-M-15}$	*aac(6')-Ib-cr*	
	LC218	*E. coli*	bear	A, Na, Cp, T	0.125	8	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$	*qnrB 19*	
	EC456	*E. coli*	otter	A	0.125	0.03	I	*bla* $_{TEM-type}$, *bla*$_{AMP-C}$		
	EC536	*E. coli*	Cariama cristata	A, Ct, An, Cp, T	>8	>8	S	*bla*$_{AMP-C}$, *bla* $_{CTX-M-14}$		Class 1
Food										
	SA4810	*S.* Heidelberg	broiler carcass	A, Ct, Su, Tm	>8	0.03	S	*bla* $_{CTX-M-1}$		Class 1
	SA31501	*S.* 4,[5],12:i:-	pork sausage	A, Ct, Su, T	2	0.03	S	*bla* $_{TEM-1}$		
	SA31511	*S.* London	Hamburger swine	A, Ct	>8	0.03	S	*bla* $_{CTX-M-14}$		

[a] Minimum Inhibitory Concentration (MIC)
[b] Disk diffusion (Kirby-Bauer method)
A, Ampicillin; **Ct**, Cefotaxime; **Na**, Nalidixic acid; **Cp**, Ciprofloxacin; **FOX**, Cefoxitin; **C**, Chloramphenicol; **G**, Gentamicin; **Su**, Sulfamethoxazole; **T**, Tetracycline; **Tm**, Trimethoprim
S, Susceptible; **I**, Intermediate; **R**, Resistant
[c] β-Lactame genes
[d] Plasmid-mediated quinolone resistance

S. Havana has been previously detected in Danish feather and rapeseed meals imported from other countries (http://unsafefood.eu/notification). Although in European countries the prevalence of serovars Mbandaka and Havana in *Gallus gallus* is low (5.48% and 0.21%, respectively) (EFSA, 2014), is a matter for concern, as they are considered potentially pathogenic for humans (Scheil *et al.,* 1998; Boisrame-Gastrin *et al.,* 2011). With regard to food products, *Salmonella* I 4,[5],12:i:- and *S*. Typhimurium were the most common serotypes recovered from food products, followed by *S.* Enteritidis. Food products and animals seem to be important reservoirs for human infection. As previously reported, monophasic *S.* Typhimurium was in third place in the top 10 list of the most commonly reported serovars in human cases, in 2012, and appears to be of increasing importance in many countries, having caused a substantial number of infections in both humans, and animals bred for food (EFSA, 2014).

In this study, important differences (≥3-fold dilutions) between MIC_{50} and MIC_{90} were noticed among some species, particularly in *E. coli* isolates for ampicillin, nalidixic acid, ciprofloxacin, sulphamethoxazole, tetracycline, and trimethoprim, and also in *Salmonella* spp., for ampicillin, nalidixic acid, sulphamethoxazole and tetracycline. For each antimicrobial, MIC50 and MIC90 distributions indicate that at least two bacterial subpopulations may exist (wild and non-wild type) (Schwarz *et al.,* 2010), which corroborates other findings (de Jong *et al.,* 2009; Clemente *et al.,* 2014).

Regarding *E. coli* isolates, the frequency of non wild-type phenotypes to all antimicrobials tested (except for cefotaxime) was higher in food-producing, followed by companion and zoo animals, which might be due to the high consumption of veterinary antimicrobials, particularly tetracyclines and fluoroquinolones (EMA, 2014). Nowadays, nearly 25% of the isolates from food-producing animals are clinically resistant to ciprofloxacin, leading to the increasing usage of third-generation cephalosporins in food animals. The usage of these antimicrobial is reported to be an important factor for the emergence of extended-spectrum cephalosporins resistant *E. coli* (Liebana *et al.,* 2013). Although the consumption reported for these antimicrobials is not high (0.2mg/PCU) (EMA, 2014), it might be underestimated, as in companion animal practice, human cephalosporins are frequently prescribed.

A comparison between the results obtained in this study with a previous work (Clemente *et al.,* 2013) show that there was an increase in the frequency of non-wild type MICs for cefotaxime in *Salmonella* spp. isolates from broilers and from food products at national level from 0.44% to 1.6%, as reported in other studies [34].

In our study, CTX-M-1, CTX-M-14, TEM-1 and SHV-12 β-lactamases were found in *Salmonella*, being in agreement with findings from other European and non-European countries (EFSA, 2011; Doublet *et al.,* 2014; Rao *et al.,* 2014). The low frequency of TEM-1 and SHV-12 follows the current situation in Europe (EFSA, 2011). However, at our knowledge, SHV-12 was here firstly described in one isolate of *S.* Enteritidis from broilers, in

Portugal. Being *bla*$_{CMY-2}$ the most frequently reported PMAβ-encoding gene in other countries and in different serovars (Dierick *et al.,* 2010; EFSA, 2011; Li *et al.,* 2013), we report its occurrence here for the first time in two isolates of *S*. Havana from broilers, only resistant to β-lactams. The spread of *bla*$_{CMY-2}$ harboring *Salmonella* through the food chain has also important public health implications; like ESBLs, it encodes resistance to third-generation cephalosporins, which is an important class of antibiotics used to treat complicated human infections, including salmonellosis (Allocati *et al.,* 2013).

In all 47 *E. coli* isolates non wild-type to cefotaxime and/or cefoxitin, the ubiquitous *ampC* gene was detected and frequently associated with *bla*$_{TEM-type}$. It should be noted that *E. coli* possess a chromossomal *ampC* gene that is normally repressed or only weakly expressed.

Alterations in *ampC* gene promoter regions increase the production of AmpC and confer variable resistance levels to penicillins and cephalosporins, including cephamycins and oxyimino-cephalosporins, suggesting that this resistance mechanism might have been triggered among our isolates (Li *et al.,* 2007).

Overall, CTX-M-group enzyme isolates was detected in 15 out of 28 (53.6%) isolates exhibiting a non-wild phenotype to cefotaxime. CTX-M-1 was the major ESBL enzyme found in food-producing (*n*=4) and also detected in zoo animals (*n*=1). It has been disseminated in several countries in food-producing animals and in wildlife (Gonçalves *et al.,* 2011; veldman *et al.,* 2013; Rao *et al.,* 2014), although not commonly observed in humans. Therefore, it has been previously reported as a possible cross-contamination between humans, avian hosts and meat, highlighting the importance of its possible transmission to humans (Leverstein van Hall *et al.,* 2011). Currently, CTX-M-15 is the most common ESBL CTX-M variant detected worldwide in clinically important human pathogens (EFSA, 2011). In our work, it was detected in two isolates from companion animals (dogs) and in five isolates from dolphins. Similarly, CTX-M-15 has also been reported in *E. coli* isolates from companion (Sun *et al.,* 2010; Dierick *et al.,* 2012; Hordijk *et al.,* 2013), wild (Veldman *et al.,* 2013) and zoo animals (Wang *et al.,* 2012a; Klimes *et al.,* 2013). Due to the potentially frequent contact between pets and owners, and zoo animals, zookeepers and visitors, bacteria containing such genes might spread among these different reservoirs.

PMAβ enzymes were also found in 6 *E. coli* isolates, from food-producing (*n*=2) and companion animals (*n*=4), confirming what has been found in other studies (Dierick *et al.,* 2010; Dierick *et al.,* 2012)**.**

The presence of PMQR, which have been increasingly reported in animals (Veldman *et al.,* 2011; Wang *et al.,* 2012a; Jones-Dias *et al.,* 2013), can have an additional effect on chromosomal quinolone resistance mechanisms, which might explain the high MIC values of >512mg/l and ≥8mg/l to nalidixic acid and ciprofloxacin, respectively, observed in some of our isolates. Indeed, resistance to quinolones in *Enterobacteriaceae* is mostly linked to chromosomal mutations in the quinolone resistance determining region (QRDR) (Jones-Dias

et al., 2013; Li *et al.,* 2014).

In Europe, *qnrS* and *qnrB* variants were the most frequently detected in different *Salmonella* serotypes from animals and food (Firoozeh, *et al.,* 2012); however, no PMQR-encoding genes were detected among such isolates in our study. Contrarly, *qnrS1* and *qnrB19* genes were detected in *E. coli* isolates from food-producing animals, co-expressing bla_{CMY-2} in one of the isolates, which increases the threat of antimicrobial resistance, as they are plasmid-mediated (Firoozeh, *et al.,* 2012). In pets and zoo animals, *aac(6')-Ib-cr* was the most frequently PMQR detected, which is common in human *E. coli* spreading worlwide (Mendonça *et al.,* 2007; Firoozeh, *et al.,* 2012; Nicholas-Chanoine *et al.,* 2014). The spread of MDR isolates producing ESBL or PMAβ is a matter of concern, especially when they carry other resistant traits conferring resistance to aminoglycosides, fluoroquinolones or mobile genetic elements like integrons (Nicholas-Chanoine *et al.,* 2014).

Among the nine *Salmonella* non-wild type isolates for cefotaxime found in our study, six were MDR (66.7%); additional resistance was also detected for sulphamethoxazole, tetracycline, trimethoprim and gentamicin. Since these drugs are used in animal production, co-selection may have played a role for the arising of ESBL-producing isolates, comparing with previous studies (Clemente *et al.,* 2013).

Thirty five (35/47, 74.5%) *E. coli* isolates were MDR, comprising a significant contribution of the food-producing animals group. Twenty seven (77.1%, 27/35) carried integrons. Similarly, among six MDR *Salmonella* isolates, four also carried integrons (66.7%). This supports the hypothesis of an association between the presence of emerging MDR isolates and integrons, as well as other mobile genetic elements, contributing to the spreading of antimicrobial resistance determinants (Clemente *et al.,* 2013).

In conclusion, we report for the first time the detection of bla_{CMY-2} gene in two isolates of *S.* Havana from broilers and bla_{SHV-12} in one isolate of *S.* Enteritidis also from broilers, in Portugal.

Overall, the results shown in this study indicate that animals should be considered as potential reservoirs for ESBL-, PMAβ- and PMQR-producing isolates. Prudent usage of antimicrobials in animals should be strongly encouraged, as well as the characterization of antimicrobial resistance genes, to monitor future trends in the occurrence of resistance to oxymino-β-lactams and fluoroquinolones.

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4.4. CTX-M-15-Producing Escherichia coli in a Dolphin, Portugal

This research paper was submitted as:

Vera Manageiro, **Lurdes Clemente***, Daniela Jones-Dias, Teresa Albuquerque, Eugénia Ferreira, Manuela Caniça. Zoonotic potential of multidrug resistant CTX-M-15-producing Escherichia coli isolate of a marine dolphin, in Portugal.* ***Emerging Infectious Diseases****, 2015, 21: 2249-51.*

Contributions of the authors for the manuscript:

Vera Manageiro: conception and design of study, acquisition of laboratory data, analysis of data, drafting of the article and critical revision and final approval of the manuscript;
Lurdes Clemente: design of study, acquisition of laboratory and epidemiological data, analysis of data, drafting of the article, critical revision and final approval of the manuscript;
Daniela Jones-Dias: design of study, acquisition of laboratory, of data, drafting of the article, critical revision and final approval of the manuscript;
Teresa Albuquerque, acquisition of laboratory and epidemiological data, final approval of the manuscript;
Eugénia Ferreira, acquisition of laboratory data, final approval of the manuscript;
Manuela Caniça, conception and design of study, colaboration in drafting the article and critical revision and final approval of the manuscript;

**These authors had equal participation in this study*

ABSTRACT

The transboundary dissemination of ST131 CTX-M-15-producing *Escherichia coli* is a subject of concern. Here, we evaluated the zoonotic potential of an isolate recovered from a captive bottlenose dolphin, by comparing its genotype with the genotype of human clinical isolates, and by investigating the genetic relatedness between them. The relationship between isolates recovered from humans and animals underlines the interspecies spread of multidrug resistant ST131 *E. coli.*

Keywords: *Escherichia coli*, dolphin, CTX-M-15, *fimH3*

Main Text

The global emergence and pandemic spread of ST131 CTX-M-15-producing *E. coli* in humans and its detection in livestock, companion animals and wildlife is a major cause for concern (Caratolli *et al.,* 2008; Nicholas-Chanoine, 2014). Hence, it is imperative to identify and explore their dissemination traits. If they continue to spread among different environments, therapeutic options will be greatly narrowed in both veterinary and human medicine (Caratolli *et al.,* 2008*)*. *E. coli* is one of the most frequently Gram-negative bacteria isolated from bottlenose dolphins (Morris *et al.,* 2011). However, few studies have been published regarding antibiotic resistant bacteria associated to dolphins (Greig *et al.,* 2007; Schaefer *et al.,* 2009; Stewart *et al.,* 2014). In this study, we established a linkage of dissemination between a CTX-M-15-producing *E. coli* isolated from a marine dolphin, *Tursiops truncatus, versus* human clinical isolates collected all over the country, in the same period.

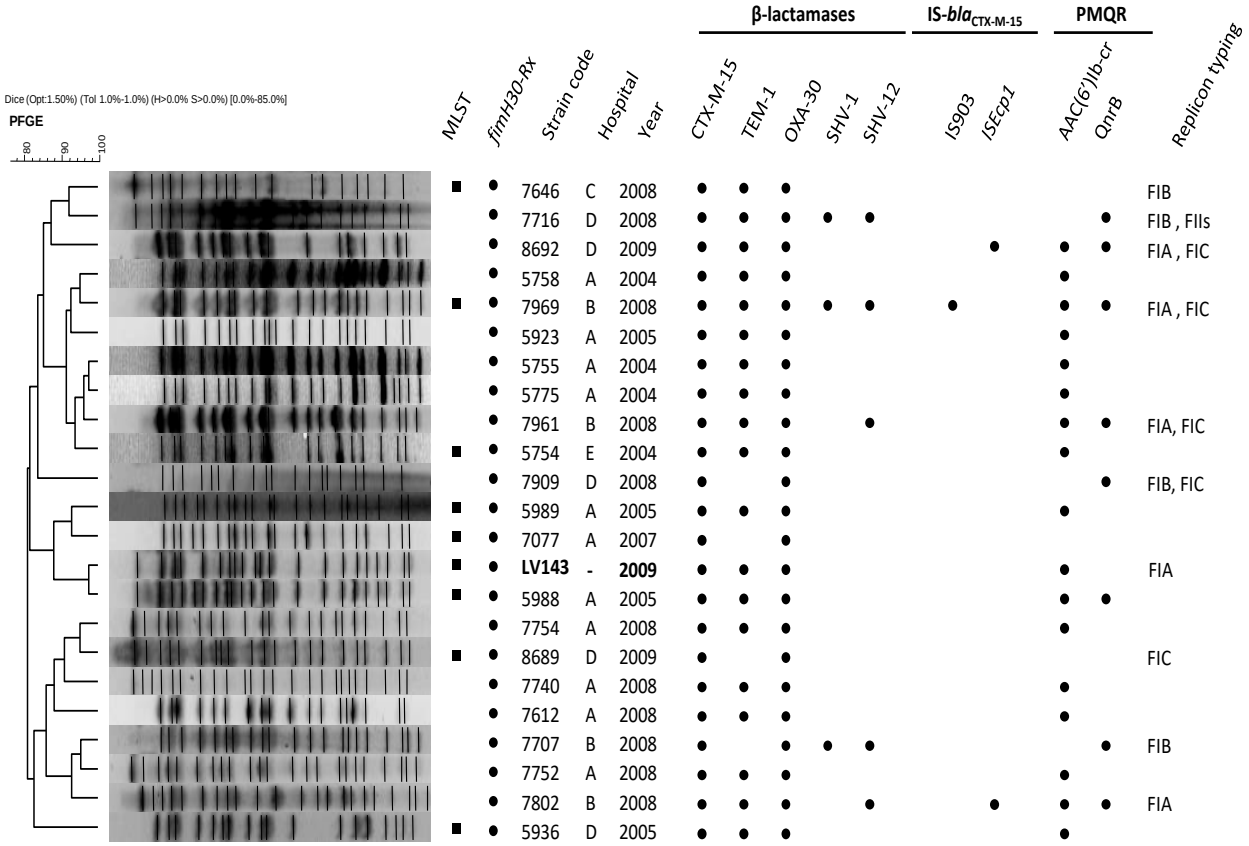
In 2009, one *Escherichia coli* strain (LV143) isolated from a respiratory exudate collected through the spiracle of a female dolphin from a Zoo Park, was sent to the National Institute of Agrarian and Veterinary Research (INIAV, Lisbon), for bacteriological, mycological analysis and antimicrobial susceptibility tests; no clinical history of the animal was evidenced. Mycological examination was negative for the detection of fungi and yeasts.

Determination of antimicrobial susceptibility of the dolphin *E. coli* strain (LV143), performed by the agar dilution method and interpreted according to European Committee of Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org/), revealed a non-wild-type phenotype to cefotaxime (MIC >8 µg/mL); it also showed a synergy towards the clavulanic acid, suggesting extended-spectrum β-lactamase (ESBL) production. LV143 was also non-wild-type to ampicillin (MIC >64 µg/mL), nalidixic acid (MIC >512 µg/mL), ciprofloxacin (MIC >8 µg/mL), gentamicin (MIC >32 µg/mL) and tetracycline (MIC >64 µg/mL). This isolate remained wild-type to chloramphenicol (MIC=4 µg/mL), florfenicol

(MIC=8 µg/mL), sulfamethoxazole (MIC=32 µg/mL), trimethoprim (MIC ≤0.25 µg/mL) and streptomycin (MIC=4 µg/mL).

For the analysis of its zoonotic potential, 61 clinical *E. coli* isolates, previously recovered from different specimens from 2004 to 2009 in seven geographically apart Portuguese hospitals (Figure 4.4.1), were selected from National Reference Laboratory of Antibiotic Resistances and Healthcare Associated Infections (NRL-AR/HAI) collection, and included in this study. Inclusion criteria for the clinical isolates were: 1) non-wild type susceptibility to cefotaxime; 2) presumptive phenotypic ESBL production; 3) genetic similarity by Pulsed-Field Gel Electrophoresis (PFGE). Genetic relatedness analysis of human and dolphin isolates determined by PFGE using *Xba*I digested DNA (7), revealed one major cluster, which included 22 (35%) clinical isolates from three different Portuguese regions, and the dolphin

Figure 4.4.1. Dendogram of PFGE profiles showing the relationship between a clonal strain of *E. coli* of animal origin (LV143, in bold), and 22 isolates of *E. coli from huma*ns. We used the method and the Dice coefficient *w*ith 1.8% optimization and band position tolerance of 1%. Isolates with a Dice band-based similarity coefficient value of ≥80% were considered to belong to the same cluster. From left to right are represented: the dendrogram, MLST (Black squares: positive for ST131), *fimH* typing, the strain code, hospital code, year of isolation, detected β-lactamases, genetic association between insertion sequences (IS) and CTX-M-15-encoding genes, detected PMQR-encoding genes, and replicon typing groups results. β-lactamases and PMQR-encoding genes, and IS combinations are indicated by black circles. *E. coli* clinical isolates which were genetically unrelated to the dolphin isolate are not shown.



The genetic characterization of the 22 clinical isolates and one dolphin strain, was performed by PCR and sequencing targeting the most prevalent ESBL (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-G1}$, *bla*$_{CTX-M}$)- and plasmid-mediated quinolone resistance [PMQR: *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *aac(6')Ib-cr*]-encoding genes, as previously described (Manageiro *et al.,* 2012). Specifically, concerning the strain recovered from dolphin, it presented *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *bla*$_{OXA-30}$, associated with a PMQR gene, the *aac(6')-Ib-cr* (Figure 4.4.1). All clinical isolates were also positive to *bla*$_{CTX-M-15}$, and *bla*$_{OXA-30}$ genes; 18 isolates presented the *bla*$_{TEM-1}$ gene, 3 *bla*$_{SHV-1}$, 5 *bla*$_{SHV-12,}$ 8 *qnrB* and 16 the *aac(6')-Ib-cr* gene. The presence of class 1 integron, IS*Ecp1*, IS*26* and IS*903* elements were also investigated as previously (Jones-Dias *et al.,* 2013). The LV143 strain was positive for the insertion sequence IS*Ecp1*, associated with *bla*$_{CTX-M-15}$, being negative for the class 1 integron (data not shown). In two clinical isolates we identified IS*Ecp1* and in one IS*903*. PCR-based replicon typing (PBRT) (Caratolli *et al.,* 2005) revealed the presence of IncF plasmid group in both, animal and in nine human isolates (a selected sample to evaluate PBRT) (Figure 4.4.1).

MLST was performed for a selected group (n=9 out of 23) of *E. coli* isolates. Referring to the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli), it was demonstrated that the dolphin and the human clinical epidemic clone exhibited the same combination of alleles across the seven sequenced *loci*, corresponding to the epidemic ST131, associated with CTX-M-15 and wide-disseminated in Portuguese hospitals (Manageiro *et al.,* 2012; Nicholas-Chanoine, 2014). Within-ST subclones were analyzed on the basis of sequence variation of the *E. coli* fimbrial adhesin gene *fimH*, as previously described (Weissman *et al.,* 2012). The *fimH30-Rx* lineage was identified in all 23 *E. coli* isolates (fluoroquinolone resistant and CTX-M-15 positive) that clustered together on the dendrogram, regardless of MLST result (Figure 4.4.1).

It is worth noting that *bla*$_{CTX-M-type}$ gene has being described in ESBL-positive *E. coli* isolates from different healthy animals, namely in mammalian animals (Caratolli, 2008). However, at our knowledge, this is the first description of an *E. coli* ESBL-producing isolate from a dolphin, and namely from CTX-M family.

In conclusion, this study illustrated the clonality among clinical isolates and a dolphin strain with common antibiotic resistance genes, specifically *bla*$_{CTX-M-15}$, *aac(6')-Ib-cr*, as well as common plasmids, such as those from group IncF. They have gone through identical evolutionary genetic events which ultimately led to the establishment of the same allelic diversity pattern (ST131 *fimH30-Rx*). Overall, the linkage found between these two reservoirs highlights the importance of the isolate's zoonotic potential.

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4.5. *New insights into resistance to colistin and third-generation cephalosporins of Escherichia coli in poultry, Portugal: novel bla$_{CTX-M-166}$ and bla$_{ESAC}$ genes*

This research paper was submitted as:

Vera Manageiro, **Lurdes Clemente***, Rafael Graça, Ivone Correia, Teresa Albuquerque, Eugénia Ferreira, Manuela Caniça. New insights into resistance to colistin and third-generation cephalosporins of Escherichia coli in poultry, Portugal: novel bla$_{CTX-M-166}$ and bla$_{ESAC}$ genes. International Journal of Food Microbiology, 2017, 263, 67–73.*

Contributions of the authors for the manuscript:

Vera Manageiro: molecular assays, bioinformatic analysis, analysis of data, critical revision and final approval of the manuscript;
Lurdes Clemente: microbiological and molecular assays, analysis of data, critical revision and final approval of the manuscript;
Rafael Graça: microbiological and molecular assays;
Ivone Correia: acquisition of laboratory data; final approval of the manuscript;
Teresa Albuquerque: acquisition of laboratory data; final approval of the manuscript;
Eugénia Ferreira: microbiological and molecular experiments
Manuela Caniça: Conception and design of the study; critical revision and final approval of the manuscript.

**These two authors had equal contribution in the study.*

ABSTRACT

The increasing incidence of intestinal colonization with extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* and Gram negative organisms that has been observed in food animals such as poultry, cattle and pigs, are suggestive that animals, food and environment are potential sources of ESBL-producing bacteria. Hence, the aim of this study was to characterized commensal *E. coli* obtained from healthy broiler and turkey flocks at slaughter for the presence of penicillinases-, ESBL-, extended-spectrum AmpC (ESAC)-, plasmid-mediated quinolone resistance- and MCR-encoding genes. Study of clonal relatedness showed genetic diversity among CTX-M-type, SHV-12 and TEM-52 producing isolates with human isolates of the same type, was also assessed. We detected that eleven (5.4%, 11/202) and forty-five (2.2%, 45/185) *E. coli* isolates from broilers and turkeys, respectively, carried bla_{ESBL} or bla_{ESAC} genes and two isolates from turkeys carried *mcr-1* gene. A new variant $bla_{CTX\text{-}M\text{-}166}$ was reported in a multidrug resistant isolate from a broiler flock. Overall, we detected a diversity of resistance mechanisms among *E. coli* from food-producing animals, all of them with high importance at a public health level.

Keywords:

Food-producing animals; ESBL; MCR-1

4.5.1. Introduction

Antimicrobials have been used in animals for treatment, prevention and control of diseases, and also as growth promoters (Marshall and Levy, 2011). Although growth promoters were banned in the European Union (EU) in 2006, this had not led to a decrease in the consumption of antimicrobials in Europe; on the contrary, an increase of metaphylactic and prophylactic use was observed (Woolhouse *et al.,* 2015).

Commensal *Escherichia coli* is typically chosen as an indicator of antimicrobial resistance in Gram-negative bacteria, as it is commonly present in animal faeces. Monitoring of antimicrobial resistance in *E. coli*, isolated from either randomly selected healthy animals or carcasses and meat derived thereof, provides valuable data, not only on the resistance pattern occurring in that population, but also in the relationship with the selective pressure exerted by the use of antimicrobials on the intestinal population of bacteria in food-producing animals (EFSA/ECDC, 2014).

E. coli is the most prevalent microorganism of gastrointestinal tract of humans and animals (food-producers, companion and wild) and one of the most frequent causes of several bacterial infections (Allocati *et al.,* 2013). Hence, it might constitute a reservoir of resistance genes, which can spread horizontally to zoonotic and other bacteria (EFSA/ECDC, 2014; Marshall and Levy, 2011). The increasing incidence of colonization with extended-spectrum

β-lactamase (ESBL)-producing *Enterobacteriaceae* and Gram negative organisms has been observed in food animals such as poultry, cattle and pigs, suggesting that animals, food and environment are potential sources of ESBL-producing bacteria (Seiffert *et al.,* 2013).

Within Europe and in most countries, the occurrence of resistance to cefotaxime in *E. coli* isolates is low, although in some countries moderate to high levels of resistance in broilers, has been observed (EFSA/ECDC, 2015). In Portugal, food-producing animals has been described as sources of ESBL-producing *E. coli* mostly associated with the spread of epidemic plasmids, within and among different farms (Rodrigues *et al.,* 2013; Clemente *et al.,* 2015; Jones-Dias *et al.,* 2016c).

Moreover, even low levels of resistance to these critically important antimicrobials are of great concern due to the spreading of multidrug resistant (MDR) ESBL- and plasmid-mediated AmpC β-lactamases (PMAβ)-producing isolates and to the use of different other antimicrobials that may co-select other resistance determinants (Dierikx *et al.,* 2010; Clemente *et al.,* 2015). This is the case of the new plasmid-mediated colistin resistance mechanism, encoded by the *mcr-1* gene (Quesada *et al.,* 2016; Skov and Monnet, 2016). Colistin has been largely used in veterinary, particularly in food-producing animals, especially as group treatment for pigs, veal calves and poultry, allowing to a high exposure of the gastrointestinal bacteria (EMA, 2016).

The purpose of this study was to characterize the new $bla_{\text{CTX-M-166}}$ variant reported in a MDR isolate collected from a broiler flock, using whole-genome sequencing (WGS). This technique is highly discriminative for typing of foodborne isolates and facilitates a rapid *in silico* analysis of the bacterial resistome, virulome and mobilome, allowing faster and deeper strain characterization (Moran-Gilad, 2017). CTX-M-166-producing *E. coli* was identified during an evaluation study of commensal *E. coli* recovered from broiler and turkey poultry flocks for antimicrobial susceptibility testing and identification of plasmid-mediated genes related to β-lactam and colistin resistance. The potential clonal relatedness between poultry and human isolates was also determined.

