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Master Thesis

INVESTIGATION OF COLISTIN RESISTANCE IN CLINICAL *Salmonella enterica* subsp. *enterica* ISOLATES IN GREECE

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ΠΑΝΕΠΙΣΤΗΜΙΟ ΔΥΤΙΚΗΣ ΑΤΤΙΚΗΣ

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ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ ΣΤΗ ΔΗΜΟΣΙΑ ΥΓΕΙΑ

ΕΙΔΙΚΕΥΣΗ: ΛΟΙΜΩΔΗ ΝΟΣΗΜΑΤΑ – ΕΡΓΑΣΤΗΡΙΑΚΗ ΔΗΜΟΣΙΑ ΥΓΕΙΑ

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Ο κάτωθι υπογεγραμμένος Γεώργιος Μάνεσης του Αντωνίου, με αριθμό μητρώου 20026 φοιτητής του Προγράμματος Μεταπτυχιακών Σπουδών στη Δημόσια Υγεία του Τμήματος Πολιτικών Δημόσιας Υγείας της Σχολής Δημόσιας Υγείας του Πανεπιστημίου Δυτικής Αττικής, δηλώνω ότι:

«Είμαι συγγραφέας αυτής της μεταπτυχιακής εργασίας και ότι κάθε βοήθεια την οποία είχα για την προετοιμασία της, είναι πλήρως αναγνωρισμένη και αναφέρεται στην εργασία. Επίσης, οι όποιες πηγές από τις οποίες έκανα χρήση δεδομένων, ιδεών ή λέξεων, είτε ακριβώς είτε παραφρασμένες, αναφέρονται στο σύνολό τους, με πλήρη αναφορά στους συγγραφείς, τον εκδοτικό οίκο ή το περιοδικό, συμπεριλαμβανομένων και των πηγών που ενδεχομένως χρησιμοποιήθηκαν από το διαδίκτυο. Επίσης, βεβαιώνω ότι αυτή η εργασία έχει συγγραφεί από μένα αποκλειστικά και αποτελεί προϊόν πνευματικής ιδιοκτησίας τόσο δικής μου, όσο και του Ιδρύματος.

Παράβαση της ανωτέρω ακαδημαϊκής μου ευθύνης αποτελεί ουσιώδη λόγο για την ανάκληση του πτυχίου μου».

Ο Δηλών

Μάνεσης Γεώργιος

Βεβαιώνω ότι η παρούσα Διπλωματική Εργασία είναι αποτέλεσμα προσωπικής μου εργασίας και δεν αποτελεί προϊόν αντιγραφής. Στις δημοσιευμένες ή μη δημοσιευμένες πηγές που αναφέρω έχω χρησιμοποιήσει εισαγωγικά όπου απαιτείται και έχω παραθέσει τις πηγές τους στο σχετικό τμήμα της βιβλιογραφίας.

Υπογραφή:.....

ABSTRACT

Salmonella enterica subsp. *enterica* is a rod-shaped, Gram-negative bacterium. The public health importance of *S. enterica* subsp. *enterica* stems from its ability to cause salmonellosis in humans and animals. Effective treatment with the appropriate antimicrobial agent is crucial for managing the disease. Antimicrobial resistance (AMR) is the ability of bacteria, viruses, fungi, and parasites to withstand the effects of medicines that are used to treat infections. *Salmonella*, along with other bacteria, have developed resistance against various classes of antimicrobials. In this frame, colistin, a polycationic peptide antimicrobial, is re-introduced in medicine to treat multidrug resistant bacteria. Of major concern is the global emergence of colistin resistant bacteria, which may also bear mobile colistin resistance (mcr) genes in plasmids, thus enabling the horizontal transmission of colistin resistance between different species of bacteria. The objectives of this study were: i) the estimation of the Minimal Inhibitory Concentration (MIC) of colistin in 120 *Salmonella enterica* human isolates using a commercial kit based on the broth microdiluton method (BDM) and ii) the investigation of the genetic basis of colistin resistance in *Salmonella enterica* isolates by employing two standardized and validated conventional multiplex PCR protocols for the detection of mcr 1-5 and mcr 6-9 plasmid-borne genes. Results showed that 10% of the isolates were colistin resistant and were predominantly belonging to S. Enteritidis serotype, revealing that the epidemiology is similar to other European countries. Mcr genes were not detected in any of the resistant strains. To comprehensively address the challenge of AMR against colistin, it is imperative to enhance our understanding of emerging resistance and the distribution of mcr genes in *Salmonella* isolates from Greece. This can facilitate the adoption of surveillance strategies and a One Health approach to manage this emerging threat.

Keywords: colistin, antimicrobial resistance (AMR), *Salmonella enterica*, human isolates, Minimal Inhibition Concentration (MIC), *mcr* genes, One Health

ΠΕΡΙΛΗΨΗ

Η *Salmonella enterica* subsp. *enterica* είναι ένα ραβδωτό, Gram-αρνητικό βακτήριο. Η σημασία του για τη δημόσια υγεία πηγάζει από την ικανότητά του να προκαλεί σαλμονέλωση σε ανθρώπους και ζώα. Η αποτελεσματική θεραπεία με το κατάλληλο αντιμικροβιακό παράγοντα είναι κρίσιμη για την αντιμετώπιση της νόσου. Η αντοχή στα αντιμικροβιακά είναι η ικανότητα των βακτηρίων, των ιών, των μυκήτων και των παρασίτων να αντιπαρέρχονται τις επιδράσεις των φαρμάκων που χρησιμοποιούνται για θεραπευτικούς σκοπούς. Η σαλμονέλα, καθώς και άλλα βακτήρια, έχουν αναπτύξει αντοχή σε διάφορες κατηγορίες αντιμικροβιακών. Σε αυτό το πλαίσιο, η κολιστίνη, ένα πολυκατιονικό αντιμικροβιακό πεπτίδιο, επανεισήχθη στην ιατρική για την αντιμετώπιση των βακτηρίων που είναι ανθεκτικά σε πολλαπλά αντιμικροβιακά. Ανησυχητική όμως είναι η παγκόσμια εμφάνιση βακτηρίων ανθεκτικών σε αυτό το αντιβιοτικό, καθώς αυτά τα βακτήρια μπορεί να φέρουν στα πλασμίδιά τους κινητά γονίδια αντίστασης στην κολιστίνη (mcr), επιτρέποντας έτσι την οριζόντια μετάδοση γονιδίων αντοχής μεταξύ διαφορετικών βακτηριακών ειδών. Κατά συνέπεια, οι στόχοι της παρούσας μελέτης ήταν: i) η εκτίμηση της ελάχιστης ανασταλτικής συγκέντρωσης (MIC) της κολιστίνης σε ένα δείγμα 120 καλλιεργημάτων *Salmonella enterica* απομονωθέντων από ανθρώπους, χρησιμοποιώντας ένα εμπορικό κιτ που βασίζεται στη μέθοδο μικροδιάλυσης σε θρεπτικό υλικό (BDM) και ii) η διερεύνηση της παρουσίας γονιδίων mcr στα ανθεκτικά στην κολιστίνη στελέχη χρησιμοποιώντας δύο τυποποιημένες συμβατικές μεθόδους PCR για την ανίχνευση των γονιδίων mcr 1-5 και mcr 6-9, αντίστοιχα. Τα αποτελέσματα έδειξαν ότι το 10% των καλλιεργημάτων ήταν ανθεκτικά στην κολιστίνη και ανήκαν κυρίως στον ορότυπο S. Enteritidis, αποκαλύπτοντας ότι η επιδημιολογία της αντοχής στην κολιστίνη είναι παρόμοια με αυτή άλλων ευρωπαϊκών χωρών. Είναι σημαντικό να αναφερθεί ότι δεν ανιχνεύθηκαν γονίδια mcr σε κανένα από τα ανθεκτικά στελέχη. Για να αντιμετωπιστεί η μικροβιακή αντοχή είναι επιτακτική ανάγκη να ενισχυθεί η κατανόησή μας για την επιδημιολογία της ανθεκτικότητας στην κολιστίνη και της κατανομής των γονιδίων mcr στα διάφορα στελέχη που προέρχονται από την χώρα μας. Αυτό είναι αναγκαίο για την υιοθέτηση μέτρων διαχείρισης και την δημιουργία μιας επιτυχημένης στρατηγικής Ενιαίας Υγείας κατά αυτού του αναδυόμενου προβλήματος.

Λέξεις κλειδιά: κολιστίνη, αντιμικροβιακή αντοχή, *Salmonella enterica*, κλινικά καλλιεργήματα, γονίδια *mcr*, Ενιαία Υγεία

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ABBREVIATIONS

LPS	**Lipopolysaccharides**
MLST	**Multilocus sequence typing**
PFGE	**Pulse field gel electrophoresis**
VNTRs	**Variable number of tandem repeats**
WGS	**Whole genome sequencing**
AMR	**Antimicrobial resistance**
MDR	**Multiple drug resistance**
SSRC	**National Salmonella Shigella Reference Centre**
MIC	**Minimal inhibitory concentration**
AST	**Antimicrobial susceptibility test**
BDM	**Broth microdiluton method**
EUCAST	**European committee on antimicrobial susceptibility testing**
CLSI	**Clinical and Laboratory Standards Institute**
CST	**Colistin susceptibility testing**

FOREWORD

My background as an animal scientist and research projects I am currently working on have led me to believe that my mission is to focus on the production of nutritious and safe food for the ever-increasing human population. This belief was further reinforced through this postgraduate program on Public Health that allowed me to better comprehend the intricate interplay of food production, ecosystems and Public Health, under the scope of One Health. Consequently, I was interested in focusing on a field which is related to food production and animal science, as well as to enhance my skills around a bacteriological laboratory. Given that colistin is primarily used in animal production and *Salmonella* is among the most common foodborne pathogens, I thought that studying the emergence of colistin resistance in *Salmonella* isolates would be the perfect opportunity to achieve these goals.

Of course, this study would never have come to fruition without the continuous support of Dr. Georgia Mandilara, the head of the National *Salmonella* and Shigella Reference Centre, her intuition in helping me design the study protocol and research hypotheses and her genuine interest in education. I would like to thank Dr. Panagiota Giakkoupi and Dr. Nikolaos Tegos for their comments and their effort in reviewing and improving this thesis. I would also like to thank the Centre for Antimicrobial Resistance (Central Public Health Laboratory, National Public Health Organization, 16672 Vari, Greece) for providing *Klebsiella pneumoniae* reference isolates that were used in the colistin susceptibility testing as well as for providing template DNA which was used as reference in PCR testing for the detection of mcr genes. I am also grateful for meeting Emmanouil (Max) Fotakis, who now I consider a friend.

Last but not least, I would like to thank my wife Evelina for always keeping me motivated, her unfaltering support through words of encouragement and delicious meals as well as her vast reserves of patience.

INTRODUCTION

Salmonella is one of the most common foodborne diseases around the globe, causing thousands of deaths annually. *Salmonella* spreading and infections are intertwined with food production and safety, farming practices, hygiene and biosecurity and modern, globalized trade. This can lead to globalized *Salmonella* outbreaks which often cannot be spatiotemporally defined. Consequently, traditional approaches for managing this disease relied on heavy use of antimicrobials. As a result, *Salmonella* serotypes have acquired resistance mechanisms to various classes of antimicrobials. The same phenomenon has been observed in other species of bacteria as well. Scientists, public health authorities and policy makers have recognized antimicrobial resistance to multiple drugs as a major public health threat which is further aggravated by the lack of newly developed antimicrobial drugs.

In an effort to manage multidrug resistance, previously marginalized antibiotics such as colistin have been reintroduced to human medicine as last resort drugs for the management of multidrug resistant pathogens. Colistin use has been limited due to concerns about potential side effects, including kidney damage and neurotoxicity, however it was, and still is in some countries, extensively used in animal production. In recent years, there has been growing concern about the emergence of colistin-resistant bacteria, including *Salmonella.* Resistance to colistin can occur through various mechanisms, including the presence of certain genes called mcr genes, which can be horizontally transferred between different bacterial strains or species. Eventually, reports on colistin resistant *Salmonella* that also bear mcr genes have emerged globally.

The aim of this thesis was to cover the existing gap on colistin resistance and mcr genes in *Salmonella enterica* subsp. *enterica* isolated from humans in Greece. To address this knowledge gap in the scientific field of antimicrobial resistance, the objectives of this thesis were: i) the estimation of the Minimal Inhibitory Concentration (MIC) of colistin in 120 *Salmonella* enterica human isolates using a commercial kit based on the broth microdiluton method (BDM) and ii) the investigation of the genetic basis of colistin resistance in *Salmonella* enterica isolates by employing two standardized and validated conventional multiplex PCR protocols for the detection of mcr 1-5 and mcr 6-9 plasmid-borne genes.

The spread of colistin resistance is a significant public health concern, as it further limits treatment options for bacterial infections. Given the importance of colistin as a last-resort antibiotic, its use is carefully monitored and regulated to prevent the emergence and spread

of resistant bacteria. The acquisition of epidemiological data on colistin resistance and the distribution of mcr genes on the Greek *Salmonella* population is the basis for designing effective control measures and evidence based One Health approaches to tackle this emerging threat. It is also accepted that better comprehension of the prevalence of mcr genes in different bacterial species can accommodate the design of countermeasures to mitigate the horizontal transmission of these genes.

Previous research has demonstrated that colistin resistance and mcr genes are widespread in *E. coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, as well as *Salmonella.* Consequently, the ECDC required from its member states to include colistin to the list of antimicrobials which are monitored for the emergence of resistant isolates. This study is a small contribution towards this goal.

This thesis is divided into two parts. Part A, the general information, is further subdivided in 4 chapters. Chapter 1 focuses on explaining the importance of *Salmonella*, its epidemiology and treatment and control options. Chapter 2 deals with concepts such as antimicrobial resistance, its drivers and methods of controlling it as well as its intricate relationship with *Salmonella*. Chapter 3 presents colistin, the resistance mechanisms and mcr genes, as well as the available antimicrobial susceptibility testing methods for colistin. Finally, chapter 4 focuses on the relationship between *Salmonella* and colistin resistance. Part B includes 5 chapters and presents the objectives of this study, the materials and methods used, the generated results, the discussion and the conclusions and suggestions for future research.

PART A. GENERAL

Chapter 1. *Salmonella enterica*

1.1 The bacterium

Salmonella spp. was observed for the first time in 1886 by Daniel E. Salmon, a veterinary pathologist in the United States. *Salmonella* spp. are rod-shaped, Gram-negative bacteria with a facultative metabolism, capable of growing in the presence or absence of oxygen (Whitman, 2015). The bacteria are usually motile and can grow on standard media such as nutrient agar and Luria-Bertani medium. Although the acquisition of the lactose operon has been described in some cases, in general the bacteria do not ferment lactose (Leonard, Lacher and Lampel, 2015). Two species have been recognized within the genus, *Salmonella bongori* and *Salmonella enterica*. *Salmonella enterica* is classified in six subspecies: i) *enterica*, ii) *salamae*, iii) *arizonae*, iv) *diarizonae*, v) *houtenae* and vi) *indica* (Monte and Sellera, 2020). Using the Kauffmann-White-Le Minor scheme, more than 2500 serovars have been classified to the six subspecies on the basis of the extensive diversity of heat-stable lipopolysaccharide (O) and heat-labile flagellar protein (H) antigens (Monte and Sellera, 2020). Salmonella serovars can be further subdivided using either phenotypic or molecular and genomic subtyping methods. Phenotypic methods include bacteriophage typing (phage typing) and antimicrobial resistance (antibiogram) typing. Genomic subtyping methods include plasmid typing, multilocus sequence typing (MLST), ribotyping, pulse field gel electrophoresis (PFGE), variable number tandem repeats (VNTRs) fingerprinting and whole genome sequencing (WGS). Approximately 99% of the identified serovars are classified to the *S. enterica* subsp. *enterica* and can cause gastroenteritis and systemic infections in warm-blooded animals. Members of the other subspecies are mainly found in cold-blooded animals or the environment (Crump and Wain, 2017).

