UNIVERSITY OF THESSALY
SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY SCIENCE
DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

DETECTION OF PATHOGENS TRANSMITTED BETWEEN WILD BIRDS AND DOMESTIC ANIMALS OR HUMANS

A thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Introduction

Wild avian species can act as vectors and reservoirs for a wide range of microorganisms that can be transmissible to humans [1]. Their global distribution, great biodiversity, unique adaptive immune system, annual long-distance migrations and habitual roosting in environments shared by other animals have given them a distinctive role in the emergence, amplification and cross-species transmission of various pathogens [2]. Wild birds are known to harbor several agents that can pose a significant risk to human health, including influenza A virus, arboviruses such as West Nile virus (WNV), *Borrelia burgdorferi*, enteric bacterial pathogens, and drug-resistant bacteria [3,4]. Notably, about 40% of emerging human diseases are thought to have originated from wild animal species [5].

Since the discovery of antibiotics, the number of human deaths attributed to infectious diseases has decreased by 70% [6]. However, microorganisms have evolved powerful mechanisms to cope with antibiotic exposure, and development of resistance to any antibiotic is nearly inevitable. Antimicrobial Resistance (AMR) occurs when microorganisms become able to adapt and grow in the presence of medications that once impacted them [7]. AMR is now considered a paramount problem of the medical community, inextricably linked to One-Health. Having been recognized as a major consequence of the use of antimicrobials, it is considered to be favored by the imprudent implementation of antibiotic therapies both in human and veterinary medicine. In particular, the excessive use or misuse of antibiotics induces a selective pressure responsible for the development of resistance mechanisms in bacterial populations. AMR transmission pathways between humans, animals and the environment are abundant and complex, even though the relative contribution of each host and pathway to the overall acquisition of AMR in humans remains largely unknown [8]. The need for in depth study of AMR carriage by animals is highlighted by reports that reveal common resistance genes, plasmids or clones between animal (both domesticated and wild) and human sources. For instance, Extended-Spectrum β-Lactamase (ESBL), AmpC- and carbapenemase producing Enterobacteriaceae are considered a significant, potentially zoonotic threat [9]. The AMR situation jeopardizes the efficacy of valuable compounds in curing disease, leads to increased mortality and causes vast economic losses [10,11]. Murray et al. estimated a median of 1.27 million deaths in 2019 directly attributable to AMR, a value that is roughly equal to HIV deaths (680.000) and malaria deaths (627.000) combined on a global scale [11,12]. AMR, if left unattended, is reckoned to lead to about 10 million human deaths per year until 2050 [13]. Hence, there is not only a need to recognize the harm of the indiscriminate use of antibiotics, but also to enforce compliance with regulations and preventive measures.

Regarding viral pathogens, several disease outbreaks among humans have been linked with wild avian vectors. A representative example is West Nile Virus (WNV), a virus that is maintained in nature in an enzootic bird-mosquito cycle [14]. This arthropod-borne, single stranded RNA virus of the genus Flavivirus and the Flaviviridae family, causes a zoonotic and epizootic infection of great importance, with wild birds serving as critical vectors because of their high viral load [15]. In Greece the first epidemic of WNV infection was reported in

2010, with 262 clinical human cases and 35 fatalities [16]. WNV lineage 2 was identified from human sera, sentinel chickens, wild birds, and *Culex* mosquitoes [17]. Since then, presence of the virus has been established, and human cases have been reported annually, except in 2015 and 2016, while 2018 was a record year, with 243 cases showing neuroinvasive disease [18]. Several reports have confirmed virus detection in mosquitoes, equines, and birds in the country throughout the years [19–22].

This thesis comprises studies that investigated the role of wild birds in the dissemination of ESBL, AmpC- and carbapenemase producing Enterobacteriaceae as well as of WNV in Greece. In detail, the following chapters are included:

Chapter 1. General information

Chapter 2. Poultry and Wild Birds as a Reservoir of CMY-2 Producing *Escherichia coli*: The First Large-Scale Study in Greece

Chapter 3. ESBL-Producing *Moellerella wisconsensis*-The Contribution of Wild Birds in the Dissemination of a Zoonotic Pathogen

Chapter 4. Occurrence and Characteristics of ESBL- and Carbapenemase- Producing *Escherichia coli* from Wild and Feral Birds in Greece

Chapter 5. Presence of West Nile virus (WNV) Lineage 2 among wild birds in the Regions of Peloponnese and Western Greece

Chapter 1. General Information

PART I. ESBL, AmpC and CARBAPENEMASE PRODUCING ENTEROBACTERIACEAE

1.1. Enterobacteriaceae

1.1.1. General characteristics

Enterobacteriaceae is a bacterial family containing a large number of genera that are biochemically and genetically related to one another. It consists of Gram-negative, aerobic or facultatively anaerobic, asporogenous, capsulated or non-capsulated, rod-shaped bacteria, with simple nutritional requirements that grow well on artificial media. Most Enterobacteriaceae are small in size, about 2–3 μm in length by 0.6 μm in width, with parallel sides and rounded ends [23]. They utilize glucose fermentatively with formation of acid or of acid and gas. The oxidase test is negative, and with a few exceptions, catalase is produced. Nitrates are reduced to nitrites [24].

Most genera and species in the family contain the enterobacterial common antigen (eca); and have a 39–59 percent guanine-plus-cytosine (G + C) content of DNA, with most of the genera in the range 49–59 mol% [25,26]. When techniques that measure evolutionary distance are used, such as DNA–DNA hybridization and 16S r-RNA sequencing, genera and species in the family are more closely related to *Escherichia coli*, the type species of the type genus of the family, than they are to organisms in other families [26].

The phylogenetic position of the family Enterobacteriaceae is in the phylum Pseudomonadota, the gamma subclass of Proteobacteria (Gammaproteobacteria) - a subclass also known as the “purple bacteria”, and the order Enterobacterales. The nomenclature and classification of the genera, species, subspecies, biogroups, and serotypes of Enterobacteriaceae are constantly being reevaluated, and the literature contains many differing opinions. In 2020, a taxonomy change was adopted to use “Enterobacterales” as the name of a new scientific order. *Enterobacteriaceae* are now a family within the “Enterobacterales” order, along with *Erwinaceae, Pectobacteriaceae, Yersiniaceae, Hafniaceae, Morganellaceae*, and *Budvicaceae*. The order Enterobacteriales had previously been suggested but did not follow the rules of nomenclature [27].

1.1.2. Members of the family Enterobacteriaceae

According to the List of Prokaryotic Names with Standing in Nomenclature (LPSN) website (https://www.bacterio.net/), a total of 68 genera and 355 species are currently listed in the Enterobacteriaceae family. These include important pathogens such as those of the genera *Salmonella*, *Shigella* and *Yersinia*, as well as opportunistic pathogens such as *Escherichia* spp., *Klebsiella* spp., *Proteus* spp. and *Enterobacter* spp.

1.1.3. Cell morphology and Antigenic structure

The cell of Enterobacteriaceae is typical of Gram-negative bacteria (Figure 1.1). It consists of a cell wall which surrounds the plasma membrane. The cell wall itself has two distinct layers; a thin (2-7nm) peptidoglycan (murein) layer covered by an outer membrane (7-8nm). Peptidoglycans are unique to prokaryotic organisms and consist of a glycan backbone of muramic acid and glucosamine (both N-acetylated), and of peptide chains that are partially cross-linked in Gram-negatives (in contrast to the high cross-linkage with bridges that is a feature of Gram-positive bacteria). Peptidoglycans confer the characteristic cell shape and provide the cell with mechanical protection. The outer membrane is comprised of lipopolysaccharides, which are large, complex molecules that consist of three parts: lipid A, the core polysaccharide and the O side chain (O antigen). Lipopolysaccharides have many important functions, contributing to the negative charge on the bacterial surface, stabilizing the outer membrane structure, helping protect pathogenic bacteria from host defenses and assisting the creation of a permeability barrier. Moreover, lipid A of lipopolysaccharides can act as an endotoxin. Passage across the outer membrane is regulated by integral membrane proteins, including one class of proteins termed porins, or through the action of specific outer membrane receptors, depending on the size of the molecule to be transported [28].

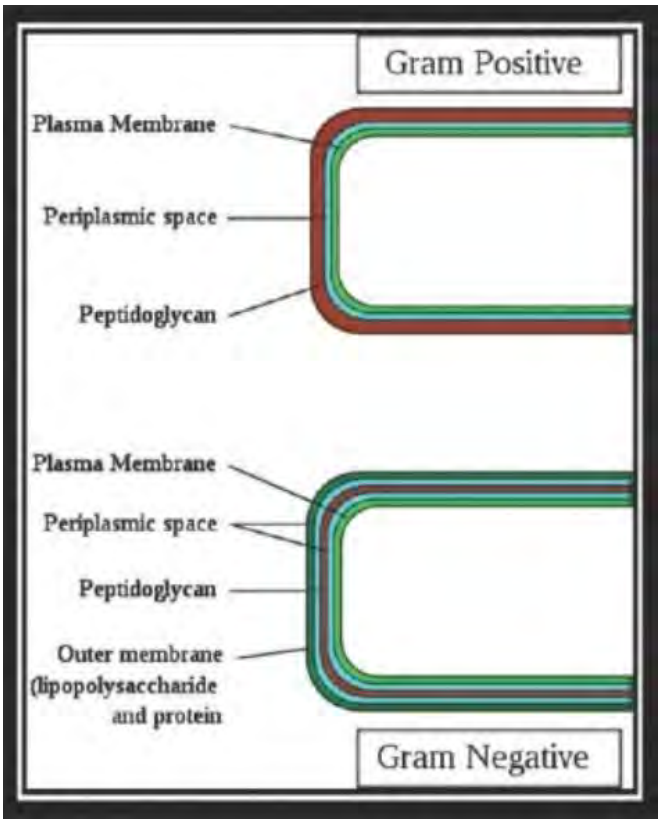


Figure 1.1. Structure of the bacterial cell envelope [10].

Some species of the family synthesize capsules, though the amount of capsular material varies significantly from strain to strain. Sometimes it forms a well circumscribed capsule around the organisms, but in other cases it becomes free in the surrounding medium as 'loose slime'. Capsulation is noted for *Klebsiella* spp. and some strains of *Enterobacter* spp. and *E. coli*. Capsules are most often composed of polysaccharides and confer several advantages for bacterial growth. They help pathogenic bacteria resist phagocytosis by host phagocytes and protect against desiccation since they contain a great percentage of water. They exclude viruses and most hydrophobic toxic substances such as detergents.

Most Enterobacteriaceae additionally synthesize flagella and are motile. The main exceptions are the genera *Klebsiella*, *Leminorella*, *Moellerella*, and *Shigella*. In the genus *Salmonella*, the only constantly nonmotile serotypes are *Gallinarum* and *Pullorum*. In the other genera, nonmotile mutants occasionally occur. Flagella are always peritrichous except in *Tatumella* spp., whose flagella tend to be polar, subpolar or lateral [23].

The genetic material is localized in the nucleoid which, although not separated from the cytoplasm by a membrane, is a distinct region of the cell. Enterobacteriaceae have a single

double stranded deoxyribonucleic acid (DNA) chromosome. In addition to the genetic material present in the nucleoid, many bacteria also contain extrachromosomal double-stranded DNA molecules, in the form of plasmids and bacteriophages (phages), which code for important proteins, such as toxins and colonization factors, that are involved in disease causation. Plasmids can also carry genes that code for a variety of enzymes involved in carbohydrate metabolism and cell wall synthesis. Plasmids' genetic information is, however, most commonly not essential to the bacterium, and cells that lack them usually function normally. Nevertheless, they often carry genes that confer a selective advantage to the bacterium in certain environments, such as antimicrobial resistance genes.