4.5.2. Materials and methods

Bacterial isolates

This study included 387 commensal *E. coli* isolates selected and recovered from 1016 poultry cecum samples [broilers, (*n*=202 out of 680) and turkeys (*n*=185, out of 336)], collected under the scope of monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria (Commission Decision 652/2013), throughout 2014. Sampling took place from April to December throughout the different slaughterhouses located in three different agrarian regions (Entre-Douro-e-Minho, Beira Litoral and Ribatejo Oeste), where the

national poultry production is concentrated. The main poultry farmers and poultry flocks raised during sampling time were covered. Each composite sample consisted of five cecum samples from five different birds of the same flock and submitted to the laboratory in sterile containers under refrigeration. Bacteriological analysis for the search of commensal *E. coli* was performed within 24 hours after collection. Each composite sample was mixed in physiological saline and 10 µL plated on MacConkey Agar, followed by incubation at 37ºC for 18 to 24hrs; no enrichment broth or selective culture medium were used. Typical lactose fermenter colonies were confirmed by means of API 20E strips (bioMérieux, France). After biochemical confirmation, all *E. coli* isolates were cryopreserved at -70ºC.

Antimicrobial susceptibility testing and phenotypic screening for β-lactamase production

Antimicrobial susceptibility testing of 387 *E. coli* isolates from broilers (*n*=202) and turkeys (*n*=185) were studied. Minimum inhibitory concentrations (MICs) were determined by agar dilution in a two-fold concentration series. The antibiotics tested were: ampicillin, cefotaxime, ceftazidime, colistin, nalidixic acid, ciprofloxacin, gentamicin, meropenem, chloramphenicol, sulphamethoxazole, tigecycline, tetracycline and trimethoprim. In order to assess the antibiotic susceptibility of the strains, interpretation of the results was done according to the epidemiological cut-off values recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://mic.eucast.org/Eucast2/).

Isolates resistant to 3rd generation cephalosporins were tested with a second panel of antibiotics, which further included cefoxitin, cefepime, temocillin, meropenem, ertapenem, imipenem, cefotaxime/clavulanate and ceftazidime/clavulanate. Commercial standardized microplates (EUVSEC2, TREK, USA), and the microdilution technique (EUCAST/ESCMID, 2003) were used accordingly to manufacturer's instructions. Isolates were considered multidrug resistant (MDR) if they presented non-wild-type phenotypes against three or more structurally unrelated antibiotics.

Isolates were characterized as presumptive ESBL, AmpC and/or carbapenemase producers, accordingly to the presence of synergy between 3rd generation cephalosporins plus clavulanate, cefoxitin and/or ceftazidime plus cloxacillin, and/or carbapenems and boronic acid or with dipicolinic acid (class A or Class B, respectively) according to previously reports (Jones-Dias *et al.,* 2014; Manageiro *et al.,* 2015d).

Molecular characterization of antimicrobial resistance and integrons

In the fifteen *E. coli* isolates from broilers (n=11) and turkeys (n=4) evidencing non susceptible phenotype to cefotaxime and ceftazidime and/or cefoxitin, *bla*$_{ESBL}$ (*bla*$_{TEM}$, bla$_{SHV}$, *bla*$_{OXA}$, *bla*$_{CTX-M}$) and *bla*$_{PMAβ}$ (*bla*$_{CMY}$, *bla*$_{MOX}$, *bla*$_{FOX}$, *bla*$_{LAT}$, *bla*$_{ACT}$, *bla*$_{MIR}$, *bla*$_{DHA}$, *bla*$_{MOR}$, *bla*$_{ACC}$) encoding genes were screened by PCR, as previously described (Clemente *et al.,* 2015). *E. coli* chromosomal *ampC* gene, including its promoter region, was also analysed by

PCR for three strains with no ESBL- and/or PMAβ-encoding gene detected by using Int-B2 and Int-H1 primers (Mammeri *et al.,* 2006). Chromosomal AmpC from *E. coli* ATCC 25922 was used as standard. Additionally, isolates evidencing decreased susceptibility to quinolones and/or colistin were screened for the presence of plasmid-mediated quinolone resistance (PMQR: *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6´)-Ib-cr*, *oqxAB*, and *qepA*)-encoding genes using primers and conditions previously described (Clemente *et al.,* 2015), and for plasmid-mediated colistin resistance (*mcr*)-encoding genes using primers and conditions first described in this study (mcr-Fint: 5′-TCCGATCATGCCAATCTAC-3′ and mcr-Rint: 5′-CAAGATACTTACGCCCAAG-3′; initial denaturation of 94ºC for 5 min; 94ºC for 30 s, 53.1ºC for 30 s and 72ºC for 1 min, for 30 cycles; final step of extension of 72ºC for 5 min).

The isolates were also subjected to the detection of class 1, 2 and 3 integrase encoding genes, as reported elsewhere (Clemente *et al.,* 2015). Positive and negative controls were used in all PCR reactions. PCR products were purified and all amplicons were further sequenced directly on both strands using automatic sequencer ABI3100 (Applied Biosystems).

Pulsed-Field Gel Electrophoresis (PFGE)

Eleven out of 15 isolates presenting a non-wild type phenotype to 3rd generation cephalosporins and/or cefoxitin, being ESBL producers from CTX-M (*n*=4), TEM (n=1) and SHV (*n*=6) families, were submitted to PFGE analysis, as previously described (Mendonça *et al.,* 2007). Furthermore, CTX-M-type (*n*=14), and TEM-52- (n=7) producing *E. coli* isolates from the biobank of the National Reference Laboratory of Antibiotic Resistances, at National Institute of Health, in Lisbon, were used to determine the genetic relatedness with human isolates. Banding patterns were analyzed by using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The unweighted-pair-group method was used to construct a dendrogram based on PFGE XbaI restriction patterns of the 32 *E. coli* isolates. The Dice band-based similarity coefficient, with a band position tolerance of 1.0% and an optimization of 1.0%, was used for clustering. Isolates with a Dice band-based similarity coefficient value of ≥ 80% were considered to belong to the same cluster.

Characterization of CTX-M-166-producing E. coli

Cloning experiments and gene location

In order to characterize the new CTX-M-166 variant, *E. coli* transformants were obtained by cloning the $bla_{\text{CTX-M-166}}$ (from LV13072) gene into the pBK-CMV phagemid vector (Stratagene) and transforming into *E. coli* TOP10 OneShot chemically competent cells

(Invitrogen). Simultaneously, the parental *bla*$_{CTX-M-1}$ (from LV21400) gene was used for comparison.

E. coli transformants were selected on MacConkey agar supplemented with 30 mg/L of kanamycin and 2 mg/L of cefotaxime. The presence and orientation of the inserted genes was confirmed by PCR; MICs of *E. coli* TOP10 recipient and transformants were determined as mentioned above. The I-CeuI technique was employed to evaluate the genomic location of *bla*$_{CTX-M-166}$ (Liu *et al.,* 1993).

Molecular characterization

CTX-M-166-producing *E. coli* was genotypically characterized by whole-genome sequencing (WGS), as previously described (Manageiro *et al.,* 2016). The assembled contigs were analysed and studied for the presence of antibiotic resistance and virulence genes, multi-locus sequence (MLST) types, *fim* type, serotype, and plasmid replicon types using bioinformatics tools (https://cge.cbs.dtu.dk/services/). PHAST (http://phast.wishartlab.com/) and ISsaga (http://issaga.biotoul.fr/issaga_index.php) tools were applied to identify prophage and insertion sequences (ISs), respectively. The genetic location of *bla*$_{CTX-M-166}$ was determined by extracting the contig harbouring the *bla* gene and compared with sequences deposited in the GenBank sequence database provided by NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Nucleotide sequence accession number

The new *bla*$_{CTX-M}$ nucleotide sequence was submitted to the GenBank Database as *bla*$_{CTX-M-166}$ with accession number KU978909.

4.5.3. Results and Discussion

The rapid dissemination of CTX-M enzymes in human and veterinary settings, among commensal bacteria of humans and animals, and in the environment, is a major contribution for both the increase of resistance to 3rd generation cephalosporins among *Enterobacteriaceae*, and the use of carbapenems and colistin (Madec *et al.,* 2017).

In this study, from the 387 *E. coli* isolates, reduced susceptibility to 3rd generation cephalosporins (cefotaxime) was observed in 5.4% (11/202) of the isolates recovered from broilers and 2.2% (4/185) from turkeys (Table 4.5.1), which values were not high percentages comparing with other European countries (EFSA/ECDC, 2014). The frequency of non-wild type *E. coli* isolates from broilers and turkeys to the other antimicrobials was respectively: ciprofloxacin (90.6 and 79.5%), nalidixic acid (88.6 and 73.5%), ampicillin (75.7 and 80%), sulfamethoxazole (69.3 and 71.9%), tetracycline (66.3 and 85.9%), trimethoprim (54.5 and 49.7%), chloramphenicol (34.2 and 52.4%), colistin (3 and 27%) and gentamicin (10 and 9.7%). MDR was observed in 81.3% of the isolates.

Table 4.5.1. MIC_{50} and MIC_{90} for 387 *Escherichia coli* isolates: broilers (*n*=202) and turkeys (*n*=185).

Antimicrobials	*Escherichia coli*		Breakpoints (mg/L)	
	Broilers (*n*=202)	**Turkeys** (*n*=185)	**DS**[a]	**R**[b]
Am			>8	
MIC_{50}	>64	>64		
MIC_{90}	>64	>64		
% DS [a]	75.7	80		
Ct			> 0.25	> 2
MIC_{50}	≤0.25	≤0.25		
MIC_{90}	≤0.25	≤0.25		
% DS [a]	5.4	2.2		
% R[b]	2	0.5		
TZ			> 0.5	> 4
MIC_{50}	≤0.5	≤0.5		
MIC_{90}	≤0.5	≤0.5		
% DS [a]	5.4	2.7		
% R[b]	3	2.7		
Na			>16	
MIC_{50}	>128	>128		
MIC_{90}	>128	>128		
% DS [a]	88.6	73.5		
Cp			> 0.06	> 1
MIC_{50}	2	2		
MIC_{90}	>8	>8		
% DS [a]	90.6	79.5		
% R[b]	51.5	50.3		
GM			> 2	
MIC_{50}	≤0.5	≤0.5		
MIC_{90}	2	16		
% DS [a]	10	9.7		
CL			> 2	> 2
MIC_{50}	≤1	≤1		
MIC_{90}	≤1	16		
% DS [a]	3	27		
% R[b]	3	27		
C			>16	
MIC_{50}	≤8	32		
MIC_{90}	>128	>128		
% DS [a]	34.2	52.4		
MP			> 0.125	> 8
MIC_{50}	≤0.03	≤0.03		
MIC_{90}	≤0.03	≤0.03		
% DS [a]	0	0		

Antimicrobials	Escherichia coli		Breakpoints (mg/L)	
	Broilers (n=202)	Turkeys (n=185)	DS[a]	R[b]
TGC			> 1	
MIC_{50}	0.5	0.5		
MIC_{90}	1	1		
% DS [a]	0	0		
Su			> 64	
MIC_{50}	>1024	>1024		
MIC_{90}	>1024	>1024		
% DS [a]	69.3	71.9		
Te			> 8	
MIC_{50}	>64	>64		
MIC_{90}	>64	>64		
% DS [a]	66.3	85.9		
T			> 2	
MIC_{50}	>32	1		
MIC_{90}	>32	>32		
% DS [a]	54.5	49.7		

Am, ampicillin; **Ct**, cefotaxime; **TZ**, ceftazidime; **Cp**, ciprofloxacin; **CL**, colistin; **C**, chloramphenicol; **T**, trimethoprim; **Su**, sulfamethoxazole; **GM**, gentamicine; **Te**, tetracycline; **TGC**, tigecycline; **MP**, meropenem; **Na**, nalidixic acid
[a] Decreased susceptibility - EUCAST epidemiological breakpoints
[b] Resistance - EUCAST clinical breakpoints

Furthermore, we found ESBL-producing isolates presenting co-resistance to other non-β-lactam antimicrobials: with quinolones, sulfamethoxazole, tetracycline, chloramphenicol (6/15, 40%); quinolones, sulfamethoxazole, tetracycline and trimethoprim (2/15, 13.33%); quinolones, sulfamethoxazole, tetracycline, trimethoprim, chloramphenicol and colistin (1/15, 6.6%); and quinolones, sulfamethoxazole, tetracycline, trimethoprim, chloramphenicol, gentamicin and colistin (4/15, 26.7%), as observed in other studies (Schink *et al.,* 2013). Certainly, the high selective pressures exerted by the massive use of antibiotics (e.g. colistin), particularly in the poultry sector, combined with an efficient gene capture and spread of resistance determinants by mobile genetic elements (e.g. third-generation cephalosporin resistant genes), are also factors to be considered. Indeed, all these factors contribute for the co-selection of CTX-M producing strains in the different settings and environment (Madec *et al.,* 2017).

Molecular characterization of the fifteen isolates non susceptible to cefotaxime and/or cefoxitin (Table 4.5.2) allowed the detection of a high diversity of resistance mechanisms, such as the penicillinases TEM-1 (*n*=4) and OXA-type (*n*=1), and ESBLs TEM [TEM-52 (*n*=1)], SHV [SHV-12 (*n*=7)] and CTX-M [CTX-M-1 (*n*=1); CTX-M-32 (*n*=2); and CTX-M-166 (*n*=1)]. CTX-M-166 differed from CTX-M-1 by the amino acid substitution Ala120Val. PMQR

Aac(6')-Ib-cr (*n*=1) and QnrB19 (*n*=1) were also identified, alone or in co-expression. All isolates presented the ubiquitous AmpC enzyme; in two isolates (LV10909 and LV19991), this β-lactamase was the only one produced (Table 4.5.2). Moreover, in two phenotype and genotype different isolates recovered from turkeys, in the same slaughterhouse agrarian region, but being separated by 5 months (Table 4.5.2), *mcr-1* gene was detected; only one of them co-expressed the SHV-12, TEM-1 and OXA-type enzymes. As reported in other studies, *mcr-1* gene has been detected in various bacterial species, from different food-producing animals, from the environment including rivers and from various types of meat and vegetables (Jones-Dias *et al.,* 2016d; Quesada *et al.,* 2016; Skov and Monnet, 2016). Indeed, though underdetected, this gene has been present for a long time, having been detected in three *E coli* isolates from 1980s, when colistin first started to be used in food-producing animals in China (Shen *et al.,* 2016).

The sequencing of the chromosomal *ampC* gene characteristic of the *E. coli* species was performed for the three *E. coli* isolates with reduced susceptibility to extended-spectrum cephalosporins, without any ESBL detected. Results revealed highly conserved mutations in the promoter/attenuator region of *E. coli* LV9211 at positions -42, -18, -1, +58 and +81, with respect to the *ampC* open reading frame, found in strong *ampC* promoters (Table 4.5.3) (Tracz *et al.,* 2007; Guillouzouic *et al.,* 2009). Indeed, these mutations have previously been described to cause hyperproduction of AmpC in French cattle and Finnish food-producing animal *E. coli* isolates (Haenni *et al.,* 2014; Päivärinta *et al.,* 2016).

Full sequencing of the *E. coli* LV10908 *ampC* gene also showed that amino acid substitutions of the AmpC enzyme were mainly detected in positions associated with narrow-spectrum AmpCs, with some exceptions (Q7K, S236I, L254V, and I284V). In *E. coli* LV19991 the exceptions were at positions P5S, Q7K, A220T and R232C (Table 4.5.3). In fact, these AmpCs showed mutations within two specific locations responsible for the conformational modifications in ESAC β-lactamases (Nordmann & Mammeri, 2007; Mammeri *et al.,* 2008): the H-9 helix (I284V, in LV10908) and the Ω-loop (A220T, in LV19991).

This is, at our knowledge, the second description of ESACs-type in animals. Indeed, these β-lactamases were mainly reported in human isolates, with the exception of ESAC-producing *E. coli* strains detected in cattle, in France (Haenni *et al.,* 2014). Thus, this is, at our knowledge, the second description of ESACs-type in animals.

Table 4.5.2. Characteristics of 15 *E. coli* isolates displaying non wild-type phenotypes to 3rd generation cephalosporins and or cephamycins.

Isolate Nº	Sampling Agrarian Region	Date of Isolation	Animal species[b]	Resistance phenotype[c]	Decreased susceptibility (µg/mL)[d]								Genetic profile[e]		
					Ct	CCt	TZ	CTZ	CX	CFP	Cp	CL	β-lactam genes	PMCR[f] gene	Integrons
9211	RO	15/04/2014	B	NaCpAmCtTZCXSuTeT	1	2	8	4	>64	0.25	8	≤1	*bla*$_{AmpC}$; *bla*$_{TEM-1b}$		Class 1
9370	RO	23/04/2014	T	NaCpAmCtTzCCLSuTeT	2	≤0.06	>8	≤0.125	8	0.5	>8	8	*bla*$_{AmpC}$; *bla*$_{TEM-1}$; *bla*$_{OXA}$; *bla*$_{SHV-12}$	*mcr-1*	Class 1
10599	RO	06/05/2014	T	AmCtTzCSuTe	4	≤0.06	>8	≤0.125	4	1	≤0.015	≤1	*bla*$_{AmpC}$; *bla*$_{SHV-12}$		Class 1
10908	RO	13/05/2014	B	NaCpAmCtTZGMSuTeT	1	≤0.06	4	≤0.125	8	2	8	≤1	*bla*$_{AmpC}$		
13059	RO	02/06/2014	B	NaCpAmCtTzCSuTeT	1	≤0.06	>8	≤0.125	8	2	8	≤1	*bla*$_{AmpC}$; *bla*$_{SHV-12}$		Class 1
13072	BL	02/06/2014	B	NaCpAmCtTzSuTeT	>4	≤0.06	1	≤0.125	8	4	2	≤1	*bla*$_{AmpC}$; *bla*$_{TEM-1}$; *bla* $_{CTX-M-166}$		Class 1
13456	RO	05/06/2014	B	AmCtTZ	2	≤0.06	>8	≤0.125	8	1	≤0.015	≤1	*bla*$_{AmpC}$; *bla*$_{SHV-12}$		Class 1
14966	RO	22/06/2014	B	NaCpAmCtTZ	2	≤0.06	>8	0.25	8	2	0.25	≤1	*bla*$_{AmpC}$; *bla*$_{SHV-12}$		
14968	BL	23/06/2014	B	NaCpAmCtTzSuTe	>4	0.125	4	0.25	4	>32	8	≤1	*bla*$_{AmpC}$; *bla* $_{CTX-M-32}$		
15015	RO	23/06/2014	B	NaCpAmCtTzCSuTeT	1	≤0.06	8	≤0.125	8	2	4	≤1	*bla*$_{AmpC}$; *bla*$_{TEM-1}$; *bla*$_{SHV-12}$		Class 1
15211	BL	27/06/2014	B	NaCpAmCtTzSuTe	>4	0.125	8	0.125	8	32	8	≤1	*bla*$_{AmpC}$; *bla*$_{CTX-M-32}$		
15402	RO	03/07/2014	T	NaCpAmCtTzCSu	2	≤0.06	>8	≤0.125	4	2	>8	≤1	*bla*$_{AmpC}$; *bla*$_{SHV-12}$		Class 1
16968	EDM	21/07/2014	B	NaCpAmCtTzCLSu	2	≤0.06	4	≤0.125	8	2	4	16	*bla*$_{AmpC}$; *bla*$_{TEM-52}$	Negative	
19991	RO	06/09/2014	T	NaCpAmCtTzCCLSuTe	1	≤0.06	>8	≤0.125	8	0.5	8	16	*bla*$_{AmpC}$	*mcr-1*	
21400	EDM	25/09/2014	B	NaCpAmCtTzSuTe	>4	≤0.06	1	≤0.125	4	>32	0.125	≤1	*bla*$_{AmpC}$; *bla*$_{CTX-M-1}$		

[a] **RO**, Ribatejo Oeste; **BL**, Beira Litoral; **EDM**, Entre-Douro-e-Minho

[b] **B**, broiler; **T**, turkey

[c] **Am**, ampicillin; **Ct**, cefotaxime; **CCt**, cefotaxime/clavulanic acid; **TZ**, ceftazidime; **CTZ**, ceftazidime/clavulanic acid; **CX**, cefoxitin; **CFP**, cefepime; **Cp**, ciprofloxacin; **CL**, colistin; **T**, trimethoprim; **Su**, sulfamethoxazole; **C**, chloramphenicol; **GM**, gentamicin; **Te**, tetracycline.

[d] Epidemiological cut-off values, EUCAST (http://mic.eucast.org/Eucast2/).

[e] Here is included the following plasmid-mediated quinolone resistance genetic profile: *aac(6')-Ib* in 9211 isolate and *qnrB19* in 15015 isolate.

[f] Plasmid-mediated colistin resistance.

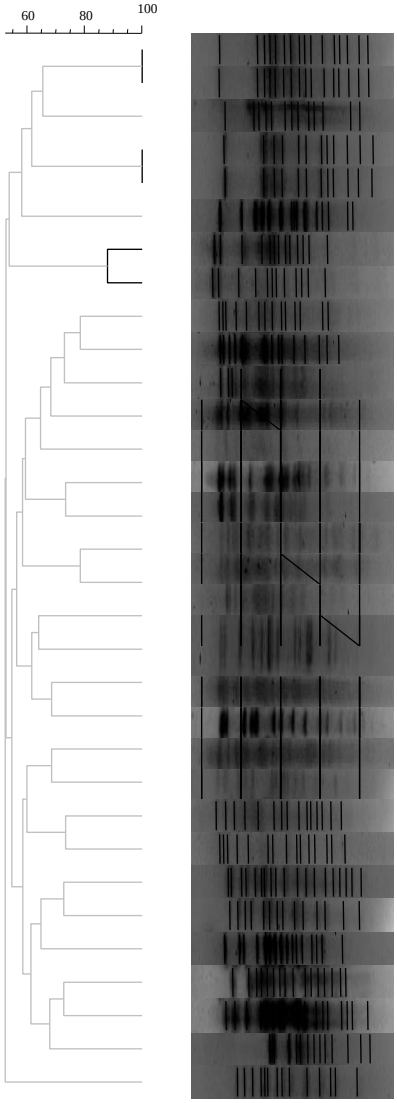
Table 4.5.3. *ampC* promoter and attenuator mutations and amino acid sequences of AmpC β-lactamases in the three *E. coli* strains.

Strain[a]	Promoter/ attenuator region[b] (bp)	Amino acid at position[c]:																							
		5	**7**	**41**	**89**	**141**	**175**	**193**	**194**	**220**	**232**	**235**	**236**	**238**	**239**	**241**	**245**	**254**	**282**	**284**	**288**	**296**	**300**	**351**
ATCC 25922		P	Q	F	T	A	K	S	A	A	R	Q	S	L	K	L	E	L	I	I	D	R	P	A
EC1	ND	-	-	Y	-	T	Q	P	-	-	-	-	-	-	-	-	-	-	S	-	-	-	A	D
EC2	ND	-	-	-	A	-	Q	P	P	-	-	R	-	M	N	R	D	-	S	-	G	H	A	-
9211	-42 (C→T), -18 (G→A), -1 (C→T), +58 (C→T), +81 (A→G)	-	-	-	-	T	Q	P	P	-	-	R	-	M	N	R	D	-	S	-	G	H	A	-
10908	+81 (A→G)	-	K	-	A	-	Q	P	-	-	-	-	I	-	-	-	-	V	-	V	-	-	A	-
19991	+81 (A→G)	S	K	-	A	-	Q	P	-	T	C	R	-	M	N	R	D	-	S	-	G	-	A	-

[a] AmpC EC1 (KR010370) and EC2 (EU497239) are narrow-spectrum cephalosporinases that were included as reference (Mammeri *et al.,* 2006); AmpC from *E. coli* ATCC 25922 (AY899338) was used as standard.
[b] chromosomal *ampC* sequence classifications as reported by Tracz et al. (Tracz *et al.,* 2007).
[c] - Indicate residues identical to those of the *E. coli* ATCC 25922 AmpC sequence.
ND, not determined

The genetic relatedness analysis of the ESBL (*bla*$_{CTX-M}$, *bla*$_{SHV-12}$ and *bla*$_{TEM-52}$)-producing *E. coli* isolates from poultry showed a high heterogeneity of PFGE profiles, since they have < 80% of homology between each other and with human isolates (Figure 4.5.1). *E. coli* LV14966 was not typeable by this method. The results showed that the occurrence of ESBL-producing *E. coli* in the Portuguese poultry population was not due to one specific clone. This contrasted with the results obtained for the clinical isolates, were 76% of the isolates corresponded to a single epidemic strain (ST131-*fimH*30 *E. coli*-producing CTX-M-15) (Mendonça *et al.,* 2007; Manageiro *et al.,* 2015a).

Figure 4.5.1. Dendrogram of PFGE profiles of 31 *E. coli* isolates presenting a non-wild type phenotype to 3rd generation cephalosporins and/or cefoxitin (11 of animal origin, and 21 isolates from humans). We used the method and the Dice coefficient with 0.9% optimization and band position tolerance of 0.8%. Isolates with a Dice band-based similarity coefficient value of ≥80% were considered to belong to the same cluster. From left to right are represented: the dendrogram, the strain code, origin, year of isolation, detected ESBL, and PFGE profiles.



Strain code	Origin	Year isolation	ESBL produced	PFGE profile
INRSA6249	Hospital A	2005	TEM-52	0001
INRSA6251	Hospital A	2005	TEM-52	0001
LV10599	Turkey	2014	SHV-12	0002
INRSA6254	Hospital A	2005	TEM-52	0003
INRSA6256	Hospital A	2005	CTX-M-32; TEM-52	0003
LV13059	Broiler	2014	SHV-12	0004
INRSA20996	Hospital B	2014	CTX-M-27	0005
INRSA21050	Hospital C	2014	CTX-M-27	0005
INRSA20823	Hospital C	2014	CTX-M-15	0006
INRSA20954	Hospital D	2014	CTX-M-27	0007
INRSA20964	Hospital D	2014	CTX-M-27	0008
INRSA21427	Hospital E	2014	CTX-M-15	0009
INRSA20638	Hospital F	2014	CTX-M-15	0010
LV13072	Broiler	2014	CTX-M-166	0011
LV15402	Turkey	2014	SHV-12	0012
INRSA20990	Hospital B	2014	CTX-M-15	0013
INRSA21027	Hospital C	2014	CTX-M-15	0014
INRSA21015	Hospital C	2014	CTX-M-27	0015
LV9370	Turkey	2014	SHV-12	0016
INRSA20979	Hospital D	2014	CTX-M-15	0017
LV21400	Broiler	2014	CTX-M-1	0018
INRSA20985	Hospital B	2014	CTX-M-15	0019
INRSA5929	Hospital A	2005	TEM-52	0020
INRSA20634	Hospital F	2014	CTX-M-15	0021
INRSA20955	Hospital D	2014	CTX-M-15	0022
INRSA5809	Hospital G	2004	TEM-52	0023
LV16968	Broiler	2014	TEM-52	0024
LV13456	Broiler	2014	SHV-12	0025
LV14968	Broiler	2014	CTX-M-32	0026
LV15211	Broiler	2014	CTX-M-32	0027
LV15015	Broiler	2014	SHV-12	0028
INRSA6265	Hospital A	2005	CTX-M-15; TEM-52	0029

The CTX-M-166 enzyme was detected in an *E. coli* isolated from a cecum sample recovered in May 2014, from a six-week broiler flock located in an industrial broiler poultry unit in Tomar, in the Central Region of Portugal. The horizontal transfer of the $bla_{CTX\text{-}M\text{-}166}$ gene was attempted by bacterial conjugation and through the direct transformation of plasmid DNA, without success (data not shown). However, further analysis by the I-CeuI technique revealed that $bla_{CTX\text{-}M\text{-}166}$ was not chromosomally encoded in isolate LV13072 suggesting that the new gene was contained in a non-transferable plasmid. Cloning experiments showed that *E. coli* transformants T13072 (CTX-M-166) and *E. coli* T21400 (CTX-M-1) strains were non-wild type to amoxicillin, cefuroxime, cefotaxime, cefepime, ceftiofur and aztreonam, while the broiler isolates were also non-wild type to ceftazidime and ciprofloxacin (*E. coli* LV21400), and to ceftazidime, ciprofloxacin plus trimethoprim (*E. coli* LV13072) (Table 4.5.4). The addition of clavulanate drastically reduced the MIC value of cefotaxime in both transformants and broiler strains, confirming the presence of an ESBL enzyme (Table 4.5.4).