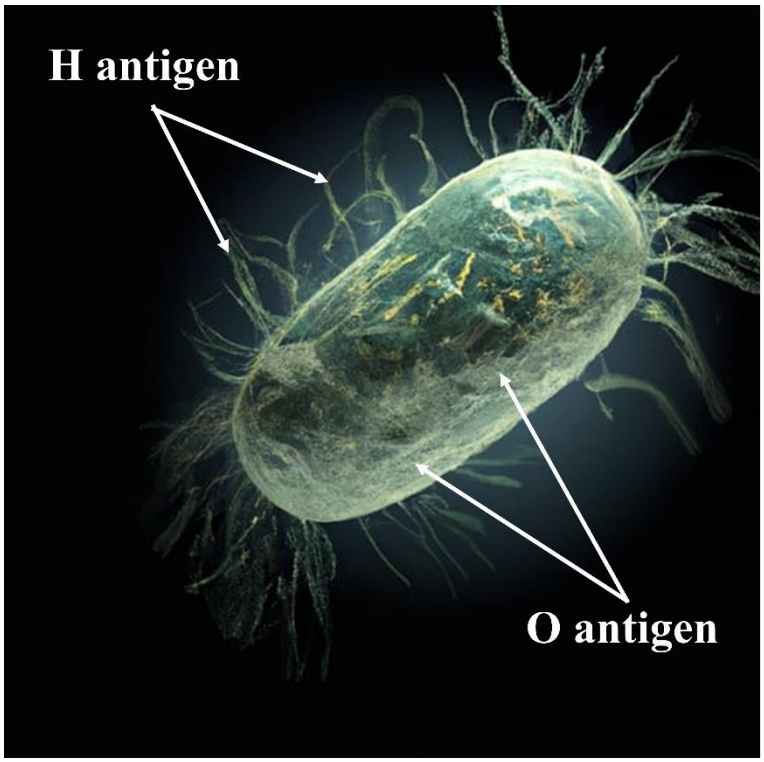

Figure 1. *Salmonella* cell with flagellar protein (H) and lipopolysaccharide (O) antigens.

The importance of *S. enterica* subsp. *enterica* stems from its ability to cause salmonellosis, a common foodborne illness worldwide. Salmonellosis is primarily acquired through the consumption of contaminated food, such as undercooked poultry, eggs, dairy products, meat, and fresh produce. The clinical manifestation of salmonellosis can vary from mild gastroenteritis to severe systemic infection, depending on the serovar, the infectious dose, and the host's immune status. Beyond the human health impact, *S. enterica* subsp. *enterica* is also a significant concern in animal health and veterinary medicine. It can cause diseases in various domestic and wild animal species, leading to economic losses in agriculture and food production industries. In livestock, such as poultry, pigs, and cattle, *Salmonella* spp. infections can result in reduced growth rates, decreased productivity, and increased mortality rates. The zoonotic potential of *S. enterica* subsp. *enterica* poses a public health risk, further highlighting its importance (Crump and Wain, 2017).

1.2 The classification of *S. enterica* subsp. *enterica*

The classification of *S. enterica* subsp. *enterica* into different serovars is critical. Understanding the diversity and distribution of *Salmonella* serovars aids in epidemiological investigations, identifying outbreaks, tracing the sources of infection, implementing targeted control measures, and monitoring the effectiveness of interventions. Additionally, the

characterization of S. enterica subsp. enterica strains helps to elucidate their virulence factors, antimicrobial resistance profiles, and host specificity, contributing to the development of diagnostic tools, vaccines, and improved treatment strategies (Crump and Wain, 2017).

The *S. enterica* subsp. *enterica* serovars can be classified to two broad categories, i) the typhoidal and paratyphoidal serovars and ii) the non-typhoidal serovars. Typhoidal serovars, such as S. Typhi, and paratyphoidal serovars, for example, S. Paratyphi A, are responsible for systemic illnesses. Symptoms include fever, headache, malaise, abdominal pain, and rash. Moreover, S. Typhi and S. Paratyphi A are serovars restricted to humans, meaning they solely cause disease in and are transmitted by humans. Contaminated food and water, resulting from inadequate sanitation practices and human fecal contamination, typically serve as the transmission route for these serovars. Due to the systemic nature of typhoidal and paratyphoidal serovars, they have historically been considered a higher public health concern compared to non-typhoidal serovars (Cohn *et al.*, 2021).

On the contrary, non-typhoidal serovars are transmitted through multiple routes, including direct contact with animals, fomites and the consumption of contaminated food. Contamination is often associated with animal hosts and products, such as raw meat, poultry, eggs, raw milk, and dairy products (Gal-Mor, Boyle and Grassl, 2014). However, it is important to note that a wide range of foods, including fruits, vegetables, dry products like spices and chocolate, have been identified as sources of non-typhoidal *Salmonella* infections and outbreaks. Furthermore, non-typhoidal *Salmonella* has demonstrated the ability to survive for extended periods in various environments outside of the host, including soil and food processing plant facilities (Cohn *et al.*, 2021). Typically, infection with non-typhoidal serovars is associated with self-limiting gastroenteritis. However, non-typhoidal *Salmonella* serovars exhibit significant variability in their tendency to cause bacteremia as opposed to diarrhea. For instance, *Salmonella* spp. serovars Dublin, Sandiego, Schwarzengrund, Panama, and Heidelberg are more frequently isolated from blood samples rather than stool samples when compared to *Salmonella* Typhimurium (Crump *et al.*, 2011). In addition to causing invasive disease, non-typhoidal *Salmonella* spp. can lead to localized infections in various organs and body parts, including the viscera, meninges, bones, joints, and serous cavities. The risk of invasive disease is influenced by factors such as the infective dose of the bacteria and host-related factors. Within low-resource countries, particularly in sub-Saharan Africa, non-typhoidal *Salmonella* infections are prominent and can be the primary

cause of bloodstream infections acquired within the community. Studies consistently show that serovars Typhimurium and Enteritidis are the most prevalent in these regions (Reddy, Shaw and Crump, 2010). Despite differences between serovars in their capacity to cause disease in humans, non-typhoidal serovars are considered to pose a similar public health risk to typhoidal serovars (Cohn *et al.*, 2021).

Although regulations in many countries treat all non-typhoidal *Salmonella* serovars as equally hazardous with regards to public health, substantial scientific evidence indicates variations among these serovars and clonal groups in terms of the associated risk. This evidence is supported by several factors: "i) the presence or absence of virulence genes encoding fully functional virulence factors, ii) phenotypic data derived from tissue culture or animal models and iii) epidemiological evidence, such as the under-representation of certain serovars or clonal groups in human clinical cases compared to their presence in food, raw materials, or animals" (Cohn *et al.*, 2021). It is important to consider geographical associations, as certain *Salmonella* serovars or clonal groups may exhibit strong regional correlations. Taking these factors into account when evaluating epidemiological evidence allows for a more comprehensive assessment of hypo- or hypervirulence among specific serovars or clonal groups (Cohn *et al.*, 2021).

1.3 Global epidemiological data on *S. enterica* subsp. *enterica*

Understanding the epidemiology of *Salmonella* is crucial for disease management and control, and in some cases for the effective treatment of patients. Typhoid and paratyphoid fevers significantly contribute to illness and elevated death rates among children and adults in developing countries. These fevers remain endemic in regions of Africa, South and Southeast Asia, as well as being frequently reported in the Middle East, South and Central America, the Pacific Islands, and certain countries in Southern and Eastern Europe. In contrast, developed countries like the United Kingdom or the United States experience low incidence of *Salmonella* Typhi infections, with the majority of cases being observed among individuals who have traveled to or returned from areas where the diseases are endemic (Lynch *et al.*, 2009). The epidemiology of paratyphoid fever is not as extensively documented as that of typhoid fever. Nonetheless, *Salmonella* Paratyphi A is responsible for approximately 25% of enteric fevers, and its prevalence has been on the rise in several Southeast Asian countries, including Vietnam, India, and Nepal.

On the other hand, it was estimated that non-typhoidal *Salmonella* caused approximately 1 million illnesses, resulting in 19,000 hospitalizations and 378 deaths in the United States in 2006 (Scallan *et al.*, 2011). Among *Salmonella* isolates that were serotyped through active foodborne disease surveillance in the United States in 2014, the top five serovars, ranked in descending order of prevalence, were Enteritidis, Typhimurium, Newport, Javiana, and Infantis. *Salmonella* Enteritidis is commonly associated with shell eggs and poultry as major sources of infection in the United States. Control measures implemented to address this issue have included interventions at the farm level, consumer education initiatives, and increased emphasis on proper refrigeration of eggs (Chai *et al.*, 2012). Due to the close association of various non-typhoidal *Salmonella* serovars with food-producing animals, numerous outbreaks have been attributed to foods derived from animals or to food and water contaminated with animal feces. Moreover, the extensive international trade in food, involving both developed and developing nations, has resulted in widespread distribution of products contaminated with *Salmonella* organisms. Non-typhoidal *Salmonella* outbreaks have been documented in various developing countries as well. Due to limitations in epidemiological investigations, the source and mode of transmission are often incompletely characterized. In addition to community-wide outbreaks, there have been instances of outbreaks in hospital settings, particularly in neonatal and pediatric wards. The clinical presentation of the disease can be severe, manifesting as diarrhea and septicemia. Invasive non-typhoidal *Salmonella* infections in such settings have been associated with case fatality rates exceeding 20% (Ao *et al.*, 2015).

1.4. *Salmonella* spp. in Greece

In the largest study for *Salmonella* monitoring ever conducted in Greece, the National Salmonella Shigella Reference Centre (SSRC) serotyped 10.513 samples in the period 2003-2020 (Mellou *et al.*, 2021). Among these isolates, 10,065 were attributed to *Salmonella enterica* subspecies *enterica*, while 157 were associated with (para)typhoidal isolates, and 291 were linked to other subspecies, including *S. enterica salamae* (252 isolates) and *S. enterica diarizonae* (39 isolates). The data revealed a noteworthy decrease in the count of non-typhoidal isolates from 2003 to 2020. The frequency of isolations displayed seasonality, with the highest rates occurring during the summer, particularly in the months of August and September (Mellou *et al.*, 2021).

Out of the 10,065 isolates belonging to *Salmonella enterica enterica* subspecies, a total of 193 distinct serotypes were detected. The most prevalent serotype among these isolates was *Salmonella enterica enterica* serotype Enteritidis (S. Enteritidis), constituting nearly 53% of the overall isolates. *Salmonella enterica enterica* serotype Typhimurium (S. Typhimurium) accounted for 12%, while its monophasic variant, *Salmonella enterica* subsp. *enterica* with antigenic type 1,4,[5],12:i:- (monophasic S. Typhimurium), made up 4% of the isolates. Among the top five most frequently identified serotypes were *Salmonella enterica enterica* serotype Bovismorbificans (S. Bovismorbificans) and *Salmonella enterica enterica* serotype Oranienburg (S. Oranienburg), representing 3.4% and 2.4% of the isolates, respectively. Additionally, *Salmonella enterica* subsp. *salamae* with antigenic type 1,4,[5],12,[27]:b:- was consistently present in Greece, although the yearly count of isolates remained relatively low (Mellou *et al.*, 2021).

Between 2003 and 2005, S. Enteritidis accounted for an average of 72% of the serotyped isolates. Over the subsequent four years (2006–2009), this average decreased to 55%, and for the next 11 years, there was a further decline, reaching as low as 35%. This reduction was expected, as Greece implemented the National Salmonella Control Programmes (NSCPs) in poultry populations in 2008. These programs primarily targeted S. Enteritidis and S. Typhimurium, in compliance with Regulation (EC) No 2160/2003. According to the European Food Safety Authority (EFSA), eggs and poultry meat are the primary sources of S. Enteritidis transmission, and the positive impact of implementing control measures against S. Enteritidis has been well-documented in Greece (Mellou *et al.*, 2021).

Salmonella enterica enterica serotype Typhimurium displayed minor fluctuations during the examined period, but it consistently held the position of the second most prevalent serovar. This trend aligns with observations made in other European Union (EU) countries, as indicated by the EU's annual summary reports on zoonoses. On average, from 2004 to 2019, S. Typhimurium ranked as the second most frequently reported serotype, accounting for approximately 18% of serotyped isolates. In 2007, Greece witnessed the emergence of the monophasic variant of S. Typhimurium (1,4,[5],12:i:-), which has since maintained its status as one of the five most common serotypes, as reported in earlier studies.

Both S. Typhimurium and its monophasic variant (1,4,[5],12:i:-) are primarily linked to pigs (meat), accounting for 42% and 72% of their respective cases. This association is largely due to their widespread presence in pork production. Notably, Greece lacks a national Salmonella control program for pigs, which is why the prevalence of these serotypes is expected to

remain relatively stable. Additionally, in Greece, there are other frequently identified serotypes such as S. Bovismorbificans, S. Oranienburg, and S. Kottbus. These serotypes are not consistently reported in EU countries, with occurrences below 0.6%. According to available literature, these serotypes have associations with various food products, including sesame seeds, ham, fruits, eggs, chocolate, and vegetables.

Altogether, the largest proportion of Salmonella isolates was obtained from young children aged less than 5 years old, accounting for 42.5% of the total. These findings align with research conducted in other European Union regions. Notably, Salmonella enterica subsp. salamae with antigenic type 1,4,[5],12,[27]:b:- was particularly prevalent among children under 5 years old. This discovery warrants further investigation to explore the potential association with a specific food source, as existing literature lacks relevant information (Mellou *et al.*, 2021).

1.5 Treatment and control

Salmonella spp. infections are treated based on the clinical manifestation of the disease and patient status (healthy, pregnant, immunocompromised, old, young etc.). Effective treatment with the appropriate antimicrobial agent is crucial and can be life-saving for enteric fever. In certain situations, treatment may need to commence before the results of antimicrobial susceptibility testing (AST) are available. Therefore, it is important to have knowledge of treatment options and potential challenges prior to initiating therapy. In cases of uncomplicated diarrhea caused by non-typhoidal *Salmonella* in otherwise healthy individuals, antimicrobial therapy is generally avoided, with rehydration therapy being the primary approach to management. However, in patients with invasive non-typhoidal *Salmonella* disease, antimicrobial treatment is essential for saving lives. Multidrug resistance (MDR), defined as resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole, is frequently encountered in non-typhoidal *Salmonella.* Furthermore, some *Salmonella* serovars are exhibiting increased resistance to fluoroquinolones, extended-spectrum cephalosporins, and azithromycin (Crump *et al.*, 2011).