Important serotypes of each Enterobacteriaceae species can be differentiated by their characteristic antigens, which interact with specific antibodies. A bacterial cell surface carries one or more of the following antigens: the somatic or O (Ohne Hauch) antigens of the outer membrane's lipopolysaccharides, the H (Hauch) antigens of the flagella which are proteins and K (Capsular) antigens from polysaccharides of the cell capsule. A fourth antigen F (fimbriae) is occasionally used for serotyping.

1.1.4. Isolation

Most clinically relevant or environmental members of Enterobacteriaceae can grow on nonselective media such as blood or chocolate agar at 35–37°C without specific atmospheric requirement, while some require lower temperature. Isolation from environments with mixed bacteria may require enrichment in selective media. Selective/differential media such as MacConkey agar or Eosin methylene blue agar can be used. Enrichment broths such as selenite broth and Gram-negative broth may also be useful, the latter particularly for the recovery of *Salmonella* and *Shigella* from stool samples. For the isolation of Enterobacteriaceae from food products for human consumption and the feed of animals, as well as from environmental samples in the area of food production and food handling, Enterobacteriaceae enrichment (EE) broth is recommended by the ISO 21528-1:2004 standard for their detection and enumeration by the most probable number method [29].

1.1.5. Distribution

Members of the Enterobacteriaceae family are ubiquitous, found worldwide in various sources. One of their most common ecological niches is the intestine of humans and other animals [29]. Other common sources include plants, fruits, vegetables, grains, soil and water. Some species, though, occupy very limited ecological niches.

The Enterobacteriaceae family is recognized as a group of bacteria with great medical, public health and veterinary impact on the global community [26]. The family is not only associated with a wide range of human clinical syndromes but is also a major causative agent of foodborne enteritis and zoonotic infections. Widely dispersed in nature in many naturally occurring ecosystems, members of the family are increasingly being implicated as pathogens, amplifying the need for continuous study of their occurrence and AMR.

1.2. Antimicrobial Agents

1.2.1. Brief history

The use of antibiotic-producing microbes to prevent disease dates back more than 2000 years, with traditional poultices of moldy bread being used to treat open wounds in Serbia, China, Greece and Egypt [30]. Bioactive compounds of plants were also medically used as, for example, by indigenous people of South America that used a powder made of the cinchona tree bark (*Cinchona pubescens* or *C. officinalis*) against fever and malaria, which had been introduced to South America by Spanish conquistadors [31]. However, the beginning of the modern "antibiotic era" with the development of anti-infective drugs is associated with the names of Paul Ehrlich and Alexander Fleming [32]. In 1909, Ehrlich developed a synthetic arsenic-based pro-drug, salvarsan, and the more soluble and less toxic Neosalvarsan against *Treponema pallidium*, the causative agent of syphilis [32,33]. These molecules were the most frequently prescribed drugs until their replacement by penicillin in the 1940s.

1.2.2. Mechanisms of action

Antimicrobial agents can be divided into groups based on the mechanism of action against antimicrobials. The main groups are agents that:

- inhibit cell wall synthesis
- depolarize the cell membrane
- inhibit protein synthesis
- inhibit nuclei acid synthesis
- inhibit metabolic pathways in bacteria.

Table 1.1 and Figure 1.2 provide examples of drugs from each of these groups.

Table 1.1. Antimicrobial groups based on mechanism of action. [34,35]

Mechanism of Action	Examples of Antimicrobial Groups	Examples of Antimicrobial Agents
Inhibition of Cell Wall Synthesis	β-Lactams	Penicillins
		Cephalosporins
		Carbapenems
		Monobactams
	β-lactam with β-lactamase inhibitor	Amoxicillin/clavulanic acid
	Glycopeptides	Vancomycin
Depolarization of Cell Membrane Structure	Polymixins	Colistin
	Lipopeptides	Daptomycin
Inhibition of Protein Synthesis by binding to the 50S	Macrolides	Erythromycin

	Subunit of the ribosome	Chloramphenicol	Chloramphenicol
		Lincosamides	Clindamycin
		Streptogramins	Quinupristin-dalfopristin
		Oxazolidanones	Linezolid
	by binding to the 30S Subunit of the ribosome	Aminoglycosides	Gentamycin
		Tetracyclines	Tetracycline
Interference with nucleic acid synthesis	by inhibiting DNA synthesis	Flouroquinolones	Ciprofloxacin
	by inhibiting RNA synthesis	Rifampicin	Rifampicin
Inhibition of metabolic pathway		Sulfonamides	Sulfamethoxazole
		Folic acid analogues	Trimethoprim

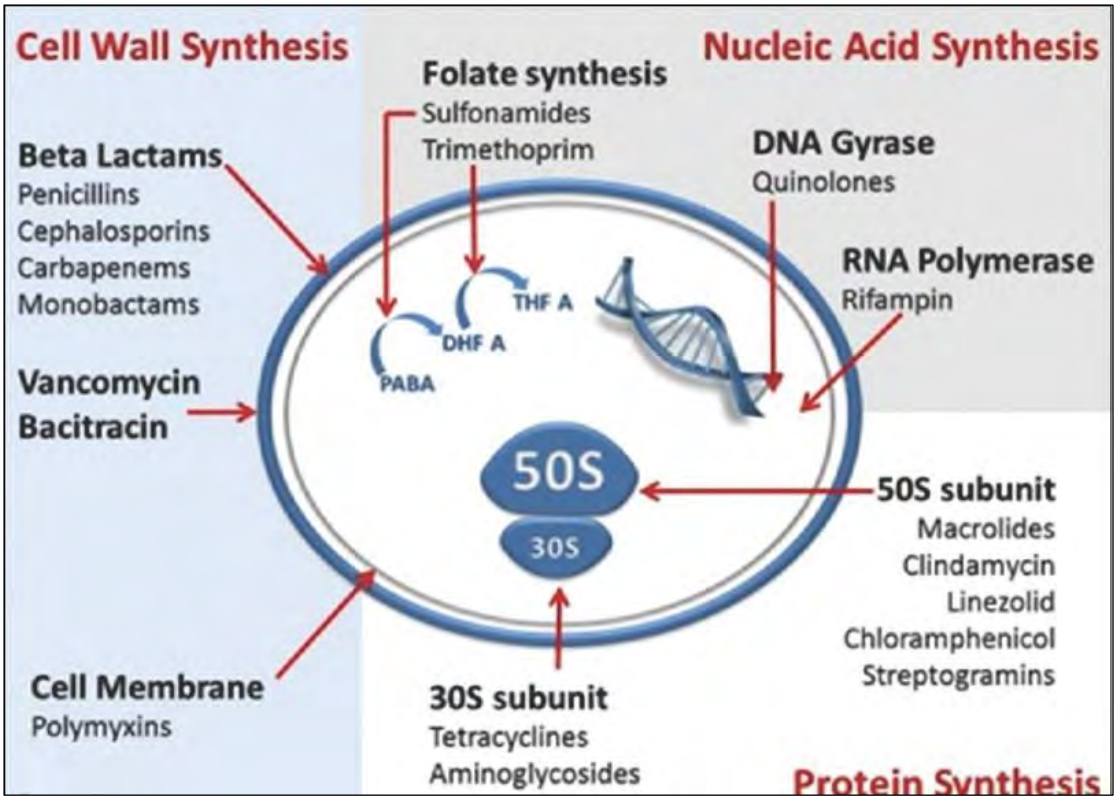


Figure 1.2. Classification of Antibiotics based on their mechanism of action [36].

1.2.2.1 Agents Acting Through Cell Wall Synthesis

The cell wall supports the underlying plasma membrane, which is subject to an internal osmotic pressure of about 5 atmospheres in gram-negative organisms [37]. Since mammalian cells are devoid of a cell wall, inhibition of its biosynthesis is a key antimicrobial target that minimizes impact on normal host tissue [38].

B-lactam antibiotics

β-lactams are the most widely used class of antibiotics in human and veterinary medicine [39,40]. β-Lactam antibiotics' bactericidal action is based on their ability to interfere with the final stage of cell wall synthesis, and in particular with the crosslinking of peptidoglycan polymers [41]. This is accomplished by obstruction of the activity of certain bacterial enzymes, the penicillin-binding-proteins (PBPs). β-lactams, being chemically affine to PBPs, bind with them, resulting in the acylation of their active site serine and subsequently in their inactivation [42–45] (Figure 1.3). PBPs are transpeptidases, carboxypeptidases and endopeptidases that, when inhibited, coherence of the bacterial cell wall cannot be achieved, thus leading to bacterial death and lysis [46].

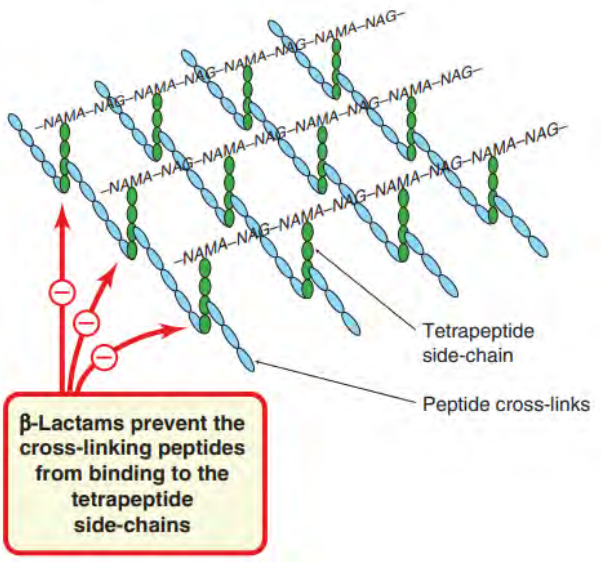


Figure 1.3. Schematic diagram of a single peptidoglycan layer from a bacterial cell, showing the site of action of a β-lactam antibiotic. NAG – N-acetyloglucosamine; NAMA – N-acetylmuramic acid (adapted from [37]).

β-lactam antibiotics are a broad group of molecules that are naturally produced by different organisms. In particular, penicillins and cephalosporins were first isolated from fungi belonging to *Penicillium* spp. and *Cephalosporium* spp., respectively, while carbapenems and monobactams from bacteria belonging to different species. Nowadays, the class consists of a large number of mostly semi-synthetic compounds.

All agents of the class share the common feature of containing in their chemical structure a four-membered ring with an amidic function, the β-lactam ring, which is the structure mainly responsible for their antibacterial properties. They can be classified into bicyclic

penicillins, cephalosporins and carbapenems, among which the β-lactam ring is fused to another 5- or 6-member ring, and in monocyclic monobactams [39] (Figure 1.4).

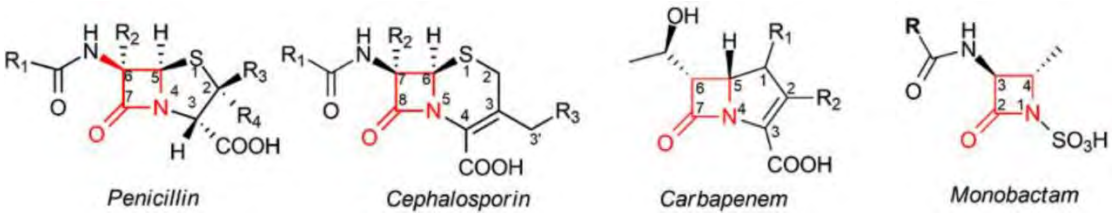


Figure 1.4. Core structures of different classes of β-lactam antibiotics. In penicillins the β-lactam ring is fused to a five-member thiazolidine ring; in cephalosporins the β-lactam ring is fused to a six-member dihydrothiazine ring; in carbapenems the β-lactam ring is fused to a pyrroline ring; in monobactams the β-lactam ring is not fused to any other ring. In all classes, the β-lactam ring is highlighted in red; the standard numbering of the nucleus for each class is also reported (adapted from [47]).