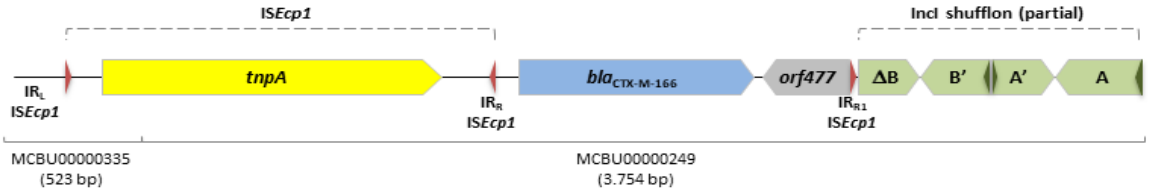
Table 4.5.4 – Phenotypic and genotypic context of CTX-M-producing *E. coli* clinical isolates, transformants and the recipient strain[a].

Antimicrobial drug[b]	MIC (μg/mL) for strains:				
	***E. coli* TOP10**	**LV13072 (CTX-M-166, TEM-1)**	**T13072 (CTX-M-166)**	**LV21400 (CTX-M-1)**	**T21400 (CTX-M-1)**
Amoxicillin	≤2	>2048	>2048	>2048	>2048
Cefuroxime	≤0.5	512	512	>512	>512
Cefoxitine	4	8	4	4	4
Ceftazidime	0.25	1	0.5	1	0.5
Ceftazidime plus clavulanate*	0.25	0.5	0.25	0.5	0.25
Cefotaxime	0.125	32	32	>128	64
Cefotaxime plus clavulanate*	0.125	≤0.125	≤0.125	≤0.125	≤0.125
Aztreonam	0.06	2	0.5	8	4
Cefepime	0.06	1	0.5	>32	4
Ceftiofur	1	32	32	64	64
Imipenem	0.125	0.125	0.125	0.125	0.125
Meropenem	0.03	0.5	0.06	0.06	0.06
Doripenem	0.06	0.125	0.06	0.06	0.06
Ertapen	0.03	0.06	0.03	0.06	0.03
Ciprofloxacin	≤0.03	8	≤0.03	0.5	≤0.03
Gentamicin	0.5	2	1	4	1
Trimethoprim	0.5	>32	1	1	1
Colistin	0.25	0.5	0.25	1	0.25
Tigecycline	0.125	0.125	0.125	0.5	0.125

[a]*E. coli* TOP10 T13072 (harboring CTX-M-166) and *E. coli* TOP10 T21400 (harboring CTX-M-1) were transformants of *E. coli* LV13072 (harboring CTX-M-166 and TEM-1) and *E. coli* LV21400 (harboring CTX-M-1), respectively; *E. coli* TOP10 was the recipient strain.[b] * Clavulanate 2 μg/ml.

WGS approach has revealed a CTX-M-166-harbouring O6:H16 ST48-*fimH*34 *E. coli*. Directed sequence analyses showed an IS*Ecp1*-*bla*$_{CTX-M-166}$-*orf477* region upstream an IncI shufflon, interrupting the segment *shfB* of the site-specific recombination system (Figure 4.5.2). This interruption, due to insertion of the IS*Ecp1*-*bla*$_{CTX-M-166}$-*orf477* element, may explain the non-conjugative properties of the CTX-M-166-harbouring plasmid (Komano, 1999). The closest match (94.7% of query coverage and 100% of identity) of the *bla*$_{CTX-M-166}$-*containing* contig as identified by BLASTn analysis was the *E. coli* plasmid pIFM3804 (KF787110), a CTX-M-1 IncI1 plasmid found on a UK pig farm (Freire *et al.,* 2014).

Figure 4.5.2. Schematic representation of CTX-M-166-harboring contig.
Blue, antibiotic resistance gene; Yellow, mobile genetic elements; Green, IncI shufflon; Grey, other genes; Right and left inverted repeats (IR_R and IR_L) are indicated as red triangles



4.5.4. Conclusion

In this study, we identified a high diversity of resistance mechanisms among commensal *E. coli* obtained from healthy food-producing animals, such as broilers and turkeys flocks at slaughter. We highlighted an emerging resistance mechanism against colistin (the *mcr-1* gene) and a new one against 3rd generation cephalosporins (the *bla*$_{CTX-M-166}$ variant) associated to mobile genetic elements, as well as an unusual gene in food-producing animals (the *bla*$_{ESAC}$ gene). All those resistance mechanisms have high importance at a public health level.

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4.6. Draft genome sequence of an Escherichia coli isolated from a Gallus gallus producing the novel CTX-M-166 variant

This research paper was submitted as:

Vera Manageiro, ***Lurdes Clemente****, Sílvia Duarte, Luís Vieira, Manuela Caniça. Draft genome sequence of an Escherichia coli isolated from a Gallus gallus producing the novel CTX-M-166 variant.* ***Genome Announcement,*** *2016****,*** *4(5), e0102916*

Contributions of the authors for the manuscript:

Vera Manageiro: design of study, acquisition of laboratory data, analysis of data, drafting of the article and final approval of the manuscript;
Lurdes Clemente: analysis of data, revision and final approval of the manuscript;
Sílvia Duarte: genomic sequencing assays, analysis of data, and final approval of the manuscript;
Luís Vieira: genomic sequencing assays, analysis of data, and final approval of the manuscript;
Manuela Caniça: design of study, revision and final approval of the manuscript;

ABSTRACT

We report the draft genome sequence of the CTX-M-166-harbouring O6:H16 ST48-*fimH*34 *E. coli* recovered from a *Gallus gallus*. Sequence analyses revealed the presence of an ST103-IncI1 IS*Ecp1-bla*$_{CTX-M-166}$-*orf477* plasmid region and of diverse antibiotic and virulence acquired-genes.

***Keywords*:** CTX-M-166, *E. coli*, broiler

4.6.1. Main Text

Animals are considered potential reservoirs of antimicrobial resistant bacteria (Caniça *et al.,* 2015). In this study, we used whole-genome sequencing (WGS) to characterize the new CTX-M-166-harbouring *E. coli* recovered in May 2014 from a six-week *Gallus gallus* broiler flock from an industrial poultry unit in the Central Region of Portugal, carrying a new amino acid substitution, when compared with CTX-M-1.

Genomic DNA of *E. coli* LV13072 was extracted using DNeasy Blood and Tissue Kit (Qiagen) and quantified using Qubit 1.0 Fluorometer (Invitrogen). The Nextera XT DNA Sample Preparation Kit (Illumina) was used to prepare sequencing libraries from 1ng of genomic DNA according to the manufacturer's instructions. WGS was performed using 250 bp paired-end reads on a MiSeq (Illumina). Sequence reads were trimmed and filtered according to quality criteria, and *de novo* assembled into contigs by means of CLC Genomics Workbench 8.5.1 (QIAGEN), as previously described (Manageiro *et al.,* 2015).

The *de novo* assembled genome contains a total assembly length of 5,236,233 bp, with a mean coverage of about 225-fold; the GC content was 49.3%. The analysis yielded 351 contigs, ranging from 402bp to 197,210bp, with a minimum of 12-fold coverage. Overall, the structural and functional annotation with NCBI Prokaryotic annotation pipeline (PGAAP, (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/) detected 97 tRNA genes, 7 rRNA genes and identified 4,656 mRNA genes.

In silico antimicrobial resistance analyses using ResFinder v2.1 (3) with a threshold of 90% identity and a minimum length of 60%, revealed genes conferring resistance to β-lactams [*bla*$_{CTX-M-166}$ (contig 249), and *bla*$_{TEM-1}$ (contig 63)], aminoglycosides [*strA-strB* (contig 289)], tetracycline [*tetA*-type (contig 20)], sulfonamides [*sul2* (contig 289)], and trimethoprim [*dfrA14*- type (contig 257)]. Seven virulence factors were also detected using VirulenceFinder v1.5 (Kleinheinz *et al.,* 2014): *iss* (contig 232), *gad* (contigs 29 and 125), *astA* (contig 123), *iroN* (contig 232), *iha* (contig 102), *mchF*-type (contig 57), *celb*-type (contig 46), and *cma*-type (contig 318).

PlasmidFinder v1.3 and pMLST v1.4 tools (Carattoli *et al.,* 2014) revealed the presence of ST103-IncI1 and Col8282 plasmids types with an identity of 100%.

The bioinformatics analysis of the genetic relatedness SerotypeFinder v1.1 (Joensen *et al.,* 2015), MLST v1.8 (Larsen *et al.,* 2012) and FimTyper v1.0 assigned this isolate to O6:H16 ST48-*fimH*34. The total number of pathogenicity determinants present in the LV13072 genome, matching 564 pathogenic families, showed a 93.2% certainty of the isolate being a human pathogen (Cosentino *et al.,* 2013).

The *bla*$_{\text{CTX-M-166}}$ gene differed from *bla*$_{\text{CTX-M-1}}$ by one point mutation that leads to the amino acid substitution Ala120Val. It was found in a 4,218bp contig, which was manually assembled overlapping contigs 249, 329 and 334, with a mean coverage was of 36.4-fold and GC content 41.2%. An IS*Ecp1*-*bla*$_{\text{CTX-M-166}}$-*orf477* region was found upstream an IncI shufflon, interrupting the segment *shfB* of the site-specific recombination system (Brouwer *et al.,* 2015). The closest match (94.7% of query coverage and 100% of identity) of the *CTX-M-166-containing* contig as identified by BLASTn analysis was the *E. coli* plasmid pIFM3804 (KF787110), a CTX-M-1 IncI1 plasmid found on a UK pig farm (Freire *et al.,* 2014).

The information presented herein will enable further studies about the genetic background of *bla*$_{\text{CTX-M-166}}$ and functional characterization of CTX-M-166 β-lactamase aiming to assess the potential impact of this new variant in veterinary settings, particularly under pressure caused by antibiotic exposure.

4.6.2. Nucleotide sequence accession numbers

This Whole Genome Sequencing Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LGYB00000000. The version described in this paper is version LGYB01000000.

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4.7. Biochemical characterization of CTX-M-166, a new CTX-M β-lactamase produced by a commensal Escherichia coli isolate

This research paper was submitted as:

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Contributions of the authors for the manuscript:

Vera Manageiro: design of study, biochemical assays, drafting of the article and final approval of the manuscript;
Rafael Graça: microbiological and biochemical assays and and final approval of the manuscript;
Eugénia Ferreira: microbiological assays and final approval of the manuscript;
Lurdes Clemente: microbiological assays and final approval of the manuscript;
Richard Bonnet: final revision and final approval of the manuscript;
Manuela Caniça: design of study, colaboration in writting the article and final approval of the manuscript;

4.7.1. Main Text

Animals are potential reservoirs of antimicrobial resistant bacteria (EFSA/ECDC, 2014; Caniça *et al.,* 2015). Studies have shown that different bacterial species of animal origin carry oxyimino-β-lactam resistance determinants, including CTX-M-type β-lactamases (Trott, 2013; Nicholas-Chanoine *et al.,* 2014). Following the alarming emergence of these enzymes in veterinary isolates, the use of ceftiofur and cefquinome to treat animal infections has become compromised.

Ceftiofur is a 3rd generation cephalosporin, a critically important class of antibiotics to human health. Nevertheless, in cattle, ceftiofur is the most widely used antibiotic for the treatment of common diseases (Hornish & Kotarski, 2002). Consequently, several studies demonstrated that ceftiofur treatment resulted in increases in resistance to β-lactams and multidrug resistance (Donaldson *et al.,* 2006; Jiang *et al.,* 2006; Chambers *et al.,* 2015).

In this study, we biochemically characterized the new CTX-M-166 β-lactamase detected in a ceftiofur-resistant *Escherichia coli* recovered in May 2014 from a six-week-old *Gallus gallus* broiler flock in an industrial poultry unit in the central region of Portugal.

E. coli INSLV13072 was non-susceptible to ampicillin (MIC>64mg/L) and oxyimino cephalosporins (>32 mg/L for ceftiofur, 8 mg/L for cefotaxime, 4 mg/L for cefepime, and 1 mg/L for ceftazidime) but susceptible to carbapenems and colistin. The MICs of ceftazidime and cefotaxime were reduced by clavulanic acid (≤0.125mg/L and ≤0.06mg/L, respectively).

The $bla_{\text{CTX-M-166}}$ gene differed from $bla_{\text{CTX-M-1}}$ by one-point mutation, which led to the amino acid substitution Ala120Val. To our knowledge, this is the first recorded observation of this mutation.

The kinetic parameters of the purified CTX-M enzymes (purity rate ≥ 95%) (data not shown) and the concentrations of inhibitors required to inhibit enzyme activity by 50% (IC_{50}s) are shown in Table 4.7.1. CTX-M-166 had strong affinity to penicillin (K_m, 14 to 8 µM), piperacillin (K_m, 6 to 3 µM), cefotaxime (K_m, 127 to 69 µM) and ceftiofur (K_m, 46 to 15 µM). However, catalytic efficiency against these antibiotics was lower for CTX-M-166 than for CTX-M-1. Notably, CTX-M-166 had the least decrease in catalytic efficiency against ceftiofur (30.2%) compared to that of CTX-M-1, whose value was set at 100% (Table 4.7.1). In contrast, the new enzyme had only 2.7% of catalytic efficiency for amoxicillin in comparison with the parental enzyme. No hydrolysis was detected against ceftazidime or imipenem. Inhibition studies, as measured by determination of the IC_{50s}, showed that CTX-M-1 and CTX-M-166 were both inhibited by clavulanic acid (0.031 and 0.030 µM, respectively) and tazobactam (0.007 and 0.005 µM, respectively).

The Ala120Val amino acid substitution, although at distance of the catalytic site, it is located in an alpha helix involved in the positioning of the loop harbouring the conserved element SDN,

which plays a major role in proton transfer during the catalytic pocket in class A enzymes (Matagne *et al.,* 1998). The Ala120 residue is highly conserved in all CTX-M groups except for CTX-M-25-group, were it is replaced by a glycine (D'Andrea *et al.,* 2013). The alanine-to-valine substitution represents an alteration to a non-reactive amino acid that is often associated with binding/recognition of hydrophobic ligands such as lipids, and thus involved in increasing the flexibility of protein (Betts & Russel, 2007). The impact of this alteration could become more relevant with the accumulation of mutations affecting enzyme activity and resistance phenotype which might arise due to antibiotic selection pressure.

Table 4.7.1. Kinetic parameters of CTX-M-166 and CTX-M-1 β-Lactamases.

	CTX-M-1[a]			**CTX-M-166[a]**			
Substrate	***kcat*** **(s^{-1})**	***Km*** **(μM)**	***kcat/Km*** **($\mu M^{-1}.s^{-1}$)**	***kcat*** **(s^{-1})**	***Km*** **(μM)**	***kcat/Km*** **($\mu M^{-1}.s^{-1}$)**	**Efficiency[b] (%)**
Penicillin G	87.7 ± 1.8	14 ± 0.5	6.453	8.2 ± 0.2	8 ±0.03	0.996	15.4
Amoxicillin	31.4 ± 0.6	10 ± 0.3	3.097	3.1 ± 0.1	37 ± 0.6	0.084	2.7
Ticarcillin	7.3 ± 0.4	21 ± 0.1	0.354	0.5 ± 0.002	21 ± 0.03	0.024	6.8
Piperacillin	32.7 ± 1.2	6 ± 0.5	5.512	2.4 ± 0.01	3 ± 0.2	0.685	12.4
Cephalothin	598.4 ± 95.1	57 ± 3.0	10.683	81.1 ± 1.4	85 ± 2.3	0.954	8.9
Cefuroxime	77.6 ± 2.7	17 ± 0.5	4.543	8.0 ± 0.7	36 ± 0.5	0.225	5.0
Cefotaxime	129.9 ± 0.6	127 ± 1.9	1.021	8.3 ± 0.3	69 ± 1.8	0.124	12.2
Ceftazidime	<0.01	170 ± 2.5	0.000	<0.01	ND	ND	ND
Ceftiofur	5.5 ± 0.4	46 ± 1.1	0.120	0.6 ± 0.004	15 ± 0.3	0.036	30.2
Cefepime	2.3 ± 0.6	26 ± 0.6	0.089	1.6 ± 0.2	102 ± 3.0	0.015	17.3
Aztreonam	2.1 ± 0.006	29 ± 0.7	0.073	0.2 ± 0.005	41 ± 0.1	0.005	7.0
Imipenem	<0.01	107 ± 8.7	<0.001	<0.01	ND	ND	ND

[a] Values are means ± standard deviations.
[b] Efficiency of CTX-M-166 compared to that of CTX-M-1, which was set at 100%.
ND, not determinable because the hydrolysis rates were too low.

4.7.2. Experimental Procedure

Antibiotic Susceptibility and Molecular Characterization

Minimum inhibitory concentrations (MICs) of the clinical *E. coli* I INSLV13072 isolate were determined by both agar dilution and microdilution methods to: ampicillin, cefotaxime, ceftazidime, cefotaxime/clavulanate, ceftazidime/clavulanate, cefepime, imipenem,

meropenem, ertapenem, ciprofloxacin, gentamicin, chloramphenicol, trimethoprim, colistin and tigecycline. The interpretation of nonsusceptibility results was performed according to the epidemiological cut-off values (ECOFFs) of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://mic.eucast.org/Eucast2/). β-lactamase-encoding genes were identified by PCR and confirmed by sequencing, as previously described (Clemente *et al.,* 2013).

Cloning experiments

For comparison, CTX-M-166 (from LV13072) and CTX-M-1 (from LV21400) were expressed in an isogenic background. The Zero Blunt® PCR Cloning Kit (Invitrogen) was used to clone CTX-M-type PCR fragments into plasmid *pCR®-Blunt*. Recombinant pCR-CTX-M-type plasmids were transformed by heat-shock transformation of chemically competent *E. coli* One Shot®TOP10 cells. *E. coli* transformants were selected on MacConkey agar supplemented with 30 mg/L of kanamycin and 2 mg/L of cefotaxime. The presence and orientation of the inserted genes was confirmed by PCR as above described.

Purification of β-lactamases

CTX-M-166 and CTX-M-1 β-lactamases were produced overnight, at 37ºC, from *E. coli* One Shot®TOP10 in LB broth, supplemented with 2 mg/L cefotaxime. Both enzymes were extracted by ultrasonic treatment, and the clarified supernatant was purified by ion exchange and gel filtration chromatography as described elsewhere (Manageiro *et al.,* 2012).

Determination of β-lactamase kinetic constants

The Michaelis constant (*Km*) and catalytic activity (*kcat*) of CTX-M-1 and CTX-M-166 and the concentrations of the inhibitors (clavulanate and tazobactam) required to inhibit enzyme activity by 50% (IC_{50}) were determined by a computerized microacidimetric method, as described elsewhere (Manageiro *et al.,* 2012). Specific activity and IC_{50} were monitored with penicillin G (200 µM) as the reporter substrate.

Nucleotide sequence accession number

The $bla_{CTX\text{-}M\text{-}166}$ nucleotide sequence was submitted to DDBJ/EMBL/GenBank with accession number NG_048951.

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4.8. QnrS1- and Aac(6')-Ib-cr-producing Escherichia coli among isolates from animals of different sources: susceptibility and genomic characterization

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Contributions of the authors for the manuscript:

Daniela Jones-Dias: conception and design of study, acquisition of laboratory data, analysis of data, drafting of article and critical revision of the manuscript, final approval of manuscript;
Vera Manageiro: acquisition of laboratory data, analysis of data, critical revision of the manuscript, final approval of manuscript;
Rafael Graça: acquisition of laboratory data, final approval of manuscript;
Daniel Ataíde Sampaio: acquisition of laboratory data, final approval of manuscript;
Teresa Albuquerque: acquisition of laboratory, final approval of manuscript;
Patrícia Themudo: acquisition of laboratory data, final approval of manuscript;
Luís Vieira: acquisition of laboratory data, final approval of manuscript;
Eugénia Ferreira: acquisition of laboratory data, critical revision of the manuscript, final approval of manuscript;
Lurdes Clemente: acquisition of laboratory and epidemiological data, critical revision of the manuscript, final approval of manuscript;
Manuela Caniça: conception and design of study, critical revision of the manuscript, final approval of manuscript.

ABSTRACT

Salmonella enterica and *Escherichia coli* can inhabit humans and animals from multiple origins. These bacteria are often associated with gastroenteritis in animals, being a frequent cause of resistant zoonotic infections. In fact, bacteria from animals can be transmitted to humans through the food chain and direct contact. In this study, we aimed to assess the antibiotic susceptibility of a collection of *S. enterica* and *E. coli* recovered from animals of different sources, performing a genomic comparison of the plasmid-mediated quinolone resistance (PMQR)-producing isolates detected.

Antibiotic susceptibility testing revealed a high number of non wild-type isolates for fluoroquinolones among *S. enterica* recovered from poultry isolates. In turn, the frequency of non-wild-type *E. coli* to nalidixic acid and ciprofloxacin was higher in food-producing animals than in companion or zoo animals.

Globally, we detected two *qnrS1* and two *aac(6')-Ib-cr* in *E. coli* isolates recovered from animals of different origins. The genomic characterization of QnrS1-producing *E. coli* showed high genomic similarity (O86:H12 and ST2297), although they have been recovered from a healthy turtle dove from a Zoo Park, and from a dog showing symptoms of infection. The *qnrS1* gene was encoded in a IncN plasmid, also carrying $bla_{\text{TEM-1}}$-containing Tn*3*. Isolates harboring *aac(6')-Ib-cr* were detected in two captive bottlenose dolphins, within a time span of two years. The additional antibiotic resistance genes of the two *aac(6')-Ib-cr*-positive isolates ($bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, *catB3, aac(3)-IIa* and *tetA*) were enclosed in IncFIA plasmids that differed in a single transposase and 60 single nucleotide variants. The isolates could be assigned to the same genetic sublineage – ST131 fimH30-Rx (O25:H4), confirming clonal spread.

PMQR-producing isolates were associated with symptomatic and asymptomatic hosts, which highlight the aptitude of *E. coli* to act as silent vehicles, allowing the accumulation of antibiotic resistance genes, mobile genetic elements and other relevant pathogenicity determinants. Continuous monitoring of health and sick animals towards the presence of PMQR should be strongly encouraged in order to restrain the clonal spread of these antibiotic resistant strains.

***Keywords*:** pathogenicity, *E. coli*, clone, PMQR, multidrug resistance, veterinary

4.8.1. Introduction

Antibiotic resistance has been critically increasing over time and now constitutes one of the major health concerns worldwide. The uncontrolled use of antibiotics in human and veterinary practices, animal production and agriculture and the increasingly easiness in

global transportation contributed to the dissemination of multidrug resistant pathogens that constitute a risk for humans, animals and the environment (Marshall and Levy, 2011; EFSA, 2015). Nowadays, antibiotic resistant *Salmonella enterica* and *Escherichia coli* are among the most problematic zoonotic bacteria, causing severe gastroenteritis in animals and humans (EFSA, 2015).

Fluoroquinolones constitute a group of broad spectrum antibiotics of critical importance, presenting applications in both human and veterinary medicines (Poirel *et al.,* 2012b). Therefore, resistance might easily emerge in animals and get transferred to humans through the food chain and direct or indirect contact. Several examples of such transmission have already been documented (Gomes-Neves *et al.,* 2014; Damborg *et al.,* 2015; Schmithausen *et al.,* 2015). Fluoroquinolone resistance has emerged rapidly due to two main types of mechanisms: mutation of the chromosomal quinolone targets DNA gyrase and topoisomerase IV, and acquisition of the transferable plasmid-mediated quinolone resistance (PMQR) determinants *qnr*, *qepA*, *aac(6')-Ib-cr*, and *oqxAB* (Veldman *et al.,* 2011; Poirel *et al.,* 2012b). The alteration of chromosomal quinolone targets can lead to higher levels of resistance than PMQRs that are only able to guarantee low-level quinolone resistance. However, the ability of the latter to be spread by horizontal gene transfer constitutes a serious concern that should be addressed (Poirel *et al.,* 2012b). In fact, antibiotic resistance genes are frequently associated to mobile genetic elements such as insertion sequences (ISs), phages, transposons and plasmids, which enhance their ability to efficiently spread among different bacterial species (Stokes and Gillings, 2011). The most worrying mechanisms of resistance, which also show a transboundary spread between animals, humans and the environment, are, in fact, encoded by mobile antibiotic resistance genes. The occurrence of mobile genetic elements harboring multiple antibiotic resistance genes is also frequent, and enables the development of bacterial multidrug resistance, which may be responsible for therapeutic failures in animals or humans (Poirel *et al.,* 2012b).

In animals, as well as in humans, several factors can affect the progression and severity of an acute infection. The synchronized presence of antibiotic resistance genes, virulence factors, mobile genetic elements and other pathogenicity determinants, is ideal to the successful spread of these microorganisms in any environment (Cosentino *et al.,* 2013).

In this study, PMQR-producing *E. coli* isolates were gathered from a collection of *S. enterica* and *E. coli* recovered from food-producing, companion and zoo animals, in the scope of their phenotypic and genotypic characterization. To further explore the genetic diversity of these isolates, as well as to understand the molecular features contributing to their spread and ability to cause infection, complete genomic sequencing was performed.

4.8.2. Materials and Methods

Collection of bacterial isolates

This study included 89 *Salmonella enterica* isolates recovered from breeders (n=12), broilers (n=33), layers (n=33), swine (n=6) and food products of animal origin (n=5) (Table 4.8.1). In poultry farms, samples were collected from feces and environment using sterile boots/sock swabs. Food products included uncooked fresh products such as minced meat, hamburgers, meat cuts, sausages and table eggs, randomly recovered at a variety of retail stores. Samples from other animal species (pigeons, partridges, ducks, pets and exotic animals) consisted of blood cultures and organs (lung, liver, spleen, kidneys and intestine) collected during post-mortem examination. All samples were examined according to ISO norm 6579:2002 applied to *Salmonella* detection in food and animal feeding stuffs. After biochemical confirmation, *Salmonella* spp. isolates were sent to the *Salmonella* National Reference Laboratory (INIAV, Lisbon) in triple sugar iron slopes or SMID plates.

This study also included 91 *E. coli* isolates (Table 4.8.1) collected from food-producing animals [(bovine, swine and poultry), (n=32)], pets [(dogs, cats, horses and cage birds), (n=37)] and zoo animals [(terrestrial and aquatic mammals, birds and reptiles), (n=22)]. Samples consisted of swabs from organic fluids and cavities, fecal samples, urine samples, blood cultures and organs collected during post-mortem examination and submitted for bacteriological analysis. Suspected *E. coli* colonies obtained in MacConkey agar plates were confirmed by API 20E strips (bioMérieux, Marcy-l'Étoile, France).