Control measures for *Salmonella* disease can be implemented at three levels: the individual, the community (or herd), and the environment. Control strategies may involve vaccination, addressing the source of infection, or interrupting transmission routes. It is essential to consider factors that contribute to the emergence and spread of specific strains, including the indiscriminate use of antimicrobials, as these can significantly hamper the effectiveness of

control efforts. Various vaccines are currently accessible for the prevention of typhoid fever. These include the oral, live attenuated Ty21a vaccine and the Vi polysaccharide capsular vaccine (Jackson, Iqbal and Mahon, 2015). The Vi conjugate vaccines have demonstrated the potential to provide protection at a younger age and for an extended duration compared to both Vi capsular polysaccharide and possibly Ty21a vaccines. However, Vi conjugate vaccines are not widely available. Vaccines are also available for certain non-typhoidal *Salmonella* strains in animal husbandry. For instance, vaccines have proven effective in controlling *Salmonella* Enteritidis infections in poultry within the United Kingdom.

In numerous outbreaks, control measures have been implemented through either the eradication of infection reservoirs, such as infected poultry flocks, or the removal of contaminated food products. For instance, a worldwide withdrawal of contaminated confectionery products occurred after contamination with *Salmonella* Montevideo. The presence of an international rapid response network has played a crucial role in effectively managing such situations (Fisher and Threlfall, 2005). The significance of ensuring the safety of water and food cannot be overstated. In developing countries, contaminated water continues to serve as a significant reservoir for *Salmonella* organisms, leading to numerous outbreaks of typhoid and paratyphoid fever associated with fecal contamination of drinking water and food. Similarly, in industrialized nations, outbreaks have been attributed to the contamination of animal products and produce by animal feces. Proper cooking practices and adherence to kitchen hygiene protocols also play a crucial role in preventing the transmission of *Salmonella.* Practicing fundamental hygiene measures, including hand washing after interacting with pets, whether they are exotic or not, is of utmost importance. Building awareness among food producers and the general public regarding the potential hazards associated with *Salmonella,* a widespread and potentially life-threatening pathogen, can significantly contribute to reducing the burden of infection.

Chapter 2. Antimicrobial resistance

2.1 Antibiotics

Antibiotics are defined as organic compounds, produced by secondary metabolites of microbial metabolism, or synthesized artificially or semi-artificially and cause either the death of microorganisms or interfere with basic biochemical processes of their metabolism. Antibiotics are categorized based on various criteria such as their chemical composition, their mechanism of action, the organism that produces them, and the pathway of their biosynthesis. The chemical structure of different antibiotics and therefore their effectiveness against different bacteria varies.

Based on their mode of action, antibiotics are classified into "bacteriostatic" which inhibit bacterial growth (e.g., sulfonamides, tetracyclines) and "bactericidal" which selectively cause bacterial death (e.g., penicillins, cephalosporins). Additionally, antibiotics can be classified based on their spectrum of activity, into "broad-spectrum" when they act on many species of bacteria and "narrow-spectrum" when they act on one species or a group of bacteria. This classification is the most popular among clinicians and veterinarians to select the appropriate antibiotic for treatment purposes. The main categories of antibiotics are penicillins, cephalosporins, tetracyclines, sulfonamides, quinolones, aminoglycosides, macrolides, lincosamides, aminopenicillins, polymyxins, carbapenems, and imidazoles.

Antibiotics are widely used in human medicine to treat bacterial infections. They are an essential tool in the field of healthcare and have saved countless lives since their discovery. The primary purpose of antibiotics is to treat various bacterial infections, including respiratory, skin, urinary tract and gastrointestinal infections as well as sexually transmitted infections. Antibiotics are sometimes used prophylactically before surgery or dental procedures to prevent bacterial infections, particularly in individuals at higher risk or may be prescribed when complications arise from other medical conditions. Antibiotic misuse including overprescribing, self-medication, incomplete treatments or inappropriate selection of antibiotics can have significant consequences, including the development of antibiotic-resistant bacteria, which are more challenging to treat.

Antibiotics have been used extensively in the past as growth promoters to enhance animal growth and increase production due to their effect on the normal gut flora (Costa et al, 2017). In some countries in Asia, Africa, and Latin America, they are still used to promote animal

growth by incorporating them in low, subtherapeutic doses to animal feed, whereas in the European Union, this use has been banned since 2006 with Regulation (EC)1831/2003.

2.2 Antimicrobial resistance (AMR)

Antimicrobial resistance (AMR) is the ability of bacteria, viruses, fungi, and parasites to withstand the effects of medicines that are used to treat infections (WHO, 2022). This means that the medicines are no longer effective in killing or stopping the growth of the microorganisms. As a result, infections become increasingly difficult or impossible to treat, and the risk of disease spread, severe illness, or death increases (WHO, 2022). AMR has emerged as a significant threat to public health, impacting the effectiveness of antimicrobial drugs, leading to increased mortality rates, prolonged illnesses, higher healthcare costs and increasing the global burden of infectious diseases. One of the major concerns of AMR is the limited treatment options available for infections caused by multidrug-resistant organisms. Previously effective antibiotics, once considered the drugs of choice for disease treatment, are increasingly losing their efficacy (O'Neill, 2016). This situation has led to a critical need for the development of new antimicrobial agents and alternative treatment approaches. In 2019, the World Health Organization (WHO) warned that AMR is "one of the biggest threats to global health, food security, and development today." AMR is estimated to cause 1.27 million deaths each year, and that number is expected to rise to 10 million by 2050 if no action is taken (O'Neill, 2016).

Antimicrobial resistance arises through various mechanisms that allow bacteria and other microorganisms to withstand the effects of antimicrobial drugs. These mechanisms include: i) *Enzyme inactivation and modification*. Some microorganisms produce enzymes that can inactivate or modify antimicrobial drugs, making them ineffective. For example, some bacteria produce β-lactamases, which can break down the β-lactam ring of antibiotics, such as penicillins and cephalosporins (Egorov, Ulyashova and Rubtsova, 2018), ii) *Modification of the antibiotics target site.* Microorganisms can modify the target of an antimicrobial drug, making it less susceptible to the drug. For instance, some bacteria can change the structure of their ribosomes, which are the targets of many antibiotics (Blair *et al.*, 2015), iii) *Overproduction of the target.* Bacteria can produce more of the target of an antimicrobial drug, making it more difficult for the drug to bind to the target (e.g., bacteria that can produce more of the enzyme penicillin-binding protein 2 which is the target of penicillin) (Egorov, Ulyashova and Rubtsova, 2018), iv) *Replacement of the target site.* Some microorganisms

can replace the target of an antimicrobial drug with a different molecule that is not affected by the drug. For example, some bacteria can replace their ribosomes with ribosomes that are not sensitive to antibiotics (Blair *et al.*, 2015), v) *Efflux and reduced permeability.* Bacteria possess efflux pumps, which are specialized transporters that actively pump out antimicrobial agents from within the bacterial cell. These efflux pumps can efficiently remove drugs from the cell, reducing their intracellular concentrations. Resistant bacteria can also reduce the permeability of their cell membranes to the drugs, preventing the antimicrobial agents from reaching their intracellular targets (Blair *et al.*, 2015) and vi) *Biofilm formation*. Bacterial biofilms, which are complex communities of microorganisms encased in a protective matrix, contribute to antimicrobial resistance. Biofilms provide a physical barrier that limits the penetration of antimicrobial agents, making them less effective in eradicating the bacteria within the biofilm. Additionally, the slow growth and altered metabolic activity of bacteria within biofilms can render them less susceptible to the action of antimicrobial drugs (Yan and Bassler, 2019). Understanding these resistant mechanisms is crucial for developing effective strategies to combat AMR.

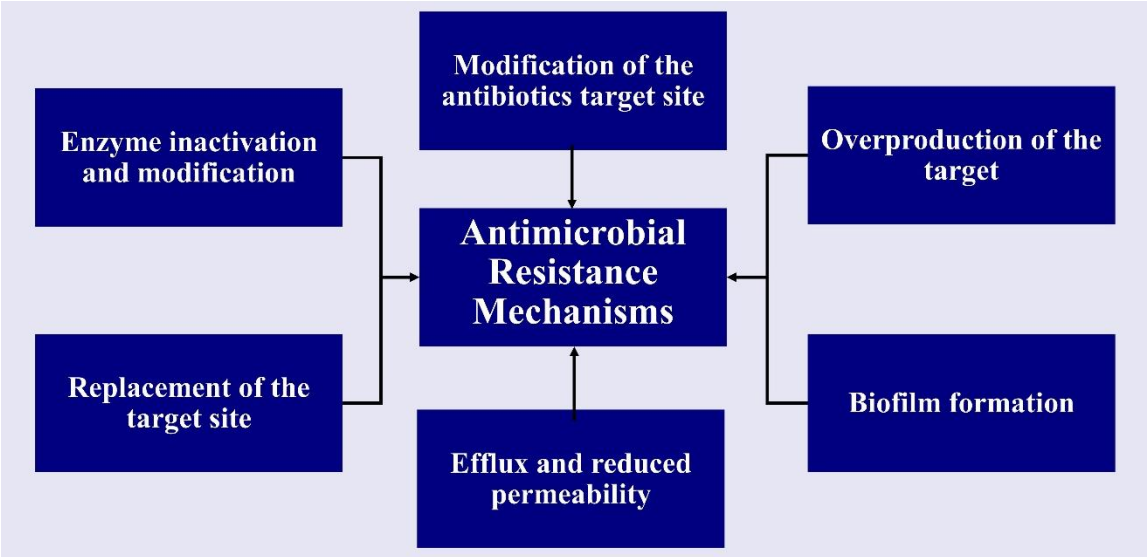

Figure 2. Categories of antimicrobial resistance mechanisms

Bacteria can acquire resistance through genetic mutations, horizontal gene transfer and selective pressure. Genetic mutations usually occur spontaneously over time and can alter the target sites of antimicrobial drugs, rendering them less susceptible to the drugs' effects. Genetic mutations can also affect the uptake or efflux of drugs, leading to reduced drug accumulation or increased drug expulsion (O'Neill, 2016). One of the most significant contributors to the spread of resistance is horizontal gene transfer. Bacteria can exchange genetic material, including resistance genes, with other bacteria through mechanisms such

as conjugation, transformation, and transduction. This allows the rapid dissemination of resistance genes within bacterial populations, promoting the emergence of multidrug-resistant strains (Tao *et al.*, 2022). The selective pressure exerted by the use and misuse of antimicrobial drugs plays also a critical role in the development and spread of resistance. When exposed to antimicrobials, susceptible bacteria are eliminated, while resistant bacteria survive and multiply, leading to the dominance of resistant strains. Inappropriate and unnecessary use of antimicrobials in both human and animal health, including over-prescription, misuse, and non-compliance, accelerates the selection and proliferation of resistant bacteria (O'Neill, 2016).

2.3 Drivers of antimicrobial resistance

There are a number of factors that contribute to the development of AMR. One of the most important is the misuse and overuse of antimicrobials. This can happen when antibiotics are prescribed unnecessarily, when they are not taken as prescribed, or when they are used in animal production. Other factors that contribute to AMR include poor infection control practices, the lack of clean water and sanitation, and the emergence of new pathogens. These drivers and factors can be broadly categorized into the following:

1. Inappropriate use of antimicrobials: The inappropriate use of antimicrobial drugs is a significant driver of antimicrobial resistance. Overprescribing antibiotics for conditions that do not require them, such as viral infections, contributes to the selective pressure on bacteria. This pressure favors the survival and multiplication of resistant strains, leading to the emergence of antimicrobial-resistant bacteria. Additionally, non-compliance with prescribed treatment regimens, stopping antibiotics prematurely, or sharing medications with others also contributes to the development of resistance (Almagor *et al.*, 2018).
2. Agricultural use of antimicrobials: The use of antimicrobial agents in agriculture, particularly in food-producing animals, is a major contributor to AMR. In many countries, antibiotics are used as growth promoters and for prophylactic purposes in animal husbandry to prevent infections in crowded and unsanitary conditions. This widespread use of antibiotics in animal agriculture promotes the development of resistant bacteria in animals, which can then be transmitted to humans through the food chain or direct contact (Thanner, Drissner and Walsh, 2016).

3. Inadequate infection prevention and control: Poor infection prevention and control practices in healthcare settings can lead to the transmission of resistant pathogens. Inadequate hand hygiene, improper disinfection of equipment, and the lack of effective isolation protocols can facilitate the spread of resistant bacteria among patients and healthcare workers. This not only increases the risk of infections caused by resistant organisms but also contributes to the dissemination of resistance genes within healthcare facilities (Pittet *et al.*, 2000).
4. Lack of access to diagnostics: Limited access to rapid and accurate diagnostic tests can lead to the overuse of broad-spectrum antimicrobial agents when the causative pathogen is unknown. Without proper diagnostics, clinicians may resort to prescribing broad-spectrum antibiotics as a precautionary measure to cover a wide range of possible pathogens, even if the infection is caused by a susceptible organism. This inappropriate use of antimicrobials provides selective pressure for the development of resistance (Ferreyra *et al.*, 2022).
5. Global travel and trade: The global interconnectedness through travel and trade facilitates the rapid spread of resistant pathogens across borders. Resistant strains can emerge in one region and be carried to other parts of the world by travelers. Additionally, imported food products from countries with high antimicrobial use in agriculture can introduce resistant bacteria into new environments, contributing to the dissemination of AMR (Memish, Venkatesh and Shibl, 2003).
6. Environmental contamination: The discharge of antimicrobial drugs and resistant bacteria into the environment can contribute to the development of environmental reservoirs of resistance genes. Inadequate waste management systems, pharmaceutical manufacturing waste, and agricultural runoff can all lead to the release of antimicrobial residues and resistant bacteria into soil and water sources, potentially impacting human and animal populations (Haenni *et al.*, 2022; Kaiser, Taing and Bhatia, 2022).
7. Inadequate new antibiotic development: The lack of new antibiotic development has resulted in a limited pipeline for effective antimicrobial agents. As bacteria continue to develop resistance to existing antibiotics, the need for new and innovative drugs becomes more urgent. The slow pace of antibiotic discovery and development

hampers treatment options for infections caused by resistant organisms (Piddock *et al.*, 2022).

8. Patient factors: Patient-related factors can also contribute to the development of antimicrobial resistance. Non-adherence to prescribed treatments, such as skipping doses or not completing the full course of antibiotics, can create conditions conducive to the survival of resistant bacteria. Furthermore, self-medication and the availability of antibiotics without a prescription in some regions can lead to inappropriate use and the emergence of resistance (Almagor *et al.*, 2018).