1.3. Antimicrobial Resistance

1.3.1. Brief history

Penicillin, discovered by Alexander Fleming in 1928 [48], was the first antibiotic successfully used to control bacterial infections in soldiers during World War II. However, even before its use in 1940, the first penicillin resistant bacterial strains had already been described [49]. The finding of a penicillinase before the use of penicillin can now be explained by the fact that a large number of antibiotic resistance determinants are components of natural microbial populations [50]. To counteract the first penicillinases, methicillin was introduced in 1959 and one year later, in 1960, a methicillin resistant *Staphylococcus* strain was reported. Vancomycin was introduced in 1958 for the treatment of methicillin-resistant staphylococci. After a couple of decades, in 1979, coagulase-negative staphylococci resistant to vancomycin were reported and ten years after that resistance in enterococci was described, followed by the report of less-susceptible *S. aureus* (vancomycin-intermediate *S. aureus*, VISA) strains in Japan in 1997.

In contrast to the rapid discoveries and developments that characterized the years 1950–1980 when antibiotic research blossomed and during which virtually all existing drugs were produced, the discovery of new antibiotics has dramatically decreased, with only few novel antibiotics introduced since 1980 [51]. As a consequence of the increasing occurrence of AMR and the weak new antimicrobial pipeline, bacterial infections due to multidrug-resistant or extensively drug-resistant pathogens have become a major concern worldwide.

In a terminological aspect, AMR today refers to resistance demonstrated by microbes which include bacteria, viruses, fungi, and parasites, whereas the term antibiotic resistance is limited to bacteria [52].

1.3.2. Origin of resistance

1.3.2.1 Natural resistance

Natural resistance may be intrinsic or induced. Intrinsic resistance is always expressed in a certain bacterial species, is independent of previous antibiotic exposure and is not associated to horizontal gene transfer (HGT) [53]. The most common bacterial mechanisms involved in intrinsic resistance are reduced permeability of the outer membrane (most specifically the lipopolysaccharide, LPS, in gram negative bacteria) and the natural activity of efflux pumps. In the case of induced resistance, AMR genes naturally occur in a bacterial species, but are only expressed to resistance levels after exposure to an antibiotic. Multidrug-efflux pumps are also a common mechanism of induced resistance [35].

1.3.2.2 Acquired resistance

Acquisition of genetic material that confers resistance is possible through all of the main routes by which bacteria acquire any genetic material: transformation, transposition, and conjugation, all termed HGT. Such an acquisition may be temporary or permanent. Plasmid-mediated transmission of AMR genes is the most common route for acquisition of outside genetic material, while bacteriophage-borne transmission is rare. Additionally, bacteria may experience mutations to their own chromosomal DNA due to stressors (starvation, UV radiation, chemicals, etc.). These mutations conferring resistance usually occur in specific types of genes; those encoding drug targets, drug transporters, regulators that control drug transporters, and antibiotic-modifying enzymes [54].

1.3.3. Mechanisms of resistance

AMR mechanisms are classified into four main categories: (1) limitation of drug uptake, (2) active drug efflux, (3) change of drug's targets and (4) modification of drug. Intrinsic resistance is achieved via limiting drug uptake, drug efflux and drug modification, while acquired resistance via drug efflux, target and drug modification. Gram negative bacteria such as Enterobacteriaceae utilize all four main mechanisms, whereas gram positive bacteria less commonly limit the uptake of a drug (since not possessing a lipopolysaccharide outer membrane), and don't have the capacity for certain types of drug efflux mechanisms [35].

1.3.3.1 Limitation of drug uptake

Many of the antibiotics used in clinical practice have intracellular bacterial targets or, in the case of Gram-negative bacteria, targets located in the cytoplasmic membrane (the inner membrane). Therefore, these compounds must penetrate the outer and/or cytoplasmic membrane to exert their antimicrobial action. Bacteria have developed mechanisms to prevent the antibiotic from reaching its intracellular or periplasmic target by decreasing the influx of the antimicrobial molecule [55]. This mechanism is particularly important in Gram-negative bacteria. The outer membrane of these bacteria acts as the first line of defense against the penetration of multiple toxic compounds, including several antimicrobial agents. A representative example of the efficiency of this natural barrier is the fact that vancomycin, a glycopeptide antibiotic, is not active against Gram-negative organisms due to the lack of penetration through the outer membrane. Hydrophilic molecules such as β-lactams, tetracyclines, and some fluoroquinolones are particularly affected by changes in the permeability of the outer membrane, since they often use water-filled diffusion channels, the porins, to cross this barrier [56]. There are two main ways in which porin changes can

limit drug uptake: a decrease in the number of porins and mutations that change the selectivity of the porin channel. For instance, Enterobacteriaceae are known to become resistant to carbapenems by reducing the number of porins [57].

Another phenomenon that can control the accumulation of an antibiotic is the formation of biofilm by a bacterial community. Formation of biofilms shields bacteria from the host immune system, while also providing protection from antimicrobial agents due to its thick, sticky consistency which contains polysaccharides, proteins and DNA from the resident bacteria. Thus, for an antibiotic to be effective, much higher concentrations are necessary. In addition, bacterial cells in the biofilm tend to be sessile (slow metabolism rate, slow cell division), so antimicrobials that target growing, dividing bacterial cells have little effect. Notably, it is also considered that horizontal transfer of genes is facilitated among bacteria forming biofilms due to the proximity of their cells [35].

1.3.3.2 Active drug efflux

Efflux pumps are transport proteins involved in the extrusion of toxic substrates, including all classes of clinically relevant antibiotics, from within cells to the external environment. Pumps may be specific for one substrate or may transport a range of structurally different compounds, thus being associated with multidrug resistance (MDR). Five major families of efflux pumps have been recognized: i) the major facilitator superfamily (MFS), ii) the small multidrug resistance family (SMR), iii) the resistance-nodulation-cell-division family (RND), iv) the ATP-binding cassette family (ABC), and v) the multidrug and toxic compound extrusion family (MATE). These families differ in terms of structural conformation, energy source, range of substrates that they are able to extrude and types of bacteria in which they occur. All utilize the proton motive force as an energy source, apart from the ABC family, which utilizes ATP hydrolysis to attain the export of substrates [55,58]. Although genes encoding efflux pumps can be located on MGEs, more commonly these genes are carried on the chromosome, providing the bacterium an intrinsic mechanism that allows survival in hostile environments. Some efflux pump genes are expressed constitutively, and others are induced or overexpressed under certain environmental stimuli or when a suitable substrate is present. High-level resistance is usually achieved via a mutation that modifies the transport channel. Mutant bacteria that overexpress efflux pump genes can subsequently be selected without the acquisition of new genetic material [35,58].

Tetracycline resistance is one of the classic examples of efflux-mediated AMR. Tet efflux pumps, belonging to the MFS family, are the predominant tetracycline resistance mechanism in many Gram-negative bacteria. Currently, more than 20 different *tet* genes have been described, most of which are harbored in MGEs. The Tet efflux proteins typically export and provide resistance to tetracycline, oxytetracycline and chlortetracycline, but not minocycline and tigecycline, as they are not able to use these compounds as substrates [59,60]. In addition to the tetracycline-specific transport systems, several MDR efflux pumps belonging to the RND family are able to extrude tetracyclines (including tigecycline) as part of their contribution to multidrug resistance [59].

1.3.3.3 Change of drug's targets

Multiple components in the bacterial cell may be targets of antimicrobial agents (Table 1.1). To achieve resistance, bacteria have evolved different mechanisms to protect these targets (avoiding the antibiotic to reach its binding site) or to modify them (decreasing affinity of the antibiotic molecule to the target site).

Target protection

The majority of genes involved in this resistance mechanism are located on MGEs. Examples of drugs affected by this mechanism include fluoroquinolones (via the action of *Qnr*), tetracycline (via the action of *Tet*[M] and *Tet*[O]) and fusidic acid (via the action of *Fus*B and *Fus*C).

A representative paradigm of target protection regards the fluoroquinolone resistance determinant *Qnr*. Most bacterial species encode two distinct, but homologous, type II topoisomerases, DNA gyrase and topoisomerase IV. Each of those enzymes is a heterotetramer, with gyrase being composed of two GyrA and two GyrB subunits and topoisomerase IV being composed of two ParC and two ParE subunits, in Gram-negative bacteria [61]. In order to accomplish their crucial physiological functions in most nucleic acid processes, DNA gyrase and topoisomerase IV generate transient double-stranded breaks in the bacterial chromosome. During this process genomic integrity is preserved with the formation of covalent bonds between the enzymes' active site tyrosine residues and the cleaved DNA. These complexes are called "cleavage complexes". Quinolones, also called "topoisomerase poisons", are synthetic antibacterial drugs that bind to the cleavage complexes, and as a result block the resealing of the DNA double-strand break. Quinolones not only inhibit enzymes' activity but also stabilize the complexes which subsequently serve as a physical barrier to the movement of the DNA replication fork, creating permanent double-strand DNA breaks. Those breaks additionally activate the bacterial DNA stress response. In the aforementioned ways quinolones exert their bactericidal activity [62,63]. *Qnr* genes encode proteins that compete with DNA binding to DNA gyrase and topoisomerase IV and subsequently hinder quinolones attachment to the cleavage complexes. As a result, the number of double-stranded breaks on the chromosomes is diminished and quinolone toxicity to the chromosomes restricted [64,65].

Modification of the target site

Introducing modifications to the target site is one of the most common mechanisms of antibiotic resistance in bacteria, affecting almost all families of antimicrobial compounds. Such target modifications may consist of i) point mutations in the genes encoding the target site, ii) enzymatic alterations of the binding site (e.g., addition of methyl groups), and/or iii) complete replacement or bypass of the original target [55].

i. mutations in the genes encoding the target site

A well-characterized example of mutational resistance involves DNA gyrase and topoisomerase IV, the two enzymes targeted by quinolones. Mutation in single amino acids in either one of the enzymes reduces their affinity for quinolones, which results to decreased drug binding to the cleavage complexes and further in decreased quinolone susceptibility of the bacterium. Mutations are primarily located on the amino terminal

domain of GyrA or of ParC, a region often referred to as the "quinolone-resistance-determining region (QRDR)" [66–68]. Combined mutations in both GyrA and ParC generally provide progressively higher levels of resistance. Changes in specific domains of GyrB and ParE have also been shown to cause quinolone resistance but are substantially less common in resistant isolates. The degree of resistance caused by mutation of a single amino acid in the subunits of gyrase or topoisomerase IV varies and depends on the relative sensitivities that those enzymes of a certain bacterial species present to a given quinolone [64].

ii. enzymatic alterations of the binding site

Glycopeptides, polymyxins, and most ribosome-targeting antibiotics are affected by this resistance strategy. Resistance to glycopeptides and polymyxins is achieved through the activity of enzymes that chemically alter elements of the cell envelope required for antibiotic binding. On the other hand, methyltransferases that modify rRNA elements on the ribosome confer resistance to aminoglycosides, lincosamides, streptogramins, macrolides and oxazolidinones [69].

For instance, in the case of macrolide resistance mediated by enzymatic modification of the target site, an enzyme encoded by the *erm* genes (*e*rythromycin *r*ibosomal *m*ethylation) catalyzes the mono- or demethylation of an adenine residue in position A2058 of the domain V of the 23rRNA of the 50S ribosomal subunit. Subsequently, the binding of the antimicrobial molecule to its target is impaired. Of note, since macrolides, lincosamides, and streptogramin B antibiotics have overlapping binding sites in the 23S rRNA, expression of the *erm* genes confers cross-resistance to all of them. It shall, however, be underlined that *erm*-mediated resistance carries an important bacterial fitness cost due to the less efficient translation by the methylated ribosome. Hence, although the resistant phenotype can be constitutively expressed, in most cases it is subject to strict control via a complex posttranscriptional gene regulation. Through this mechanism, bacteria growing in the absence of antibiotic produce an inactive mRNA transcript that cannot be translated into the desired methylase. Conversely, in the presence of antibiotic, the transcript becomes active, and the system is primed to confer rapid resistance. The array of compounds capable of inducing the resistant phenotype varies among different *erm* genes, but as a general rule the best inducer is erythromycin [55,70].

iii. complete replacement or bypass of the target site

Via this strategy, bacteria are capable of evolving new targets that accomplish similar biochemical functions as the original target but are not inhibited by the antimicrobial molecule. A relevant clinical example of replacement of the antibiotics' target is methicillin resistance in *S. aureus* resulting from the acquisition of the *mecA* gene. This gene is often located in a large DNA fragment designated staphylococcal chromosomal cassette *mec* (SCC*mec*) and encodes PBP2a, a PBP that has lower affinity for all β-lactams (except for last generation cephalosporins) in comparison to sensitive PBP enzymes, rendering these agents useless against MRSA [71]. Similarly, vancomycin resistance in enterococci can be achieved via modifications of the peptidoglycan structure mediated by *van* genes. These genes code for a biochemical machinery that remodels the synthesis of peptidoglycan by, *i*) changing the last D-Ala for either D-lactate (high-level resistance) or D-

serine (low-level resistance), and *ii*) destroying the "normal" D-Ala-D-Ala ending precursors to prevent vancomycin binding to the cell wall precursors [55].