Serotypes of *S. enterica*

S. enterica isolates were serotyped by the slide agglutination method, using the method of Kauffmann-White scheme (Grimont and Weill, 2007).

Table 4.8.1. Distribution of the *S. enterica* (n = 89) and *E. coli* (n =91) isolates.

Source	*S. enterica*			Source	*E. coli*
	Enteritidis	**Other serotypes[a]**	**Total**		
Breeders	12	0	12	Food	32
Layers	24	9	33	Companion	36
Broilers	32	1	33	Zoo	23
Swine	0	6	6		
Food of animal origin	3	2	5		
Total	71	18	**89**	**Total**	**91**

[a]*Salmonella* 4,5:i:- (n=1), *Salmonella* 6,7,14:-:1,2 (n=1), *Salmonella* Bradenburg (n=1), *Salmonella* Gallinarum (n=1), *Salmonella* Give (n=1), *Salmonella* Hadar (n=1), *Salmonella* Heidelberg (n=1), *Salmonella* IIIa 48:z10:- (n=1), *Salmonella* Mbandaka (n=1), *Salmonella* Rissen (n=2), *Salmonella* Typhimurium (n=3), *Salmonella* Virchow (n=4).

Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by agar dilution following standard recommendations, using a panel of ten antimicrobial compounds: ampicillin, cefotaxime, nalidixic acid, ciprofloxacin, gentamicin, streptomycin, chloramphenicol, tetracycline, sulfamethoxazole and trimethoprim (Table 4.8.2). Isolates harboring PMQR determinants were further studied by determination of the MICs to a larger panel of fluoroquinolones, which included moxifloxacin, gatifloxacin, levofloxacin, ofloxacin, enrofloxacin and norfloxacin. To assess non-wild-type isolates, interpretation of results was performed according to the epidemiological cut-off values suggested by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://mic.eucast.org/Eucast2/). For *Salmonella* spp., the cut-off value used for sulfamethoxazole was that for sulfonamides from Clinical Standards Laboratory Institute (http://clsi.org). MIC_{50} and MIC_{90} were calculated as reported elsewhere (Schwarz *et al.,* 2010). *E. coli* ATCC 25922 was used as the quality control strain. Isolates were considered multidrug resistant (MDR) if they presented non-wild-type phenotypes against three or more structurally unrelated antibiotics (Magiorakos *et al.,* 2011).

Molecular characterization of resistance

All isolates were evaluated regarding the presence of *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, and *qepA* genes, using primers and conditions previously described (Jones-Dias *et al.,* 2013), and *oqxAB* genes using primers and conditions first described in this study (oqxA-F, 5'-AGAGTTCAAAGCCACGCTG-3' and oqxb-R, 5'-CTCCTGCATCGCCGTCACCA-3'; initial denaturation of 94ºC for 5 minutes; 94ºC for 30 seconds, 64ºC for 30 seconds and 72ºC for 1 minute, for 30 cycles; final step of extension of 72ºC for 5 minutes). PMQR-producing isolates were also characterized regarding the production of β-lactamase-encoding genes and conventional Multilocus sequence typing (MLST), as described elsewhere (Jones-Dias *et al.,* 2015).

Genomic characterization of PMQR-producing *E. coli*

The genomes of the four PMQR-producing *E. coli* (LV46221, LV46743, LV36464 and LV27950) were characterized. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Aarhus) and quantified using Qubit 1.0 Fluorometer (Invitrogen, Waltham). The Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA) was used to prepare sequencing libraries from 1ng of genomic DNA, according to the manufacturer's instructions. Paired-end sequencing of 150 bp reads was performed on a MiSeq (Illumina). Sequence reads were then trimmed and filtered according to quality criteria, and assembled *de novo* using CLC genomics workbench version 8.5.1 (QIAGEN, Aarhus). RAST (Rapid Annotation using Subsystem Technology) was used for subsystem annotation of the genomes (Aziz *et al.,* 2012; Overbeek *et al.,* 2014).

Identification of pathogenicity-related genes

Pathogenicity-related genes were detected using a variety of online web tools. PathogenFinder 1.1, ResFinder 2.1, VirulenceFinder 1.4, SerotypeFinder 1.1, MLST 1.8, pMLST 1.4 and PHAST were used to estimate the pathogenicity determinants, acquired antibiotic resistance genes, virulence factors, serotypes, MLST, plasmid MLST and phage regions, respectively in the genomes of PMQR-producing *E. coli* (Zhou *et al.,* 2011; Larsen *et al.,* 2012; Zankari *et al.,* 2012; Cosentino *et al.,* 2013; Carattoli *et al.,* 2014; Joensen *et al.,* 2014; Joensen *et al.,* 2015). ISsaga was also used to detect and annotate insertion sequences in the draft genomes of the *E. coli* isolates (Varani *et al.,* 2011). Specific analysis of antibiotic resistance genes and respective flanking regions was carried out with *CLC genomics workbench version* 8.5.1 (Qiagen, Aarhus). Contigs carrying antibiotic resistance genes were manually assembled whenever necessary and blasted against GenBank to identify their genetic location.

Nucleotide Sequence GenBank Accession Numbers

The draft genomes of isolates LV46221, LV46743, LV36464 and LV27950 have been deposited at DDBJ/EMBL/GenBank under the accessions LRXG00000000, LRXH00000000, LRXI00000000 and LRXJ00000000, respectively. The versions described in this paper are version LRXG01000000, LRXH01000000, LRXI01000000 and LRXJ01000000, respectively.

4.8.3. Results

Serotypes of *Salmonella spp.*

S. enterica serotype Enteritidis is one of the most common serotype in humans (EFSA, 2015) and it was the most frequently detected among the 89 *S. enterica* isolates (71/89, 79.8%), being present in all food animals except swine. The remaining *Salmonella* serotypes were detected in a less extent and were comprised of *Salmonella* 4,5:i:- (n=1), *Salmonella* 6,7,14:-:1,2 (n=1), *Salmonella* Bradenburg (n=1), *Salmonella* Gallinarum (n=1), *Salmonella* Give (n=1), *Salmonella* Hadar (n=1), *Salmonella* Heidelberg (n=1), *Salmonella* IIIa 48:z10:- (n=1), *Salmonella* Mbandaka (n=1), *Salmonella* Rissen (n=2), *Salmonella* Typhimurium (n=3) and *Salmonella* Virchow (n=4).

Antimicrobial susceptibility of *S. enterica* and *E. coli* isolates

Susceptibility profiles of *S. enterica* and *E. coli* isolates differed with the animal group (Table 4.8.2). Although high rates of non wild-type *S. enterica* were detected for nalidixic acid (from 82% to 100%) and ciprofloxacin (from 64% to 100%) in all groups, they were particularly evident in poultry, and predominant in breeders. *S. enterica* isolates recovered from other sources (swine and food products, n=11), showed higher non-wild-type phenotypes for ampicillin (36%), streptomycin (64%), tetracycline (45%), sulfamethoxazole (36%) and

trimethoprim (27%) (Table 4.8.2). The poultry groups of breeders and broilers were mainly susceptible to ampicillin (100%), cefotaxime (100%), gentamicin (100%) and streptomycin (100%).

Table 4.8.2. MIC_{50} and MIC_{90} for *S. enterica* (n =89) and *E. coli* (n=91) isolates

Antibiotic	*S. enterica*				*E. coli*		
	Food animals						
	Breeders (n=12)	Broilers (n=33)	Layers (n=33)	Others[a] (n=11)	Food animals (n=32)	Zoo Animals (n=23)	Companion Animals (n=36)
Na							
MIC_{50}	128	128	128	128	8	4	4
MIC_{90}	128	128	128	128	128	128	128
% Wt	0	12	6	18	59	77	78
%N-Wt	100	88	94	82	41	23	22
Cp							
MIC_{50}	0.25	0.25	0.25	0.25	0.03	0.015	0.015
MIC_{90}	0.25	0.25	0.25	0.5	8	8	8
% Wt	0	3	18	36	59	77	72
%N-Wt	100	97	82	64	41	23	28
A							
MIC_{50}	2	4	0.5	8	8	8	8
MIC_{90}	4	4	8	64	64	64	64
% Wt	100	100	94	64	53	50	53
%N-Wt	0	0	6	36	47	50	47
Ct							
MIC_{50}	0.125	0.06	0.125	0.125	≤0.06	0.06	≤0.06
MIC_{90}	0.125	0.125	0.125	2	0.125	0.125	0.125
% Wt	100	100	94	82	100	95	94
%N-Wt	0	0	6	18	0	5	6
G							
MIC_{50}	0.25	0.25	0.25	0.5	0.5	0.5	0.5
MIC_{90}	0.5	0.5	0.5	1	2	1	1
% Wt	100	100	100	100	91	91	100
%N-Wt	0	0	0	0	9	9	0
St							
MIC_{50}	2	2	4	32	4	4	4
MIC_{90}	8	4	128	64	256	256	128
% Wt	100	100	82	36	69	64	86
%N-Wt	0	0	18	64	31	36	14
T							
MIC_{50}	2	2	2	4	32	2	2
MIC_{90}	4	4	4	64	64	64	64
% Wt	92	100	91	55	47	59	75
%N-Wt	8	0	9	45	53	41	25
C							
MIC_{50}	8	4	8	8	4	8	8
MIC_{90}	8	8	8	16	64	8	16
% Wt	100	97	100	91	84	100	92
%N-Wt	0	3	0	9	16	0	8
Su							
MIC_{50}	128	128	128	128	16	32	16
MIC_{90}	128	128	128	>512	>512	>512	>512
% Wt	92	100	100	64	63	59	78
%N-Wt	8	0	0	36	38	41	22
Tp							
MIC_{50}	0.5	0.5	0.5	0.25	0.5	0.5	0.5
MIC_{90}	0.5	0.5	0.5	32	32	32	32
% Wt	92	100	100	73	81	68	81

[a] Others, pigs (n=6) and food products of animal origin (n=5).
Na, Nalidixic acid; **Cp**, Ciprofloxacin; **A**, Ampicillin; **Ct**, cefotaxime; **G**, Gentamicin; **St**, Streptomycin; **T**, Tetracycline; **C**, Chloramphenicol; **Su**, sulfamethoxazole; **Tp**, trimethoprim

The frequency of non wild-type isolates was globally higher for *E. coli* than for *S. enterica* against ampicillin (minimum value of 47% versus 0%, respectively), tetracycline (minimum value of 25% versus 0%, respectively), sulfamethoxazole (minimum value of 22% versus 0%, respectively) and trimethoprim (minimum value of 19% versus 0%, respectively). Although no major discrepancies were noticed for *E. coli* in rates of non-wild-type isolates for the different animal groups, isolates recovered from food animals still presented more non-wild-type phenotypes than zoo or companion animals against nalidixic acid (41%), ciprofloxacin (41%), tetracycline (53%) and chloramphenicol (16%).

In *S. enterica* isolates from poultry, similar MIC_{50} and MIC_{90} values were observed for the majority of the antibiotics tested; major differences (≥3 fold dilutions) were observed for the group "others" for ampicillin (8 and 64mg/L), tetracycline (4 and 64mg/L), sulfamethoxazole (128 and >512mg/L) and trimethoprim (0.25 and 32mg/L). For *E. coli*, the most significant differences in MIC_{50} and MIC_{90} values were observed for nalidixic acid (4 and 128mg/L), ampicillin (8 and 64 mg/L), streptomycin (4 and 128mg/L), tetracycline (2 and 64mg /L), sulfamethoxazole (16 and >512mg/L) and trimethoprim (0.5 to 32 mg/L).

While for *S. enterica* only 9.0% (8/89) MDR isolates were detected, for *E. coli*, MDR was registered in 38.9% (35/90) of the isolates, which were distributed among 16/90 isolates from food-producing animals, 9/90 isolates from companion animals, and 10/90 isolates from zoo animals.

Molecular characterization of *S. enterica* and *E. coli* isolates

Overall, among the 180 isolates studied, we have detected and identified four PMQR determinants in *E. coli* isolates: two *qnrS1* were detected in isolates recovered from a captive turtle dove (LV46221) and a pet dog (LV46743), and two *aac(6´)-Ib-cr* were isolated from *E. coli* recovered from captive bottlenose dolphins (LV36464 and LV27950). The detection of β-lactamase-encoding genes showed the presence of bla_{TEM-1} in isolates LV46221 and LV46743, and bla_{TEM-1}, bla_{OXA-1} and $bla_{CTX-M-15}$ in LV36464 and LV27950. No other PMQR- or β-lactamase-encoding genes were identified in the collection of *E. coli* and *S. enterica* isolates.

Genomic characterization of QnrS1-producing *E. coli*

The assembly of the genome sequences of the two *qnrS1*-harboring *E. coli,* LV46221 and LV46743, yielded 200 and 199 contigs (each >200 bp long), which together comprised 4,799,985 bp and 4,801,518 bp, respectively. The average coverage of LV46221 was 135.9, while LV46743 displayed 114.1 fold. The maximum contig length obtained for these genomes was 398,205 bp and 333,601 bp, respectively (Table 4.8.3).

The automated annotation of the draft genomes showed that LV46221 (63%, 2,879/4,618) and LV46743 (63%, 2,873/ 4,609) presented a similar number of sequences attributed to specific subsystems. General annotation of both genomes showed 109 coding sequences

associated with virulence, disease and defence, as well as 143 sequences coding for functions related with mobile genetic elements, such as phages, prophages, transposable elements and plasmids. Globally, the proportion of each subsystem was equally represented in the genomes of the two isolates.

Table 4.8.3. Genome analysis of *E. coli* LV46221, LV46743, LV36464 and LV27950.

Isolates	LV46221	LV46743	LV36464	LV27950
Genome size (bp)	4,799,985	4,801,518	5,180,399	5,156,819
Number of contigs	200	199	136	209
Average coverage	135.9	114.1	178.7	150.1
N50 (bp)[a]	119,356	119,356	158,975	158,977
Maximum contig (bp)	398,205	333,601	399,998	369,918
Minimum contig (bp)	208	201	486	218
Protein-coding genes	4618	4609	5107	5069
RNAs	77	86	77	76

[a] Minimum contig length of at least 50% of the contigs.

According to RAST annotation system, LV46221 and LV46743 isolates carried 77 and 86 RNAs, respectively. The bioinformatics analysis of the genetic relatedness was carried out with regard to serotype and MLST: the serotypes of both isolates were defined as O86:H12, and they also shared the assigned MLST - ST2297 (Table 4.8.4).

In silico analysis of the antibiotic resistance genes (90% identity and 40% minimum length) confirmed the presence of a *qnrS1*, and identified $bla_{\text{TEM-1}}$ gene in both isolates (Table 4.8.4). *qnrS1* was detected in a contig with an approximate length of 11,000 bp in both cases, showing 99% of homology with a resistance region from *S. enterica* subsp. *enterica* serovar Infantis pINF5 plasmid.

By mapping all contigs against this plasmid, we detected $bla_{\text{TEM-1}}$- containing T*n3*, and a disrupted IS*2*-like element upstream of *qnrS1,* as well as IS*26* transposase downstream of the gene. Other contigs showed complementary regions, revealing the presence of a fragment encoding conjugation transfer genes upstream of Tn*3* that showed homology with *S.* Virchow plasmid pVQS1 (99%).

LV46221 and LV46743 showed no additional PMQR or other acquired antibiotic resistance genes. Moreover, no mutations were detected in the quinolone resistance determining region (QRDR) of genes *gyrA*, *gyrB*, *parC* and *parE,* which are known to confer high level resistance to fluoroquinolones (Veldman *et al.,* 2011). The isolates were also characterized with regard to specific mobile genetic elements of different classes. The screening of typable plasmids (>98% homology) enabled the identification of IncN plasmids, which were further typed as ST1 by pMLST (Table 4.8.4). ISsaga allowed the specialized annotation of insertion sequences and revealed a different distribution of the same elements for LV46221 and LV46743: IS*1* (3.23% and 1.96%, respectively), IS*200*_IS*605* (3.23% and 3.92%, respectively), IS*21* (3.23% and 3.92%, respectively), IS*3* (24.19% and 21.57%, respectively), IS*4* (3.23% and 3.92%, respectively), IS*481* (1.61% and 1.96%, respectively), IS*5* (1.61% and 1.96%, respectively), IS*6* (1.61% and 1.96%, respectively), IS*As1*(27.42% and 27.45%, respectively), IS*Kra4* (6.45% and 5.88%, respectively), IS*L3* (12.9% and 9.8%, respectively), ISNCY (9.68% and 11.76%, respectively) and finally Tn*3* (1.61% and 1.96%, respectively). IS*As1* was the most frequent element detected in both isolates, and IS*66* was exclusively detected in LV46743 (1.96%). Moreover, in LV46221 we identified ten prophage regions among which three were questionable but seven were intact. The latter included prophage regions reaching up to 90.6Kb, containing 133 coding sequences (Table 4.8.5). In turn, in LV46743, 14 different prophages were detected that included one questionable, four incomplete and nine intact phage regions; intact zones ranged between regions of 10.4Kb carrying 12 coding sequences, and 70.3Kb with 88 protein coding DNA fragments. Overall, among the prophages showing higher scores for both genomes were serotype-converting *Shigella flexnerri* bacteriophage and Enterobacteria lambda phages (Table 4.8.5).

The total number of pathogenicity determinants, which according with PathogenFinder includes, for instance, virulence factors, antibiotic resistance genes and mobile genetic elements, detected a similar number of sequences in the genomes of *E. coli* LV46221 and LV46743: 607 and 611 different pathogenic families showed a 93.5% certainty of the isolates being human pathogens. Finally, the virulence factor glutamate decarboxylase (*gad*) was detected in both isolates, while the increased serum survival factor *iss* was exclusively identified in LV46221 (Table 4.8.4).

Table 4.8.4. General features of PMQR-harboring *E. coli* isolates recovered from animals of different sources.

Isolate	Origin	Year	Serotype	MIC (mg/L)								PMQR	Other resistance genes	Virulence factors	MLST	Plasmids	pMLST
				Mx	Cp	Ga	Le	Of	Ef	Na	Nx						
LV46221	Dove	2008	O86:H12	0.75	0.38	0.75	0.38	1	1.5	8	0.75	*qnrS1*	*bla*$_{TEM-1}$	*gad*, *iss*	ST2297	IncN	ST1
LV46743	Dog	2008	O86:H12	0.5	0.38	0.5	0.5	1.5	2	8	1.5	*qnrS1*	*bla*$_{TEM-1}$	*gad*, *iss*	ST2297	IncN	ST1
LV36464	Dolphin	2009	O25:H4	>32	>32	8	>32	>32	>32	>256	>256	*aac(6')-Ib-cr*	*aac(3)-IIa*, *bla*$_{CTX-M-15}$ *bla*$_{TEM-1}$, *bla*$_{OXA-1}$ *catB3, tetA*	*iss*, *sat*, *gad*	ST131	IncFIA, IncX	ST1
LV27950	Dolphin	2011	O25:H4	12	>32	8	8	>32	>32	>256	>256	*aac(6')-Ib-cr*	*aac(3)-IIa*, *bla*$_{CTX-M-15}$ *bla*$_{TEM-1}$, *bla*$_{OXA-1}$ *catB3, tetA*	*iss*, *sat*	ST131	IncFIA	ST1

Mx, moxifloxacin; **Cp**, ciprofloxacin; **Ga**, gatifloxacin; **Le**, levofloxacin; **Of**, ofloxacin; **Ef**, enrofloxacin; **Na**, nalidixic acid; **Nx**, norfloxacin.
PMQR, Plasmid-mediated quinolone resistance
MLST, Multilocus sequence typing

Table 4.8.5. Representation of intact phage regions detected in the draft genome of LV46221, LV46743, LV36464 and LV27950.

Phage regions	Region length (Kb)	Score	Number of coding sequences	Accession number
LV46221				
1	30.4	100	36	NC_003315
2	19.4	100	23	NC_016158
3	14.7	100	17	NC_001416
4	58.9	150	67	NC_021857
5	54.1	150	50	NC_010463
6	37.9	150	39	NC_001416
7	90.6	150	133	NC_001416
LV46743				
1	10.4	96	12	NC_001609
2	19	100	21	NC_001416
3	24.7	100	35	NC_010463
4	18.6	110	28	NC_028943
5	54.5	100	37	NC_022747
6	48.4	150	56	NC_021857
7	21.9	130	29	NC_016158
8	43	150	44	NC_001416
9	70.3	150	88	NC_001416
LV36464				
1	33.9	150	46	NC_001895
2	46.8	120	34	NC_026014
3	36.8	93	54	NC_009237
4	31.8	150	36	NC_001416
5	49.4	150	61	NC_019522
6	17.4	140	24	NC_004813
7	44.7	150	38	NC_004813
8	51.1	150	88	NC_019716
LV27950				
1	68.5	150	80	NC_019522
2	34.7	150	26	NC_019716
3	33.9	150	44	NC_022750
4	40.6	120	32	NC_026014
5	34.7	150	46	NC_005882
6	25.3	150	29	NC_004813
7	25.2	110	38	NC_001416
8	20.6	150	30	NC_001416
9	58.3	150	90	NC_019716

Genomic characterization of Aac(6')-Ib-cr-producing *E. coli*

The genome sequences of isolates LV36464 and LV27950, which were known to produce Aac(6')-Ib-cr and CTX-M-15, were also compared. Their *de novo* assembly yielded 5,180,399 bp for LV36464 and 5,156,819 bp for LV27950 and displayed a mean coverage of 178.6 and 150.1 fold, respectively. Approximately, 136 and 209 contigs (each >200 bp long)

were recovered for LV36464 and LV27950 with a maximum contig length of 399,998 bp and 369,918 bp, respectively (Table 4.8.3).

The automated annotation of the genomes showed a total number of coding sequences of 5,107 for LV36464 and 5,069 for LV27950, excluding 77 and 76 annotated RNA molecules. The distribution of the annotated coding sequences by subsystem showed an identical representation of functions in both isolates (LV36464: 368%, 3,062/5,107; LV27950: 61%, 3,081/5,069).

The serotypes of the LV36464 and LV27950 isolates obtained upon the analysis of *fliC*, *wzy* and *wzx* genes, were defined as O25:H4. The epidemiology and diversity of *E. coli* isolates was also explored, assigning both of them to ST131 and to sublineage fimH30-Rx.

Globally, in isolates LV36464 and LV27950 seven different acquired antibiotic resistance genes were detected: *aac(6')Ib-cr, bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM-1,}}$ *bla*$_{\text{CTX-M-15}}$, *catB3, aac(3)-IIa* and *tetA*. By mapping, the main difference between the plasmids carried by these isolates was the deletion of a 2,400 bp sequence that displayed 99.7% homology with the transposase of Tn*5403*. Moreover, 60 single nucleotide variants have been detected between them. Both plasmids displayed an IncF plasmid from a ST131 *E. coli* isolate (JJ2434, unpublished) as its best blast hit. The comparative analysis with JJ2434 showed the absence of two regions of 9,329 bp and 1,740 bp that corresponded to deletions of genes coding for unknown functions, replication proteins, endonucleases, transcriptional regulators, and conjugation transfer proteins in LV36464 and LV27950 plasmids. The analysis of the QRDR of genes *gyrA* (from 67 to 106 aminoacids), *gyrB* (from 415 to 470 aminoacids), *parC* (from 47 to 133 aminoacids) and *parE* (from 450 to 528 aminoacids) revealed the presence of amino acid substitutions in *gyrA* (S83L and D87N) and *parC* (S80I and E84V) in both isolates.

A high number of mobile genetic elements was detected in the draft genomes of these isolates. Both harboured a plasmid (>98% homology) from incompatibility group IncFIA, which according to PlasmidFinder was classified as an IncFIA type 1. LV36464 accommodated an additional IncX plasmid. The distribution of insertion sequences present in LV36464 and LV27950 genomes was also globally similar: IS*1* (5.77% and 5.66%, respectively), IS*110* (3.85% and 1.89%, respectively), IS*1380* (1.92% and 1.89%, respectively), IS*200*_IS*605* (5.77% and 3.77%, respectively), IS*21* (3.85% and 3.77%, respectively), IS*3* (23.08% and 26.42%, respectively), IS*30* (1.92% and 3.77%, respectively), IS*4* (5.77% and 3.77%, respectively), IS*481* (3.85% and 3.77%, respectively), IS*6* (1.92% and 1.89%, respectively), IS*66* (11.54% and 11.32%, respectively), IS*As1* (1.92% and 1.89%, respectively), IS*L3* (19.23% and 18.87%, respectively) and IS*NCY* (9.62% and 9.43%, respectively). It is worth mentioning that the worldwide disseminated Tn*3* was only represented in the genome of LV36464 (3.39%), and IS*92* (1.89%) in LV27950. The specialized annotation of phage and prophages revealed that LV36464 harboured 17 regions: 8 intact, 6 incomplete and 4 questionable. These intact prophage regions ranged

between 17.4Kb and 51.5Kb, showing different numbers of coding sequences that varied between 24 and 88. In turn, LV27950 harboured 13 prophage regions: it displayed ten intact regions spanning between 20.6 to 86.1Kb. Globally, regions from five phages were present in the genomes of both and two were exclusive of each isolate (Table 4.8.5).

The detection of virulence factors in the genome of LV27950 revealed the presence of an increased serum survival factor provided by an ISS-encoding gene and a secreted autotransporter toxin denominated *sat* (Table 4.8.4). LV36464 shared the same virulence factors and, in addition, harboured a glutamate descarboxilase-encoding gene (*gad*). The overall estimation of pathogenicity factors present in the genome of the isolates, using known proteins with recognized involvement in pathogenicity as reference, enabled us to determine that the assembled contigs of LV36464 and LV27950 matched 553 and 544 pathogenic families, which resulted in the estimation of both isolates being human pathogens (93.1% and 93.3%), confirming their zoonotic potential.

4.8.4. Discussion

The prevalence of antibiotic resistance genes in isolates from animal origin has been fairly assessed (Szmolka *et al.,* 2011; Tamang *et al.,* 2011a; Tamang *et al.,* 2011b; Bardoň *et al.,* 2013; Clemente *et al.,* 2015). However, taking in account the current availability of genomic characterization tools, we are now able to proceed with more detailed characterizations of these genes, in a broader context. In this study, we characterized the genome of PMQR-producing *E. coli*. To understand the antibiotic susceptibility background of these specific isolates we have also evaluated the antibiotic susceptibility phenotypes of a collection of *S. enterica* and *E. coli* recovered from animals of different origins, in which the isolates were originally included.

The levels of non-wild type phenotypes revealed to be very distinct among *S. enterica* and *E. coli*. Non wild-type isolates for fluoroquinolones were particularly evident among poultry isolates recovered from *S. enterica*. Regarding *E. coli* isolates, the frequency of non-wild-type phenotypes to nalidixic acid and ciprofloxacin was higher in food-producing animals than in companion and zoo animals, which might be due to the high consumption of veterinary antibiotics in animal industrial units, particularly tetracyclines, sulphonamides and fluoroquinolones (EFSA, 2015). Portugal still represents a European country with high antibiotic use in animals. This fact raises concerns regarding antibiotic resistance in veterinary settings (EMA, 2014). Different MIC_{50} and MIC_{90} (3-fold dilutions) were noted for some groups of each species: *E. coli* isolates for nalidixic acid, ciprofloxacin, ampicillin, streptomycin, tetracycline, sulfamethoxazole and trimethoprim, and *S. enterica* for ampicillin, cefotaxime, tetracycline and trimethoprim.