2.4 *Salmonella spp.*, Antibiotics and AMR

Salmonella is commonly encountered in food producing animals, in fact is part of the natural enteric microbiota of some species (e.g., poultry), most times without manifesting any clinical signs. The use of antibiotics in animal production, especially when used in subtherapeutic concentration as growth promoters or for prophylaxis, may impose selective pressure on *Salmonella* causing resistant strains to dominate this "ecological" niche. Apart from this direct route of promoting AMR in *Salmonella*, the extensive use of antibiotics in agriculture may lead to the emergence of other resistant bacterial species which could transfer horizontally resistance genes to *Salmonella*, due to its wide distribution. Moreover, *Salmonella* is the most common foodborne illness causing millions of infections worldwide, often resulting in hospitalizations. Improper use of antibiotics in medicine poses the same AMR risks with the extensive antimicrobial use in animal production. Finally, common disinfectants used in farms or in hospital settings have been found to contribute to the expansion of antimicrobial resistance due to the activation of efflux pumping mechanisms (Nhung *et al.*, 2015) or due to the co-selection of resistance to certain drugs by the use of biocides (Davies and Wales, 2019).

Resistance to crucial antimicrobial agents is a pressing concern in the treatment of infections caused by *Salmonella* Typhi and *Salmonella* Paratyphi A. Before the mid-1970s, chloramphenicol was the primary treatment for enteric fever. However, reports of chloramphenicol-resistant isolates began to surface before 1970, leading to outbreaks of chloramphenicol-resistant *Salmonella* Typhi in Central America and subsequently in South and Southeast Asia. The resistance determinant for chloramphenicol was found on a transmissible plasmid of the HI1 incompatibility type, which often carried genes conferring resistance to streptomycin, sulfonamides, and tetracyclines (Crump and Wain, 2017). As

chloramphenicol resistance grew, ampicillin and trimethoprim-sulfamethoxazole became the main treatments. However, trimethoprim-sulfamethoxazole resistance was reported in 1975 in France, and by the late 1980s, multidrug resistance was reported in different countries (Rowe, Ward and Threlfall, 1997). With the emergence and spread of multidrug-resistant *Salmonella* Typhi, the fluoroquinolone drug ciprofloxacin became the first-line treatment for enteric fever. Nonetheless, *Salmonella* Typhi with intermediate susceptibility and resistance to ciprofloxacin began to be observed from 1992. An epidemic of fluoroquinolone-resistant *Salmonella* Typhi was reported from Tajikistan in 1997 (Threlfall *et al.*, 1998). Decreased fluoroquinolone susceptibility is also emerging in *Salmonella* Paratyphi A. In regions where fluoroquinolone intermediate susceptibility and/or resistance are common, extended-spectrum cephalosporins have become pivotal in managing severe and complicated enteric fever, while azithromycin is used for uncomplicated disease (Effa and Bukirwa, 2008). Although uncommon to date, there have been reports of *Salmonella* Typhi displaying resistance to extended-spectrum cephalosporins, such as ceftriaxone. Resistance mechanisms have included extended-spectrum beta-lactamases (ESBL) and AmpC beta-lactamases. Similarly, there have been sporadic reports of azithromycin resistance (Crump and Wain, 2017).

The rise of multiple drug resistance in serovars other than Typhi has had a significant impact on managing *Salmonella* septicemia, particularly among infants and young children in developing countries. For the past three decades, multiple drug-resistant strains have been implicated in numerous outbreaks, both in the community and in hospital pediatric units (Kariuki *et al.*, 2015). In numerous low and middle-income countries, the sources and transmission modes of non-typhoidal *Salmonella* remain poorly understood. In low-resource areas, particularly in sub-Saharan African countries, invasive non-typhoidal *Salmonella* disease without diarrhea is prevalent. This invasive form of the disease is severe and often fatal, making antimicrobial therapy critical for saving lives (Gilchrist and MacLennan, 2019). In industrialized nations, animals serve as the primary source of non-typhoidal *Salmonella* infections, and transmission typically occurs through the foodborne route. When antimicrobial resistance is present, it is often acquired before the organism is transmitted through the food chain to humans (Eng *et al.*, 2015). In many countries, the most common non-typhoidal *Salmonella* serovars are Enteritidis and Typhimurium. Multidrug resistance has emerged in *Salmonella* Typhimurium and other non-typhoidal serovars. In the early 1980s, multidrug resistance *Salmonella* Typhimurium was first reported in the United

Kingdom, closely associated with PT DT104. These isolates displayed resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines (R-type ACSSuT). By the 1990s, this resistance phenotype was observed in several other countries, including the United States, Canada, Israel, Turkey, and Japan, although it has since declined in many areas (Poppe *et al.*, 1998). A concerning trend is the emergence of decreased susceptibility and resistance to fluoroquinolones in various non-typhoidal *Salmonella* serovars. This resistance has increased rapidly in some serovars and countries since the mid-1990s (Li *et al.*, 2018). In certain industrialized countries, decreased fluoroquinolone susceptibility has been prevalent in a significant proportion of *Salmonella* serovars Enteritidis, Typhimurium, Virchow, and Hadar. Particularly high prevalence of decreased fluoroquinolone susceptibility and resistance has been reported in some southeast Asian countries (Crump and Wain, 2017). Resistance to extended-spectrum cephalosporins in non-typhoidal *Salmonella* strains has been recognized since the mid-1980s and is often mediated through beta-lactamases of the ESBL and AmpC type. These strains were frequently reported in North Africa from the mid-1980s and in Southeast Asian countries, including Singapore, the Philippines, and Thailand (Pietsch *et al.*, 2021). Of major concern is the emergence of non-typhoidal *Salmonella* expressing both multidrug resistance and ceftriaxone resistance in various locations. Moreover, non-typhoidal *Salmonella* strains with extensive drug resistance to six or seven antimicrobial classes, including carbapenems, have been reported in Malaysia and Vietnam. Carbapenem resistance has also been observed in non-typhoidal *Salmonella* from multiple countries, such as China, Colombia, Pakistan, and the United States. The most common mechanism for this resistance was *Klebsiella pneumoniae* carbapenemase and New Delhi metallo-beta-lactamase. Some of these isolates also exhibit resistance to trimethoprim-sulfamethoxazole and azithromycin (Fernández, Guerra and Rodicio, 2018).

2.5 Addressing the AMR

The ramifications of AMR extend far beyond mere loss of life. It can result in escalated healthcare costs, decreased productivity, and disruption of social dynamics. Additionally, AMR's adverse effects on food security make it more challenging to raise and manage livestock. To effectively address AMR, a comprehensive approach of antimicrobial stewardship and conservation strategies is essential to ensure responsible and appropriate antimicrobial usage (Van Katwyk *et al.*, 2020). Furthermore, fostering substantial international cooperation in the regulation and surveillance of antimicrobial usage must

remain a top priority (Hoffman *et al.*, 2015). Research plays a pivotal role in generating evidence on the impact and effectiveness of various AMR policies, as well as ensuring that health system investments in AMR are based on robust evidence. However, existing research has yet to provide definitive clarity on the most effective interventions for achieving AMR goals across diverse contexts, cultures, and health systems (Van Katwyk *et al.*, 2020). Consequently, further research is necessary to identify and evaluate suitable interventions that can effectively combat AMR in different settings and circumstances.

Addressing AMR necessitates a comprehensive and coordinated approach, involving various stakeholders such as governments, healthcare professionals, veterinarians, researchers, industries, and the general public. A crucial aspect of this approach involves implementing antimicrobial stewardship programs within healthcare settings to foster responsible antimicrobial drug use. These programs require the implementation of guidelines and protocols that ensure appropriate prescription practices, optimal dosages, and the correct duration of antimicrobial therapy (Doron and Davidson, 2011). By reducing unnecessary use and optimizing treatment, the development of resistance can be minimized. Furthermore, enhancing infection prevention and control measures in healthcare facilities is essential to prevent the transmission of resistant bacteria. Strict adherence to hand hygiene, proper disinfection of equipment, and the implementation of effective isolation protocols play a pivotal role in limiting the spread of resistant pathogens (Musoke *et al.*, 2021). By collectively adopting these measures, we can take significant steps towards combating AMR and preserving the effectiveness of antimicrobial drugs.

The establishment of robust surveillance systems to monitor the occurrence of AMR holds utmost importance. Surveillance plays a critical role in identifying emerging resistance patterns and facilitates timely responses to address the issue. The data collected through surveillance serve as a guide for formulating treatment guidelines and informing public health interventions (Tacconelli *et al.*, 2018). Moreover, enhancing access to rapid and accurate diagnostic tests is vital in identifying the causative pathogen and its resistance profile swiftly. This expedites targeted treatment, avoiding unnecessary or inappropriate use of antimicrobial drugs (Shanmugakani *et al.*, 2020). In addition to surveillance and diagnostics, widespread vaccination can significantly contribute to preventing infections, thereby reducing the reliance on antimicrobial treatment. Vaccines targeting specific bacterial infections, such as those caused by *Salmonella* and *Streptococcus pneumoniae*, can effectively mitigate the risk of resistance development (Micoli *et al.*, 2021). Lastly,

encouraging research and development of new antibiotics becomes paramount to replenish the dwindling supply of effective antimicrobial drugs. Providing incentives to pharmaceutical companies and increasing funding for research can stimulate innovation in the discovery of novel antibiotics (Gould and Bal, 2013). By pursuing these multi-pronged strategies, we can combat AMR effectively and safeguard the effectiveness of antimicrobial drugs for future generations.

Embracing a One Health approach, which acknowledges the interconnectedness of human health, animal health, and the environment, is of utmost importance in tackling AMR. Given that AMR can disseminate among humans, animals, and the environment, coordinated endeavors are indispensable to address the issue holistically (McEwen and Collignon, 2018). It is crucial to raise awareness among the public, healthcare professionals, and policymakers about AMR. Equipping the public with knowledge about the appropriate use of antimicrobials, the risks associated with resistance, and preventive measures can play a pivotal role in curbing its spread (Chukwu *et al.*, 2020). Additionally, governments must enact robust regulations and policies to govern the use of antimicrobials in human medicine, animal agriculture, and aquaculture. These policies may encompass restrictions on over-the-counter antibiotic sales, guidelines for the responsible use of antibiotics in agriculture, and measures to combat environmental contamination (Van Katwyk *et al.*, 2019). By implementing such measures, we can work collectively to safeguard the efficacy of antimicrobials and address the challenges posed by AMR. Every year, significant financial resources are dedicated to global public programs aimed at increasing awareness about AMR, providing education to healthcare professionals on appropriate prescribing practices, and reducing antimicrobial usage in both the health and agricultural domains (Van Katwyk *et al.*, 2019). Despite substantial investments in terms of finances and political commitment, it has proven challenging to establish direct correlations between these programs and tangible improvements in antimicrobial utilization, resistance rates, or overall health outcomes (Davey *et al.*, 2013). This difficulty is compounded by notable gaps in surveillance and information, which hinder the effectiveness of the global response to AMR (Wernli *et al.*, 2017). Efforts to combat AMR require improved mechanisms for identifying and prioritizing critical research questions that can drive effective actions. Considerable research has already been conducted to understand the underlying social and microbial factors contributing to AMR (Michael, Dominey-Howes and Labbate, 2014). However, the focus must now shift towards determining the effectiveness of interventions aimed at addressing

the root causes of AMR, understanding the reasons behind their success, identifying essential elements for their effectiveness, and discerning the specific contexts and circumstances in which these interventions work best (Davey *et al.*, 2013; Van Katwyk *et al.*, 2019). Given that the bulk of current research mainly addresses interventions in high-income settings (Davey *et al.*, 2013; Van Katwyk *et al.*, 2019), there is a pressing need for further research on these subjects that can specifically benefit low- and middle-income countries and other resource-limited settings. Such research would aim to identify policy interventions that can be customized to address local needs and priorities. AMR is a global challenge that demands international cooperation. By fostering collaboration in research, data sharing, and the implementation of coordinated strategies, we can effectively tackle AMR on a global scale.

Chapter 3. Colistin, resistance, *mcr* genes and antimicrobial susceptibility testing

3.1 Colistin

Colistin, also known as Polymyxin E, is a polycationic peptide antimicrobial that was discovered in Japan in 1949. It is produced by *Bacillus polymyxa* and belongs to the polymyxin class of antibiotics, which exhibit both hydrophilic and lipophilic properties. Within the polymyxin group, there are five different chemical compounds (polymyxins A, B, C, D, and E) (Falagas and Rafailidis, 2008; Gallardo-Godoy *et al.*, 2016), but only two of them, polymyxin B and colistin (polymyxin E), are utilized for clinical purposes (Cassir, Rolain and Brouqui, 2014). Colistin is available in two forms for clinical use: colistin methanesulfonate sodium (CMS), which is a prodrug for parenteral administration, and colistin sulfate (CS), used for oral, inhalation, or topical applications (Brink *et al.*, 2014).

Colistin, containing L-diaminobutyric acid and carrying a positive charge, interacts with the negatively charged phosphate groups of lipid A, a critical element of the lipopolysaccharide (LPS) found in Gram-negative bacteria (Deris *et al.*, 2014). Lipid A plays a pivotal role in bacterial permeability and communication with the cell exterior (Velkov *et al.*, 2010). Colistin competitively displaces divalent cations like calcium (Ca2+) and magnesium (Mg2+), which results in the disruption of the three-dimensional structure of LPS and its function within the bacterial outer membrane. Subsequently, colistin inserts its hydrophobic terminal acyl fat chain, leading to the enlargement of the external outer membrane monolayer. This expansion causes permeabilization of the outer membrane, enabling colistin to penetrate through. This process explains the synergistic effect observed when colistin is used in combination with other antimicrobials possessing hydrophilic properties, such as b-lactamics, gentamicin, rifampicin, meropenem, and tigecycline (Bolla *et al.*, 2011). As colistin acts by incorporating hydrophilic groups into the fatty acid chains of the phospholipid bilayer of the inner membrane, the stability of the membrane is compromised, causing a change in its integrity and ultimately resulting in its destruction. This disruption leads to the inability of the inner membrane to maintain cellular content, leading to cell lysis (Velkov *et al.*, 2010). Additionally, colistin's binding to lipid A allows it to exert an anti-endotoxin activity (Falagas and Rafailidis, 2008), preventing the induction of shock by endotoxins. Overall, colistin works by essentially solubilizing the bacterial cell membrane, which leads to a bactericidal effect.