Alternatively, bacteria can overproduce the antibiotic target so that higher concentrations of the antibiotic would be required to bind to the excess target in order to decrease its biological activity to a level that prevents bacterial growth. One of the best examples of this mechanism is development of resistance to trimethoprim/sulfamethoxazole (TMP-SMX). This drug impairs bacterial synthesis of purines and some important amino acids by altering the production of folate, exploiting the fact that most bacteria are unable to incorporate folate from external sources. Therefore, bacteria rely on their own biochemical machinery for folate synthesis. The synthetic pathway of folate involves two major enzymes, namely *i)* dihydropteroic acid synthase (DHPS), which forms dihydrofolate from para-aminobenzoic acid (inhibited by SMX), and *ii)* dihydrofolate reductase (DHFR), which catalyzes the formation of tetrahydrofolate from dihydrofolate (inhibited by TMP). Although development of resistance to TMP-SMX can be achieved through several strategies, a "clever" bypass strategy is the overproduction of DHFR or DHPS through mutations in the promoter region of the DNA encoding these enzymes. These mutations result in the production of increased quantities of the above enzymes, "overwhelming" the ability of TMP-SMX to inhibit folate production and permitting bacterial survival [55].

1.3.3.4 Modification of drug

One of the most successful bacterial survival strategies in the presence of antibiotics is to produce enzymes that either inactivate the drug by adding to it specific chemical groups or destroy the antibiotic molecule itself, rendering it unable to interact with its target.

Chemical alterations of the antibiotic

Enzymes that introduce chemical changes to the antimicrobial molecule mostly affect antibiotics that exert their action by inhibiting protein synthesis in the ribosome. Many types of modifying enzymes have been described, and the most frequent biochemical reactions they catalyze include *i)* acetylation (aminoglycosides, chloramphenicol, streptogramins), *ii)* phosphorylation (aminoglycosides, chloramphenicol), and *iii)* adenylation (aminoglycosides, lincosamides) [72]. Regardless of the biochemical reaction, the resulting effect is often related to steric hindrance that decreases the avidity of the drug for its target, which, in turn, is reflected in higher bacterial minimum inhibitory concentrations (MICs).

In this category of resistance mechanisms, a representative example is aminoglycoside modifying enzymes (AMEs). These enzymes covalently modify the hydroxyl (-OH) or amino (-NH_2) groups of the aminoglycoside molecule. The nomenclature to classify the multiple AMEs is based on their biochemical activity [acetyltransferase (ACC), adenyltransferase (ANT) or phosphotransferase (APH)], the site of the modification, which is represented by a number from 1 to 6 corresponding to the particular carbon on the sugar ring, and a single or double apostrophe to symbolize that the reaction occurs in the first or in the second sugar moiety, respectively. In addition, whenever there is more than one enzyme catalyzing the exact same reaction, a roman numeral is used for their differentiation. Usually, genes coding for AMEs are harbored in MGEs, but chromosomal location has also been described in certain bacterial species.

Destruction of the antibiotic

This category of resistance encompasses β-lactamases, which are discussed in detail in the following section ("1.3.4 B-lactamases").

1.3.4 B-lactamases

β-lactamases are enzymes capable of disrupting the amide bond of the β-lactam ring and therefore of rendering β-lactam antimicrobials ineffective [73]. Particularly, the cyclic amid of the β-lactam ring system is hydrolyzed and, following the opening of the nitrogen hetero cycle, the carbonyl moiety is lost, leaving the remaining structure inactive and not capable of binding to the PBP (Fig. 1.5). Of note, the hydrolytic cleavage of the β-lactam ring system is the most common resistance mechanism against β-lactams amongst Enterobacteriaceae spp. The β-lactamases, either intrinsic or acquired, that can be found in Enterobacteriaceae are presented in Figure 1.6.

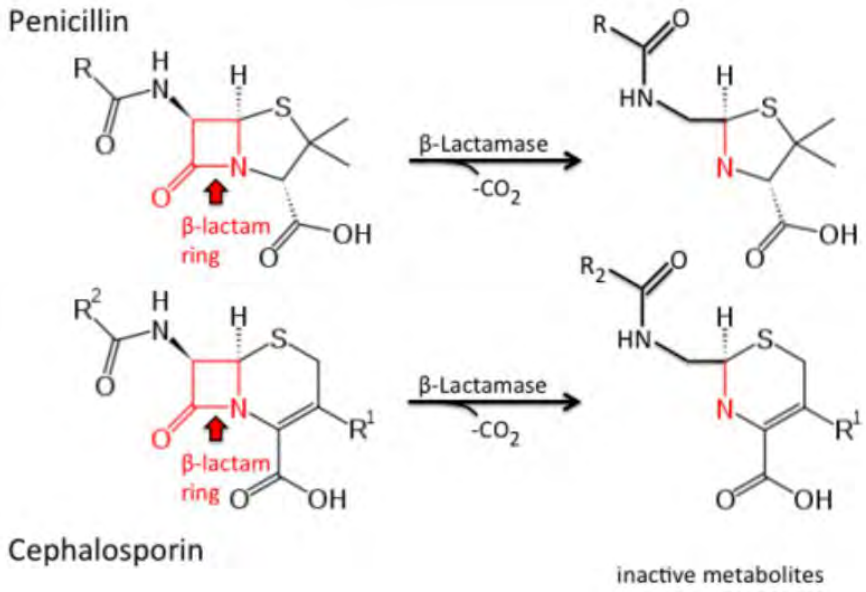


Figure 1.5. Core structures for penicillin and cephalosporin, and molecular mechanism of β-lactamase hydrolysis.

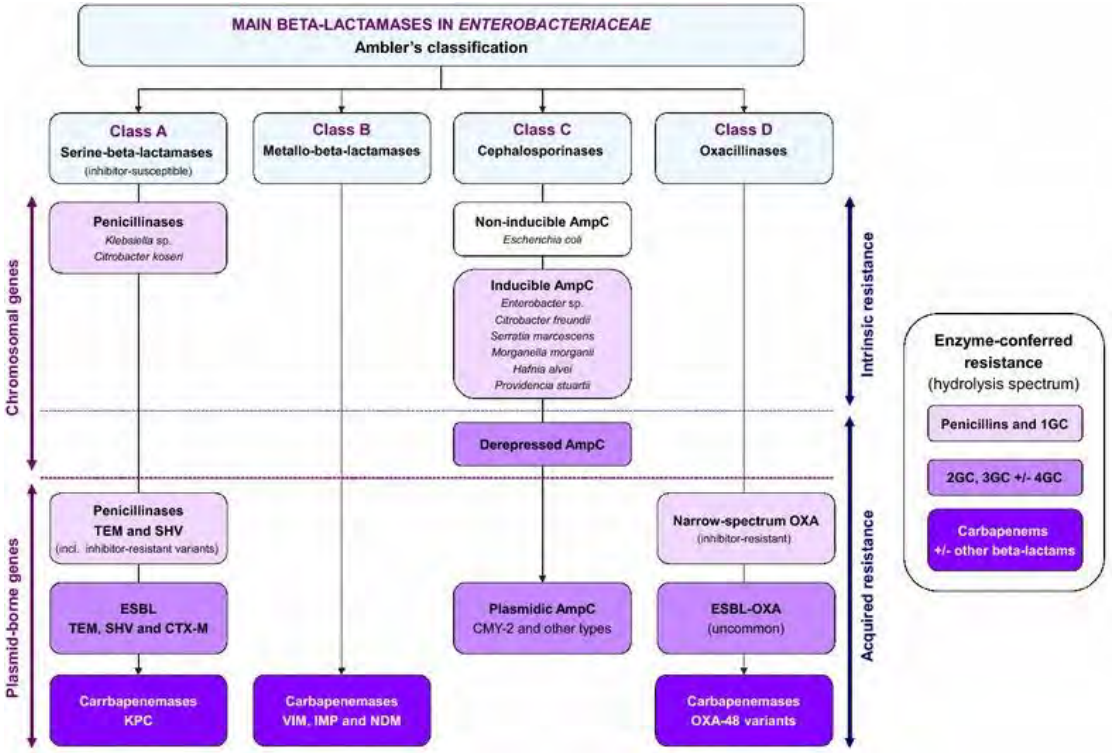


Figure 1.6. Schematic representation of the intrinsic and acquired β-lactamases in Enterobacteriaceae [74].

The first β-lactamase was identified in *Bacillus* (*Escherichia*) *coli* in 1940, prior to the release of penicillin for use in medical practice, i.e., in the absence of pressure by therapeutic antibiotics [49]. To date, more than 2000 distinct β-lactamases have been reported. Regarding nomenclature, it shall be noted, that β-lactamase numbers are assigned based on amino acid sequences and only naturally occurring β-lactamases (as opposed to laboratory-generated mutants) receive a new number [75]. Allele numbering for most β-lactamase families is designated and tracked by NCBI (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047).

Beginning in the 1970s, there have been multiple attempts to classify β-lactamases according to their substrate profile, functional and molecular characteristics [42,76–89], with the classification schemes proposed by Bush K., Jacoby G. A. and Medeiros A.A. [85] and by Ambler R.P. [79] being the most commonly used ones. The simplest classification by Ambler R.P. is based on protein sequence, grouping β-lactamases into four molecular classes, A, B, C and D, according to conserved and distinguishing amino acid motifs. Classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine, whereas class B β-lactamases are metalloenzymes that utilize at least one active site zinc ion to facilitate β-lactam hydrolysis [79,86]. Such a structural approach to classify this diverse set of enzymes is easy and least controversial, however, a functional classification, even if more subjective, provides the opportunity to corelate the enzymes to their clinical role, i.e., describe the selective resistance they provide to different classes of β-lactam antibiotics. The functional classification of β-lactamases, as revised in

2010 by Bush K. and Jacoby G. A. [86], and in comparison with previous classification schemes, is provided in Table 1.2.

Table 1.2. Classification of β-lactamases [86].

Bush-Jacoby group	Bush-Jacoby-Medeiros group	Ambler Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	NI	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	NI	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	NI	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
		B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	4	Unknown					

CA - clavulanic acid, TZB – tazobactam, NI - not included.

1.3.4.1 Extended spectrum β-lactamases (ESBLs)

Extended spectrum β-lactamases (ESBLs) are enzymes that hydrolyze most β-lactam antibiotics, including penicillins, cephalosporins and aztreonam, but not cephamycins (e.g., cefoxitin) or carbapenems and that are traditionally inhibited by β-lactamase inhibitors, including clavulanic acid, sulbactam, tazobactam, avibactam, relebactam, and vaborbactam.

The term ESBLs refers to Ambler class A, C, or D enzymes, which may be categorized as groups 1e, 2be, 2de, and 2e group, according to Bush-Jacoby classification [86,90]. Nevertheless, to date there is no consensus regarding the precise definition of ESBLs [89,91].