Although PMQR determinants are typically responsible by low level resistance, their presence has been increasingly reported in animals, resulting in an additional effect on the

nonsusceptibility of fluoroquinolones (Ahmed and Shimamoto, 2013; Donati *et al.,* 2014; Jamborova *et al.,* 2015). The high MIC values of 128mg/L against nalidixic acid and 8 mg/L against ciprofloxacin observed in some of the isolates of our collection may be associated with amino acid alterations in the quinolone resistance-determining region (QRDR). Indeed, although the fluoroquinolone nonsusceptibility is frequently compromised by target modification, the PMQR-encoding genes have the potential to spread and promote co-selection of other antibiotic resistance genes (EMA, 2014). Late reports even suggest that the spread of PMQR may not be triggered by selection pressure, which justifies the low rates of these determinants in animals, despite the high use of fluoroquinolones (Veldman *et al.,* 2011).

Considering the high level MICs, most likely caused by QRDR chromosomal mutations that might mask the presence of PMQR, we decided in this study to retrospectively search for these determinants in all isolates of the collection, regardless of the MIC value. We have detected four PMQR-encoding genes (4/180) (two *qnrS1* and two *aac(6')-Ib-cr*) in *E. coli* LV46221, LV46743, LV36464 and LV27950 recovered from animals of different origins: a healthy turtle dove from a Zoo Park (2008), a diseased pet dog (2008), a bottlenose dolphin from a Zoo Park showing signs of respiratory infection (2009), and a second but healthy bottlenose dolphin from the same Zoo Park (2011) (Table 4.8.4).

The comparison of the genomes of QnrS1-producing *E. coli* revealed that isolates LV46221 and LV46743 were very similar in terms of their global pathogenicity potential, although they were recovered from animals of different classes and completely different backgrounds (Table 4.8.4). The absence of chromosomal mutations in the QRDR of isolates LV46221 and LV46743 corroborated the low fluoroquinolone MIC values obtained, which spanned between 0.38g/L for ciprofloxacin and 8g/L for nalidixic acid, highlighting the low level resistance conferred by QnrS1 determinants (Cavaco and Aarestrup, 2009). The plasmid region in which the *qnrS1* was enclosed in both isolates, that included the association with Tn*3*, has already been described in association with *qnrS1* genes in plasmids from *Shigella flexneri* recovered from food products, *Salmonella* Infantis from avian origin, and human clinical *Klebsiella pneumoniae* isolates, respectively (Hata *et al.,* 2005; Chen *et al.,* 2006; Kehrenberg *et al.,* 2006). Moreover, we have previously detected other *qnrS1* from animals in Portugal, associated with a similar genetic environment, exclusively in food-producing animals (Jones-Dias *et al.,* 2013). IncN plasmids harbored by LV46221 and LV46743 were assigned to ST1 by pMLST, which have also been associated with chickens and wild bird water in Czech Republic and the Netherlands, respectively (Ben Sallem *et al.,* 2014).

Few genomic differences were noticed between the two *aac(6')-Ib-cr*- and $bla_{CTX\text{-}M\text{-}15}$-harboring *E. coli*. In fact, the isolates could be assigned to the same genetic sublineage – ST131 fimH30-Rx, confirming clonal spread. Although samples have been recovered within a reasonable time span of 2 years, their origin refers to two bottlenose dolphins of the same

species held captive in the same Zoo Park. The presence of four chromosomal alterations in the QRDR region of isolates LV36464 and LV27950 was reflected in the high levels of fluoroquinolone MICs, which ranged between 8mg/L and >256mg/L. All antibiotic resistance genes detected in LV36464 and LV27950 (*aac(6')Ib-cr, bla*$_{OXA-1}$, *bla*$_{TEM-1,}$ *bla*$_{CTX-M-15}$, *catB3, aac(3)-IIa* and *tetA*) could be traced back to a single multidrug resistance IncFIA plasmid that showed 99.9% of homology with a plasmid submitted this year to Genbank in U.S.A (JJ2434, unpublished). Although 60 single nucleotide variants have been detected between the LV36464 and LV27950 plasmids, the main difference consisted of a single deletion that involved part of a transposase-encoding gene. The absence of a set of conjugation transfer proteins (*tra* genes), among other genes, highlighted the preponderance of clonal spread over horizontal gene transfer in ST131 *E. coli* (Nicolas-Chanoine *et al.,* 2014). Although several isoforms of identical plasmids have been detected worldwide, the simultaneous resistance to β-lactams, fluoroquinolones, aminoglycosides, chloramphenicol and tetracyclines has been a permanent feature, which reinforces the advantage that it confers (Boyd *et al.,* 2004; Zhou *et al.,* 2015). The detection of a ST131 fimH30-Rx *E. coli* in two dolphins, which are continuously in contact with a live audience, constitutes a public health concern. These clinically relevant multidrug resistant *E. coli* isolates have been on the rise for years (Nicolas-Chanoine *et al.,* 2014). Initially restricted to clinical contexts, recent findings suggest that their prevalence in non-clinical settings is maintained by the constant exchange of isolates throughout the time, as verified in this study (Mathers *et al.,* 2015).

Although *E. coli* is a common inhabitant of the gastrointestinal tract of humans and animals, the detected transposons, plasmids and bacteriophages are essential to the acquisition of pathogenicity factors that enlarge their ability to adapt to new niches, allowing bacteria to increase the capacity to cause a broad spectrum of diseases (Bien *et al.,* 2012). All isolates displayed genomic factors that may be critical to cause a zoonotic infection and that were reflected in high probabilities for the isolates to be human pathogens (>93%). Concerning virulence factors, we detected the presence of glutamate decarboxylase, increased serum survival gene and a secreted autotransporter toxin, irregularly distributed across the four isolates (Table 4.8.3), which did not denote any relation with the conditions of their respective hosts. These virulence factors confer resistance to extreme acid conditions of the intestines, enable the isolate to survive complement system and cause defined damage to kidney epithelium, being indicative of their ability to cause disease (Johnson *et al.,* 2008; Becker Saidenberg *et al.,* 2012). Indeed, *E. coli* isolates can frequently encode a number of virulence factors, which enable the bacteria to colonize the urinary tract and face highly effective host defenses (Bien *et al.,* 2012).

Although fluoroquinolones are consistently used in veterinary medicine, results presented in this study indicate that PMQR determinants occurred at a low frequency in these isolates (2.2%), as previously reported (Donati *et al.,* 2014; Jamborova *et al.,* 2015). However, the

studied groups of animals should still be considered potential reservoirs for PMQR-producing isolates, especially because there is the inherent potential for transboundary dissemination. These isolates presented a set of genetic features essential to promote their own successful spread: multiple antibiotic resistance genes carried by well-known mobile genetic elements, virulence factors adequate to zoonotic transmission and numerous other pathogenicity factors.

The analysis of many bacterial genomic features showed us great genetic relatedness between the two *qnrS1*- and *aac(6')-Ib-cr*-harboring isolates. The data gathered throughout this study illustrates two scenarios: the presence of the same strain in different hosts inhabiting remote locations and the persistence of a unique strain in a single niche during a long period of time. The strains were each associated with a case of symptomatic infection (LV46743 and LV36464) and with a report of microbiological control of an asymptomatic host (LV46221 and LV27950), which reinforces the ability of *E. coli* isolates to act as silent vehicles, allowing the accumulation of antibiotic resistance determinants, mobile genetic elements and other relevant pathogenicity determinants (Mathers *et al.,* 2015). It is not certain whether these bacteria spread from humans to animals, between different animals or from the environment to animals. However, in the case of companion animals, but particularly zoo animals, surveillance is essential to prevent continuous dissemination. The contact between animals and owners, zookeepers, visitors and handlers raises concerns, considering that these bacteria might easily spread to humans and to other animals (Veldman *et al.,* 2011; Ewers *et al.,* 2012).

Overall, permanent surveillance of health and sick animals should be strongly encouraged, regardless of their origin, in order to monitor future trends in the dissemination of resistance to fluoroquinolones and other antibiotics.

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4.9. *Draft genomic analysis of an avian multidrug resistant Morganella morganii isolate carrying qnrD1*

This research paper was submitted as:

Daniela Jones-Dias, ***Lurdes Clemente****, Inês Barata Moura, Daniel Sampaio, Teresa Albuquerque, Luís Vieira, Vera Manageiro*, Manuela Caniça. Draft genomic analysis of an avian multidrug resistant Morganella morganii isolate carrying qnrD1.* ***Frontiers in Microbiology,*** *2016****,*** *7: 1660, doi: 10.3389/fmicb.2016.01660.*

Contributions of the authors for the following manuscript:

Daniela Jones-Dias: conception and design of study, acquisition of laboratory data, analysis of data, drafting of article and critical revision of the manuscript, final approval of manuscript;
Lurdes Clemente: acquisition of laboratory and epidemiological data, critical revision of the manuscript, final approval of manuscript;
Inês Barata Moura: analysis of data, critical revision of the manuscript, final approval of manuscript;
Daniel Ataíde Sampaio: acquisition of laboratory data, final approval of manuscript;
Luís Vieira: acquisition of laboratory data, critical revision of the manuscript, final approval of manuscript; Vera Manageiro: analysis of data, drafting of article and critical revision of the manuscript, final approval of manuscript;
Manuela Caniça: conception and design of study, drafting of article and critical revision of the manuscript, final approval of manuscript.

ABSTRACT

Morganella morganii is a commensal bacterium and opportunistic pathogen often present in the gut of humans and animals. We report the 4.3Mbp draft genome sequence of a *M. morganii* isolated in association with an *Escherichia coli* from broilers in Portugal that showed macroscopic lesions consistent with coliseptisemia. The analysis of the genome matched the multidrug resistance phenotype and enabled the identification of several clinically important and potentially mobile acquired antibiotic resistance genes, including the plasmid-mediated quinolone resistance determinant *qnrD1*. Mobile genetic elements, prophages and pathogenicity factors were also detected, improving our understanding towards this human and animal opportunistic pathogen.

Keywords**:** *qnrD1*, plasmid, multidrug resistance, *Morganella morganii*, WGS

4.9.1. Introduction

The Gram negative *Morganella morganii* belongs to the tribe *Proteeae* of the family *Enterobacteriaceae* (O'Hara *et al.,* 2000). This species, along with other elements of *Proteus* and *Providencia* genera can be found in the normal flora of humans, reptiles and in the wider environment (O'Hara *et al.,* 2000; Lee *et al.,* 2006; Dipineto *et al.,* 2014). However, *M. morganii* isolates also constitute clinically relevant opportunistic pathogens, which can cause a variety of infections. Nosocomial outbreaks have been reported, suggesting that infections caused by *M. morganii* can lead to major clinical problems, such as wounds, urinary tract infections and septicemia (Nicolle, 2001; Tsanaktsidis *et al.,* 2003; Falagas *et al.,* 2006; Lee *et al.,* 2006; Lin *et al.,* 2015).

This bacterium has also been associated with infections in animals and with human animal bite wound infections, which suggests that *M. morganii* may also cause zoonotic infectious diseases (Ono *et al.,* 2001; Choi *et al.,* 2002; Abrahamian and Goldstein *et al.,* 2011; Zhao *et al.,* 2012; Di Ianni *et al.,* 2015).

Several factors can affect the progression and severity of an infection. The presence of pathogenicity determinants is essential to the success of *M. morganii* in any environment, particularly in food animal farms, where the pressure caused by antibiotic treatments and the lack of prophylactic measures to avoid the spread of infectious diseases are usually noteworthy (Chen *et al.,* 2012; Lin *et al.,* 2015). It is globally accepted that horizontal gene transfer plays an important role in the dissemination of antibiotic resistance genes and pathogenicity factors (Huddleston, 2014). Considering that *M. morganii* may share the habitat with other clinically relevant pathogens, the investigation of any multidrug resistant isolate recovered from poultry is an important assignment.

Resistance to quinolones and fluoroquinolones has been increasingly reported among human and veterinary isolates, very likely as a consequence of the great usage of those antibiotics (Tamang *et al.,* 2011a). The *qnrD* gene, now denominated *qnrD1* due to the report of a second variant of the gene (Abgottspon *et al.,* 2014), is a relatively uncommon antibiotic resistance gene, which has been described in members of the *Proteeae* family from different origins (Mazzariol *et al.,* 2012; Zhang *et al.,* 2013; Nasri Yaiche *et al.,* 2014). This plasmid-mediated quinolone resistance (PMQR) determinant encodes a protein that protects DNA gyrases and topoisomerases from quinolone inhibition (Cavaco *et al.,* 2009; Jacoby *et al.,* 2014). Carriage of PMQR-encoding genes frequently confers modest increases to the minimum inhibitory concentrations (MIC) of fluoroquinolones (Poirel *et al.,* 2012b). Current studies have identified the environment, particularly animals and aquatic habitats, as a reservoir of PMQR genes (Poirel *et al.,* 2012b).

The aim of this study was to investigate the molecular background sustaining the multidrug resistance and pathogenicity of a *M. morganii* isolate. In this study, we report the antibiotic susceptibility and the draft genome sequence of a *qnrD1*-harboring avian isolate. The data gathered from bioinformatics analysis may improve our understanding towards this opportunistic pathogen.

4.9.2. Material and Methods

Bacterial isolation, antibiotic susceptibility and molecular characterization

M. morganii INSRALV892a was recovered in association with *E. coli* INSRALV892b in 2012 from a 13-days old broiler, recovered from a poultry industrial unit in Portugal. Samples consisted of organs (macerates of liver and spleen) collected during post-mortem examination that were submitted for bacteriological analysis. During post-mortem examination, the birds showed macroscopic lesions consistent with coliseptisemia: aerosaculitis, acute enteritis, perihepatitis and fibrinous peritonitis. Suspected *Enterobactericeae* colonies obtained in MacConkey agar plates were isolated in non selective media and identification was performed using API 20E strips (BioMérieux, Marcy-l'Étoile).

MICs were determined for both isolates by agar dilution method to ten antibiotics: ampicillin, cefotaxime, ceftazidime, meropenem, ciprofloxacin, gentamicin, chloramphenicol, trimethoprim, colistin and tigecycline. To assess nonsusceptibility, interpretation of results was performed according to the clinical breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org).

PMQR [QnrA, QnrB, QnrC, QnrD, QnrS, QepA, OqxAB and Aac(6')-Ib-cr]-, β-lactamase (TEM, SHV, OXA-G1 and CTX-M)-, and integrase (class 1, 2 and 3)-encoding genes wereidentified by PCR and confirmed by sequencing using DNA of both isolates, as previously described (Clemente *et al.,* 2013).

The transference ability of specific antibiotic resistance genes from *M. morganii* INSRALV892a and *E. coli* INSRALV892b was assessed by broth mating out assays using *E. coli* J53 $NaN3^R$ as recipient strain, as described elsewhere (Jones-Dias *et al.,* 2016). Resistant J53 *E. coli* transconjugants were then selected on MacConkey agar plates containing amoxicillin (100 mg/l) or ciprofloxacin (0.06 mg/l) together with sodium azide (200 mg/l), according with the antibiotic susceptibility profile of the donor isolates. To confirm the acquisition of the antibiotic resistance genes, we detected and identified the determinants in the transconjugants, following the methodology described above in this section.

Genome sequencing and analysis

Genomic DNA of *M. morganii* INSRALV892a was extracted using DNeasy Blood and Tissue Kit (Qiagen, Aarhus), and DNA quantification was performed by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Carlsbad), according to the manufacturer's instructions. Libraries were prepared from 1 ng of genomic DNA using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego), also following manufacturer's instructions. Whole Genome Sequencing (WGS) was performed using 150 bp paired-end reads on a MiSeq (Illumina, San Diego).

Sequence reads were then trimmed and filtered according to quality criteria. Briefly, reads were assembled *de novo* using CLC genomics workbench version 8.5 (Qiagen, Aarhus), which is based on Smith and Waterman algorithm. The raw FASTQ reads were first processed by quality score trimming (quality score limit = 0.05), removing all reads containing more than 2 ambiguous nucleotides or shorter than 50 bp. Trimmed reads were then de *novo* assembled with automatic bubble, word size and paired distance detection, using mapping mode “map reads back to contigs” (including scaffolding, and minimum contig length of 400 nucleotides). The NCBI prokaryotic genome automatic annotation pipeline (PGAAP) was used for annotation (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). All *de novo* contigs were BLAST searched against the GenBank’s non-redundant nucleotide collection (nr/nt). PathogenFinder 1.1, ResFinder 2.1 and PlasmidFinder 1.3 were used to estimate the number and type of pathogenicity determinants, antibiotic resistance genes and plasmids, respectively, within the genome (Zankari *et al.,* 2012; Cosentino *et al.,* 2013; Carattoli *et al.,* 2014). PHAST search web tool was used to identify and annotate any prophage sequence present in the draft genome (Zhou *et al.,* 2011). ISsaga semi-automatic annotation system was also applied to detect the presence of insertion sequences (IS) (Varani *et al.,* 2011).

Contigs containing antibiotic resistance genes were searched for identity through blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nr/nt NCBI database, and further mapped against the closest bacterial plasmids or genomes using CLC Genomics Workbench version 8.5.

Nucleotide Sequence GenBank Accession Numbers

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LGYC00000000. The version described in this paper is the LGYC01000000 (http://www.ncbi.nlm.nih.gov/nuccore/LGYC00000000.1).

4.9.3. Results and Discussion

M. morganii INSRALV892a was found to be nonsusceptible to ampicillin (>64mg/L), cefotaxime (>4mg/L), ceftazidime (2mg/L), ciprofloxacin (>8mg/L), chloramphenicol (16mg/L), gentamicin (>32mg/L), trimethoprim (>32mg/L), colistin (>16 mg/L) and tigecycline (0.5 mg/L). However, it is important to highlight that *M. morganii* is intrinsically resistant to colistin, while tigecycline has also been shown to have poor activity against this species (http://www.eucast.org). Among the antibiotics tested, the isolate was susceptible only to meropenem (0.125mg/L). The *E. coli* INSRALV892b was also characterized with regard to antibiotic susceptibility and found to be nonsusceptible to ampicillin (>64mg/L), cefotaxime (>4mg/L), ceftazidime (2mg/L) and trimethoprim (>32mg/L), and susceptible to meropenem (≤0.03mg/L), ciprofloxacin (0.125mg/L), chloramphenicol (≤8mg/L), gentamicin (≤0.5mg/L), colistin (≤1 mg/L) and tigecycline (0.5mg/L). The molecular characterization of the isolates showed the presence of *qnrD1* and a class 2 integron in *M. morganii* INSRALV892a, and $bla_{\text{CTX-M-1}}$ gene flanked by an IS*Ecp1* and *orf477*, as well as a class 1 integron in *E. coli* INSRALV892b. Conjugation experiments only revealed the transference of $bla_{\text{CTX-M-1}}$ from *E. coli* INSRALV892b to isogenic J53 *E. coli* strain.

The WGS assembly of *M. morganii* INSRALV892a yielded 74 contigs (each >200 bp long and >100-fold coverage), which together comprised 4,267,817bp, showing a GC content of 50.6%. The largest contig was 523,676bp long and the N50 statistic, which stands for the minimum contig length of at least 50% of the contigs, was 342,352bp. The average length of the obtained contigs was 34,190bp. Among the obtained data, six contigs, ranging from 802 to 8,575 in length and showing a minimum coverage of 117.7 fold, matched plasmid sequences of different species. Overall, the genome sequence comprised 4,116 putative genes, among which 3,950 consisted of protein encoding sequences.

In silico analysis of the antibiotic resistance genes (90% identity and 40% minimum length) revealed the presence of loci for acquired resistance to aminoglycosides (*aadA1y*, *aph(3')-Ic*, and *strA-strB*), β-lactam ($bla_{\text{OXA-1}}$), fluoroquinolones (*qnrD1*, ***aac(6')-Ib-cr***), phenicols (*catA2 and catB3*), rifampicin (Δ*arr*), sulphonamides (*sul2*), trimethoprim (*dfrA1*), tetracycline (*tetY)* and streptothricin (*sat2*). Nonsusceptibility to third generation cephalosporins such as cefotaxime and ceftazidime was not associated to any extended-spectrum β-lactamase, suggesting the involvement of inducible or stably derepressed *M. morganii* chromosomal *ampC* gene, the $bla_{\text{DHA-type}}$ gene (Harris & Ferguson, 2012).

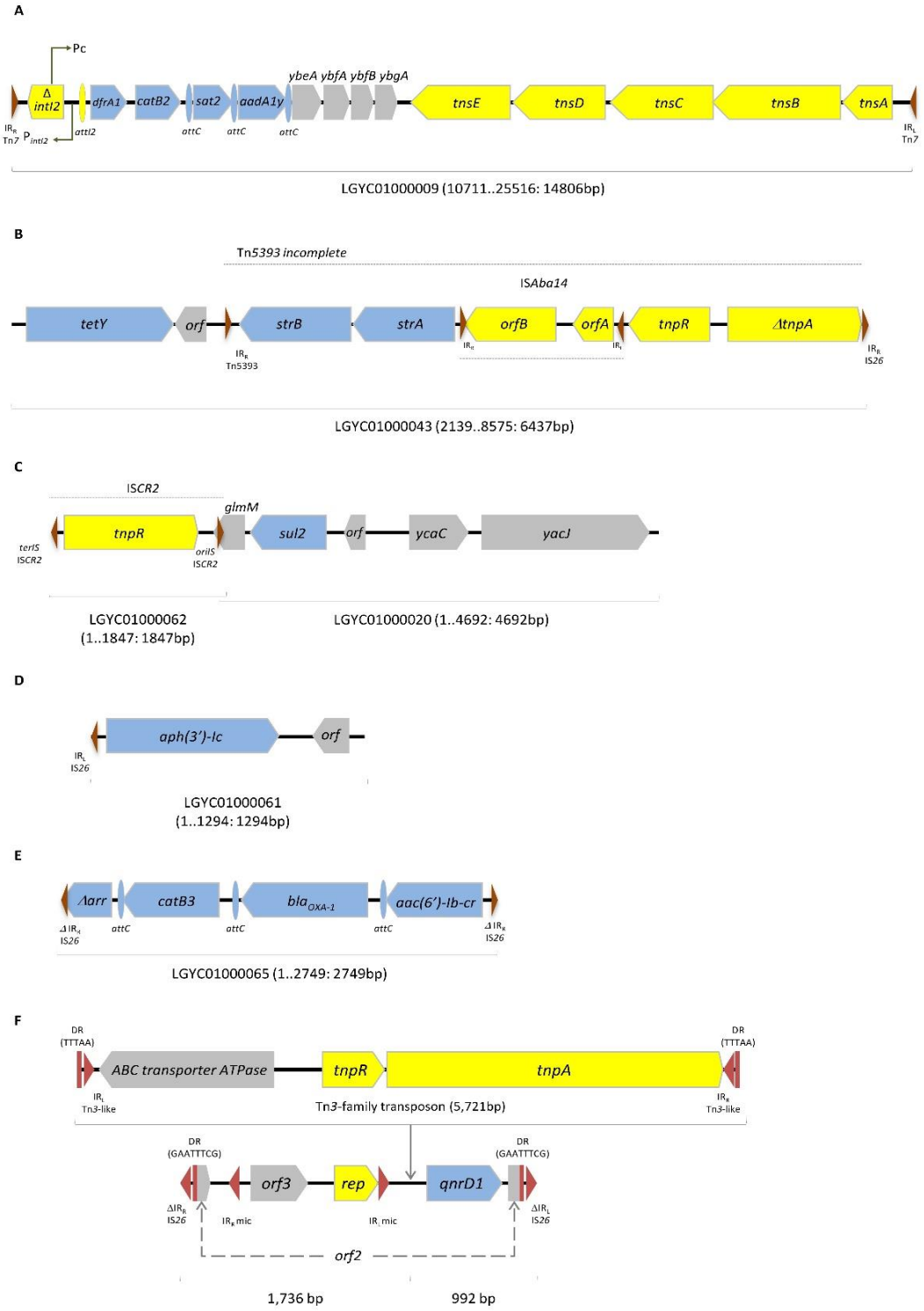
The *dfrA1*, *catB2*, *sat2*, and *aadA1y* genes were enclosed in an In*2-17* class 2 integron that has already been described, for instance, in *Proteus vulgaris* isolates from China (HQ386830) (Figure 4.9.1A). Genes encoding resistance to tetracycline (*tetY*) and streptomycin (*strA-strB*) were detected in association with each other, and with proteins linked to DNA transfer processes, such as IS*Aba14* and an incomplete Tn*5393* (Figure 4.9.1B). The sulphonamide resistance gene *sul2* was flanked upstream by a *glmM*-containing region and IS*CR2*, while the downstream region consisted of a chromosomal region typical of *M. morganii* (Figure 4.9.1C); the *glmM* gene (formerly called *ureC*) encodes a phosphoglucosamide mutase that is considered a housekeeping gene essential for the cell wall synthesis (Tavares *et al.,* 2003).

The aminoglycoside resistance gene *aph(3')-Ic* was associated to an IS*26* IR and a unknown *orf* (Figure 4.9.1D), and the genes Δ*arr*, *catB3*, *bla*$_{OXA-1}$ and *aac(6´)-Ib*-cr were enclosed together, as gene cassettes of an integron variable region that has been previously found, for instance, in *S. enterica* from livestock (Figure 4.9.1E). However, in the latter, the array was flanked up and downstream by truncated inverted repeats of IS*26* while no conserved integron regions were found. The genetic regions where antibiotic resistance genes were incorporated were highly similar to other plasmid-borne structures, previously described in different Gram-negative bacteria, suggesting acquisition of resistance determinants through horizontal gene transfer (Figure 4.9.1).

The *qnrD1* gene was enclosed in an 8,449bp length contig (LGYC01000051: mean coverage of 183.9-fold and a total read count of 13,382), matching a Col3M plasmid. Indeed, the *qnrD1* gene is frequently located on small nonconjugative plasmids harbored by *Proteeae*, which was corroborated by our conjugation assay (Zhang *et al.,* 2013). Furthermore, the *qnrD1* gene has been located in plasmids showing similarities with a specific *Providencia vermicola* plasmid, suggesting that these small nonconjugative plasmids might be the product of recombination between an unknown *qnrD*-bearing region and a native plasmid from *Proteeae*.

This contig accommodated a 2,683bp sequence showing 99% identity with previously described *qnrD1*-harboring plasmids, such as pGHS09-09a (HQ834473) and pCGS49 (JQ776507), reported in France and China, respectively. The three *qnrD1*-encoding sequences shared a *rep* gene (also reported as *orf4*) and two additional *orfs* (Figure 4.9.1F) (Guillard *et al*., 2012; Zhang *et al.*, 2013). Six single nucleotide variants (SNVs) were detected between *qnrD1*- INSRALV892a and the pGHS09-09a plasmid, two within the *rep* gene. Only one SNP was found with relation to pCGS49 in a non-coding region. The *qnrD1* gene was located within a mobile insertion cassette (mic) element bracketed by two inverted repeats, as previously described (Guillard *et al*., 2014) (Figure 4.9.1F).

Figure 4.9.1. Examples of contigs containing antibiotic resistance genes in *M. morganii* INSRALV892a. **(A)** selection of contig 9 shows a complete In*2-17* class 2 integron encoding *dfrA1*, *catB2*, *sat2*, and *aadA1y* ; **(B)** selection of contig 43 encodes *tetY* and *strA-strB*, in addition to mobile genetic element and hypothetical proteins; **(C)** selection of contig 20 encodes *sul2* flanked by *M. morganii* genes; **(D)** Contig 61 consists of a small sequence accommodating *aph(3')-Ic*; **(D)** Contig 69 displays an integron variable region encoding *arr-2*, *catB3*, *bla*OXA-1 and *aac(6')-Ib-cr*. Blue, antibiotic resistance genes; Yellow, mobile genetic elements; Grey, other genes.