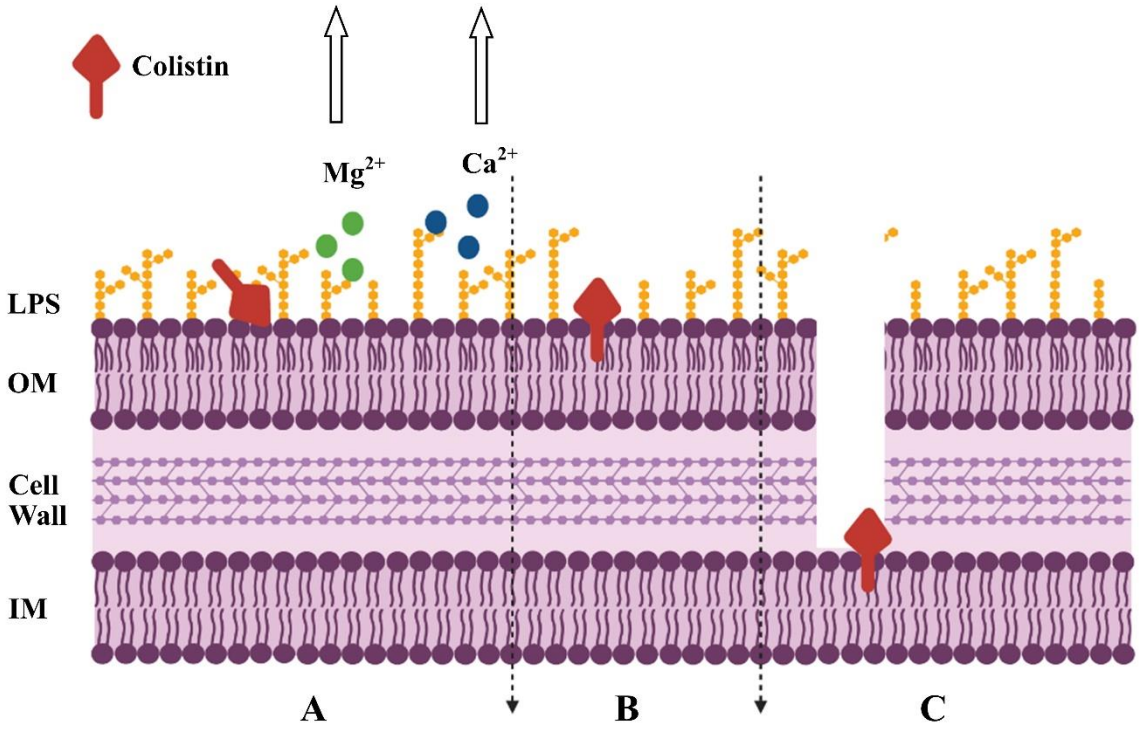

Figure 3. Colistin mode of action. A) Colistin interacts with lipid A and competitively displaces divalent cations like calcium (Ca^{2+}) and magnesium (Mg^{2+}), which results in the disruption of the three-dimensional structure of LPS, B) Colistin inserts its hydrophobic terminal acyl fat chain, leading to the enlargement of the external outer membrane monolayer. This expansion causes permeabilization of the outer membrane, enabling colistin to penetrate through and C) Colistin acts by incorporating hydrophilic groups into the fatty acid chains of the phospholipid bilayer of the inner membrane, the stability of the membrane is compromised, causing a change in its integrity and ultimately resulting in its destruction.

Colistin was initially employed in both human and veterinary medicine back in 1952. However, from the 1970s to the 1980s, its medical use declined significantly due to concerns over nephrotoxicity and neurotoxicity. As a result, colistin continued to be primarily used in veterinary settings during this period. In recent times, the emergence of multidrug-resistant Gram-negative bacilli, particularly those producing carbapenemase, has led to a resurgence in colistin's use in human medicine as a last-resort treatment option (Falagas and Rafailidis, 2008; Velkov *et al.*, 2010; Biswas *et al.*, 2012; Azzopardi *et al.*, 2013). Recognizing its critical role, esteemed organizations such as the World Health Organization (WHO) have reclassified colistin under the category of "very high importance for Human Medicine" (WHO, 2018). Colistin falls under Category B of antibiotics critically important in human medicine, as classified by the European Medicines Agency (EMA). The designation "Restrict" implies that its use in veterinary medicine should be restricted to minimize potential risks to public health. This category also includes quinolones (fluoroquinolones and other quinolones), third and fourth-generation cephalosporins (excluding those with beta-lactamase inhibitors), and polymyxins. Antibiotics in Category B should be reserved

for treatment only when antimicrobials in Categories C or D are deemed clinically ineffective, and there are no viable alternatives available. In such cases, the utilization of these antibiotics should be guided by the results of AST, particularly those included in Category B (European Medicines Agency, 2019). Recent data on colistin as monotherapy have shed light on its pharmacodynamics and pharmacokinetics. However, relying solely on monotherapy raises concerns about achieving adequate plasma levels, potentially leading to the development of colistin resistance. As a result, caution should be exercised in its administration to mitigate the risk of resistance emergence.

Currently, colistin remains a commonly used antibiotic in veterinary medicine, particularly in pigs, to treat intestinal infections caused by Enterobacterales (Burow *et al.*, 2019). The administration of antibiotics like colistin to animals has facilitated the expansion of modern farm animal production practices. It has enabled improved weaning rates, higher animal density, and likely improved economic control of pathologies resulting from *E. coli* infections, including those caused by verotoxigenic *E. coli* (VTEC) (Rhouma, Beaudry and Letellier, 2016). Colistin exhibits poor absorption through the gastrointestinal tract, which highlights the potential for colistin resistance to emerge due to selective pressure on the intestinal microbiota (Rhouma, Beaudry and Letellier, 2016). Studies have shown that pigs treated with colistin generally have higher proportions of resistant bacterial isolates compared to untreated pigs (Burow *et al.*, 2019). Similarly, colistin is administered orally to calves for the treatment of gastrointestinal diseases caused by Gram-negative bacteria. This practice could also contribute to the isolation of colistin-resistant bacteria from calves, although conclusive data on the use of colistin in these animals is lacking (Haenni *et al.*, 2016). The oral route is the most common method of administering colistin in animal production worldwide, especially when used for prophylactic purposes (Trauffler *et al.*, 2014). Colistin is primarily administered through feed but can also be given via drinking water (European Medicines Agency, 2019). The practice of using colistin for prophylaxis or as a growth promoter for farm animals, which is still prevalent especially in Asia, should be prohibited. The use of low sub-inhibitory concentrations of antibiotics for prophylaxis or to enhance animal growth has been associated with the development of antibiotic resistance (Rhouma, Beaudry and Letellier, 2016). It is noteworthy that antimicrobials used for animal growth promotion can often be obtained without veterinary oversight, even within the European Union. Instead, its clinical use should be restricted to treating enteric infections caused by susceptible (supported by an AST if possible), non-invasive *E. coli* (European

Medicines Agency, 2016). To effectively address the misuse of antimicrobials, maintaining a high level of hygiene and controlling the microbial load on the farm is crucial.

3.2 Colistin resistance mechanisms and mcr genes

Colistin resistance can be attributed to various mechanisms. Previously, it was believed that resistance resulted solely from chromosomal point mutations. Since colistin targets the lipopolysaccharide (LPS) in bacteria, any alteration in this component can affect colistin's effectiveness (Biswas *et al.*, 2012). *Salmonella* and *E. coli* have the ability to modify their LPS by incorporating 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (PEtn) in lipid A through biosynthesis. This alteration in the LPS is linked to resistance acquired through chromosomal-mediated mechanisms. These mechanisms rely on two-component response regulators and sensor kinase systems: PmrA/PmrB and PhoP/PhoQ (Falagas, Rafailidis and Matthaiou, 2010; Needham and Trent, 2013; Olaitan, Morand and Rolain, 2014). The first system, PmrA/PmrB, also regulates the pmr HIJKLM operon, which facilitates the synthesis of N4-aminoarabinose. The chemical bonding with lipid A fractions alters the cell membrane's charge by neutralizing the negatively charged phospholipids. This specific resistance mechanism is observed in *Pseudomonas aeruginosa* (Ly *et al.*, 2012). The PhoP/PhoQ system regulates the expression of genes involved in the biosynthesis of LPS, including lipid A. When a bacterium is exposed to colistin or other stress conditions, the PhoQ sensor kinase is activated in response to changes in the outer membrane caused by colistin. Upon activation, PhoQ transfers a phosphate group to the PhoP response regulator, leading to the activation of specific genes under the control of PhoP. One of the genes activated by PhoP encodes a small transmembrane protein called MgrB (membrane-associated regulator of PhoP). MgrB acts as a negative regulator of PhoP by inhibiting its phosphorylation or promoting its dephosphorylation. When MgrB is active, it prevents the activation of genes involved in lipid A modification, including the biosynthesis of molecules like 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (PEtn) on lipid A. The inactivation of the mgrB gene in bacteria, such as *Klebsiella pneumoniae*, prevents the production of MgrB, which, in turn, leads to the overexpression of genes responsible for lipid A modifications. This overexpression alters the negative charge of lipid A and decreases colistin's binding affinity, thereby conferring resistance to colistin (Lopez-Camacho *et al.*, 2014; Poirel *et al.*, 2015). On the other hand, *Acinetobacter baumannii* exhibits colistin resistance by suppressing the production of lipopolysaccharide (LPS). This lack of LPS production may arise from the

inactivation of a gene involved in lipid A biosynthesis, such as lpxA, lpxC, or lpxD. Consequently, this absence of lipid A leads to colistin resistance in *Acinetobacter baumannii* (Moffatt *et al.*, 2010).

The mcr-1 gene, a plasmid-mediated colistin resistance gene, was initially reported in China in 2015 (Liu *et al.*, 2016), and later found in various regions across Asia, Africa, Europe, and America (Giamarellou, 2016; Rhouma, Beaudry and Letellier, 2016; Schwarz and Johnson, 2016). This gene encodes an enzyme that alters the lipid A component of LPS, replacing it with a metabolite of phosphoethanolamine, which prevents its binding to colistin. The concerning aspect is that bacterial resistance to colistin can be transferred along with resistance to broad-spectrum cephalosporins, as both the mcr-1 gene and extended-spectrum beta-lactamase gene (ESBL) can be carried on a single plasmid. This poses significant challenges in treating infections caused by Gram-negative bacteria. Additionally, a chromosomally-located mcr-1 gene was detected in two colistin-resistant *E. coli* isolates collected from calves (Veldman *et al.*, 2016).

Subsequently, a new colistin resistance gene called mcr-2 was found in Belgium. This gene was carried by a plasmid in *E. coli* isolates obtained from samples of porcine and bovine origin. Notably, these isolates also co-harbored ESBL genes (Xavier *et al.*, 2016). Since then, the discovery of seven more mcr homologues (mcr-3 to mcr-9) in Enterobacterales has been reported (Yang *et al.*, 2018; Carroll *et al.*, 2019). PCR tests have been developed to facilitate the detection of these resistance genes (Rebelo *et al.*, 2018). This mechanism can be acquired during therapy and is easily transmitted, thereby contributing to the rapid spread of resistance.

Interestingly, the presence of multiple mcr genes in *E. coli* does not necessarily result in a significant difference in minimum inhibitory concentration (MIC) when compared to resistant *Salmonella* isolates carrying only the single plasmid mcr-1 gene (Quesada *et al.*, 2016). In resistant Enterobacterales isolated from swine, the mcr-1 gene was often associated with a low level of resistance, with most isolates showing MICs of 4 or 8 mg/L. These values are only 2-4 times higher than the clinical breakpoint of 2 mg/L set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical Laboratory Standard Institute (CLSI) (Anjum *et al.*, 2016; Liu *et al.*, 2016; Quesada *et al.*, 2016). Strains with MICs below 2 mg/L are considered susceptible according to the EUCAST protocol and intermediate according to CLSI; however, the susceptible category was recently eliminated by CLSI. Bacteria that are resistant to colistin often exhibit resistance to other commonly

used antibiotics, including aminoglycosides, tetracycline, sulfonamide and trimethoprim, lincosamide, beta-lactams, quinolones, and third-generation cephalosporins. These resistant strains employ various mechanisms, such as enzymatic activity, efflux pumps, reduced permeability, or point mutations, to evade the effects of these antibiotics (Anjum *et al.*, 2016; Falgenhauer *et al.*, 2016; Haenni *et al.*, 2016; Malhotra-Kumar *et al.*, 2016; Poirel *et al.*, 2016).

The rise of resistance to colistin, which serves as one of the limited treatment options for patients infected with carbapenem-resistant *K. pneumoniae* and other crucial antimicrobial groups, is a major challenge, particularly in human infections. In 2017, the European Centre for Disease Prevention and Control (ECDC) reported that colistin-resistant isolates accounted for 8.5% (2.4% of all reported *K. pneumoniae* isolates and sporadic cases in E. coli). Greece and Italy were responsible for the majority (88.5%) of these reported cases (European Centre for Disease Prevention and Control, 2017). Conversely, the same data source revealed that in 2016, only 51.3% of all *P. aeruginosa* isolates exhibited susceptibility to colistin. Regarding *Acinetobacter* spp., colistin susceptibility data were observed in up to 51.3% of all isolates (European Centre for Disease Prevention and Control, 2017). However, the ECDC warned in 2018 that these findings might not be fully representative of Europe as a whole and should be approached with caution. The caution arises due to the low number of isolates tested, the relatively high proportion of isolates from regions with high resistance, and the technical complexities involved in colistin susceptibility testing (European Centre for Disease Prevention and Control, 2017).

The potential transmission of colistin-resistant *E. coli* between different species is a viable concern, especially from swine (Olaitan *et al.*, 2015) or pets (Zhang *et al.*, 2016) that have close interactions with humans. The transmission of mcr-1 resistance from animals to humans raises important questions about the implications of using colistin in veterinary medicine, including pet treatments and farm animal production, and its potential entry into the human food chain (Olaitan *et al.*, 2015). The presence of mcr-1 in the environment and its ability to be transmitted through various routes to humans further highlight the possibility of the gene transferring from animals to humans. However, these transmission routes necessitate further in-depth research and comprehensive studies for a better understanding.

3.3 Colistin antimicrobial susceptibility testing

Accurate AST data is crucial for both individual patient management and epidemiological studies, especially when dealing with bacteria isolated from infected humans and animals. However, colistin's binding to various laboratory materials poses several technical challenges, leading to potentially misleading or incorrect susceptibility results. A survey conducted in 2017 among laboratories providing data revealed that a significant number of them either did not conduct local colistin susceptibility testing or used methods that were not recommended (European Centre for Disease Prevention and Control, 2017).

The Clinical & Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), organizations responsible for standardizing laboratory protocols for antimicrobial susceptibility testing, collaborated to establish guidelines for colistin susceptibility testing. They jointly issued recommendations affirming that, currently, microdilution is the only valid method for determining colistin susceptibility. Assessing susceptibility to colistin poses several challenges related to the methodology. The reference method for performing an antimicrobial susceptibility test AST is the microdilution method (BMD) outlined in the 20776-1 standard, which is currently validated for Enterobacterales, *P. aeruginosa*, and *Acinetobacter* spp. Standardization efforts have been made regarding culture media, colistin formulation, and the type of plastic used in microplates. However, this method is laborious and time-consuming, requiring a minimum of 24-48 hours to produce results. Other susceptibility testing methods, such as agar dilution, disk diffusion, gradient diffusion, and automated methods (e.g., Vitek2, Phoenix), are not recommended for colistin susceptibility testing, rendering much of the available epidemiological data inaccurate.

EUCAST has established clinical breakpoints for colistin susceptibility testing in different bacterial species. For Enterobacteriaceae, which includes *Escherichia coli* and Klebsiella spp. (excluding *Proteus* spp., *Morganella morganii*, *Providencia* spp., and *Serratia* spp.), and for *Acinetobacter baumannii*, the clinical breakpoints are currently set at ≤2 μg/mL for colistin-susceptible isolates and >2 μg/mL for colistin-resistant strains. For *Pseudomonas aeruginosa*, the values are ≤4 μg/mL for a colistin-susceptible isolate and >4 μg/mL for a colistin-resistant isolate. These breakpoints are currently being reviewed and may be subject to changes in the future. For non-clinical surveillance purposes, the epidemiological cut-off (ECOFF) value for colistin can vary within a bacterial genus. This is particularly relevant

for certain intrinsically less susceptible *Salmonella* serovars, such as *Salmonella* Dublin and *Salmonella* Enteritidis (Catry *et al.*, 2015).