Among the different ESBL types, enzymes of the CTX-M (Cefotaximase-first isolated in Munich) family are the most prevalent in human, livestock and wildlife isolates [92–95]. CTX-M-type enzymes have originated from mobilization of chromosomal *bla* genes of *Kluyvera spp.* [96]. In particular, $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-3}}$ are likely to have originated from *K. ascorbate* [97,98], $bla_{\text{CTX-M-14}}$ from *K. georgiana* [99], and $bla_{\text{CTX-M-37}}$ from *K. cryocrescens* [100]. CTX-Ms subsequently diverged by mutations over time and many different variants arose. Most enzymes of the family can be clustered into five groups based on sequence homologies: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. The most common CTX-M-1 group is CTX-M-15, followed by CTX-M-3 and CTX-M-1, whereas in the CTX-M-9 group, CTX-M-9, CTX-M-14 and CTX-M-27 are often reported [92].

In contrast to the CTX-M enzymes, which are, as aforementioned, closely related to bacterial chromosomal β-lactamases, the SHV-, TEM- and OXA-type ESBL enzymes derived from the classical (European and North American) plasmid-encoded penicillinases SHV-1, TEM-1 and TEM-2 and OXA-10, respectively. Until the end of the 1990s most of the ESBLs detected in human isolates were SHV and TEM types, however during the last few years their occurrence has diminished.

The first β-lactamase of the SHV family was identified in 1972 in *E. coli* and was denominated SHV-1, referring to sulfhydryl-variable, as it was assumed that the inhibition of its activity by *p*-chloromercuribenzoate was substrate-related, and was variable according to the substrate used for the assay [101]. However, this was never confirmed. As for the first SHV ESBL, SHV-2, was identified in 1983, when $bla_{\text{SHV-2}}$ was detected and characterized in a German clinical strain of *Klebsiella ozaenae* [102]. The most prevalent SHV ESBL variant today is SHV-12.

TEM-type ESBLs are derivatives of TEM-1 and TEM-2 β-lactamases. TEM-1 was first reported in 1965 from an *E. coli* isolate from a patient in Athens, Greece, named Temoneira [103]. Under selective pressure, TEM genes evolved, giving rise to point mutant derivatives that confer resistance to expanded-spectrum cephalosporins or to β-lactam – clavulanic acid combinations. In 1984, *K. pneumoniae* isolates harboring a novel plasmid-mediated β-lactamase were detected in France [104,105]. The enzyme was originally named CTX-1 because of its enhanced activity against cefotaxime, but is now termed TEM-3, since it differs from TEM-2 by only two amino acid substitutions [106]. TEM-3 was thought to be the first TEM-type β-lactamase that displayed the ESBL phenotype. Revision of the data, though, reveals that TEM-3 may not have been the first TEM-type ESBL. *K. oxytoca* harboring a gene located on a plasmid encoding ceftazidime resistance, was first isolated in Liverpool, England, in 1982 [107]. The responsible β-lactamase was the today called TEM-12 and is most probably the first TEM ESBL ever detected.

In 1974 Hedges et al. [108] supported that plasmid-encoded β-lactamases in Gram-negative bacilli could be divided into two types, the TEM type, and a type with a distinguishable ability to hydrolyze oxacillin. Among this second type of enzymes three distinct oxacillinases

(OXA-1, OXA-2, OXA-3) were later identified, composing the first variants of the OXA family of β-lactamases [78]. At the same time, Jacoby G.A. [109] identified a group of plasmid-borne β-lactamases in *Pseudomonas aeruginosa,* which were named PSE (Pseudomonas-specific enzymes) as they were falsely considered to be specific to *Pseudomonas aeruginosa* [110,111]. The PSE group constituted of four enzymes, PSE-1, PSE-2, PSE-3 and PSE-4. In 1983 Philippon et al. [112] noted that PSE-2 bears the ability to hydrolyze oxacillin and displayed a close relationship to OXA β-lactamases based on genome sequencing. PSE-2 had some homology to OXA-2 [81] but was much closer in structure to OXA-5 [113]. In 1993, a report about an enzyme of a *Pseudomonas aeruginosa* isolate from blood cultures of a burn patient in Turkey was published [114]. This new enzyme conferred high levels of resistance to ceftazidime. Resistance was transferable, and when sequenced, the enzyme had two amino acid changes compared to the PSE-2. Given the data which indicated that PSE-2 really was an OXA enzyme [81], Hall et al. suggested that PSE-2 was renamed to OXA-10 and the new enzyme they detected was named OXA-11 [114]. OXA-11 was the earliest OXA enzyme that had become, through mutation, an extended-spectrum β-lactamase (ESBL). OXA-type ESBLs differ from the CTX-M, SHV and TEM enzymes as they are not inhibited by clavulanic acid [86].

Other, less commonly identified ESBL families are summarized in Table 1.3.

Table 1.3. Uncommon ESBL families (adapted from [92]).

Family	Nomenclature	Characteristics
IRT	Inhibitor-resistant TEM	TEM variants that are resistant to inhibition by clavulanate and sulbactam, but do not have ESBL phenotype
CMT	Complex mutant derived from TEM-1	TEM variants that are resistant to inhibition by clavulanate and sulbactam and also have ESBL phenotype
GES	Guiana-extended spectrum	More prevalent in *P. aeruginosa* than Enterobacterales Some variants also hydrolyze carbapenems
PER	*Pseudomonas* extended resistant	More prevalent in *P. aeruginosa* and A. baumannii than Enterobacterales Inhibition by newer β-lactamase inhibitors is variable
VEB	Vietnam extended-spectrum β-lactamase	Preferentially hydrolyses ceftazidime and aztreonam compared with cefotaxime Inhibition by newer β-lactamase inhibitors is variable
BEL	Belgium extended β-lactamase	Preferentially hydrolyses ceftazidime and aztreonam compared with cefotaxime
TLA	Named after the Tlahuica Indians (Mexico), from whom the first isolate was obtained	Preferentially hydrolyses ceftazidime and aztreonam compared with cefotaxime
SFO	From *Serratia fonticola*	Inducible
OXY	From *Klebsiella oxytoca*	Chromosomally encoded

1.3.4.2 AmpC β-lactamases

AmpC β-lactamases belong to Bush-Jacoby-Medeiros functional group 1 and Ambler class C [79,85]. These enzymes confer resistance to penicillins, early and oxyimino- cephalosporins (such as ceftazidime, cefotaxime and ceftriaxone), monobactams, as well as cephamycins (such as cefoxitin). Although they do not hydrolyze carbapenems effectively (with some

exceptions, such as CMY-10 or ACT-28), when the AmpC enzyme is hyper-produced in strains with additional resistance mechanisms (e.g., repression or inactivation of porins), clinically important resistance levels can be achieved [115]. Most class C enzymes are not inhibited by inhibitors such as clavulanic acid, sulbactam, and tazobactam, however, they are susceptible to the newer inhibitors avibactam, relebactam, and vaborbactam [116]. In addition, these enzymes are inhibited by cloxacillin and boronic acid [117]. ACC-1 is an exceptional AmpC β-lactamase as it does not confer resistance to cephamycins and is actually cefoxitin inhibited.

In some bacterial species AmpCs are resident enzymes, encoded by chromosomal genes (cAmpCs). These species are typically characterized by a low production of the enzyme because the AmpC gene is regulated by a weak promoter and a strong attenuator system (repression state). Certain β-lactams can, more or less effectively, induce AmpC production, however when the compound disappears such induction ceases. Mutations in regulator genes (mostly *ampD* and *ampR*) can lead to high-level production of the enzyme, even if there is no inducing β-lactam (derepressed strain) [115]. Enterobacteriaceae with inducible chromosomal AmpC β-lactamases include *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens*, *Morganella morganii*, and *Providencia stuartii*. *E. coli* also possess a cAmpC β-lactamase which is, however, regulated by a growth rate-dependent attenuation mechanism and not by induction, since *ampR* is missing [118].

Enterobacteriaceae can acquire cephamycin/cephalosporin-resistance via plasmid-mediated AmpC β-lactamases (pAmpCs), derived from the mobilization of cAmpCs [118]. CMY-type cephamycinases are the most commonly encountered pAmpCs. Six current variants (CMY-1, -8, -9, -10, -11, and -19) are related to the cAmpC of *Aeromonas* spp., while the remainder (including CMY-2, the most common pAmpC β-lactamase worldwide) are related to the cAmpC of *Citrobacter freundii*. Other pAmpC families include FOX (providing resistance to cefoxitin), MOX (providing resistance to moxalactam), LAT (providing resistance to latamoxef), ACT (AmpC type β-lactamase), ACC (Ambler class C β-lactamase), MIR-1 (Miriam Hospital in Providence, Rhode Island), DHA (Dhahran hospital in Saudi Arabia), BIL-1 (patient of first detection named Bilal).

1.3.4.3 Carbapenemases

Carbapenemases belong to Bush-Jacoby functional groups 2f, 2df and 3, and to Ambler classes A, B, and D (Table 1.2, Table 1.4) [119]. Apart from hydrolyzing penicillins, cephalosporins and monobactams, they present the important ability of degrading carbapenems. Carbapenemases' hydrolytic mechanisms involve either β-lactam acylation at an active site serine for group 2f and 2df enzymes, or the presence of at least one divalent Zn^{2+} atom at the active site for group 3 enzymes [120].

Table 1.4. Substrate and inhibition profiles of carbapenemases (adapted from [119]).

Molecular class (Ambler)	Functional group (Bush-Jacoby)	Enzyme Family	Hydrolysis profile[1]					Inhibition profile[2]	
			Penicillins	Early cephalosporins	Extended spectrum cephalosporins	Aztreonam	Carbapenems	EDTA	Clavulanic acid
A	2f	NMC-A	+	+	+	+	+	–	+
		IMI	+	+	+	+	+	–	+

		SME	+	+	±	+	+	−	+
		KPC	+	+	+	+	+	−	+
		GES	+	+	+	−	±	−	+
B1	3	IMP	+	+	+	−	+	+	−
		VIM	+	+	+	−	+	+	−
		GIM	+	+	+	−	+	+	−
		SPM	+	+	+	−	+	+	−
D	2df	OXA	+	+	±	−	±	−	±

[1] +, strong hydrolysis ±, weak hydrolysis; −, no measurable hydrolysis

[2] +, inhibition; ±, variable inhibition among β-lactamase family members; −, no inhibition

Serine (class A) carbapenemases include the following major families: IMI (Imipenemase), NMC-A (Non-metallo-carbapenemase A), SME (*Serratia marcescens* enzyme), GES (Guiana extended spectrum) and KPC (*Klebsiella pneumoniae* carbapenemase). Most of these enzymes are inhibited by the clinically available β-lactamase inhibitors, such as clavulanic acid, tazobactam and sulbactam [121]. Among class A β-lactamases, KPC is the only non-species-specific family of enzymes. Notably, following its emergence in the late 1990s, the KPC family disseminated remarkably, becoming the most common carbapenemase detected globally nowadays [116]. Despite its broad-spectrum hydrolytic capabilities that encompass all classes of β-lactams, including monobactams, KPC is never found as a single enzyme in an isolate, but commonly co-occurs with TEM-1, SHV-11 and/or SHV-12. Less frequently, KPC can be co-produced with OXA enzymes or ESBLs in the CTX-M family [120].

Metallo-β-lactamases (MBLs) or class B carbapenemases include the VIM (Verona integron-encoded metallo-β-lactamase), IMP (Imipenemase), GIM (German imipenemase), NDM (New Delhi metallo-β-lactamase) and SIM enzymes (Seoul imipenemase). These enzymes possess the characteristic hallmark of being universally inhibited by EDTA as well as other chelating agents of divalent cations [122]. As aforementioned for KPC, MBLs are never reported as a single β-lactamase in an organism. Regardless of the MBL, the most common companion enzymes are TEM-1, followed by SHV or CTX-M β-lactamases [120].