Comparative bioinformatics analyses revealed the disruption of *orf2* caused by the insertion of IS*26* left and right inverted repeats flanking a region containing *orf3* and *rep*, within a mic, followed by *qnrD1*. In fact, this shows that is possible that LGYC01000051 contig could be either a Col3M plasmid missing an IS*26*-flanked region or a *qnrD1*-containing region that has become incorporated into a larger plasmid.

In addition, this region (Figure 4.9.1F) included three additional open reading frames: besides an ABC transporter-encoding gene perfectly matching a protein from *Aeromonas hydrophila*, this region harbored Tn*3*-like resolvases- and transposase-encoding genes, displaying *E. coli* plasmid pH226B (KX129784) as its best blast hit.

Mobile genetic elements are crucial tools for the acquisition of genetic diversity (Huddleston, 2014). Thus, we decided to search for and characterize the elements detected in the *M. morganii*'s genome. We identified 10 prophage regions, among which six were incomplete and four were intact, comprising 381 prophage-related genes. Intact prophage regions presented between 24.2Kb and 41.7Kb and harbored 13 to 56 coding DNA sequences. The intact phages showing highest scores were assigned to Enterobacteria phage SfV, which is associated with O-antigen modification and serotype conversion in *Shigella flexneri*, and Enterobacteria phage mEp235 that consists of an unclassified Lambda-like virus (Sun *et al.,* 2013). The bioinformatics detection of IS resulted in the identification of seven transposable elements: IS*3*, Tn*3,* IS*L3*, IS*256*, IS*6*, IS*91* and IS*As1*. Besides the already mentioned Col3M no other typable plasmids were detected within the *M. morganii* genome, according with the PlasmidFinder tool.

Based on the probability scores assigned by PathogenFinder web-server (Consentino *et al.,* 2013), the isolate has a probability of acting as a human pathogen of 68.9%, which is in line with the opportunistic nature of this species. *M. morganii*'s genome matched 22 pathogenic families and 5 non-pathogenic families. Pathogenic factors showed diversity of functions and hosts, and included, for instance, transposase *insA* from IS*91* of *Salmonella enterica*, transposition protein *tnsE* of the Tn7 transposon of *Shigella flexneri*, and transcriptional regulator LysR family protein from *S. enterica*.

Multidrug resistant *M. morganii* isolates are rare and normally associated with non invasive nosocomial opportunistic infections in humans (Nicolle, 2001; Falagas *et al.,* 2006). The detection of an avian *M. morganii* isolate harboring multiple and mobile antibiotic resistance genes and pathogenicity factors raises concerns regarding the dissemination of infection in birds and potential risk of zoonotic transmission. Several factors may affect the susceptibility of poultry to bacterial diseases, namely environmental stressors and previous antibiotic treatments, which are crucial to the development of infections involving different *Enterobacteriaceae* (Burkholder *et al.,* 2008). The detection of an avian *M. morganii* isolate harboring multiple and mobile antibiotic resistance genes and pathogenicity factors raises

concerns regarding the dissemination of infection in birds and potential risk of zoonotic transmission.

M. morganii is a well characterized opportunistic pathogen (Lee *et al.,* 2006). However, its detection in poultry flocks, co-habiting the same hosts as other clinically important pathogens, makes it susceptible to the acquisition and donation of pathogenicity factors by horizontal gene transfer (Huddleston, 2014). To the best of our knowledge this report represents the first genome analysis of an isolate from animal origin carrying *qnrD1*. This genome sequence represents a valuable resource for studies on the epidemiology of zoonotic *M. morganii* isolates, and its features may be used as markers for the study of antibiotic resistance.

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Chapter 5

OTHER RESISTANCE MECHANISMS

5.1. Salmonella Enteritidis *isolate harboring multiple efflux pumps and pathogenicity factors, shows absence of O antigen polymerase gene*

This research paper was submitted as:

Daniela Jones-Dias, **Lurdes Clemente***, Conceição Egas, Hugo Froufe, Daniel Sampaio, Luís Vieira, Maria Fookes, Nicholas Thompson, Vera Manageiro*, Manuela Caniça. Salmonella Enteritidis isolate harboring multiple efflux pumps and pathogenicity factors, shows absence of O antigen polymerase gene.* ***Frontiers in Microbiology,*** *2016, 7:1130.*

Contributions of the authors for the manuscript:

Daniela Jones-Dias: design of study, molecular laboratory assays, analysis of data, drafting of article and final approval of manuscript;
Lurdes Clemente: laboratory microbiological assays and final approval of manuscript;
Conceição Egas: genomic sequencing assays, analysis of data and final approval of manuscript;
Hugo Froufe: genomic sequencing assays, analysis of data and final approval of manuscript;
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Maria Fookes: analysis of data and final approval of manuscript;
Nicholas Thompson: analysis of data and final approval of manuscript;
Vera Manageiro: design of study, análise dos dados, revisão e aprovação final do manuscrito
Manuela Caniça: desenho do estudo, analysis of data, colaboration in drafting of article, final revision and approval of manuscript, and eddition of manuscript

**These authors had equal participation in the study*

BACKGROUND

Salmonella enterica is one of the most important causes of gastrointestinal infection in humans, being the great majority of infections related to the consumption of poultry meat and eggs (EFSA/ECDC, 2015; Foley & Lynne, 2008).

In animals, infections caused by serotype Enteritidis are rarely responsible for severe disease with animals frequently becoming asymptomatic carriers, except in the case of young chicks and poults, where outbreaks exhibiting clinical disease are often accompanied by high mortality rates (Foley *et al.,* 2008; Foley *et al.,* 2013). Indeed, *S. enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) has been responsible for severe disease in industrial poultry farming facilities worldwide, posing a potential hazard for public health (Lutful Kabir, 2010).

In order to be infectious, *Salmonella* needs to adapt to different niches and conditions, where virulence and heavy-metal-tolerance factors play an important role, through co-selection events and the formation of pathogenicity islands, respectively (Hensel, 2004; Medardus *et al.,* 2014). Furthermore, antibiotic resistance determinants can also facilitate their survival, with ubiquitous chromosomally encoded efflux mechanisms, playing an important role in both intrinsic and acquired multidrug resistance. Other resistance mechanisms, such as changes in the membrane permeability, enzymatic modification and target alterations may increase the levels of bacterial resistance, contributing to the success of the infection (Poole, 2004; Delmar *et al.,* 2014; Li *et al.,* 2015).

Both antibiotic susceptibility determination and serotyping constitute very useful tools for the epidemiologic classification of *Salmonella enterica* isolates. Indeed, in *S. enterica*, the resistance rates fluctuate according to the serotype and with the antibiotic (Clemente *et al.,* 2015). Classically, serotyping is based on the antigenic reactivity of lipolysaccharide (O antigen) and flagellar proteins (H antigen), followed by a designation using names or formulas (Grimont & Weill, 2007). In this study, we aimed to analyze the genome of a *S*. Enteritidis isolate responsible for omphalitis in chicks, exploring the molecular features associated with antibiotic resistance and pathogenicity, as well as the ability to spread the respective determinants.

Keywords**:** *Salmonella* Enteritidis, omphalitis, *wzy* deletion, epidemiology, pathogenicity factors, MGE, Metal tolerance

5.1.1. Methods

Bacterial isolate, antibiotic susceptibility testing and serotyping

The isolate (LV60) was recovered from a sample collected from the yolk sac of a chick with omphalitis, under the scope of the *"Salmonella* National Control Programme in food-producing animals and food of animal origin for bacteriological diagnosis, serotype identification and antibiotic susceptibility testing". The guidelines of the Commission Decision (CD), 2007/407/EC were followed. LV60 was tested for its antimicrobial resistance through the determination of minimum inhibitory concentrations (MICs) using the agar dilution method, as previously described (Clemente *et al.,* 2013) and according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org/). Briefly, a panel of eleven antibiotic compounds was tested in a two-fold concentration series over the following ranges: ampicillin and tetracycline (0.5 - 64µg/mL), gentamicin and trimethoprim (0.25 - 32µg/mL), ciprofloxacin (0.008 - 8µg/mL), cefotaxime (0.06 - 8µg/mL), nalidixic acid and streptomycin (2 - 512µg/mL), chloramphenicol (2 - 256µg/mL), florfenicol (1 - 128µg/mL) and sulphamethoxazole (8 - 1024µg/mL). The epidemiological cut-off values recommended by EUCAST to *Salmonella* spp. were used for the interpretation of susceptibility testing results. Quality control was performed using the *Escherichia coli* ATCC 25922 strain. LV60 isolate was then serotyped by the slide agglutination method for its O and H antigens using the method of Kauffman-White scheme (Grimont & Weill, 2007).

Whole Genome Sequencing (WGS), assembly and annotation

Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen), and DNA quantification was performed by Qubit Fluorometric Quantitation (Life Technologies), according to with the manufacturer's instructions. The genome was sequenced using a double strategy of 454 (Roche) and MiSeq (Illumina) sequencing.

Five hundred nanograms of bacterial DNA were fragmented by nebulization, followed by adaptor ligation to create double stranded DNA libraries and sequenced on a 454 GS FLX Titanium according to the standard manufacturer's instructions (Roche-454 Life Sciences, Brandford, CT, USA). The second genome library was prepared from 1ng of genomic DNA using the Nextera XT DNA Sample Preparation Kit (Illumina, CA) and sequenced on the Illumina MiSeq sequencer (Illumina) using paired-end 2x150 bp reads.

First quality evaluation of raw read sequences and their corresponding quality values were assigned by the FastQC software. Reads were then trimmed and filtered according to quality criteria, and *de novo* assembled with Ray, version 2.3.1 (Boisvert *et al.,* 2010). Contigs were searched for identity through blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nr/nt NCBI database to identify the closest bacterial genome and/or plasmid. Therefore, LV60 genome was mapped against the bacterial genome of *S*. Enteritidis strain p125109 and its

plasmid (NC_011294 and HG970000, respectively) using GS Mapper version 2.9 (Roche). Additionally SNV (single nucleotide variants) and structural variants were also detected with the GS Mapper (Roche, version 2.9).

Structural and functional annotation was performed using PGP (Prokaryotic Genome Prediction) (Egas *et al.,* 2014), an in-house developed pipeline. Taxonomy identification was performed by BLASTP search against the NCBI GenBank non-redundant (nr) database of the 16s rRNA sequence gene, identified in the previous step and confirmed using RNAmmer v1.2 (Lagesen *et al.,* 2007).

The final data was submitted in the DDBJ/EMBL/GenBank databases, using the Sequin software tool (http://www.ncbi.nlm.nih.gov/Sequin/). This dataset, which includes files in Genbank (LIHI01.1.gbff.gz), Fasta (LIHI01.1.fsa_nt.gz) and ASN.1 (LIHI01.1.bbs.gz) formats, can be accessed and/or reused at http://www.ncbi.nlm.nih.gov/nuccore/LIHI00000000.

In silico analyses

CLC genomics workbench 8.0 (QIAGEN, Aarhus), PathogenFinder 1.1, ResFinder 2.1, PlasmidFinder 1.3 and MLST 1.8 (MultiLocus Sequence Typing) were used to estimate the number of pathogenicity determinants, acquired antibiotic resistance genes, plasmids and the MLST using the *S.* Enteritidis genome (Larsen *et al.,* 2012; Zankari *et al.,* 2012; Cosentino *et al.,* 2013; Carattoli *et al.,* 2014). SeqSero tool was used for *Salmonella* serotyping by whole genome sequencing (Zhang *et al.,* 2015).

PHAST search web tool was applied to detect, identify and annotate prophage sequences (Zhou *et al.,* 2011). ISsaga was used for the high throughput identification and semiautomatic annotation of insertion sequences in the genome (Varani *et al.,* 2011). The presence of molecular determinants of antimicrobial resistance was predicted based on homology and SNP models using the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/analyze/rgi), through Resistance Gene Identifier software (RGI; McArthur *et al.,* 2013).

5.1.2. Results

LV60 isolate was serotyped as *S.* Enteritidis, using the method of Kauffman-White scheme, and found to be wild-type to all the antibiotics tested, except tetracycline.

The *de novo* assembly yielded 4.977Mbp distributed in 83 contigs (largest contig with 970,921bp) with a N50 of 491,005bp. Overall, the structural and functional annotation with PGP detected 97 tRNA genes, 7 rRNA genes and identified 4,656 mRNA genes.

From mapping against the bacterial genome of *S*. Enteritidis strain p125109, the main difference between the two genomes was the absence of the O-antigen polymerase gene *wzy* in the LV60 isolate, which in *S.* Enteritidis is located outside the O antigen gene cluster (Liu *et*

al., 2014). The coding sequence of *wzy* gene was searched against the assembled genome using blastn, confirming its absence. The flanking regions of *wzy* gene, which coded for a disrupted membrane and a hypothetical protein, were also absent. The *wzy* gene is involved in the Wzx/Wzy-dependent pathway, which constitutes the predominant pathway for O-antigen production in Gram-negative bacteria, specifically in *Salmonella* (Hong *et al.,* 2015).

However, in this study, the absence of the *wzy* gene did not compromised the use of a high-throughput genome sequencing serotype determination method (Zhang *et al.,* 2015), which corroborated the result obtained by the gold standard method. Indeed, this method, based on the detection of O and H antigens encoding genes, predicted an antigenic profile 9:g,m:- based on the O-9,46 *wba*V gene, which encodes to the O-antigen tyvelosyl transferase. Furthermore, the *S.* Enteritidis serotype was confirmed by the presence of *sdf gene* (*Salmonella* difference fragment virulence gene), a characteristic marker of commonly circulating *S. enterica* Enteritidis (Agron *et al.,* 2001).

Sixty-one SNVs were detected between LV60 and the *S*. Enteritidis strain p125109. The SNVs that resulted in amino acid substitutions are represented in Table 5.1.1. *In silico* analysis with ResFinder tool did not reveal the presence of any acquired antibiotic resistance genes (90% identity and 40% minimum length) or plasmids (95% identity). However, the RGI analysis, using the *perfect algorithm*, showed the presence of a *Salmonella*-specific MerR-like gold (Au) sensor- GolS - involved in Au resistance (Pontel *et al.,* 2007). This constitutes a matter of concern since antibacterial biocides and metals can contribute to the development and maintenance of antibiotic resistance in bacterial communities through mechanisms of cross- or co-resistance (Baker-Austin *et al.,* 2006; Lemire *et al.,* 2013; Pal *et al.,* 2015).

Furthermore, the RGI strict algorithm, which detects previously unknown variants of known antimicrobial resistance genes, identified 52 genes involved in efflux, transport, and permeability, which might justify the low-level tetracycline resistance identified by phenotypic methods (Table 5.1.2). Resistance to additional classes of antibiotics such as fluoroquinolones, aminoglycosides and chloramphenicol were bioinformatically predicted. Indeed, efflux pumps are often associated with discrete decreases in antibiotic susceptibility that may not necessarily reflect an alteration in interpretation categories (Fernández & Hancock, 2012).

Genes responsible for the intrinsic resistance to benzylpenicillin, glycopeptides, macrolides, and rifampicin were also detected.

The total number of pathogenicity determinants present in the genome of *S.* Enteritidis LV60, matching 1164 pathogenic families, showed a 94.1% certainty of the isolate being a human pathogen. Here we highlight the presence of *Salmonella* Pathogenicity Island 4, which usually encodes a non-fimbrial adhesion and the cognate type 1 secretion system (Gerlach *et al.,* 2007).

The use of complementary web tools assigned this isolate to ST11, which according with MLST data (http://mlst.warwick.ac.uk/) is commonly found among CTX-M-14 and CTX-M-15-producing *S.* Enteritidis human isolates (Kim, 2011; Bado *et al*., 2012;). In this study, the identification of ST11 in an isolate of animal origin, together with other pathogenicity determinants may suggest its zoonotic potential.

We also identified 6 prophage regions, among which three were incomplete and three were intact. The last included prophage regions reaching the lengths of 64.3Kb, 49.2Kb and 31.7Kb, and encoding 42, 78 and 66 DNA coding sequences, respectively.

Overall, 33 different IS were detected within the genome, which were distributed as follows: 27.03% of IS*3* family, 18.92% of IS*256* family, 13.51% of IS unclassified elements, 10.81% of IS*200*/IS*605* complex and of IS*L3* family, 8.11% of IS*481* family, 5.41% of IS*630* family, and 2.7% of IS*1* and IS*110* families. All identified structures (pathogenicity island, prophages, ISs) constitute a multiplicity of pathogenicity factors in LV60 *S*. Enteritidis isolate and contribute for the fitness of the isolate in different environments; its presence may also suggest the possibility of acquisition of other factors by different mechanisms, including resistance genes e.g. by horizontal gene transfer, contributing to its biological diversity and genetic evolution.

Table 5.1.1. Single nucleotide variants that represent amino acid substitutions in *S.* Enteritidis LV60 using *S*. Enteritidis strain p125109 as the reference genome.

Reference Position	Reference	Allele	Gene (Product)	Amino acid change	Coverage
40158	C	T	SEN_RS00180 (arylsulfatase)	Pro92Ser	155
55278	C	A	*ileS* (isoleucine-tRNA ligase)	Ala557Glu	144
93979	G	A	SEN_RS00415 (hypothetical protein)	Ala96Thr	127
156264	G	A	SEN_RS00685 (peptidase M23)	Gly299Asp	123
353437	T	C	SEN_RS01600 (isopropylmalate isomerase)	Val454Ala	119
357149	A	T	SEN_RS01625 (hypothetical protein)	Leu1Met	177
401018	C	A	*prpE* (acetyl-CoA synthetase)	Arg9Ser	132
411602	T	G	SEN_RS01845 (hypothetical protein)	Trp209Gly	58
561577	T	C	SEN_RS02560 (MFS transporter)	Ser333Pro	68
659902	T	G	*dpiB* (sensor histidine kinase)	Tyr3Asp	52
988620	G	C	SEN_RS04610 (hypothetical protein)	Ala89Pro	130
1044895	G	T	*helD* (DNA helicase IV)/Mobile element	Ala204Ser	75
1156702	G	C	*sirA* (virulence gene transcriptional regulator)	Val181Leu	112
1325689	A	G	SEN_RS06450 (hydrogenase-1 operon protein HyaF)	Tyr209His	93
1427037	T	A	SEN_RS06930 (diguanylate phosphodiesterase)	Asp16Glu	92
1787654	A	G	SEN_RS08735 (transporter)	Arg348Gly	79
1807289	G	A	SEN_RS08820 (lipoprotein)	Ala14Val	79
1931818	C	T	SEN_RS09505 (NAD-dependent deacetylase)	Met37Ile	82
2419980	G	A	SEN_RS11950 (NADH: ubiquinone oxidoreductase subunit M)	Leu474Phe	130

Reference Position	Reference	Allele	Gene (Product)	Amino acid change	Coverage
2426844	A	G	SEN_RS11980 (NADH dehydrogenase subunit G)	Val610Ala	125
2463887	T	C	SEN_RS12170 (amino acid transporter)	Ile452Val	34
2647060	G	A	SEN_RS12985 (outer membrane protein RatA)	Pro459Ser	108
2647626	G	T	SEN_RS12985 (outer membrane protein RatA)	Ala270Glu	111
2672592	A	C	SEN_RS13070 (hypothetical protein)	Ile313Ser	61
2956057	C	A	SEN_RS14420 (2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase)	Arg53Leu	123
3185834	C	A	SEN_RS15495 (D-mannonate oxidoreductase)	Asn151Lys	81
3659470	G	T	SEN_RS17815 (membrane protein)	Gln71Lys	122
3802073	G	A	*coaD* (phosphopantetheine adenylyltransferase)	Val116Ile	127
4051393	T	C	SEN_RS19620 (DNase TatD)	Ser141Pro	150
4059155	G	A	*fadB* (3-ketoacyl-CoA thiolase)	Ala395Val	84
4348398	A	G	SEN_RS20980 (membrane protein)/ *Salmonella* Pathogenicity Island 4	Asn2902Asp	158
4402123	C	T	SEN_RS21190 (sugar:sodium symporter)	Ala350Val	77
4476625	T	C	SEN_RS21580 (hypothetical protein)	Lys76Glu	170
4555382	C	T	SEN_RS21985 (DNA polymerase III subunit chi)	Asp10Asn	110

Table 5.1.2. Perfect and strict best hit results, by predicted gene, obtained using the Resistance Gene Identifier (RGI).

Predicted gene	evalue	Identity (%)	Contig	Average coverage	Start	Stop	RGI Cut-off	RGI Protein Model_type	Antibiotic Resistance Ontology (ARO) category
golS	1,41E-108	100	4	147,97	80575	81039	Perfect	homolog	efflux pump conferring AR; chloramphenicol RG; beta-lactam RG; gene modulating antibiotic efflux
acrF	0	99	4	147,97	73608	76775	Strict	homolog	efflux pump conferring AR; beta-lactam RG; fluoroquinolone RG
sdiA	0	99	2	127,7	1179091	1179813	Strict	homolog	chloramphenicol RG; gene modulating antibiotic efflux; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG
crp	1,30E-151	99	7	160,37	388833	389465	Strict	homolog	efflux pump conferring AR; macrolide RG; beta-lactam RG; gene modulating antibiotic efflux; fluoroquinolone RG
mdsA	0	98	4	147,97	76772	77977	Strict	homolog	efflux pump conferring AR; chloramphenicol RG; beta-lactam RG
mdsC	0	98	4	147,97	72134	73624	Strict	homolog	efflux pump conferring AR; chloramphenicol RG; beta-lactam RG
aac(6')-Iy	2,36E-101	97	2	127,7	808040	808477	Strict	homolog	antibiotic inactivation enzyme; aminoglycoside RG
cpxR	1,24E-160	97	3	152,34	67603	68301	Strict	homolog	efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux
bacA	0	97	14	155,64	142061	142882	Strict	homolog	peptide AR gene; gene conferring AR via molecular bypass
cpxA	0	96	3	152,34	66233	67606	Strict	homolog	efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux
baeR	5,11E-165	96	2	127,7	107261	107983	Strict	homolog	efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux
emrY	0	95	8	158,13	93935	95473	Strict	homolog	efflux pump conferring AR; tetracycline RG

Predicted gene	evalue	Identity (%)	Contig	Average coverage	Start	Stop	RGI Cut-off	RGI Protein Model_type	Antibiotic Resistance Ontology (ARO) category
marA	1,35E-82	95	2	127,7	702301	702690	Strict	homolog	chloramphenicol RG; gene modulating antibiotic efflux; gene modulating permeability to antibiotic; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG
H-NS	9,89E-75	94	2	127,7	965098	965511	Strict	homolog	gene modulating antibiotic efflux; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; beta-lactam RG
mexD	0	94	5	135,43	37513	40626	Strict	homolog	chloramphenicol RG; trimethoprim RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; beta-lactam RG
phoP	6,18E-151	93	2	127,7	417112	417786	Strict	homolog	efflux pump conferring AR; polymyxin RG; macrolide RG; gene modulating antibiotic efflux; gene altering cell wall charge conferring AR
emrR	7,58E-115	93	8	158,13	92089	92619	Strict	homolog	efflux pump conferring AR; gene modulating antibiotic efflux; fluoroquinolone RG
mexD	0	93	4	147,97	209028	212177	Strict	homolog	chloramphenicol RG; trimethoprim RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; beta-lactam RG
mdtH	0	92	2	127,7	349496	350704	Strict	homolog	efflux pump conferring AR
mdtK	0	92	2	127,7	607306	608679	Strict	homolog	efflux pump conferring AR; fluoroquinolone RG
mexN	0	92	2	127,7	113873	116995	Strict	homolog	efflux pump conferring AR; chloramphenicol RG
mexN	0	91	2	127,7	110792	113872	Strict	homolog	efflux pump conferring AR; chloramphenicol RG
emrD	0	90	7	160,37	11534	12718	Strict	homolog	efflux pump conferring AR
mdtG	0	90	2	127,7	339682	340896	Strict	Homolog	efflux pump conferring AR

Predicted gene	evalue	Identity (%)	Contig	Average coverage	Start	Stop	RGI Cut-off	RGI Protein Model_type	Antibiotic Resistance Ontology (ARO) category
emrA	0	89	8	158,13	92719	93918	Strict	homolog	efflux pump conferring AR; fluoroquinolone RG
pmrE	0	89	2	127,7	174573	175739	Strict	homolog	polymyxin RG; gene altering cell wall charge conferring AR
baeS	0	89	2	127,7	107980	109383	Strict	homolog	efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux
tolC	0	89	14	155,64	163404	164879	Strict	homolog	chloramphenicol RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; aminocoumarin RG; tetracycline RG; rifampin RG; beta-lactam RG
acrE	0	88	1	155,02	4223	5380	Strict	homolog	efflux pump conferring AR; beta-lactam RG; fluoroquinolone RG
mexD	0	88	1	155,02	1098	4211	Strict	homolog	chloramphenicol RG; trimethoprim RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; beta-lactam RG
mdfA	0	87	13	131,07	105101	106333	Strict	homolog	efflux pump conferring AR
pmrF	0	87	5	135,43	231615	232598	Strict	homolog	polymyxin RG; gene altering cell wall charge conferring AR
mdtM	0	86	11	163,1	148308	149549	Strict	homolog	efflux pump conferring AR
ramA	1,93E-71	86	4	147,97	311233	311622	Strict	homolog	chloramphenicol RG; gene modulating antibiotic efflux; gene modulating permeability to antibiotic; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG
mdtD	0	86	2	127,7	109383	110795	Strict	homolog	efflux pump conferring AR
acrA	0	85	4	147,97	212200	213393	Strict	homolog	chloramphenicol RG; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG

Predicted gene	evalue	Identity (%)	Contig	Average coverage	Start	Stop	RGI Cut-off	RGI Protein Model_type	Antibiotic Resistance Ontology (ARO) category
pmrB	0	85	9	160,96	118002	119081	Strict	homolog	polymyxin RG; gene altering cell wall charge conferring AR
mdtA	0	82	2	127,7	116995	118332	Strict	homolog	efflux pump conferring AR; aminocoumarin RG
pmrC	0	82	9	160,96	119747	121390	Strict	homolog	polymyxin RG; gene altering cell wall charge conferring AR
acrR	1,83E-124	82	4	147,97	213535	214188	Strict	variant	chloramphenicol RG; gene modulating antibiotic efflux; fluoroquinolone RG; efflux pump conferring AR; antibiotic resistant gene variant or mutant; tetracycline RG; rifampin RG; beta-lactam RG
robA	0	81	11	163,1	77518	78387	Strict	homolog	chloramphenicol RG; gene modulating antibiotic efflux; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG
arnA	0	79	5	135,43	229636	231618	Strict	homolog	polymyxin RG; gene altering cell wall charge conferring AR
mdtL	0	77	16	156,65	44691	45878	Strict	homolog	efflux pump conferring AR
rosB	0	74	4	147,97	230248	231924	Strict	homolog	polymyxin RG
rosA	0	71	4	147,97	232128	233348	Strict	homolog	efflux pump conferring AR; polymyxin RG
rpoB	0	58	19	154,2	4220	8248	Strict	variant	rifampin RG; antibiotic resistant gene variant or mutant
katG	0	56	3	152,34	121560	123740	Strict	variant	antibiotic resistant gene variant or mutant; isoniazid RG
gyrB	0	55	16	156,65	54369	56783	Strict	homolog	aminocoumarin RG; antibiotic resistant gene variant or mutant

Predicted gene	evalue	Identity (%)	Contig	Average coverage	Start	Stop	RGI Cut-off	RGI Protein Model_type	Antibiotic Resistance Ontology (ARO) category
macB	0	50	13	131,07	143618	145564	Strict	homolog	efflux pump conferring AR; macrolide RG
vanG	8,15E-81	38	4	147,97	113335	114447	Strict	homolog	glycopeptide RG; AR gene cluster, cassette, or operon; gene conferring AR via molecular bypass
macA	2,30E-51	35	13	131,07	142503	143621	Strict	homolog	efflux pump conferring AR; macrolide RG

RG: resistance gene; **AR**: antibiotic resistance

5.1.3. Conclusion

The detection of an avian *S.* Enteritidis isolate harboring multiple efflux pumps, pathogenicity factors, a variety of mobile genetic elements and heavy-metal-tolerance genes raises concerns regarding the dissemination of infection in birds and potential risk of zoonotic transmission. This study demonstrated the added value of WGS as a routine tool for surveillance programs directed to food-producing animals, which might complement sanitary measures, essential to prevent the spread of *Salmonella* infections among animals. It also proved to have an added value as a complementary typing method. Moreover, the simultaneous detection of putative Au resistance, intrinsic antibiotic resistant genes, and mobile genetic elements, underline this method as a helpful resource to follow the spread and evolution of antibiotic resistance in this species by genomic comparison studies.