Optimizing newer methods, including molecular approaches, is still necessary as they can currently detect only a limited number of known resistance genes, making them insufficient for a formal susceptibility assay. For instance, the presence of resistance genes like mcr indicates resistance to colistin, but the absence of such genes does not guarantee susceptibility. A promising novel method based on flow cytometry has been developed, enabling AST determination within 2 hours instead of the conventional 2 days when using positive blood cultures or colonies (reducing it to 1 day). This advancement has the potential to revolutionize the diagnostic paradigm (Fonseca E Silva *et al.*, 2019; Van Belkum *et al.*, 2020). Given the increasing antimicrobial resistance, there is an urgent need for microbiological laboratories to provide quick AST reports to aid in timely and effective treatment decisions.

Chapter 4. Colistin resistance and *Salmonella enterica*

In *Salmonella enterica*, the development of chromosomal colistin resistance is linked to the activation of two-component regulatory systems, namely PmrA/PmrB and PhoP/PhoQ. These systems are responsible for the biosynthesis of L-Ara4N and PEtn, and their activation is triggered by environmental stimuli, such as a low concentration of Mg2+, or specific mutations in the genes encoding these regulatory systems (Lima, Domingues and Da Silva, 2019). Mutations in the PmrA/PmrB and PhoP/PhoQ systems result in their constitutive expression, leading to the continuous activation of the arnBCADTEF and pmrCAB operons, respectively. This, in turn, leads to the permanent addition of L-Ara4N and PEtn to lipid A, making it less susceptible to the action of colistin (Olaitan, Morand and Rolain, 2014). Other alterations that can contribute to colistin resistance in *S. enterica* include deacylation of lipid A by PagL and activation of the transcription of genes involved in bacterial adaptation and survival by RpoN. However, these mechanisms are less commonly observed.

Various serovars of *Salmonella enterica* have been found to carry plasmid-mediated colistin resistance genes, including mcr-1, mcr-2, mcr-3, mcr-4, mcr-5, and mcr-9 (Lima, Domingues and Da Silva, 2019). Similar to other bacterial species, mcr-like genes have been detected in isolates from various sources, such as food-producing animals, food products, and human samples. These genes are often located within diverse genetic environments and plasmids. Interestingly, the presence of mcr genes may not always result in high levels of colistin resistance. In some cases, it has been associated with low levels of resistance, which could allow the mcr bearing strains to persist undetected (Lima, Domingues and Da Silva, 2019). In fact, the direct link between the presence of mcr genes and colistin resistance in *Salmonella* is not entirely clear. These genes have also been identified in susceptible strains. It is worth noting that only a small number of resistant strains actually carry these genes. This observation suggests that other mechanisms of resistance to colistin may be at play (Bertelloni *et al.*, 2022).

Salmonella Typhimurium is the predominant serotype carrying mcr genes, and it is also known for causing a significant number of human infections (Eng *et al.*, 2015). Monophasic variants of S. Typhimurium, such as 1,4,[5],12:i:-, are also commonly observed carrying mcr genes. Interestingly, mcr-positive Paratyphi B has been detected in animal samples, despite this serotype typically infecting humans (Eng *et al.*, 2015). Food-producing animals, particularly poultry and swine, appear to be the primary reservoir for mcr-positive *S. enterica* strains. China has seen the highest number of mcr-positive *S. enterica* strains, which

correlates with the extensive use of colistin in livestock and veterinary medicine in the country, leading to the emergence of resistance (Sun *et al.*, 2018). In some European countries, like Italy and Portugal, where colistin is frequently used for therapeutic and metaphylactic purposes in animal husbandry, there have also been reports of emerging isolates carrying the mcr genes (Lima, Domingues and Da Silva, 2019).

It is reasonable to assume that the continuous use of colistin in poultry and swine likely leads to a positive selective pressure for colistin-resistant bacteria to develop (Portes *et al.*, 2022). The mcr resistance genes appear to have been horizontally transmitted to *Salmonella* through contact with *E. coli*, which was the first bacterial species to exhibit this gene (Liu *et al.*, 2016). Animal husbandry involving resistant strains may contaminate the final products, such as meat and eggs (Hu *et al.*, 2019). These resistant microorganisms can then reach humans through contaminated food (Ferrari, Panzenhagen and Conte-Junior, 2017). Transmission can occur through the fecal-oral route, resulting in the human-to-human spread of colistin-resistant *Salmonella* strains (Gopinath, Carden and Monack, 2012). Additionally, the movement of asymptomatic humans with salmonellosis between different countries has contributed to the global spread of these strains (Arcilla *et al.*, 2016). Hence, the presence of mcr genes in *Salmonella* should not be underestimated, as it is a zoonotic pathogen of significant concern for public health (Portes *et al.*, 2022).

The presence of colistin resistance genes integrated into mobile genetic elements, such as plasmids, is a significant concern due to their ability to horizontally transfer between different bacteria. Moreover, these mcr genes can be found alongside other resistance genes, such as blaCTX-M, floR, and/or qnr, leading to strains resistant to multiple classes of antibiotics, including polymyxins, most beta-lactams (including broad-spectrum cephalosporins and monobactams), amphenicols, and quinolones (Lima, Domingues and Da Silva, 2019). For example, in a study, mcr-1 and blaCTX-M-1 genes were found on a plasmid of type IncHI2, and they were co-transferred from *S. enterica* isolated from swine retail meat through conjugation under colistin selection (Figueiredo *et al.*, 2016). The co-occurrence of resistance genes can compromise the treatment of complicated gastroenteritis and invasive infections caused by *S. enterica*, as it leads to limited treatment options and challenges in managing infections.

Currently, the epidemiological data concerning colistin resistance and the spread of mcr genes in *Salmonella* are incomplete, making it challenging to establish clear connections between colistin usage in human and veterinary medicine. A significant obstacle is the

limited number of *Salmonella* isolates tested for colistin resistance (and mcr genes) across different regions and countries. This scarcity of data can be attributed to the difficulties and cost associated with conducting colistin susceptibility testing, with only the broth microdilution method being deemed acceptable. However, there is a growing awareness of the importance of monitoring AMR, including colistin resistance, and the European Centre for Disease Prevention and Control (ECDC) has included colistin in the list of monitored antimicrobials. This increased attention and surveillance efforts could potentially provide more insight into the intricate relationship between colistin resistance and *Salmonella* isolates of human origin. By obtaining a more comprehensive understanding of this issue, it will be possible to address the challenges posed by colistin resistance more effectively in both human and veterinary medicine.

PART B. THE STUDY

Chapter 5. Objectives

This study aimed to provide the first data on the distribution and the mechanisms of colistin resistance in *Salmonella enterica* isolates of human origin in Greece, which were deposited in the sample bank of the National *Salmonella* and Shigella Reference Centre (SSRC) in 2022. The key objectives of this study were: i) the estimation of the Minimal Inhibitory Concentration (MIC) of colistin in *Salmonella enterica* human isolates using a commercial kit based on the broth microdiluton method (BDM) and ii) the investigation of the genetic basis of mcr-mediated colistin resistance (if detected) in *Salmonella enterica* isolates by employing two standardized and validated conventional multiplex PCR protocols for the detection of mcr 1-5 and mcr 6-9 plasmid-borne genes, respectively.

Chapter 6. Materials & Methods

6.1 Samples

Surveillance of *Salmonella* strains originating from humans is conducted by the National *Salmonella* and Shigella Reference Centre (SSRC) in Greece. During the year 2022, the SSRC received a total of 660 specimens of *Salmonella* from various hospitals across Greece. These specimens were cultured on XLD agar (XLD AGAR ISO FORM, Biolife, Milan, Italy) and then placed in a CO_2 incubator (MCO-17A, Sanyo, Japan) for a duration of 20 hours to confirm the presence of *Salmonella.* Subsequently, individual colonies were introduced into nutrient agar (NUTRIENT AGAR, Biolife, Milan, Italy) and cultivated for 20 hours under the same CO_2 incubator conditions. Following this, the samples were preserved in glycerol at a temperature of -80°C. Within this collection of samples, a subset of 120 were chosen at random to specifically monitor colistin resistance. These selected samples were collected from 18 different prefectures across Greece. The majority of these samples were subjected to serotyping, and relevant information such as antigenic type, along with patient age (structured at 4 Groups, Group A: 0 – 5 years old, Group B: 6 – 14 years old, Group C: 15 – 64 years old, Group D: ≥ 65 years old) and gender data, was duly recorded. Descriptive statistics were produced in SPSS v23 software (IBM Corp., Armonk, NY, USA).

6.2 Colistin susceptibility testing

The procedure of introducing the samples which were selected for colistin susceptibility testing (CST) into XLD and nutrient agars was replicated, following the method outlined earlier. The CST was executed using a commercially available kit (ComASP Colistin, Liofilchem®, Roseto degli Abruzzi (Te), Italy) that adheres to the broth microdilution technique and has been sanctioned by EUCAST. The kit was used according to the manufacturer's instructions. In a concise overview of the process, the samples were diluted in saline and standardized to McFarland 0.5 turbidity. The standardized suspension was then further diluted in saline at a ratio of 1:20 (Solution A). Subsequently, 0.4 ml of Solution A was introduced into pre-filled vials containing Mueller Hinton II Broth (Solution B). A total of 100 μl of Solution B was placed in each well of a designated row within the test panel. This panel encompassed desiccated colistin at 7 incremental dilutions (ranging from 0.25 μg/ml to 16 μg/ml), as outlined in the provided table (Table 1). Following this, the panels were incubated at a temperature of 37°C for a duration of 20 hours.

Table 1. Configuration of colistin test panel.

Test	Colistin Concentration (μg/ml)							
A	Growth	0.25	0.50	1.00	2.00	4.00	8.00	16.00
B	Growth	0.25	0.50	1.00	2.00	4.00	8.00	16.00
C	Growth	0.25	0.50	1.00	2.00	4.00	8.00	16.00
D	Growth	0.25	0.50	1.00	2.00	4.00	8.00	16.00

Growth was evident either as turbidity or as a sediment at the well's base (Figure 4). When the incubation period concluded, growth patterns were observed within the wells, and the Minimum Inhibitory Concentration (MIC) was determined as the lowest colistin concentration that hindered visible growth. In accordance with EUCAST guidelines, the MIC breakpoints for Enterobacterales are as follows: i) susceptible when ≤ 2 μg/ml and ii) resistant when > 2 μg/ml. To ensure testing accuracy, two strains of *Klebsiella pneumoniae*, one that was colistin-susceptible (with an MIC of 0.5 μg/ml) and the other colistin-resistant, were procured from the Centre for Antimicrobial Resistance (Central Public Health Laboratory, National Public Health Organization, 16672 Vari, Greece) and employed as controls to assess test and user quality. Antibiotic resistance data were also recorded for colistin resistant samples.

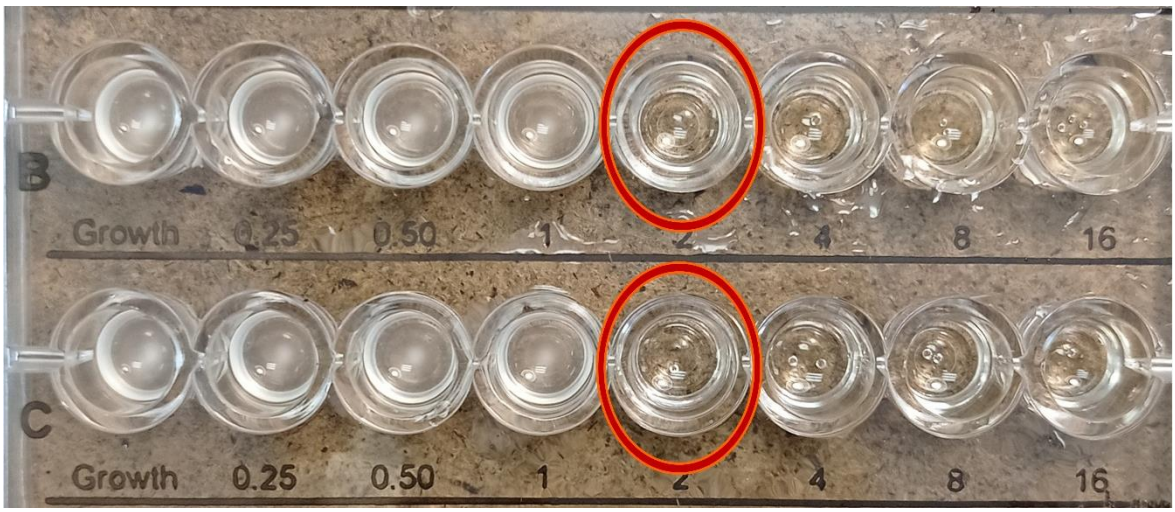

Figure 4. Turbidity showing the growth of *Salmonella* in the kit's wells. Turbidity is firstly hindered in the wells in the red circles, corresponding to MIC values of 2 μg/ml, thus the samples are colistin susceptible.

6.3 Conventional multiplex PCR assays for mcr genes detection

DNA was extracted from fresh overnight agar cultures of colistin resistant *Salmonella enterica* isolates using the thermal cell lysis method. In brief, the bacterial cells were suspended in 100 μl of water into a sterile Eppendorf tube. The tubes were then subjected in

a boiling water bath at 100°C for 15 minutes. Following this, the tubes were promptly transferred to an ice bath for 5 minutes to cool the resulting lysate. Lysates were centrifuged at high speed for 10 minutes to pellet cellular debris. Supernatants, containing the extracted DNA, were placed into fresh tubes and stored at -20°C for PCR testing.

Each crude DNA sample was tested with two conventional multiplex PCR assays targeting mcr 1-5 and mcr 6-9 genes, respectively, using the primer sets and protocols suggested by EUCAST. The primer sets, sequences and amplicon lengths are presented in the tables below. All conventional PCR assays were performed in a total volume of 25 µL, consisting of 12.5 µL 2× KAPA 2G Fast Multiplex PCR Mix (Kapa Biosystems Pty (Ltd), Cape Town, South Africa) and 2 µl of crude DNA samples. For the first multiplex PCR (mcr 1-5) the rest of the volume was made of 5 µl primer mix (consisting of 0.5 µl of 10µM forward primer solution and 0.5 µl of 10µM reverse primer solution for each mcr gene, a total of 5 mcr genes were targeted) and 5.5 µl of H_2O. For the second multiplex PCR (mcr 6-9) the rest of the volume was made of 4 µl primer mix (consisting of 0.5 µl of 10µM forward primer solution and 0.5 µl of 10µM reverse primer solution for each mcr gene, a total of 4 mcr genes were targeted) and 6.5 µl of H_2O.

Table 2. Multiplex PCR primer sequences and expected amplicon sizes for the detection of mcr 1-5 genes.