Within the class D OXA family of serine β-lactamases, only a small fraction has a functional role as a carbapenemase. Among these are OXA-23, OXA-40, and the increasingly prevalent OXA-48, with its related variants (OXA-162, OXA-181 and OXA-232). The OXA carbapenemases generally have hydrolytic activity against penicillins and broad-spectrum carbapenems and are poorly inhibited by β-lactamase inhibitors, except for avibactam. OXA-48, in particular, hydrolyzes a narrower spectrum of β-lactams, with clinically relevant hydrolysis of penicillins and imipenem and lower hydrolysis of meropenem [123]. OXA-48 producers may be fully susceptible to cephalosporins [124]. Overall, the vast majority of OXA carbapenemases have been discovered in the opportunistic gram-negative pathogen *Acinetobacter baumannii* [119].

1.4. Antimicrobial Susceptibility Testing

The performance of antimicrobial susceptibility testing (AST) is important to confirm susceptibility of an isolate to chosen antimicrobial agents or to detect resistance. It is also particularly significant among species that may possess acquired resistance mechanisms, such as Enterobacteriaceae [125]. Organisms are classified as "susceptible", "susceptible-increased exposure" (formerly termed intermediate), or "resistant" [126].

The methods most commonly used for AST are outlined below.

1.4.1. Disk diffusion test

Initially, an inoculum of the organism to be tested is prepared by the direct colony suspension method, mixing colonies from an overnight culture (on non-selective media) in saline to the density of a 0.5 McFarland turbidity standard, which corresponds to approximately $1–2\times10^{8}$CFU/mL. Subsequently, using a sterile cotton swab, inoculum is evenly spread over the entire surface of a Mueller-Hinton agar plate, ensuring that there are no gaps between streaks. Commercially available, fixed concentration, paper antibiotic disks are placed on the inoculated agar surface and the plates are incubated for 16-24 h at 35 ± 1°C. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. Consequently, the zone diameters of each drug are interpreted into susceptibility categories according to breakpoint tables published by organizations (CLSI, EUCAST).

The results of the disk diffusion test are "qualitative" since the MICs are not determined by this method [125]. The test is well-standardized and widely used due to its low cost, ease of performance and applicability for numerous bacterial species and antibiotics. Its main disadvantages are the inability to determine the MIC and delays in issuing the results [127].

1.4.2. Broth and agar dilution tests

Broth macro- and microdilution and agar dilution methods are used to determine the MICs of antimicrobial agents (i.e., the lowest concentrations at which the agents completely inhibit the growth of microorganisms). The MIC value serves as the basis for assessing the susceptibility category of the pathogen to a given antibiotic. Broth dilution methods additionally allow the determination of the minimum bactericidal concentration, which is the lowest concentration of an antimicrobial substance that kills 99.9% of bacteria [127].

The macrodilution method, also known as the in-tube dilution test, uses serial two-fold dilutions of an antimicrobial substances (e.g., 1, 2, 4, 8, and 16 μg/mL) in liquid media dispensed in tubes. A bacterial suspension of $1–5\times10^{5}$CFU/mL is added to the tubes and following a 24h incubation at 37 °C bacterial growth is measured by turbidity, allowing visual determination of the MIC values. The lowest concentration of the antibiotic that prevents growth represents the MIC. The advantage of this technique is its quantitative result (i.e., the MIC). The main disadvantages are the time-consuming, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large number of reagents and space required for each test [125,127].

Another macrodilution method is the time-kill test. This test allows monitoring of the effect of different concentrations of antimicrobial substances by examining the rate at which antimicrobials lead to bacterial death - i.e., the bactericidal activity of antimicrobial agents is determined depending on the concentration and time. For 24 h, colonies of the tested organism are counted on agar plates at regular intervals in order to determine bacterial viability. For 24 h, changes in log CFU/mL are also monitored to determine the rate of bacterial growth. Based on the results, experimental curves which represent the absence of growth or the killing effect can be constructed and provide insight into the interaction between the bacteria and the antimicrobial agent [127].

MIC determination can also be conducted by broth microdilution, a standardized, accurate, and inexpensive method. The test constitutes a miniaturization of broth macrodilution and is performed in 96-well microtiter plates, allowing testing of several antimicrobial substances in a row and eight series of two-fold dilutions of antimicrobial agents in one plate. The procedure followed for performing a broth microdilution test is depicted in Fig 1.7. Several broth microdilution systems are commercially available. Thus, the reproducibility and the economy of reagents and space are the major advantages of the method.

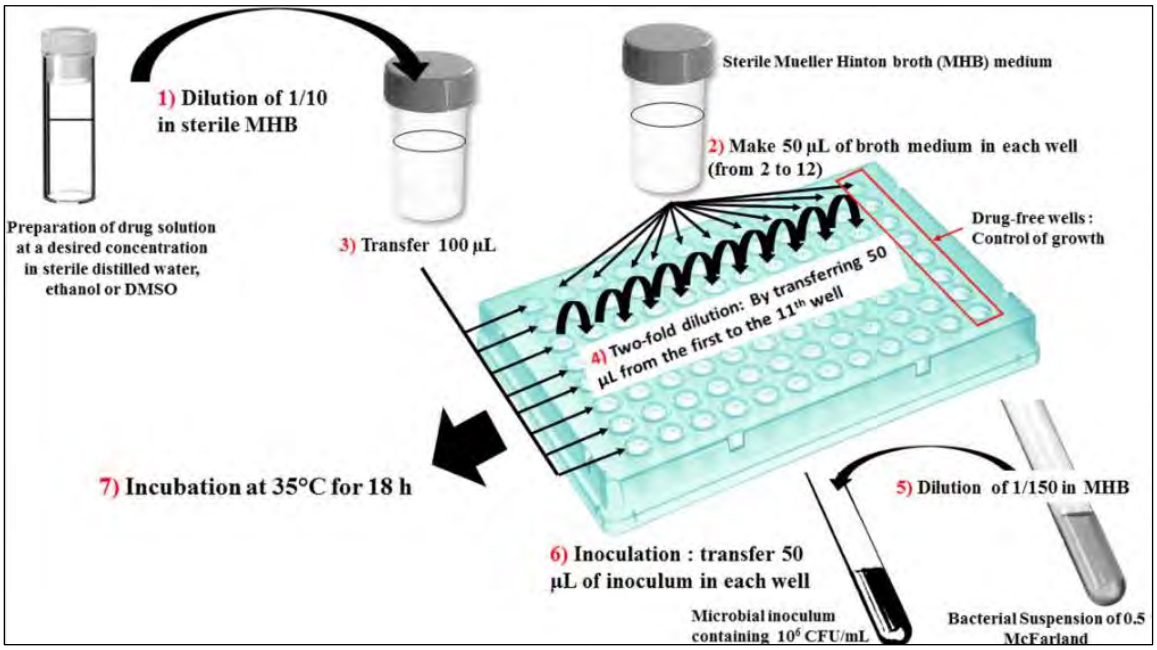


Figure 1.7. Broth microdilution for AST [128].

The agar dilution method involves adding different concentrations of an antimicrobial agent to a non-selective medium before solidification. Afterwards, a standardized bacterial inoculum is inoculated on the agar surface. Following overnight incubation, plates are evaluated visually, determining whether growth has occurred at the inoculated sites. The lowest concentration of antibiotic that prevents bacterial growth is considered to be the MIC. This method allows simultaneous testing of different bacterial isolates against a single compound and is particularly preferred over broth dilution if the compound tested masks the detection of microbial growth in the liquid medium with its coloring. It is also often recommended as a standardized method for fastidious organisms [128].

1.4.3. Antimicrobial gradient method

The antimicrobial gradient method combines the principles of dilution methods with those of diffusion methods, in order to determine the MIC value. A short plastic or paper strip containing a gradient of antimicrobial from low to high concentrations is placed on inoculated agar. On the standardized 100 mm Petri dish, two strips may be placed, while on the larger 150 mm Petri dish, up to six antibiotics may be tested simultaneously. Following incubation, the apex of the teardrop zone of inhibition indicates the MIC of the organism for the tested agent and is measured using the labelled concentrations on the strip (Fig. 1.8). Plenty of advantages of the gradient test are known including its simple performance, flexibility in the testing of any combination of antibiotics, and the fact that it does not require expertise and special technologies. However, its use is suited when only few antibiotics are to be tested per strain, since the price of each strip is relatively high, compared with the price of disks used in the disk diffusion method. The incubation time of 16–24 h for gradient tests may represent another disadvantage, as more rapid automated systems are available for the determination of MIC [128,129].

Figure 1.8. Antimicrobial gradient method of a *Staphylococcus aureus* strain showing results for vancomycin (VA) and teicoplanin (TP). The MICs are read off of the strip where the bottom portion of the ellipse intersects with the strip (white arrows) [130].

1.4.4. Chromogenic Agar Media

The main purpose of the use of chromogenic media is to enable rapid detection of resistant microorganisms. The target organisms are characterized by specific enzyme systems that metabolize the substrates to release the chromogen. The chromogen can then be visually detected by direct observation of a distinct color change in the medium. Thus, these selective and differential media enable target pathogens to grow as colored colonies. Compared with the use of conventional culture media, the use of chromogenic agar often reduces the costs and labor time. The sensitivity and specificity of chromogenic media depend on the manufacturer and the type of microorganism detected. Therefore, additional confirmation of the resistant bacteria is usually needed [127]. A variety of different

chromogenic media for the detection of clinically important resistant pathogens including MRSA, VRE, ESBL, CPE and colistin-resistant Gram-negative bacteria have been developed and are commercially available.

1.4.5. Automated and Semi-Automated Devices

Due to the need for fast and reliable microbial identification and AST, automated and semi-automated devices are becoming increasingly common to reduce time to result, increase efficiency, and improve cost-effectiveness. Various test systems such as the VITEK 2 (bioMérieux, France), MicroScan Walkaway (Dade-Behring MicroScan, Deerfield, IL, USA), and Phoenix system (BD Diagnostic Systems, Baltimore, MD, USA) are widely used. These instruments use optical systems for recording and determining bacterial growth and antimicrobial susceptibility and can produce results in a shorter time (6–12 h) than conventional manual AST methods [127].

The VITEK 2 System, for example, is an AST system based on broth microdilution that uses 64-well plastic cards containing 17–20 antimicrobial agents (AST card). It measures changes in turbidity over time (growth curve), comparing a growth control well with wells containing various drug concentrations. If the bacterial isolate is not previously identified, an ID card that identifies the microorganism based on biochemical reactions and nutrient usage can be used. AST results are reported in 4–18 h, containing MIC and category of susceptibility, while the detection of AMR is facilitated by the Advanced Expert System (AES). This expert system uses specific rules or algorithm to classify isolates' susceptibility profile or phenotype, provide result interpretations and recommend correction if it detects a single MIC inconsistency with the bacterial identification compared to the internal database [131].

1.4.6. Molecular-Based Techniques

The most widely used nucleic acid amplification-based method for the detection of specific resistance genes is polymerase chain reaction (PCR). Both conventional and real-time PCR rely on the amplification of nucleic acid sequences that encode resistance to an antibiotic. Multiplex assays for simultaneous testing of multiple genetic determinants in various bacterial species have also been developed, such as multiplex assays for identifying numerous cephalosporinase and carbapenemase encoding genes.

DNA-microarrays are also frequently employed to identify the presence of specific nucleic acid sequences using complementary short oligonucleotides immobilized on a solid surface. Since these oligonucleotides can be assembled onto solid surfaces in close proximity, this method detects numerous sequences in a single assay, which allows concurrent, in parallel detection of different pathogens and of vast numbers of different resistance genes, as well as detection of numerous distinct resistance mechanisms or variants of a single mechanism present in bacterial isolates, as opposed to PCR-based approaches [132].