5.1.4. Data Access

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LIHI00000000. The version described in this paper is version LIHI01000000.

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5.2. The novel MCR-1.9 variant within colistin-resistant Enterobacteriaceae isolates from food-producing animals and meat

This research paper was submitted as:

Lurdes Clemente*, Vera Manageiro**, Raquel Romão, Catarina Silva, Luís Vieira, Ivone Correia, Ana Amaro, Teresa Albuquerque, Patrícia Themudo, Eugénia Ferreira, Manuela Caniça. The novel MCR-1.9 variant within colistin-resistant Enterobacteriaceae isolates from food-producing animals and meat. Submitted to **International Journal of Antimicrobial Agents**, 2017*

Contributions of the authors for the manuscript:

Lurdes Clemente: microbiological and molecular assays, analysis of data, drafting of the manuscript, final approval of the manuscript;
Vera Manageiro: molecular assays, analysis and interpretation of data, drafting of the manuscript, final approval of the manuscript;
Raquel Romão: microbiological and molecular assays;
Catarina Silva: genomic sequencing assays (Ilumina);
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**These authors had equal participation in the study*

ABSTRACT

We screened 1840 *Enterobacteriaceae* isolates from food-producing animals, meat, meat products and animal feed, for the detection of plasmid-mediated colistin resistance, during 2010-2015. The *mcr-1* gene was detected in 8% *Escherichia coli* and in 0.47% *Salmonella enterica* isolates, with a high number of *mcr-1* positive *E. coli* isolates (45.7%) being extended-spectrum β-lactamase or plasmid-mediated AmpC β-lactamase co-producers. Here we describe the detection and characterization of a novel *mcr-1* variant, *mcr-1.9*, in an *E. coli* from a swine, co-producing $bla_{\text{CTX-M-8}}$. Our findings highlight the spread of *mcr-1* genes over food-producing animals and meat, in Portugal.

Keywords

Enterobacteriaceae; food-producing animals; meat; *mcr-1*; *mcr-1.9*; ESBL; PMAβ

5.2.1. Introduction

Following the original report of plasmid-mediated colistin resistance (PMCR) in China by the end of 2015 (Liu *et al.,* 2016), several studies in different countries reported a worldwide distribution of the *mcr-1* gene in *Enterobacteriaceae* isolates from humans, food and companion animals, meat and environment (Campos *et al.,* 2016; Figueiredo *et al.,* 2016; Hasman *et al.,* 2015; Jones-Dias *et al.,* 2016a; Perrin-Guyomard *et al.,* 2016; Quesada *et al.,* 2016; Zurfuh *et al.,* 2016; Zhang *et al.,* 2016). More recently, two novel variants, *mcr-2* and *mcr-3*, were detected in colistin resistant *E. coli* isolates: from sick calves and piglets in Belgium (Xavier *et al.,* 2016), and from a faecal sample of an apparently healthy pig at a conventional farm in China (Yin *et al.,* 2017), respectively (Table 5.2.1). MCR-1 variants were also detected, namely MCR-1.2, identified in a human isolate of *Klebsiella pneumoniae*, in Italy (Di Palato *et al.,* 2016); MCR-1.3 in *E. coli* from chickens from China (Yang *et al.,* 2017); MCR-1.5 from clinical *E. coli* isolates previously described in Argentina (Tijet *et al.,* 2017); and MCR-1.6 in *Salmonella enterica* serovar Typhimurium isolate from a healthy person in China (Lu *et al.,* 2017) (Table 5.2.1). In this study, we analysed several colistin-resistant *E. coli* and *S. enterica* isolates from food-producing animals, meat, meat products and animal feed, for the presence of PMCR-encoding genes.

Table 5.2.1. Comparison of amino acid substitutions, and epidemiology of first reports of MCR-producing strains.

MCR variant	Amino Acid substitutions	Specie	Host	Country	GenBank accession no.
MCR-1	-	*E. coli, K. pneumoniae*	Pigs, retail meat, human	China	KP347127
MCR-1.2	Gln3Leu	*K. pneumoniae*	Human	Italy	KX236309
MCR-1.3	Ile38Val	*E. coli*	Chickens	China	NG_052861
MCR-1.4	Asp440Asn	*E. coli*	Sewage	China	KY041856
MCR-1.5	His452Tyr	*E. coli*	Human	Argentina	KY283125
MCR-1.6	Arg536His	*Salmonella* Typhimurium	Healthy human	China	NG_052893
MCR-1.7	Ala215Thr	*E. coli*	Sewage	China	KY488488
MCR-1.8	Gln3Arg	*E. coli*	Poultry	Brunei	KY683842
MCR-1.9*	Val413Arg	*E. coli*	Swine	Portugal	KY780959
MCR-2	80.7% identity to MCR-1	*E. coli*	Sick calves, piglets	Belgium	NG_051171
MCR-3	32.5% and 31.7% identity to MCR-1 and MCR-2, respectively	*E. coli*	Pig	China	KY924928

5.2.2. Material and Methods

Antimicrobial susceptibility testing

A total of 1206 *E. coli* and 634 *S. enterica* isolates from healthy food-producing animals, meat and animal feed (Tables 5.2.2 and 5.2.3), were submitted to colistin, cefotaxime and ceftazidime susceptibility testing for the determination of Minimum Inhibitory Concentrations (MIC), using the agar dilution method (EUCAST/ESCMID, 2003). Results were interpreted according to the epidemiological cut-off values of the European Committee on Antimicrobial Susceptibilty Testing (EUCAST, http://mic.eucast.org/Eucast2/). Non-wild type isolates towards cefotaxime and ceftazidime, were tested for the phenotypic detection of extended-spectrum β-lactamases (ESBL) and plasmid-mediated AmpC β-lactamases (PMAβ), by the microdilution method (TREK, diagnostic systems).

Genetic detection of PMCR

Colistin-resistant *E. coli* and *S. enterica* isolates were screened for the presence of PMCR-encoding genes (*mcr-1* and *mcr-2*), using a multiplex PCR (Cavaco *et al.,* 2016), followed by sequencing of the amplicons.

Identification of ESBL and PMAβ

Identification of variants of bla_{ESBL} and $bla_{PMA\beta}$ genes of all *Enterobacteriaceae* isolates harbouring *mcr* genes and concomitantly exhibiting an ESBL or PMAβ phenotype, was performed as previously (Jones-Dias *et al.,* 2016b).

Characterization of E. coli LV23529

Transfer experiments

Conjugation experiments were performed using sodium azide-resistant *E. coli* J53 as a recipient strain. Transconjugants were selected on McConkey agar supplemented with sodium azide (150µg/mL), cefotaxime (2µg/mL) and colistin (2µg/mL). Plasmid DNA was extracted from *E. coli* LV23529 using a NucleoBond Xtra Plus kit (Macherey-Nagel), and transformed into *E. coli* TOP10 OneShot chemically competent cells (Invitrogen), accordingly to manufacture's protocol. *E. coli* transformants were selected on MacConkey agar supplemented with 2µg/mL of colistin. MICs of recipients and transformants were determined as mentioned above.

Genetic environment of *mcr-1.9* gene

Plasmid DNA was extracted from *E. coli* LV23529, using a NucleoBond Xtra Plus kit (Macherey-Nagel), and quantified using Qubit 1.0 Fluorometer (Invitrogen).

The Nextera XT DNA Sample Preparation Kit (Illumina) was used to prepare sequencing libraries from 1ng of genomic DNA according to the manufacturer's instructions. Plasmid sequencing was performed using 150 bp paired-end reads on a MiSeq (Illumina), as previously described (Manageiro *et al.,* 2017). Sequence reads were trimmed and filtered according to quality criteria, and de novo assembled into contigs by means of CLC Genomics Workbench 9.0 (Qiagen). The contig carrying the *mcr-1.9* gene and respective genetic environment was manually annotated after blasted against GenBank.

5.2.3. Results and Discussion

Overall, we detected *mcr-1*-like genes in 100 colistin-resistant *Enterobacteriaceae* isolates (*E. coli*, *n*=97 and *S. enterica*, *n*=3) (Tables 5.2.2 and 5.2.3). All amplicons excepting one, exhibited a sequence with 100% homology to the recently described *mcr-1* (Liu *et al.,* 2016); one amplicon (of a commensal isolate LV23529 from a swine), hereafter named *mcr-1.9*, differed from *mcr-1* by one-point mutation (T1238C), leading to Val413Ala substitution (Table 5.2.1).

To our knowledge, this was the most wide-ranging study conducted in Portugal, reporting for the first time the occurrence of *mcr* genes in *E. coli* isolates, from food-producing animals [turkeys (27%), swine (10.1%) and broilers (1.5%)] and swine meat (5.1%). Noteworthy, this is the high frequency of *mcr*-positive *E. coli* isolates from turkeys, when comparing with other European countries (Haenni *et al.,* 2016). No colistin-resistant isolates were detected in bovine animals and bovine meat; these findings agree with some studies (EFSA/ECDC, 2017), though contradicting others, reporting a higher frequency of *mcr-1*-positive isolates from veal calves, particularly ESBL-producing isolates (Haenni *et al.,* 2016; Xavier *et al.,* 2016). Regarding *S. enterica*, *mcr-1* gene was confirmed in three isolates (8.6%, 3/35), one in *S*. Reading from bovine meat, and two in serotype 4,5,12:i:- from bovine and swine meat.

In our country, PMCR had been detected in *S. enterica* isolates from humans and food (Campos *et al.,* 2016; Figueiredo *et al.,* 2016), and more recently in one *E. coli* strain isolated from fresh vegetables (Jones-Dias *et al.,* 2016a). The presence of colistin resistance gene in food represents a potential public health threat, as it is located in mobile genetic elements that have the potential to spread horizontally.

Worryingly, we observed that a high number of *mcr-1* positive *E. coli* isolates (45.7%, 42/92) were ESBL or PMAβ co-producers (Table 5.2.2): *bla*$_{\text{CTX-M-1}}$, *n*=14; *bla*$_{\text{CTX-M-32}}$, *n*=13; *bla*$_{\text{CTX-M-14}}$, *n*=5; *bla*$_{\text{CTX-M-27}}$, *n*=1; *bla*$_{\text{SHV-12}}$, *n*=3; *bla*$_{\text{CMY-2}}$, *n*=3; *bla*$_{\text{AmpC,}}$ *n*=2 (Table 5.2.2). Bioinformatics analysis of the MCR-1.9-producing isolate revealed genes conferring resistance to β-lactams (*bla*$_{\text{CTX-M-8}}$ and *bla*$_{\text{TEM-1}}$), sulphamethoxazole (*sul3*), trimethoprim (*dfrA12-type*), chloramphenicol (*cmlA1-type*) and colistin (*mcr-1.9*).

Table 5.2.2. *Escherichia coli* (*n*= 1206), from food-producing animals, meat and meat products.

Isolate origin/ Animal species	Isolates tested for MIC[a] (*n*)	Year	Colistin resistant isolates (%) [MIC range mg/L]	*mcr* positive isolates (%) [MIC range mg/L]	Frequence of *mcr* positive isolates (%)	Frequence of ESBL/PMAβ *mcr* positive isolates (%)	Genetic profile *bla* genes
Clinical cases							
Food animals	20	2010-2013	2 [8 - 16]	2 [8 - 16]	2/20	0	
Cecum samples							
Broilers	202	2014	6/202 (3) [8 - 16]	3/6 (50) [8 - 16]	3/202 (1.5)	0	
Turkeys	185	2014	50/185 (27) [8 - >16]	50/50 (100) [8 - >16]	50/185 (27)	2/50 (4)	*bla*$_{TEM-1}$, *bla*$_{SHV-12}$ (*n*=1) *bla*$_{AMPC}$ (mutation in promotor) (*n*=1)
Bovine	350	2015	0 (0)				
Swine	398	2015	42/398 (10,6) [8 - >16]	40/42 (95,2) [8 - >16]	40/398 (10,1)	38/40 (95)	*bla*$_{TEM-type}$, *bla*$_{CTX-M-1}$ (*n*=11) *bla*$_{CTX-M-1}$ (*n*=1) *bla*$_{TEM-1}$, *bla*$_{CTX-M-8}$ (*n*=1) [b] *bla*$_{TEM-type}$, *bla*$_{CTX-M-14}$ (*n*=5) *bla*$_{TEM-type}$, *bla*$_{CTX-M-27}$ (*n*=1) *bla*$_{TEM-type}$, *bla*$_{CTX-M-32}$ (*n*=13) *bla*$_{TEM-1}$, *bla*$_{SHV-12}$ (*n*=2) *bla*$_{AMPC}$ (mutation in promotor) (*n*=1) *bla*$_{CMY-2}$ (*n*=3)
Meat samples							
Bovine	12	2015	0 (0)				
Swine	39	2015	3/39 (7,7) [8 - 16]	2/3 (66,6) [16]	2/39 (5,1)	2/2 (100)	*bla*$_{TEM-1}$; *bla*$_{CTX-M-1}$ (*n*=2)
Total	**1206**		**103/1206 (8,6)**	**97/103 (94,2)**	**97/1206 (8)**	**42/92 (45,7)**	

[a] MIC: minimum inhibitory concentration, range [1 - >16mg/L]
[b] *mcr-1.9* gene positive isolate.

Table 5.2.3. *Salmonella* spp (*n*= 634), from food-producing animals, meat, meat products and animal feed.

Serotypes	Isolate origin/ Animal species	Isolates tested for MIC[a] (*n*)	Year	Colistin resistant isolates (%) [MIC range mg/L]	*mcr* positive isolates (%) [MIC range mg/L]	Frequence of *mcr* positive isolates (%)
	Faeces/ Environment					
	Poultry					
Enteritidis		47	2013-2015	11/47 (23,4) [4 - 8]	0	
Typhimurium		11	2014-2015	1/11 (9,1) [8]	0	
4,5,12:i:-		5	2014-2015	0		
Other		325	2011-2015	1/325[b] (0,3) [>16]	0	
	Bovine					
Typhimurium		1	2014-2015	0		
4,5,12:i:-		4	2014-2015	0		
	Animal feed					
Enteritidis		6	2014-2015	6/6 (100) [4 - 16]	0	
Other		7	2014-2015	0		
	Meat and meat products					
Enteritidis	Poultry; Swine	20	2014-2015	6/20 (30) [4 - 8]	0	
Typhimurium	Poultry; Swine	37	2014-2015	0		
4,5,12:i:-	Bovine; Swine; Poultry	54	2014-2015	6/54 (11,1) [8 - 16]	2/6 (33,3)[c]	2/54 (3,7)
Other	Bovine; Swine; Poultry	117	2014-2015	4/117 (3,4) [4 - >16]	1/4 (25)[d]	1/117 (0,9)
	Total	**634**		**35/634 (5,5%)**	**3/35 (8,6)**	**3/634 (0,47)**

[a] MIC: minimum inhibitory concentration, range [1 - >16mg/L]; [b] *S.* Havana; [c] Bovine and swine; [d] *S*. Reading (swine meat); [c,d] Non-ESBL or non-PMAβ producers.

Table 5.2.4. Phenotypic and genotypic context of CTX-M-8 and MCR-1.9 producing *E. coli* clinical isolate, transformant, transconjugant and the recipient strains.

Antibiotic [a]	LV23529 [b] (bla$_{CTX-M-8}$, *mcr-3*)	Transformation		Conjugation	
		E. coli TOP10 [c]	T 23529 [d] (*mcr-1.9*)	ECJ53AZNa [e]	ECJ53AzNa T LV23529[f] *bla*$_{CTX-M-8}$
Ampicillin	>64	4	8	2	>64
Cefoxitine	4	4	4	4	8
Ceftazidime	2	0.5	0.5	≤0,5	1
Ceftazidime plus clavulanate*	≤0,125/4	0.5	0.5	≤0,125/4	≤0,25/4
Cefotaxime	32	≤0.25	≤0.25	≤0,25	2
Cefotaxime plus clavulanate*	≤0,06/4	0.125	0,125	≤0,06/4	≤0,06/4
Cefepime	8	0.125	0,125	≤0,06	2
Imipenem	0,25	0.5	0,5	0,25	0,25
Meropenem	≤0,03	0.06	0,06	≤0,03	≤0,03
Ertapenem	≤0,015	≤0,015	≤0,015	≤0,015	≤0,015
Nalidixic acid	≤4	≤4	≤4	≤4	≤4
Ciprofloxacin	≤0.015	≤0.015	≤0.015	≤0,015	≤0,015
Chloramphenicol	>128	≤8	≤8	≤8	≤8
Sulphamethoxazole	>1024	≤8	≤8	≤8	≤8
Tetracycline	>64	≤2	≤2	≤2	≤2
Trimethoprim	>32	≤0.25	≤0.25	≤0,25	≤0,25
Gentamicin	≤0,5	≤0.5	≤0,5	≤0,5	≤0,5
Colistin	4	≤1	2	≤1	≤1
Tigecycline	≤0,25	≤0,25	≤0,25	≤0,25	≤0,25
Azythromycin	4	≤2	≤2	≤2	4

[a] * Clavulanate 4 µg/ml.
[b] *E. coli* LV23529 was the clinical isolate harbouring *bla*$_{CTX-M-8}$ and *mcr-1.9*.
[c] *E. coli* TOP10 was the recipient strain in the transformation experiment.
[d] TOP10 T23529 is a transformant of LV23529 (harbouring *mcr-1.9*).
[e] E. coli J53AZNa was the recipient strain in the conjugation experiment.
[f] ECJ53AzNa T LV23529 is a transconjugant of LV23529 (harbouring *bla*$_{CTX-M-8}$).

The *mcr-1.9* genetic background was characterized by an IS*26* element upstream of the *mcr-pap2* element and by the absence of *IS*Apl1. Plasmid analysis revealed the presence of IncF [F2:A-:B-], IncX4-harbouring *mcr-1.9* (pLV23529-MCR-1.9), and IncI1-ST113-carrying the $bla_{CTX\text{-}M\text{-}8}$. The new *mcr-1.9* positive isolate, co-harbouring $bla_{CTX\text{-}M\text{-}8}$ and $bla_{TEM\text{-}1}$ genes, is here reported for the first time in an *E. coli* isolate of animal origin. In fact, $bla_{CTX\text{-}M\text{-}8}$ gene is rarely detected in Europe in isolates of animal origin (Börjesson *et al.,* 2016), but in humans seems to be emerging (Eller *et al.,* 2014). Only the transferability of the $bla_{CTX\text{-}M\text{-}8}$ gene was achieved by conjugation, with TcLV23529 ($bla_{CTX\text{-}M\text{-}8}$) exhibiting the ESBL resistance phenotype from LV23529 isolate, and susceptibility to colistin (Table 5.2.4). Although conjugation assays for *mcr-1.9* were negative, the colistin resistance determinant could be transferred to *E. coli* TOP10, with transformant TLV23529 (*mcr-1.9*) showing the respective resistance to colistin (Table 5.2.4). Indeed, selection pressure exerted by broad-spectrum cephalosporins and other antimicrobials may enhance the rapid dissemination of PMCR and vice-versa.

Likewise, *mcr* genes have been associated with several plasmid incompatibility types, resulting in a potentially greater bacterial host range. IncX4 plasmid has been widely implicated in the spread of *mcr-1* gene in Europe, from human and animal isolates (Campos *et al.,* 2016; Hasman *et al.,* 2015; Veldman *et al.,* 2013); *ISApl1* was not identified in *mcr-1.9*-carrying IncX4 plasmid, which is in accordance with other studies about *mcr-1* gene (Veldman *et al.,* 2016). Indeed, initially *ISApl1* was presumably involved in the transposition of the *mcr-1* cassette and then was lost, contributing for the stability of *mcr* gene on IncX4 plasmids (Sun *et al.,* 2017).

5.2.4. Conclusions

This study corroborates a worldwide dissemination of PMCR gene, underlining the importance of its continuous monitoring. We also emphasized the benefit of employing next generation sequencing-based methods on the early detection and characterization of antimicrobial resistance, as it allowed a rapid analysis of a large dataset *in silico*, being also important when new resistance genes emerge.

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CHAPTER 6

GENERAL DISCUSSION

The emergence and spread of antibiotic resistant bacteria is currently one of the greatest threats in public health (O'Neill, 2016). The selective pressure exerted by the abusive and inadequate use of antibiotics in human and animal clinical practices, animal and agricultural production, and the environmental impact resulting from these activities, are the main causes for the emergence of antibiotic resistance. It is a dynamic, complex, multifactorial process involving humans, animals and the environment (Cantas *et al.,* 2013; Butaye *et al.,* 2014; Roca *et al.,* 2015; Woolhouse *et al.,* 2015).

The research studies developed throughout this thesis (***Chapters 3, 4*** and ***5***), intended to demonstrate the relevance of different animal species and products of animal origin as reservoirs of bacteria carrying antibiotic resistance determinants, through phenotypic and molecular characterization of Gram negative bacteria, and the mobile genetic elements involved in the spread of resistance. A total number of 4689 Gram negative strains belonging to *Enterobacteriaceae* family were analyzed, being 2406 *Salmonella enterica*, 2282 *Escherichia coli* and one *Morganella morgannii*, isolated from different animal species, samples and geographic regions, during the period 2009-2015. Although a specific discussion has been included in each manuscript, the main findings will be globally analysed and discussed in the present chapter (***Chapter 6***).

In a brief review of the state of the art, in ***Chapter 3***, the dynamics of antibiotic resistance is debated. Specifically, it is discussed how the spread of resistant bacteria is facilitated by the existence of multiple pathways between the different reservoirs (humans, animals and the environment) by the involvement of mobile genetic elements, and by the impact of environmental pollution (antibiotic molecules) (Bauer *et al.,* 2008; Heuer *et al.,* 2011; Marshall & Levy, 2011; Garcia-Alvarez *et al.,* 2012).

The selective pressure exerted by the administration of antibiotics, the accumulation of antibiotic residues in the environment, the international movement of people and trade of animals, food products and feedstuffs contribute for the exposure to commensal and pathogenic resistant bacteria (Barbosa & Levy, 2000; Martinez *et al.,* 2002; Perron *et al.,* 2008; Allen *et al.,* 2010; Gaze *et al.,* 2011). Antibiotic-producing environmental bacteria also contribute for selection of antibiotic resistant bacteria (Hemala *et al.,*2014). Studies carried out by Kreitlow *et al.,* (1999) and Martins *et al.,* (2008) showed that some cyanobacteria produce proteins that show antimicrobial activity, more effective in Gram positive than in Gram negative, due to the protective layer of lipopolysaccharides in the latter.

Mobile genetic elements show a prominent role in the dissemination of antibiotic resistance, constituting the base for horizontal gene transfer and genetic recombination within the bacterial populations; these elements contribute for the diversity and adaptability of the strains to the different niches (Leavis *et al.,* 2007; Devirgillis *et al.,* 2011; Brown *et al.,* 2012; Butaye *et al.,* 2014; Roca *et al.,* 2015; Gomes-Neves *et al.,* 2015; Woolhouse *et al.,* 2015).

The monitoring of antibiotic resistance and the implementation of surveillance programs on commensal, zoonotic and pathogenic bacteria are some of the strategic priorities to control the spread of antibiotic resistance. Phenotypic characterization of antibiotic susceptibility and the interpretation of results according to epidemiological breakpoints may lead to important findings regarding changes in resistance patterns. It may also show how resistance mechanisms are emerging and disseminating through different animal populations and products of animal origin.

In ***Chapters 4.1***, ***4.2***, ***4.3*** and ***4.8***, antibiotic susceptibility of several *Salmonella* spp serotypes identified in different animal species and samples was determined. Antibiotic susceptibility depends on animal species, serotype, strains and the antibiotic consumption during production cycle. The great variability of serotypes found in the mentioned study suggests the existence of diversity with regards to sources of infection, specifically breeding flocks, hatcheries, feed and feedstuffs, environment, human contact, animal facilities and equipment (Papadopoulou *et al.,* 2009).

Serotype variability in different countries and animal populations, and its association with specific resistance patterns may explain some of the differences in levels of resistance and multidrug resistance (EFSA/ECDC, 2015). Other factors, such as national and international trade of animals, food products and feedstuffs, management systems and the pyramidal structure of primary animal production may also influence the spread of resistant strains (EFSA/ECDC, 2015).

In our studies, the frequency of resistance towards ampicillin, tetracycline and sulfamethoxazole was particularly high in serotypes Typhimurium, Rissen and 4, [5], 12: i: -, unlike 3rd generation cephalosporins, in which the frequency of resistance was low, as described in Europe (EFSA/ECDC, 2015). The frequency of non-wild type strains to ciprofloxacin was high, particularly in poultry, in serotypes Enteritidis, Mbandaka and Havana, which may be explained by the high consumption of fluoroquinolones at national level (DGAV, 2013, EMA, 2016). Among the various serotypes, some resistant strains to ciprofloxacin and susceptible to nalidixic acid occurred, suggesting an increase on the occurrence of plasmid-mediated quinolone resistance (PMQR) mechanisms (Veldman *et al.,* 2011).

Also in ***Chapters 4.3***, ***4.4***, ***4.5***, ***4.6***, ***4.7*** and ***4.8***, *E. coli* strains isolated from food-producing, companion and zoo animals were analyzed, showing different antibiotic susceptibility patterns. For all antibiotics tested, the frequency of strains showing decreased susceptibility was higher in food-producing animals, followed by companion and zoo animals, which can be attributed to the higher antibiotic consumption in that group of animals, particularly penicillins, tetracyclines, sulfonamides and fluoroquinolones (DGAV, 2013, EMA, 2016). However, the frequency of strains with decreased susceptibility to cefotaxime was higher in companion animals (8%), followed by food-producing (3%) and zoo animals (2.7%). Although

the consumption of 3rd generation cephalosporins is low, it may be underestimated because human cephalosporins are frequently administered in clinical therapy of companion animals (DGAV, 2013; EMA, 2016).

Third generation cephalosporins and fluoroquinolones are antibiotics of critical importance in veterinary and human clinical practice (Vaarten, 2012; WHO, 2014; OIE, 2015). Research studies all over the world, using strains recovered from several animal species and products of animal origin, have been reporting a significant increase of strains that harbour ESBL, PMAβ and PMQR, showing resistance to the mentioned antibiotics (Veldman *et al.,* 2011; Dierikx *et al.,* 2012; Ewers *et al.,* 2012; Tamang *et al.,* 2012a; Liebana *et al.,* 2013; Jones-Dias *et al.,* 2013; Stefani *et al.,* 2014; Jamborova *et al.,* 2015; Michael *et al.,* 2016).