Primer name	**Sequence (5'-3')**	**Target gene**	**Amplicon size (bp)**
mcr1_320bp_fw	AGTCCGTTTGTTCTTGTGGC	*mcr-1*	320
mcr1_320bp_rev	AGATCCTTGGTCTCGGCTTG		
mcr2_715bp_fw	CAAGTGTGTTGGTCGCAGTT	*mcr-2*	715
mcr2_715bp_rev	TCTAGCCCGACAAGCATACC		
mcr3_929bp_fw	AAATAAAAATTGTTCCGCTTATG	*mcr-3*	929
mcr3_929bp_rev	AATGGAGATCCCCGTTTTT		
mcr4_1116bp_fw	TCACTTTCATCACTGCGTTG	*mcr-4*	1116
mcr4_1116bp_rev	TTGGTCCATGACTACCAATG		
mcr5_1644bp_fw	ATGCGGTTGTCTGCATTTATC	*mcr-5*	1644
mcr5_1644bp_rev	TCATTGTGGTTGTCCTTTTCTG		

Table 3. Multiplex PCR primer sequences and expected amplicon sizes for the detection of mcr 6-9 genes.

Primer name	Sequence (5'-3')	Target gene	Amplicon size (bp)
mcr6_252bp_fw	AGCTATGTCAATCCCGTGAT	*mcr-6*	252
mcr6_252bp_rev	ATTGGCTAGGTTGTCAATC		
mcr7_551bp_fw	GCCCTTCTTTTCGTTGTT	*mcr-7*	551
mcr7_551bp_rev	GGTTGGTCTCTTTCTCGT		
mcr8_856bp_fw	TCAACAATTCTACAAAGCGTG	*mcr-8*	856
mcr8_856bp_rev	AATGCTGCGCGAATGAAG		
mcr9_1011bp_fw	TTCCCTTTGTTCTGGTTG	*mcr-9*	1011
mcr9_1011bp_rev	GCAGGTAATAAGTCGGTC		

Cycling conditions for the first multiplex PCR (mcr 1-5) were: pre-denaturation at 94°C for 15 minutes, followed by 25 cycles of i) denaturation at 94°C for 30 seconds, ii) annealing at 58°C for 90 seconds and iii) extension at 72°C for 60 seconds. The final extension step was done at 72°C for 10 minutes. Cycling conditions for the second multiplex PCR (mcr 6-9) were: pre-denaturation at 95°C for 3 minutes, followed by 30 cycles of i) denaturation at 95°C for 30 seconds, ii) annealing at 55°C for 30 seconds and iii) extension at 72°C for 60 seconds. The final extension step was done at 72°C for 10 minutes. Water, substituting the DNA samples, was used as negative control for both reactions. Positive controls, i.e. purified DNA from Centre for Antimicrobial Resistance (Central Public Health Laboratory, National Public Health Organization, 16672 Vari, Greece) was available only for mcr 1-5 genes. The reactions were performed in a SimpliAmp™ thermal cycler (Applied Biosystems, Singapore). PCR products were analyzed in a 2% agarose gel. The 100 – 3000 bp DNA Rainbow Ladder (GeneON GmbH, Ludwigshafen am Rhein, Germany) was used to assess amplicon lengths.

Chapter 7. Results

7.1 Samples

A total of 120 *Salmonella* specimens were selected at random and incorporated into the research. Among these samples, serotyping information was accessible for 89 of them. The prevailing serotypes observed were S. Enteritidis (n=35, constituting 29.2% of the included samples), followed by S. Bovismorbificans (n=16, 13.3%), S. Give (n=9, 7.5%), and S. Typhimurium (n=7, 5.8%). The details regarding serotypes and their corresponding antigenic types are succinctly outlined in the provided table (Table 4) and visually depicted in the accompanying pie chart (Figure 5). The serotypes were also classified based on their prefecture of origin (Figure 6).

Table 4. List of serotypes, antigenic types and their frequencies of the samples included in the study.

Serotype	Antigenic type	Frequency	Percent (% of total samples)
Monophasic Typhimurium	4:i:- & 4,5:i:-	4	3.3
S. Hermannswerder	28:c:1,5	1	0.8
S. Typhimurium	4,5:i:1,2	7	5.8
S. Newport	6,8:e,h:1,2	2	1.7
S. Livingstone	6,7:d:l,w	1	0.8
S. Paratyphi B	4,5:b:1,2	1	0.8
S. Infantis	6,7:r:1,5	4	3.3
S. Virchow	6,7:r:1,2	1	0.8
S. Inganda	6,7:z10:1,5	1	0.8
S. Enteritidis	9,12:g,m:-	35	29.2
S. Agona	4,5:f,g,s:-	1	0.8
S. Bovismorbificans	6,8:r:1,5	16	13.3
S. Derby	4:f,g:-	1	0.8
S. Oranienburg	6,7:m,t:-	1	0.8
S. Hindmarsh	8:r:1,5	1	0.8
S. Give	3,10:l,v:1,7	9	7.5

S. Ball	4,12:y:e,n,x	3	2.5
Missing	**-**	**31**	**25.8**
Total		**120**	**100.00**

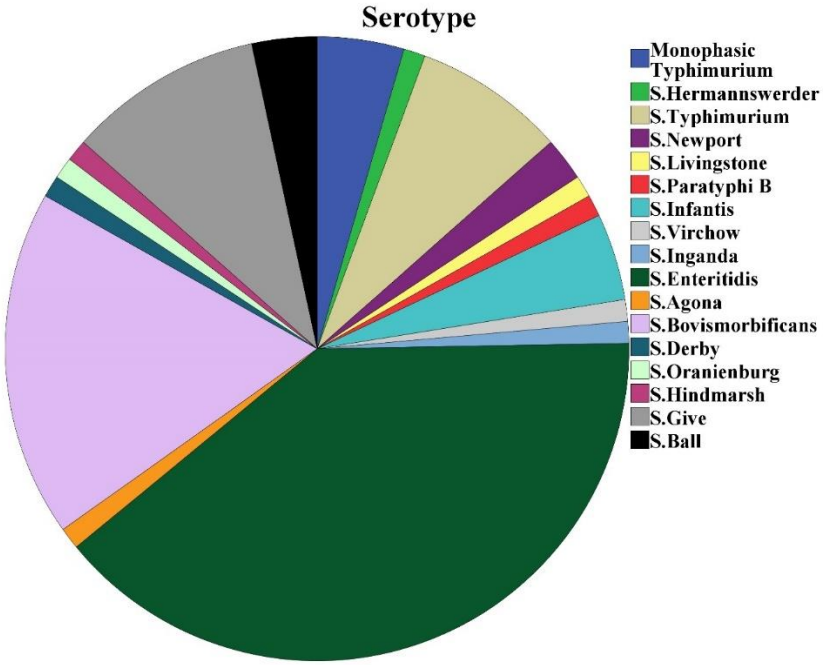

Figure 5. Pie chart representing the serotypes of the samples included in this study.

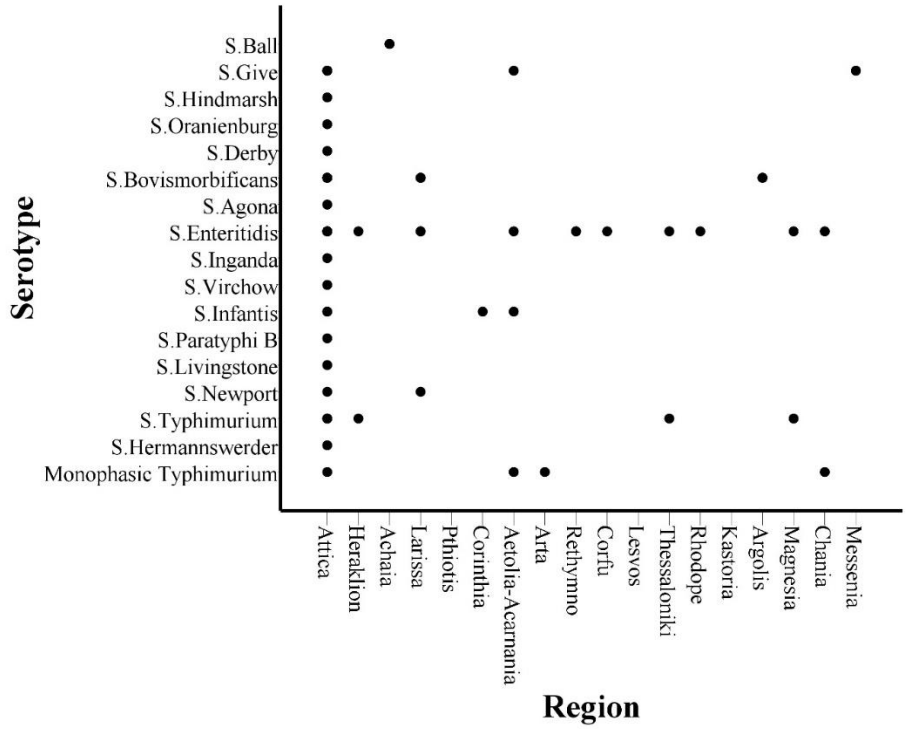

Figure 6. Scatterplot of the classification of serotypes based on their prefecture of origin.

Age and gender were additional parameters under examination. In detail, 40 samples (33.3%) fell into age Group A (0 – 5 years old), 20 samples (16.7%) were in Group B (6 – 14 years old), 27 samples (22.5%) belonged to Group C (15 – 64 years old), and 15 samples (12.5%) were in Group D (≥ 65 years old). Missing patient age data accounted for 15% of the samples. Furthermore, serotypes were categorized according to age groups. Notably, the majority of S. Enteritidis cases were detected in Group A and Group B, while a larger portion of S. Bovismorbificans cases emerged in Group C and Group D (Figure 7). Regarding sex-based classification of samples, 38.3% originated from female patients, while 55% originated from male patients. Missing patient sex data accounted for 6.7% of the samples.

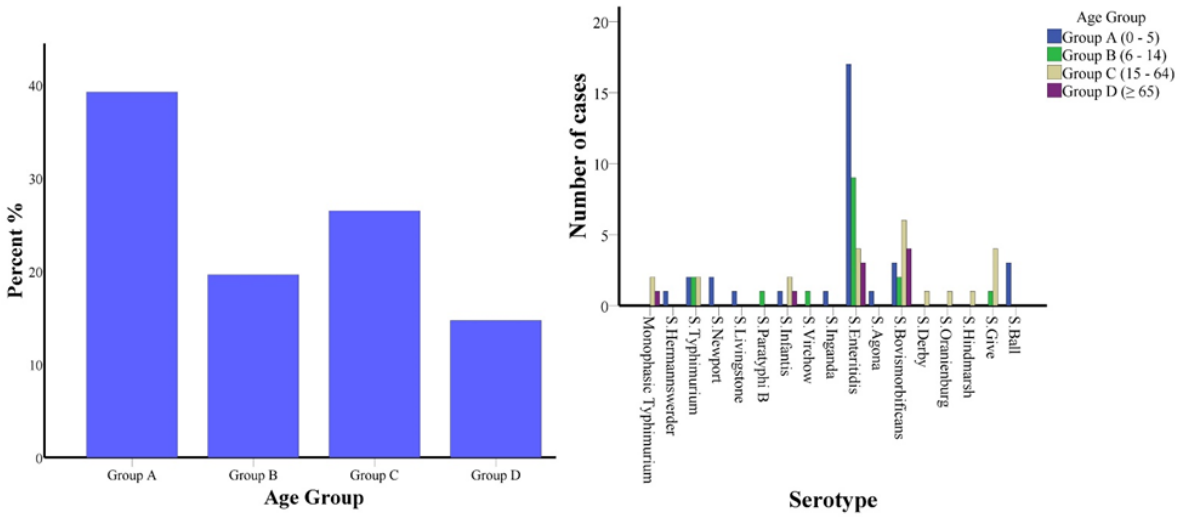

Figure 7. Number of cases per age group and number of cases per age group and serotype.

7.2 Results of colistin susceptibility testing

Results from colistin susceptibility testing indicated that 10% of the samples (12 out of the 120) exhibited resistance to colistin. Among these, nine samples were attributed to the S. Enteritidis serotype, while one belonged to the S. Typhimurium, another to the Monophasic Typhimurium (antigenic type 4,5:i-), and one to an unidentifiable serotype. The majority of these colistin-resistant isolates were sourced from Group A and Group B (young individuals), with one originating from Group C and another from Group D. Notably, two of the colistin-resistant S. Enteritidis isolates also demonstrated resistance to nalidixic acid and pefloxacin, while an additional two isolates of the same serotype displayed resistance to ampicillin and tetracycline. Key details, encompassing MIC values, age groups, and genders, are succinctly compiled within Table 5.

Table 5. Summary of serotypes, antigenic types, MIC values, age group and sex for the colistin resistant *Salmonella* isolates.

Sample ID	Serotype	Antigenic type	MIC (µg/ml)	Age group	Sex
1	*S. Enteritidis*	9,12:g,m:-	4	Group B	Female
2	*S. Enteritidis*	9,12:g,m:-	4	Group A	Male
3	-	-	8	Group C	Male
4	*S. Typhimurium*	4,5:i:1,2	4	Group A	Female
5	*S. Enteritidis*	9,12:g,m:-	4	Group B	Male
6	*S. Enteritidis*	9,12:g,m:-	4	Group B	Female
7	*S. Enteritidis*	9,12:g,m:-	4	Group B	Female
8	*S. Enteritidis*	9,12:g,m:-	4		Male
9	*Monophasic Typhimurium*	4,5:i:-	4		Female
10	*S. Enteritidis*	9,12:g,m:-	8	Group D	Male
11	*S. Enteritidis*	9,12:g,m:-	8	Group A	Male
12	*S. Enteritidis*	9,12:g,m:-	4	Group A	Female

7.3 PCR assays for mcr genes detection

Throughout this investigation, solely the *Salmonella enterica* isolates that displayed resistance to colistin underwent evaluation using the previously described conventional PCR assays to detect the presence of mcr genes. It is noteworthy that none of the samples yielded positive results for mcr genes 1-9.

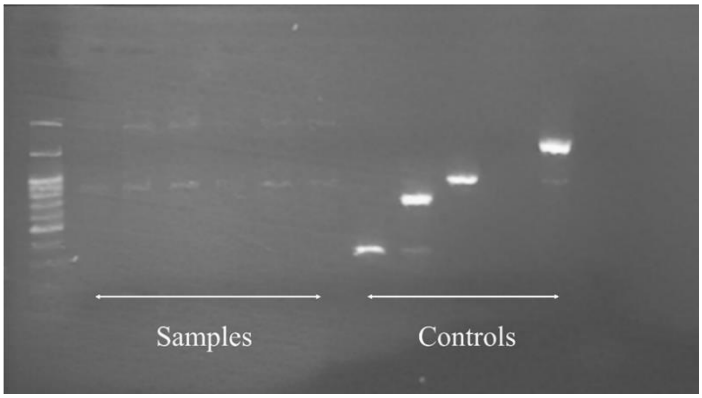

Figure 8. PCR results of six *Salmonella enterica* isolates (samples) along with the controls. The faint bands are non-specific, probably generated during the isolation of crude DNA with the boiling method.

Chapter 8. Discussion

In this study, 120 *Salmonella enterica* human isolates, collected within 2022 from SSRC, were randomly selected and tested for colistin resistance and the presence of mcr genes in resistant isolates. The outcome of the antimicrobial susceptibility testing revealed that 10% of the isolates displayed resistance to colistin; nevertheless, none of the colistin-resistant isolates exhibited the presence of mcr genes. While investigations into colistin resistance in *Salmonella enterica* isolates from animals have previously been conducted, as far as my knowledge extends, this study represents the initial endeavor to specifically examine colistin resistance in human isolates within the context of Greece.