Both the aforementioned molecular-based methods are direct, rapid, highly sensitive, and specific. However, it should be noted that the presence of a resistance marker does not necessarily correlate with phenotypic resistance. Additionally, the extent and intensity of gene expression are important parameters, as some genes need different expression levels to produce resistance. A potential solution to this issue would be the use of quantitative

reverse transcription PCR, which relies on the measurement of gene transcripts (RNA levels) instead of the presence of a gene. Another drawback is that these methods can only detect resistances that are searched for, and not novel or uncharacterized mechanisms of resistance, which could lead to false-negative results and misclassification of resistant isolates as susceptible. A final drawback is that these methods are not capable of defining MIC values and, thus, their results have to be assessed along with phenotypic data to be useful [127].

In addition, Whole-Genome Sequencing (WGS) is more and more frequently applied in AST, offering a vast amount of information for identifying and characterizing pathogens and permitting high-resolution tracing of microbial transmission events and rapid, accurate identification of outbreaks. WGS provides the opportunity to detect novel molecular markers of resistance and can therefore enhance surveillance capacity to better inform strategies to tackle AMR. This feature of WGS could, moreover, contribute to the creation of comprehensive databases of all species-specific resistance factors and make in silico AMR detection possible. Despite all of the advantages, WGS is not routinely performed in clinical microbiology mainly due to its elevated cost compared with other techniques [127].

1.4.7. Mass spectrometry

Finally, mass spectrometry analysis has also been proposed for the rapid detection of antimicrobial resistance. Two main technologies can be used, liquid chromatography-tandem mass spectrometry (LC-MS/MS) and Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS, the most commonly employed of the two, is a rapid and simple procedure that combines the universal advantages of phenotypic assays with the rapidity and accuracy of molecular assays. It can utilize three main approaches for the detection of antimicrobial resistance: measuring antibiotic modifications due to the enzymatic activity of bacteria, analysis of the peak patterns of bacteria, and semi-quantification of bacterial growth in the presence of a given antibiotic. Several assays have, thus, been developed for the detection of selected resistance mechanisms (e.g., carbapenemase production) and others for universal growth-based phenotypic AST [including MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA), Minimal profile change concentration (MPCC), Stable-isotope labeling, Direct-on-target microdroplet growth assay (DOT-MGA)]. MALDI-TOF MS has several advantages such as the possibility to use solid samples, the low cost per test, the possibility of identifying microorganisms and detecting antimicrobial resistance with the same equipment, the simplicity of the procedure and the automated interpretation of the spectra generated. The major disadvantages are the initial high cost of the equipment, the need for a high bacterial load which hinders the use of the technique with all clinical samples and the lack of commercially available certified kits [133,134].

1.5. Detection of ESBL, AmpC and CP Enterobacteriaceae

1.5.1 ESBL screening and confirmatory tests

Detection of ESBL-producing Enterobacteriaceae relies on their non-susceptibility to indicator oxyimino-cephalosporins, followed by phenotypic and, in some cases, genotypic confirmation tests.

According to EUCAST, the recommended methods for ESBL initial screening are broth dilution, agar dilution, disk diffusion or automated systems. Both cefotaxime (or ceftriaxone) and ceftazidime sould be used as indicator cephalosporins since there may be large differences in the MICs of these compounds for different ESBL-producing isolates. ESBL screening can also be conducted using chromogenic culture media, which provide presumptive identification of ESBL-producing Enterobacteriaceae directly from clinical samples within 24 h. Currently, several commercial chromogenic media are available, such as ChromID (bioMérieux), CHROMagar (CHROMagar), Brilliance ESBL (Oxoid Ltd.), BD Drigalski, Hardy CHROM ESBL agar (Hardy Diagnostics) and Colorex ESBL (E&O Laboratories Ltd) [135].

For the confirmation of ESBL production, phenotypic methods exploiting the fact that ESBLs are inhibited by traditional β-lactamase inhibitors, such as clavulanic acid, are applied. In particular, the following tests can be performed [136]:

- Combination disk test (CDT): Disks or tablets containing the cephalosporin alone (cefotaxime, ceftazidime, cefepime) and disks or tablets containing the cephalosporin in combination with clavulanic acid are applied in a petri dish. After incubation, the test is considered positive if the inhibition zone around the cephalosporin disk or tablet combined with clavulanic acid is ≥5 mm larger compared to the zone around the disk or tablet with the cephalosporin alone.
- Double disk synergy test (DDST): Disks containing cephalosporins (cefotaxime, ceftazidime, cefepime) are applied next to a disk with clavulanic acid (amoxicillin-clavulanic acid) in a petri dish. The distance between the disks should optimally be 20mm centre-to-centre for cephalosporin 30 μg disks; however it may be reduced (15 mm) or expanded (30 mm) for strains with very high or low levels of resistance, respectively. Following incubation, the test is considered positive when the inhibition zones around any of the cephalosporin disks are augmented or there is a “keyhole” in the direction of the disk containing clavulanic acid.
- Gradient test: Etests for phenotypic confirmation of ESBL production are commercially available and should be performed according to the manufacturers’ instructions. A two-sided strip that contains a cephalosporin antibiotic (cefotaxime, ceftazidime, cefepime) on the one end and the same antibiotic with clavulanic acid on the other end is applied on a petri dish. After incubation, a reduction equal to or greater than eight times in the MIC of the cephalosporin combined with clavulanic acid compared to the MIC of the cephalosporin alone refers to ESBL production. Similar to the double-disk synergy test, a phantom zone or deformed ellipse below the clavulanic end also indicates a positive result.

- Broth microdilution: Broth microdilution is performed with cation-adjusted Mueller-Hinton broth containing serial two-fold dilutions of cefotaxime, ceftazidime and cefepime at concentrations ranging from 0.25 to 512 mg/L, with and without clavulanic acid at a fixed concentration of 4 mg/L. The test is positive if a reduction equal to or greater than eight times is observed in the MIC of any of the cephalosporins combined with clavulanic acid compared with the MIC of that cephalosporin alone, while in all other cases the test result is interpreted as negative.
- Biochemical (colorimetric) tests: The colorimetric approach for the detection of ESBL activity relies on obtaining a variation in the color of the reagent medium resulting from a hydrolytic activity that modifies the chemical composition of the medium. This variation can be detected by eye [137]. The ESBL NDP test and the β-LACTA test are currently available.

It shall be noted that high-level expression of AmpC β-lactamases may mask the presence of ESBLs. As such, for some Enterobacteriaceae species (*Enterobacter* spp., *Serratia* spp., *Citrobacter freundii*, *Morganella morganii*, *Providencia* spp, *Hafnia alvei*), which commonly present cephalosporin resistance due to the derepression of their chromosomal AmpC β-lactamase, cefepime can be used in phenotypic testing with clavulanic acid given that it is stable to AmpC hydrolysis. Cefepime may be employed in the CDT, DDST, gradient test or broth dilution test formats. Alternatively, cloxacillin, a good inhibitor of AmpC enzymes, can be used on the CDT or DDST [136].

The presence of ESBLs may also be masked by carbapenemases, such as MBLs or KPCs (but not OXA-48-like enzymes), and/or by severe permeability defects. In these cases, molecular methods should be used for ESBL detection [136].

1.5.2. AmpC screening and confirmatory tests

According to EUCAST, the recommended method for AmpC initial screening consists of the evaluation of the susceptibility to cefoxitin and ceftazidime and/or cefotaxime. A cefoxitin MIC >8 mg/L (zone diameter<19mm) combined with phenotypic resistance to ceftazidime and/or cefotaxime (as defined by breakpoints) indicate AmpC production. However, this method does not detect ACC-1, a plasmid-mediated AmpC that does not hydrolyze cefoxitin. It should also be noted that cefoxitin resistance may be due to porin deficiency. Susceptibility to cefepime together with resistance to cefotaxime and/or ceftazidime is another phenotypic indicator of AmpC, although less specific [136].

Phenotypic AmpC confirmation tests are generally based on the inhibition of AmpCs by either cloxacillin or boronic acid derivatives. In particular, the following tests can be performed [136,138,139]:

- Phenyl boronic acid inhibitor-based method: A solution of 120mg of phenyl boronic acid and 3ml dimethyl sulfoxide (DMSO) and 3ml of distilled water is prepared. Then 20μl of the solution is dispensed onto disks containing 30μg of cefotetan. Disks are allowed to dry for 30 min. Mueller-Hinton agar is inoculated by the standard disk diffusion method and a disk containing 30μg of cefotetan and a disk containing 30μg of cefotetan and 400μg of boronic acid are placed onto the agar. After incubation, an organism that

demonstrates a zone diameter around the disk containing cefotetan and boronic acid ≥5mm than the zone diameter around the disk containing cefotetan alone is considered an AmpC producer.

- Cefoxitin Cloxacillin-Double disc synergy test (CC-DDS): A disks or tablet containing cefoxitin alone and a disk or tablet containing the cefoxitin in combination with cloxacillin are applied in a petri dish. After incubation, the test is considered positive if the inhibition zone around the cefoxitin disk or tablet combined with cloxacillin is ≥5 mm larger compared to the zone around the disk or tablet with cefoxitin alone.
- AmpC Tris-EDTA disc test: The test is based on the use of Tris–EDTA to permeabilize the bacterial cell and release β-lactamases. Tris-EDTA disks are prepared by adding 20µl of a 1:1 mixture of saline and Tris–EDTA to sterile filter paper disks. Disks are allowed to dry and several colonies of the organism to be tested are applied to a disk. The disk is then placed on an inoculated Mueller-Hinton agar plate along with a 30µg cefoxitin disk. The inoculated disk must be positioned nearly touching the antibiotic disk with its inoculated surface coming in contact with the agar surface. After incubation, an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin, is considered as AmpC positive.
- Disk approximation - Induction based method: A Mueller-Hinton agar plate is inoculated with 0.5 McFarland bacterial suspension. A 30µg ceftazidime disk is placed at the center of the plate as the reporter substrate, then 10µg imipenem, 30µg cefoxitin, and 20/10µg amoxicillin/clavulanate disks are placed at a distance of 20mm from the ceftazidime disk as the inducing substrates. After incubation, any blunting or flattening of the zone of inhibition between the ceftazidime disk and the inducing substrates (imipenem, cefoxitin and amoxicillin/clavulanate disk) is considered as a positive result for AmpC production.
- Gradient test: Etests impregnated with cefotetan on one end and cefotetan–cloxacillin on the other end are commercially available for phenotypic confirmation of AmpC production and should be performed according to the manufacturers' instructions.

Overall, it shall be noted that boronic acid derivatives also inhibit class A carbapenemases, as well as some class A penicillinases (e.g., K1 in *K. oxytoca*). Commercially available tests, such as the AmpC gradient test (BioMérieux), can also be used. For *E. coli* and *Shigella* spp., however, molecular methods shall be applied for the discrimination between acquired AmpC and constitutive hyperproduction of the chromosomal AmpC, since AmpC confirmation tests cannot differentiate these two.

1.5.3. CPE screening and confirmatory tests

Evaluation of susceptibility to carbapenems constitutes the initial screening step for CPE detection. Meropenem offers the best combination of sensitivity and specificity, while ertapenem is the most sensitive carbapenem but has low specificity, especially in species such as *Enterobacter* spp., due to its instability to ESBLs and AmpCs in combination with porin loss, i.e., isolates with these resistance mechanisms may be ertapenem resistant without harboring a carbapenemase. Since carbapenem MICs for carbapenemase-producing

Enterobacteriaceae may be below the clinical breakpoints, epidemiological cut-off (ECOFF) values are used for screening purposes (Table 1.5) [136].

Table 1.5. Clinical breakpoints and screening cut-off values for carbapenemase-producing Enterobacteriaceae according to EUCAST methodology.

Carbapenem	MIC (mg/L)		Disk diffusion zone diameter (mm) [1]	
	S/I breakpoint	Screening cut-off	S/I breakpoint	Screening cut-off
Meropenem	≤2	>0.125	≥22	<28 [2]
Ertapenem	≤0.5	>0.125	≥25	<25

[1] Refers to zone diameter around 10 μg disks.