Thus, in ***Chapters 4.2*** and ***4.3***, resistance mechanisms to 3rd generation cephalosporins and/or cephamycins (associated to ESBL and/or PMAβ-encoding genes), in *S. enterica* strains isolated from poultry (live animals), as well as poultry and swine food products, were investigated. We reported for the first time in Portugal the occurrence of ESBL and/or PMAβ enzymes belonging to CTX-M family [(CTX-M-1 in *S*. Havana), (CTX-M-14, CTX-M-15 nd CTX-M-32 in *S*. I4, [5], 12: i: - and in *S*. London)], CMY-2 in *S*. Havana, and SHV-12 in *S*. Enteritidis. CTX-M-1 is the most frequent enzyme in strains of *S. enterica* of animal origin, not only in Europe (Rodriguez *et al.,* 2009, Dierikx *et al.,* 2010, Freire Martin *et al.,* 2014), as in other continents (Choi *et al.,* 2015, Fitch *et al.,* 2016). However, other enzymes, namely CTX-M-14, CTX-M-15, CTX-M-32 and SHV-12 were also identified in food-producing animals (Politi *et al.,* 2005; Riäno *et al.,* 2006; Chiaretto *et al.,* 2008; Tamang *et al.,* 2011). In Portugal, $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ genes had only been described in strains of *E. coli* isolated from poultry feces and meat (Machado *et al.,* 2008a).

In *E. coli* strains isolated from food-producing animals, CTX-M-1 was also the most frequent. In humans, the occurrence of this β-lactamase is rare and seems to be associated with direct or indirect contact through the food chain (Gonçalves *et al.,* 2011; Leverstein-van Hall *et al.,* 2011, Veldman *et al.,* 2014; Day *et al.,* 2016). Companion and zoo animals are also potential reservoirs of ESBL and PMAβ-encoding genes, which can be transmitted to humans due to close contact with owners, caretakers and visitors (Ewers *et al.,* 2012; Dobiasova *et al.,* 2013; Donati *et al.,* 2014).

Regarding PMAβ enzymes, CMY-2 was identified in six *E. coli* strains isolated from food-producing (*n* = 2) and companion animals (*n* = 4), and *S.* Havana (*n* = 2) isolated from broilers. As in other studies, CMY-2 represents the most frequent PMAβ in animals (Dierikx *et al.,* 2010; EFSA, 2011a; Dierikx *et al.,* 2012; Ewers *et al.,* 2012; Li *et al.,* 2013; Liebana *et al.,* 2013) and humans, mainly in strains isolated from nosocomial and community-acquired infections (Nicolas-Chanoine *et al.,* 2014; Jamborova *et al.,* 2015; Alonso *et al.,* 2016).

In the study described on ***Chapter 4.3***, we identified CTX-M-15 in two *E. coli* strains isolated from companion animals and in seven strains from captive dolphins. This enzyme has been

widely described in companion animals (Sun *et al.,* 2010; Hordijk *et al.,* 2013; Klimes *et al.,* 2013; Veldman *et al.,* 2013; Jamborova *et al.,* 2015; Day *et al.,* 2016), and here was detected for the first time in dolphins.

E. coli CTX-M-15 producer and belonging to ST131 is one of the most widespread and successful bacterial clones among humans, isolated from hospitals, health care units and the community (EFSA, 2011a; Nicolas-Chanoine *et al.,* 2014). Detection of this enzyme in *E. coli* strains from captive dolphins, which are in permanent contact with humans, prompted us to evaluate its zoonotic potential, as described in ***Chapter 4.4***. Genetic relatedness between the *E. coli* strain isolated from a dolphin and a collection of human clinical strains isolated from different health care units, with common resistance determinants [$bla_{\text{CTX-M-15}}$ and *aac(6')-Ib-cr*] associated to the same plasmid (IncF), demonstrated the clonality between the human strains and the animal strain. Results confirmed the zoonotic potential of the isolate, suggesting the occurrence of the same genetic events in the dolphin and the human isolates, leading to the same pattern of allelic diversity, ST131 *fim*H30-Rx. Although we were alerted for the transmission of a high-risk clone, we were not able to establish the direction of the transfer of resistance and virulence determinants. Thus, we confirmed the possibility of interconnection between human and animal health, although it is unclear whether environmental intervention took place in the transmission (Robinson *et al. ,*2016).

In the study described on ***Chapter 4.5***, antibiotic susceptibility of 387 *E. coli* strains isolated from broiler and turkey cecal samples at slaughter was determined. The frequency of 3rd generation cephalosporin resistant strains was not high, levelling between 4% and 2.7%, respectively, in contrast to that observed for fluoroquinolones, 90.6% and 79.5%, respectively. Although the administration of 3rd generation cephalosporins is not authorized for poultry, the occurrence of resistant strains can be explained by co-selection, using other antibiotics, namely fluoroquinolones (Michael *et al.,* 2017). As a matter of fact, the total sales of fluoroquinolones in our country increased by 0.4% during the period 2011-2014, with a peak in 2014, when samples included in this study were collected (EMA/ESVAC, 2016).

Molecular characterization of 15 isolates nonsusceptible to cefotaxime and/or cefoxitin revealed a wide diversity of resistance mechanisms, such as penicillinases from TEM family, ESBLs from several families [SHV (-12), TEM (-52) and CTX-M (-1, -32 and -166)], being CTX-M-166, a novel variant characterized by the substitution of alanine for valine at amino acid position 120 (Ala120Val).

In three strains, only the *ampC* gene was detected or associated with the $bla_{\text{TEM-1}}$. After sequencing AmpC-encoding gene, the analysis revealed that nucleotide mutations responsible for conformational modifications in the AmpC β-lactamases were found, specifically in the Ω-loop (A220T, strain 19991) and helix-9 (I284V, strain 10908) (Kim *et al.,*2006), suggesting the occurrence of extended-spectrum AmpC β-lactamases, (ESAC); phenotypic susceptibilty assay showed sinergy with clavulanic acid. In the third strain

exhibiting no synergy with clavulanic acid, mutations were observed on well conserved regions in the promoter/attenuator gene of the chromosomal *ampC* gene, suggesting that it is not an ESAC strain.

The identification of AmpC enzymes responsible for the hydrolysis of broad-spectrum cephalosporins is rare in *E. coli* strains of animal origin. Thus, to our knowledge, this study constitutes the second description of ESAC-type enzymes in animals (Haenni *et al.,* 2014). In addition, this study revealed that the occurrence of ESBL-producing *E. coli* isolated from this poultry population is not due to a specific clone, unlike those from human clinical isolates or from animals kept in captivity (***Chapter 4.4***) (Mendonça *et al.,* 2007).

The characterization of kinetic parameters of the new ESBL enzyme detected, CTX-M-166, compared with the parental enzyme of the same group, CTX-M-1, was the main objective of the study developed in ***Chapter 4.6.*** Although the catalytic activity of the new variant has shown a strong affinity for penicillin, piperacillin, cefotaxime and ceftiofur, it is lower than that of CTX-M-1 enzyme. The aminoacid substitution identified represents a modification of a neutral aminoacid to another equally neutral, frequently associated to an increase in the protein flexibility, meaning substrate recognition and binding. Antibiotic selection pressure can cause the accumulation of mutations with important impact on the enzymatic activity and so, on the resistance phenotype.

The development of next generation technologies, namely WGS and its application in research and diagnostics, granted a rapid and complete analysis of numerous genomes, from potentially pathogenic agents, to commensal and environmental organisms. The detection of intrinsic and acquired antibiotic resistance determinants, virulence factors, mobile genetic elements, tolerance to heavy metals and others represents a valuable tool for additional characterization, which should be used in research and surveillance programs, in the present and in the near future (Zankari *et al.,* 2013; Anjum, 2015; Franzosa *et al.,* 2015; Gilad, 2017; Nado *et al.,* 2017).

Thus, using the potential offered by this technology, we intended to make a complete characterization of a multidrug resistant CTX-M-166-producing *E. coli* strain, as described in ***Chapter 4.7***. Several resistance genes, conferring resistance to β-lactams ($bla_{\text{TEM-1}}$), aminoglycosides (*strA-strB*), tetracycline (*tetA*-type), sulfonamides (*sul2*) and trimethoprim (*dfrA14-type*), virulence factors (*iss*, *gad*, *astA*, *iroN*, *iha*, *mchF-type*, *celb-type* and *cma-type*), insertion sequences (IS*Ecp1-bla*$_{\text{CTX-M-166}}$-*orf477*), plasmids (ST103-IncI1 and Col8282) and serotypes (O6:H16 ST48-*fim*H34) were identified with this approach. The study of the pathogenicity factors present in this genome indicated that there was a probability of 93.2% of this bacterial isolate acting as a human pathogen (Cosentino *et al.,* 2013). The survival of a specific bacteria in multiple ecological niches, and their ability to adapt to alternative hosts, either by capturing new virulence factors or antibiotic resistance determinants, while maintaining their fitness may be a matter of concern.

The detection of PMQR-encoding genes has been increasing in *Enterobacteriaceae* strains isolated from animals (Veldman *et al.,* 2011; Jones-Dias *et al.,* 2013; Donati *et al.,* 2014; Jamborova *et al.,* 2015). Although these determinants may be responsible for a low level of resistance, PMQR are associated to a cumulative effect when combined with chromosomal mechanisms, which may explain high MIC values to nalidixic acid (> 512mg/L) and ciprofloxacin (> 8mg/L), observed in some of our strains. This may also increase the likelihood of dissemination of other resistance genes through co-selection (Donati *et al.,* 2014; Jamborova *et al.,* 2015).

In the studies developed in ***Chapters 4.2***, ***4.3***, ***4.4***, ***4.5*** and ***4.8***, a phenotypic and genotypic characterization of *S. enterica* and *E. coli* strains isolated from food-producing, companion and zoo animals, regarding the detection of PMQR, was performed. Although the administration of fluoroquinolones in the veterinary practice is high (DGAV, 2013, ESVAC, 2016), the results obtained in these studies indicate that PMQR-encoding genes occur at low frequency, as previously reported by other authors (Veldman *et al.,* 2011; Donati *et al.,* 2014; Jamborova *et al.,* 2015).

In the *S. enterica* strains analyzed, no PMQR-encoding genes were found, unlike in *E. coli* strains where *qnrS1* (*n*=3), *qnrB19* (*n*=3) and *aac(6 ')-Ib-cr* (*n*=9) were detected. Despite the high consumption of fluoroquinolones, the low frequency of detection PMQR-encoding genes suggests that the occurrence of these determinants may not be triggered by selection pressure (Veldman *et al.,* 2011).

In ***Chapter 4.8*****,** and to explore genetic diversity between different *E. coli* strains, we analysed two strains carrying the *qnrS1* gene, isolated from a canine and a pigeon, and two strains carrying *aac(6 ')-Ib-cr* gene, isolated from two dolphins. Using WGS, and comparing both QnrS-1 genomes, a high genetic relationship was noticed between the two pairs of strains. The absence of chromosomal mutations at the level of the QRDR region corroborates with low quinolone MIC values of 0.38mg/L for ciprofloxacin and 8mg/L for nalidixic acid. Between both AAC(6')-Ib-cr- and CTX-M-15-bearing strains there were few genomic differences; the presence of four mutations in the QRDR region of both strains corroborates with high quinolone MIC values of >256mg/L for nalidixic acid and >32mg/L for ciprofloxacin. The absence of genes supporting conjugation (*tra* genes) in two, ST131 *fim*H30-Rx strains reinforces the preponderance of a clonal spread over horizontal transfer, as previously described in other *E. coli* ST131 strains (Nicolas-Chanoine *et al.,* 2014). The data gathered throughout this study illustrates two scenarios: the presence of the same strain in different hosts inhabiting remote locations and the persistence of a unique strain in a single niche during a long period of time.

Although it may be rare in isolates from animals, *qnrD* gene is common in members of the *Proteeae* family (Zhang *et al.,* 2013; Guillard *et al.,* 2016); recently it has been described in *Salmonella* spp strains isolated from food-producing and animal products (Jiang *et al.,* 2014;

Lin *et al.,* 2015). In ***Chapter 4.9***, genome analysis of a *Morganella morgannii* strain isolated from broilers aged 13 days old, and with *post-mortem* lesions identical to those observed in colisepticemia, was performed, through WGS. *M. morgannii* is a commensal bacterium occasionally causing infections in animals and humans (urinary tract, liver, skin and soft tissues) (Roels *et al.,* 2007; Zhao *et al.,* 2012; Lin *et al.,* 2015). In this thesis, this emerging opportunistic pathogen was broadly characterized due to its uncommon presence in human and animal infections and because of its multidrug resistance; thus, the complete characterization of antibiotic resistance genes, virulence factors, and mobile genetic elements responsible for the putative dissemination of the *M. Morgannii* strain was performed.

Genome characterization confirmed the phenotypic multiresistance pattern towards, aminoglycosides [*aadA1y*, *aph(3')-Ic*, and *strA-strB*], β-lactam (bla_{OXA-1}), fluoroquinolones [*qnrD1*, *acc(6')-Ib-cr*], phenicols (*catA2* and *catB3*), rifampicin (*arr-2*), sulfonamides (*sul2*), trimethoprim (*dfrA1*), tetracycline (*tetY*), and streptotrichin (*sat2*). Several virulence factors and mobile genetic elements were also detected. The presence of inverted sequences in an IS*26* suggests the occurrence of a recent event of genetic recombination in a small non-conjugative plasmid (8449pb) carrying *qnrD1* gene, corroborating with the absence of ability to conjugate (Zhang *et al.,* 2013; Guillard *et al.,* 2014).

Analysis through PathogenFinder (Cosentino *et al.,* 2013) showed a 68.9% likelihood of being a human pathogen, which is in accordance with the opportunistic nature of this species (Zhao *et al.,* 2012; Lin *et al.,* 2015). The detection of *M. morgannii* of avian origin, carrying multiple mobile resistance determinants and virulence factors is a cause for concern, due to its capacity of dissemination and infect other birds, and the potential risk of zoonotic transmission. To our knowledge, this study represented the first genomic analysis of an animal isolate, carrying *qnrD1* gene.

Infection with *Salmonella enterica* is the first cause of human food poisoning in Europe, mostly related to the consumption of poultry food products, namely meat and eggs, being serotype Enteritidis (*S*. Enteritidis) the most frequent (EFSA/ECDC, 2015); in animals, infection is generally asymptomatic except in young chicks and poults, wherein mortality is high (Foley *et al.,* 2013).

In ***Chapter 5.1***, using WGS and available bioinformatic tools, and in collaboration with Sanger Institute and Biocant, we analyzed the genome of a *S*. Enteritidis strain isolated from day-old chicks with omphalitis; molecular features associated with the serotype, antibiotic resistance, virulence, and mobile genetic elements, were explored.

Due to the absence of the *wzy* gene, which encodes O antigen in Gram negative bacteria, including *Salmonella* (Hong *et al.,*2015), confirmation of serotype Enteritidis was obtained through the detection of *sdf* gene (*Salmonella* difference fragment virulence gene), encoding a marker for most common strains of circulating *S*. Enteritidis (Agron *et al.,* 2001). In

addition, 52 genes encoding efflux, transport and permeability mechanisms, where identified, corroborating with the tetracycline phenotype. Although the isolate showed susceptibility towards fluoroquinolones, aminoglycosides and chloramphenicol, bioinformatic analysis allowed the detection of genes that have already been associated to resistance in such antibiotic classes. However, in this case, the strain maintained full susceptibility to antibiotics. (Fernandez & Hancock, 2012).

The presence of a *Salmonella*-specific MerR-like gold (Au) sensor-GolS involved in Au resistance was identified (Pontel *et al.,* 2007). Studies carried out by Pal *et al.,* (2015) showed that although the genetic co-existence of resistance determinants to antibiotics, biocides and metals does not pose an immediate risk by horizontal gene transfer, as they are mostly located in different plasmids, the coexistence of various determinants of resistance poses a risk by promoting and maintaining plasmids with potential for co-selection (Baker-Austin *et al.,* 2006; Lemire *et al.,* 2013; Pal *et al.,* 2015).Thus, the identification of genetic determinants that encode multiple efflux pumps, virulence factors, mobile genetic elements and heavy metal tolerance is a cause for concern regarding the capacity dissemination of infection to other birds, as well as the risk of zoonotic transmission.

Polymyxins, particularly colistin, have been used in human and veterinary medicine for about five decades. Due to nephrotoxic and neurotoxic effects, its use in humans is restricted to the treatment of severe infections caused by carbapenem resistant *Enterobacteriaceae*, *Acinetobacter* spp. and *Pseudomonas aeruginosa*. In veterinary medicine, colistin is widely used in preventive and curative treatments for gastrointestinal tract infections, in food-producing animals, occupying the 5th place of sales in European countries (Kempf *et al.,* 2013; EMA/ESVAC, 2016). Shortly after the first notification of occurrence of plasmid-mediated resistance (PMCR), *mcr-1*, by Liu *et al.,* (2016), numerous studies worldwide reported the detection and dissemination of the *mcr-1* gene in *Enterobacteriaceae* strains isolated from humans, different animal species, foodstuffs and the environment (Hasman *et al.,* 2006; Campos *et al.,* 2016; Jones-Dias *et al.,* 2016b; Figueiredo *et al.,* 2016; Perrin-Guyomard *et al.,* 2016; Quesada *et al.,* 2016, Zurfuh *et al.,* 2016; Zhang *et al.,* 2016).

In ***Chapter 5.2***, a retrospective study (2010-2015) on a collection of 1840 *Enterobacteriaceae* strains (*E. coli*, *n*=1206; *S*. *enterica*, *n*=634), regarding susceptibility to polymyxins (colistin) and β-lactams (3rd generation cephalosporins, cefotaxime and ceftazidime), in strains resistant to colistin, was performed. We identified 138 colistin-resistant strains (*E. coli*, *n*=103; *Salmonella* spp, n=35). The detection of *mcr* genes (*mcr-1* and *mcr-2*) in resistant strains revealed a high frequency of occurrence, particularly in turkeys (27 %) and pigs (10.6%). A new variant of the *mcr-1* gene was detected and designated as *mcr-1.9*. This gene differed from *mcr-1* in a single point mutation that consisted in a substitution of thymine by cytosine at position 1238 (T1238C), leading to the substitution of a valine by an alanine at position 413 (Val413Ala).

In addition, 45.7% (42/92) of *E. coli* strains bearing the *mcr-1* gene were also resistant to 3rd generation cephalosporins and/or cephamycins (ESBL and/or PMAβ); several other *bla* genes were also detected (*bla*$_{CTX-M-1}$, *n*=14; *bla*$_{CTX-M-32}$, *n*=13; *bla*$_{CTX-M-14}$, *n*=5; *bla*$_{CTX-M-8}$, *n*=1; *bla*$_{CTX-M-27}$, *n*=1; *bla*$_{SHV-12}$, *n*=3; *bla*$_{CMY-2}$, *n*=3; *bla*$_{ampC}$, *n*=2). We highlighted the first occurrence, in our country, of the enzyme CTX-M-8 in strains of animal origin. Although these β-lactamases are rare in European countries (Borjesson *et al.,* 2016), they seem to be emerging in human strains (Eller *et al.,* 2014).

In addition to the *mcr-1.9* and *bla*$_{CTX-M-8}$ genes, other resistance determinants were identified, namely sulfamethoxazole (*sul3*), trimethoprim (*dfrA12-type*), chloramphenicol (*cmlA1-type*) and β-lactam (*bla*$_{TEM-1}$), corroborating with the resistance phenotype observed. As previously reported (Hasman *et al.,*2015, Campos, *et al.,*2016), three plasmids were identified, among which we highlight IncX4, carrying the *mcr-1.9* gene, and IncI1 carrying the *bla*$_{CTX-M-8}$ gene; the latter have already been broadly identified in human, animals and food strains (Veldman *et al.,* 2011, Accogli *et al.,* 2013, Dierikx *et al.,* 2013). Detection of the *mcr-1* gene in two strains isolated from food-producing animals in Portugal in 2010 suggests the involvement of a silent dissemination, as reported in other studies (Campos *et al.,* 2016; Haenni *et al.,* 2016; Perrin-Guyomard *et al.,* 2016).

Indeed, it is alarming that in addition to the selection pressure exerted by colistin itself, 3rd generation cephalosporins can also act as a selection for resistance to colistin and vice-versa. Moreover, abusive and inadequate use of tetracyclines and sulfonamides in animals, may contribute to the dissemination of plasmids bearing the *mcr* gene (Haenni *et al.,* 2016).

The high frequency of *mcr-1* occurrence in isolates of veterinary origin, most likely associated with the high consumption of colistin in food-producing animals (EMA, 2016), contrary to what happens in human medical practice, suggests a gene flow from animals to humans. In fact, plasmid resistance to colistin lies in the interface between animal health and human health (Skov & Monnet, 2016).

In a One Health perspective, and recognizing the importance of colistin in human clinical practice as a last resource antibiotic in severe multidrug resistant infections, data obtained in this study and previously published by others emphasize the urgent need to control the dissemination of plasmids bearing the *mcr* gene, and reconsider the massive use of colistin in veterinary medicine worldwide. Moreover, the European Medicines Agency (EMA) issued a set of recommendations aiming to restrict the use of colistin to a second-line treatment in animals and to classify this antibiotic as a drug reserved to treat infections in animals, only when there is no alternative treatment (EMA, 2016).

Horizontal transfer of antibiotic resistance genes among Gram negative bacteria plays a major role in the spread of multidrug resistance. The emergence and spread of antibiotic resistance among pathogenic bacteria of clinical relevance has been a major concern in public health. Commensal and environmental bacteria, mobile genetic elements and

bacteriophages constitute important reservoirs of antibiotic resistance genes (resistome), which pathogenic bacteria can capture by horizontal gene transfer (von Wintersdorff *et al.,* 2016).

In the studies developed in ***Chapters 4*** and ***5***, we emphasized the importance of associations between ESBL-, PMAβ-, PMQR- and PMCR-encoding genes with mobile genetic elements, such as integrons, ISs, transposons, phages and plasmids (Partridge, 2015). ISs play an important role in the transfer of antibiotic resistance encoding-genes by encoding a transposase and providing promoters to activate silent genes, or enhance expression of downstream determinants (Zhao & Hu, 2013). IS*Ecp1-bla*$_{CTX-M}$-IS*903* and IS*Ecp1-bla*$_{CTX-M}$-orf*477* are two major genetic platforms important in the mobilization of *bla*$_{CTX-M}$ genes (Lartigue *et al.,*2004), as we found in our studies. Being IS*Ecp1* one of the most important and frequent genetic elements, others as IS903 and IS*26* were also found to be adjacent to b*la*$_{CTX-M}$ genes. Such heterogeneity may be explained by continuous recombination events with exchange of genes and genetic rearrangements (Zhao & Hu, 2013).

Other mobile genetic elements like Class 1 and Class 2 integrons were identified in a large number of *Salmonella* and *E. coli* strains. Their spread among separate microbial populations may be facilitated due to their location in transposons, such as *Tn*402 in class 1 and *Tn*7 in class 2 integrons. This association allow an increased mobility between different plasmids and between plasmids and the bacterial chromosomes (Stokes & Gillings, 2011). Overall, integrons play a relevant role as genetic reservoirs for transfer, integration and dissemination of resistance genes among bacteria (van Essen-Zandbergen *et al.,* 2007; Ramírez *et al.,* 2010; Sunde *et al.,* 2015).

CHAPTER 7

CONCLUDING REMARKS

The studies performed in this thesis included commensal, zoonotic and pathogenic bacterial strains belonging to *Enterobacteriaceae* family (*Escherichia coli*, *Salmonella* spp and *Morganella morgannii*), isolated from different animal species (food-producing, companion and zoo animals) and food products of animal origin.

The research and monitoring of antibiotic resistance, as well as the implementation of surveillance programs consist of some of the strategic priorities aiming to control the spread of antibiotic resistance. Indeed, the phenotypic characterization of antibiotic susceptibility, and respective interpretation of the results according to epidemiological breakpoints, may result in the acquisition of important information related with changes in resistance patterns. It may also be relevant to predict the emergence of new resistance mechanisms and denote possible shifts in the dynamic of resistance within different animal populations and products of animal origin.

Antibiotic susceptibility of *Salmonella* spp and *E. coli* strains is greatly related with the animal species of origin, strain's serotype (for *Salmonella*) and genotype, as well as the antibiotic consumption to which the animals are subjected to. Overall, nonsusceptibility towards all tested antibiotics was higher in food-producing animals, followed by companion and zoo animals. In contrast, cefotaxime nonsusceptibility values were higher in companion animals, followed by food-producing and zoo animals. Regarding colistin, a very high frequency of resistance was reported in *E. coli* strains isolated from food-producing animals, particularly turkeys and swine.

The diversity of hosts sampled and techniques used, which included bacteriological, classical molecular biology, and whole genome sequencing (WGS), contributed to deepen the knowledge about antibiotic resistance in *Escherichia coli* and *Salmonella* spp. Moreover, critically important molecular antibiotic resistance mechanisms present either in human or in veterinary medicine, were highlighted throughout the studies included in this thesis.

Globally, we would like to emphasize:

1. High diversity of resistance determinants to critical important antibiotics detected in *Salmonella* spp and *E. coli* strains, namely: β-lactamases [ESBL and/or PMAβ (*bla*$_{\text{TEM-1}}$, *bla*$_{\text{TEM-52}}$, *bla*$_{\text{SHV-12}}$, *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-32}}$, *bla*$_{\text{CTX-M-27}}$, *bla*$_{\text{CTX-M-166}}$, *bla*$_{\text{CMY-2}}$)], *bla*$_{\text{ESAC}}$, PMQR (*qnrB19*, *qnrS1*, *aac(6')-Ib-cr, qnrD1*), and PMCR (*mcr-1, mcr-1.9*);

2. The first occurrence of ESBL enzymes of CTX-M family in *Salmonella enterica* isolates from animal origin (*bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-32}}$), ESBL-encoding genes (*bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{CTX-M-27}}$), and *bla*$_{\text{CTX-M-166}}$ in *E. coli* strains isolated from swine and broilers, respectively; *bla*$_{\text{ESAC}}$ in *E. coli* isolated from poultry, PMQR (*qnrD1*) in *M. morganii* from broilers and PMCR (*mcr-1.9*) in *E. coli* isolated from swine;

3. Genes encoding unique efflux mechanisms that may anticipate the prediction of antibiotic and metal resistance mechanisms in *S*. Enteritidis isolated from broilers;

4. Horizontal gene transfer possibly mediated by diverse mobile genetic elements, such as insertion sequences (*IS*Ecp1, *IS*26, *IS*903, ORF477), class 1 and 2 integrons, and plasmids of several incompatibility groups (IncI1, IncF, IncX4, IncN, IncFIA, Col8282, among others).

5. High genetic similarity between bacterial isolates recovered from animals and food products of animal origin and human pathogens.

In summary, the studies performed in this thesis reinforce the need for a permanent investigation on the resistance mechanisms present in multiple ecological niches, and bring new insights into the current scenario of antibiotic resistance in *Enterobacteriaceae*, in Portugal. Prioritizing the molecular characterization of antibiotic resistance mechanisms that are critically important for humans and animals was essential, not only for understanding the emergence of new resistance mechanisms, but also to control its spread.

Globally, this work achieved the objectives initially outlined, having even surpassed them, namely by using new generation technologies as an essential complementary tool in the study of antibiotic resistance.

CHAPTER 8

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