Specimens were gathered from hospitals dispersed throughout Greece, encompassing a span of 18 distinct prefectures. The majority of these samples originated from the Attica prefecture. However, this outcome was in line with expectations, considering that approximately half of the Greek populace resides in Attica and the area boasts the highest concentration of hospitals relative to its land area within Greece. A significant proportion of patients were distributed across age Groups A, B, and D, encompassing both younger and older individuals. This pattern is interesting given that age Group C (15 – 64 years old) constitutes the largest share of the Greek population, accounting for approximately 63.43% as per 2021 data (Statista, 2023). This aligns with the recognized tendency for hospitalizations linked to *Salmonella* infections to largely manifest within the YOPI category (young, old, pregnant, and immunocompromised individuals) (Gil Prieto *et al.*, 2009). Variations in the proportions of female and male patients are likely attributed to randomness stemming from the sample size. Notably, a significant share of the examined serotypes were attributed to S. Enteritidis, a well-known instance of one of the most frequently encountered serotypes isolated from human sources as reported by the CDC (Centers for Disease Control and Prevention, 2022). Intriguingly, the second most prevalent serotype within the analyzed sample was S. Bovismorbificans. This particular serotype was primarily found in the Attica and Larissa prefectures; however, a distinct and definitive epidemiological connection between prevalence, serotype, prefecture of origin, or age group was not conclusively established. Given the limited scope of this study, no attempts were made to establish epidemiological connections among serotypes, the prefecture of origin, patient age, and gender. This precaution was taken to prevent any potential biases stemming from the size of the studied sample.

In the context of this study, the occurrence of colistin-resistant *Salmonella* strains in 2022 was found to be 10%. Information regarding colistin resistance within the European Union is generally limited. According to the most recent report available from the European Centre for Disease Prevention and Control (ECDC) on antimicrobial resistance in *Salmonella* spp. (all non-typhoidal serovars) from human sources, a total of 2957 *Salmonella* isolates were subjected to testing (European Food Safety Authority and European Centre for Disease Prevention and Control, 2022). The highest prevalence of colistin-resistant *Salmonella* isolates was documented in the Netherlands (21.5%), succeeded by Estonia (16.8%) and Denmark (6.7%). In comparison, the average prevalence across the European Union stands at 7.1%. Preliminary findings from this study indicate that the prevalence of colistin resistance in *Salmonella* isolates from human sources closely aligns with or slightly surpasses the EU average. Interestingly, data derived from the same report highlight that colistin resistance among *Salmonella* isolates from animals involved in food production is relatively low within the EU (below 3%), except for laying hen flocks where colistin resistance was observed in 7.2% of the isolates. This percentage is akin to the proportion of colistin-resistant isolates originating from human sources.

The predominant portion (9 out of 12) of isolates displaying colistin resistance in this study were identified as belonging to the S. Enteritidis serotype. Specifically, 25.7% of the S. Enteritidis serotype strains within this study exhibited colistin resistance. In contrast, in Europe, approximately 20.5% of tested S. Enteritidis serotypes isolated from humans demonstrated colistin resistance. Notably, the primary sources of colistin-resistant S. Enteritidis serotypes among food-producing animals were laying hens (15.9% of the tested serotypes), broilers (11.5%), and turkeys (9.1% of the tested serotypes) as documented by the European Food Safety Authority (EFSA) (European Food Safety Authority and European Centre for Disease Prevention and Control, 2022). Given that S. Enteritidis ranks as the most frequently isolated serotype from human cases and constitutes a major source of *Salmonella* infections linked to chicken eggs (Raspoet *et al.*, 2011), it's plausible that an epidemiological connection exists between the prevalence of colistin-resistant S. Enteritidis isolates in poultry and those in humans. The remaining colistin-resistant isolates were affiliated with serotypes S. Typhimurium, S. Monophasic Typhimurium, and an unidentified serotype. In Europe, the colistin resistance prevalence for S. Typhimurium and S. Monophasic Typhimurium isolates stands at approximately 2.9% and 1.5%, respectively. However, the

sample sizes for S. Typhimurium and S. Monophasic Typhimurium in this study were insufficient for assessing the prevalence of colistin-resistant isolates within Greece.

PCR testing for the presence of mcr genes in the identified colistin resistant isolates showed that none of them harbored any of the mcr1-9 genes. Notably, it is important to highlight the observed contrast between the high prevalence of colistin resistance and the limited presence of mcr genes within the S. Enteritidis serotype (Fortini *et al.*, 2022). This particular serotype, belonging to serogroup D, has garnered global recognition for its intrinsic predisposition to colistin resistance (Luo *et al.*, 2020). For example, EFSA reported instances of colistin resistance in various isolates of S. Enteritidis, with this particular serovar representing 33.3%, 52%, and 60.2% of colistin-resistant isolates found in broiler carcasses, broilers, and laying hens, respectively (European Food Safety Authority and European Centre for Disease Prevention and Control, 2022). Both S. Enteritidis and S. Dublin belong to group D salmonellas (serogroup O9) and tend to exhibit reduced susceptibility to colistin, even though there are no known acquired or mutational colistin resistance mechanisms associated with them (European Food Safety Authority and European Centre for Disease Prevention and Control, 2022). It has been proposed that the O-antigen epitope within Salmonella group D plays a role in determining their susceptibility to colistin (Fortini *et al.*, 2022). This is because the O-antigens of Salmonella group D differ from those of group B, primarily due to the presence of tyvelose instead of abequose as the side-branch sugar. Furthermore, increased susceptibility to colistin in Salmonella group D has been attributed to a frameshift mutation identified in the rfc gene, which encodes the O-antigen polymerase (Fortini *et al.*, 2022).

S. Typhimurium, on the other hand, has been documented as the prevailing serotype known to harbor mcr genes. Importantly, S. Typhimurium ranks among the most commonly occurring serotypes responsible for human infections (Lima, Domingues and Da Silva, 2019). Additionally, variants of S. Typhimurium, such as the monophasic type 1,4,[5],12:i:, are frequently reported to carry mcr genes. It is pertinent to mention that while mcr-positive Paratyphi B isolates have been identified in animal samples, however this serotype primarily infects humans, often leading to invasive diseases (Lima, Domingues and Da Silva, 2019). We also have to consider that the presence of mcr genes does not always correlate with elevated levels of colistin resistance. In certain instances, mcr-bearing strains have been linked to lower degrees of resistance, potentially allowing them to persist without detection (Lima, Domingues and Da Silva, 2019). The direct connection between mcr gene presence

and colistin resistance in *Salmonella* remains somewhat elusive. These genes have even been identified in strains that are susceptible to colistin. Surprisingly, only a minority of resistant strains actually carry these genes. This observation implies that alternative mechanisms of colistin resistance play a significant role (Bertelloni *et al.*, 2022). Chromosomally mediated colistin resistance is primarily described in human clinical isolates of Enterobacterales. Its prevalence is expected to rise, particularly in human medicine, where colistin is increasingly employed as a last-resort antimicrobial against carbapenemase-producing pathogens (Binsker, Käsbohrer and Hammerl, 2022). Moreover, given the extensive utilization of colistin in veterinary medicine, it's reasonable to anticipate a further increase in chromosomally mediated colistin resistance and the dissemination of mobile colistin resistance mechanisms. This expectation is exemplified by zoonotic agents like S. Enteritidis and the monophasic variant of S. Typhimurium (Fortini *et al.*, 2022).

The presence of colistin resistance genes integrated into mobile genetic elements, like plasmids, raises significant concerns due to their capacity to transfer horizontally between different bacterial species. To illustrate, the mcr-1 gene located on plasmids was initially reported in late 2015 in *E. coli* samples collected from animals in China (spanning the period 2011-2014). This same gene was also identified in *K. pneumoniae* and *E. coli* samples from Chinese patients in 2014 (Hussein *et al.*, 2021). These observations gave rise to the theory that mcr genes originated in E. coli and subsequently spread to other bacterial species, although this hypothesis lacks conclusive evidence. For example, reports of S. Enteritidis strains, isolated in Italy in 2009 and bearing the mcr1 gene have also been documented (Fortini *et al.*, 2022). Regardless, the potential for horizontal transfer between diverse bacterial species remains a tangible risk. Furthermore, these mcr genes often coexist with other resistance genes, such as blaCTX-M, floR, and/or qnr. This co-occurrence results in strains that exhibit resistance to multiple classes of antibiotics, encompassing polymyxins, a wide spectrum of beta-lactams (including broad-spectrum cephalosporins and monobactams), amphenicols, and quinolones (Lima, Domingues and Da Silva, 2019).

The acquisition of mobile elements, such as plasmids, to mediate antibiotic resistance places a fitness burden on the bacterial host. When antibiotic pressure is absent, susceptible strains have the potential to outcompete resistant strains burdened with additional genetic material (Li *et al.*, 2021). A recent investigation illuminated that the expression of mcr1 and mcr3 genes imposes fitness costs on bacteria during their initial 50 generations. Interestingly, despite these costs, these genes and associated plasmids manage to persist over time,

implying that compensatory mutations alleviate the burden over generations (Yang *et al.*, 2020). Furthermore, a study focusing on the influence of various plasmids harboring the mcr1 gene on host fitness, unveiled that plasmids belonging to the IncI2, IncHI2, and IncX4 types, which carry mcr1 genes, demonstrate stability and have minimal impact on bacterial growth. This observation could indicate that a significant proportion of the reported mcr1 plasmids fall within these specific types (Wu *et al.*, 2018; Yang *et al.*, 2020). This implies that the potential enhancement of fitness or co-selection via other antimicrobial agents may contribute to the broader dissemination of plasmids carrying the mcr1 gene (Li *et al.*, 2021).

It is logical to deduce that the ongoing utilization of colistin in animal production is likely exerting positive selective pressure, prompting the emergence of colistin-resistant bacteria (Portes *et al.*, 2022). The rearing of animals carrying these resistant strains could potentially lead to contamination in end products, including meat and eggs (Hu *et al.*, 2019). Subsequently, these resilient microorganisms may find their way to humans through contaminated food sources (Ferrari, Panzenhagen and Conte-Junior, 2017). The transmission of these resistant strains can occur via the fecal-oral route, thereby facilitating the human-to-human dissemination of colistin-resistant *Salmonella* variants (Gopinath, Carden and Monack, 2012). Moreover, the movement of asymptomatic individuals with salmonellosis across different countries has played a role in the global dissemination of these strains, contributing to their widespread distribution (Arcilla *et al.*, 2016). As a result, the presence of mcr genes in *Salmonella* warrants significant attention, given its status as a zoonotic pathogen of considerable importance to public health (Portes *et al.*, 2022).

Within this context, the need for effective management of colistin resistance, prudent colistin utilization, and *Salmonella* infections in both human and animal domains becomes imperative. Embracing a One Health framework underscores this approach—an all-encompassing perspective that acknowledges the intricate interplay between human health, animal well-being, and the environment. One Health promotes judicious antibiotic employment in both human medical care and veterinary practice. The unchecked use and improper administration of antibiotics in animals can fuel the emergence and propagation of antibiotic resistance. By instating meticulous antibiotic stewardship measures, the selective pressure leading to resistance can be curtailed. Striking a balanced and controlled application of this category of antimicrobials in human and animal medicine emerges as one of the paramount methods to curb the spread of colistin resistance. Regulations and guidelines governing antibiotic usage in animals raised for food can encompass restrictions on

antibiotic application for growth promotion and preventive purposes. These measures also advocate the exploration of non-antibiotic alternatives. Ensuring stringent standards of hygiene and biosecurity across human healthcare facilities and animal production environments acts as a safeguard against the dissemination of resistant bacteria among humans, animals, and their surroundings. The One Health approach also acknowledges the repercussions of antibiotic residues and resistant bacteria entering the ecosystem via agricultural runoff and wastewater. The implementation of regulatory measures to control the release of antibiotics and resistant bacteria into water bodies and soil can significantly mitigate environmental contamination. Moreover, vigilance and surveillance concerning antibiotic resistance, spanning human and animal populations as well as the environment, play a pivotal role in recognizing emerging resistance trends and identifying critical areas of concern. This proactive approach enables timely intervention and mitigation strategies.

While surveillance is widely acknowledged as a critical component in combating antimicrobial resistance (AMR), devising an appropriate surveillance system can prove to be a complex undertaking. It is imperative to consider benefit-risk assessments, evaluating the potential advantages of surveillance, such as enhanced public health responses, against potential drawbacks, including stigma, discrimination, and costs. This deliberation is crucial in determining the justification of engaging in surveillance activities. For instance, in this particular scenario, the samples were randomly chosen from a pool of 660 specimens forwarded to SSRC. However, this initial sample pool only represents hospitalizations attributed to *Salmonella* and individuals seeking medical care, rather than encompassing the entire population. Another potential source of bias could stem from variations in physician training or the policies of public and private hospitals regarding sample sharing with SSRC for surveillance purposes. These factors could potentially introduce significant representational disparities. Nevertheless, the implementation of an active surveillance system might be impractical due to the considerable costs and resource demands, including personnel, laboratory infrastructure, and consumables. Efforts by ECDC to address these challenges involve offering guidelines, data, and even conducting sample analyses in suspected *Salmonella* outbreak situations. Technological progress, like the development of Point of Care diagnostics capable of identifying *Salmonella* or even serotyping prevalent serotypes directly within hospital or medical professional settings, without necessitating advanced laboratory facilities, holds potential for establishing more robust surveillance networks.

Chapter 9. Conclusions & suggestions for future research

This study, a pioneering endeavor for the Greek setting, has illuminated the existence of colistin resistance within the circulating *Salmonella* isolates of the country. The prevalence of colistin resistance aligns closely with, or slightly exceeds, the average observed across the European Union. Notably, the presence of mcr genes within the colistin resistant isolates under scrutiny could not be verified in this particular sample. To comprehensively address the challenge of antimicrobial resistance (AMR) against this specific antibiotic, it is imperative to enhance our understanding of colistin resistance and the distribution of mcr genes in *Salmonella* isolates from Greece. Achieving this understanding can facilitate the adoption of surveillance strategies and a One Health approach. Such initiatives are crucial for devising effective strategies, minimizing the horizontal (and potentially vertical) transmission of mcr genes within the microbial community of Greece, and mitigating the impact of AMR.

Future investigations should concentrate on illuminating the precise landscape of colistin resistance within *Salmonella* strains in Greece, while simultaneously addressing the constraints inherent in the current study. This objective can be achieved by augmenting the sample size under scrutiny and collecting data on colistin resistance and mcr gene prevalence over successive years. Furthermore, it is prudent to delve into the investigation of colistin-susceptible isolates harboring multidrug resistance genes, in an effort to uncover any latent presence of mcr genes. These genes could potentially exist within *Salmonella* serotypes without manifesting the characteristic colistin-resistant phenotype. Integral to this endeavor is the assimilation of data from veterinary services and food safety authorities, along with the adoption of a comprehensive, multi-stakeholder approach. This collaborative approach is indispensable for effective surveillance of colistin resistance and mcr genes, extending beyond the confines of *Salmonella.* By doing so, we can unmask the authentic repercussions of colistin employment in animal husbandry and meticulously construct One Health strategies to counteract this emerging threat.

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