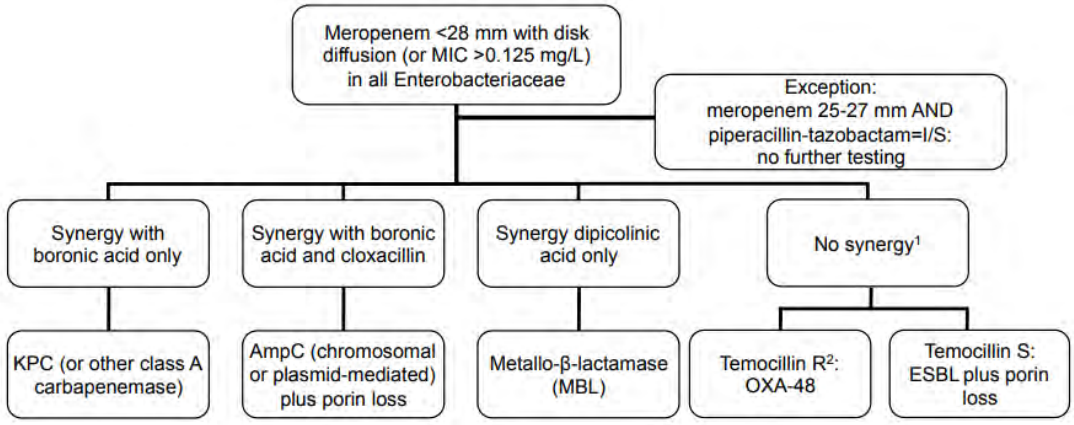
[2] Isolates with 25-27mm zone only need to be investigated for carbapenemase-production if they are resistant to piperacillin-tazobactam and/or temocillin (temocillin contributes more to the specificity). Investigation for carbapenemases is always warranted if zone diameter of meropenem is <25mm.

CPE initial screening can also be conducted using commercially available chromogenic culture media [e.g., chromID CARBA (BioMérieux), CHROMagar KPC (CHROMagar), *Brilliance* CRE (Oxoid)], which provide presumptive identification directly from clinical samples within 24 h.

After the detection of reduced susceptibility to carbapenems, phenotypic methods for detection of carbapenemases should be applied. In particular, the following tests can be performed [136]:

- Combination disk tests: Following the principals that boronic acid inhibits class A carbapenemases (although data beyond KPC are not sufficient), and dipicolinic acid as well as EDTA inhibit class B carbapenemases, test kits with disks or tablets containing meropenem and these inhibitors are commercially available. Despite avibactam is known to inhibit OXA-48, this compound has yet not been included in test panels. Temocillin high-level resistance (MIC >128 mg/L) has been proposed as a phenotypic marker for putative OXA-48-like carbapenemase producers. However, this marker is not specific for OXA-48-type carbapenemases, as other resistance mechanisms might confer this phenotype. Cloxacillin, an AmpC inhibitor, has been added to the tests to differentiate between AmpC hyperproduction with porin loss and carbapenemase production. Interpretation of the results of these tests is based on the algorithms presented in Figure 1.9 and Table 1.6 [136].

Figure 1.9. EUCAST algorithm for carbapenemase detection [136].



[1] Combination of several carbapenemases can also contribute to no synergy – e.g., MBL and KPC in combination. Molecular testing shall be applied.

[2] High-level temocillin resistance (>128 mg/L, zone diameter <11mm) is a phenotypic marker of OXA-48.

Table 1.6. EUCAST algorithm for carbapenemase detection [136].

β-lactamase	**Synergy [1]**				**Temocillin MIC >128 mg/L or zone diameter <11 mm [2]**
	DPA/EDTA	APBA/PBA	DPA + APBA	CLX	
MBL	+	-	-	-	Variable
KPC	-	+	-	-	Variable
MBL + KPC	Variable	Variable	+	-	Variable
OXA-48-like	-	-	-	-	Yes
AmpC + porin loss	-	+	-	+	Variable
ESBL + porin loss	-	-	-	-	No

MBL - metallo-β-lactamase, KPC - *Klebsiella pneumoniae* carbapenemase, DPA - dipicolinic acid, EDTA - ethylenediaminetetraacetic acid, APBA - aminophenyl boronic acid, PBA - phenyl boronic acid, CLX – cloxacillin

[1] Synergy is observed as an increase in zone diameter (mm) with 10 μg meropenem disk/tablet.

[2] Temocillin susceptibility test is performed in cases where no synergy is detected, in order to differentiate between ESBL + porin loss and OXA-48-like enzymes. In the presence of other enzymes susceptibility to temocillin is variable and does not provide any further indication of the β-lactamase present.

- Growth-based phenotypic assays: Tests of this category evaluate the presence of carbapenemases by detecting the growth of an organism in the presence of an antibiotic. For example, the Modified Hodge Test (MHT) is performed by plating a carbapenem-susceptible *Escherichia coli* strain with a carbapenem disk (usually meropenem) in the center and plating linear streaks of the carbapenem-resistant test isolate away from the carbapenem disk. The test is considered positive if there is

enhanced growth of the carbapenem-susceptible indicator strain toward the carbapenem disk along the linear streak of the test isolate, resulting in a cloverleaf-like indentation [140]. However, MHT use is not currently recommended since results are difficult to interpret, while the specificity and in some cases the sensitivity are poor [136]. Alternatively, the carbapenem inactivation method (CIM) and its modified versions can be applied, which detect enzymatic hydrolysis by incubating a carbapenem with a bacterial suspension. For the CIM-test a suspension of the isolate to be tested is prepared and is incubated with a susceptibility-testing disk containing meropenem. After a 2h incubation, the disk is removed and is placed on a Mueller-Hinton agar plate inoculated with a susceptible *E*. *coli* indicator strain. If the bacterial isolate of interest produced a carbapenemase, meropenem in the susceptibility disk will have been enzymatically inactivated, allowing the uninhibited growth of the susceptible indicator strain. Disks incubated in suspensions of organisms that do not produce carbapenemases yield a clear inhibition zone [141].

- Biochemical (colorimetric) tests: Colorimetric assays have been developed to detect carbapenemase production directly from bacterial culture isolates, using a pH indicator, which results in a color change when imipenem is hydrolyzed by carbapenemases, producing an acidic product [140]. Several tests, including the CarbaNP, the Blue-Carba test and the β Carba test are commercially available.
- MALDI-TOF MS: This method detects carbapenemases by assessing the presence of hydrolysis of a carbapenem i.e., by detecting the decrease or disappearance of certain specific peaks of carbapenems in a mass spectrum when a bacterial suspension is previously incubated with a carbapenem antibiotic for a specific length of time. The sensitivity of the method in detecting OXA-48-like producers can be improved by adding NH_4HCO_3 in the reaction.
- Lateral flow assays: These assays use nanoparticles bound to a nitrocellulose membrane with antibodies to capture epitopes specific to carbapenemase enzymes within the lateral-flow device. Tests such as the OXA-48-K-Se T, KPC-K-SeT assays (Coris BioConcept), and NDM LFA are commercially available for easy and rapid detection of OXA-48, KPC, and NDM carbapenemases [140].

It should be noted that molecular methods are generally useful to verify and distinguish the carbapenemase class present in tested isolates.

1.6. Occurrence of ESBL, AmpC and CP Enterobacteriaceae among animals

MDR organisms, previously known to be confined to hospital settings, are nowadays widely spread among livestock, poultry as well as wildlife. For instance, Guerra et al. reported the dissemination of carbapenemase producers among food-producing animals, their environment, companion animals and wild birds [142], underlining that these hosts form an interconnected system that should be holistically addressed in terms of AMR and antibiotic stewardship. The occurrence of ESBL, AmpC or CP-producing bacteria in the intestinal flora of animals is undesirable, as it might lead to their dissemination from animals and food to humans. Bacteria from animals with such resistances should also be considered as a reservoir of resistance determinants, which may be transferable to other bacteria including food-borne zoonoses (e.g., *Salmonella* spp.), potentially leading to further public health consequences [143]. Moreover, a possible infection of the animals by these resistant bacteria would be difficult to treat, adding to the overall burden of AMR.

The most recent specific monitoring of ESBL-/AmpC-/CP- *E. coli* among food producing animals in Europe was conducted in 2019-2020 by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) [143]. According to this report, the occurrence of ESBL and/or AmpC producing *E. coli* among caecal samples of fattening pigs, bovine animals under 1 year of age and broilers is depicted in Figures 1.10-1.12. Marked variations in the prevalence of presumptive ESBL and/or AmpC *E. coli* were observed among the participating countries, as it ranged from 2.1% (Cyprus) to 99.2% (Italy) for fattening pigs, from 7.8% (Denmark) to 99.6% (Italy) for bovine animals under 1 year of age and from 10.3% (United Kingdom) to 100% (Malta) for broilers. Important differences among the reporting countries were also evident when assessing the prevalence of ESBL or AmpC producers separately. The prevalence of presumptive ESBL-producing versus AmpC-producing *E. coli* from caecal samples of fattening pigs, bovine animals under 1 year of age and broilers per country is depicted in Figures 1.13-1.15. Regarding CP *E. coli*, the low number of isolates sporadically detected in some countries could not provide sufficient information on a possible trend in the prevalence of these microorganisms.

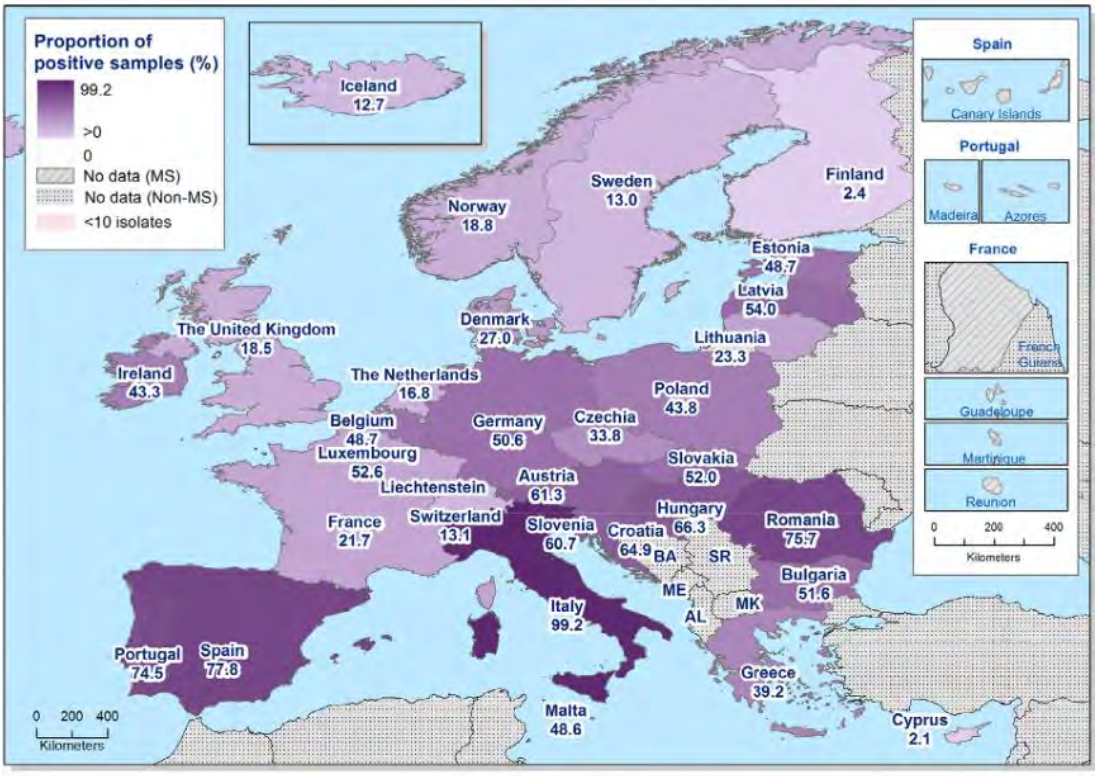


Figure 1.10. Spatial distribution of the prevalence of presumptive ESBL and/or AmpC-producing *E. coli* from fattening pigs in 2019 [143].

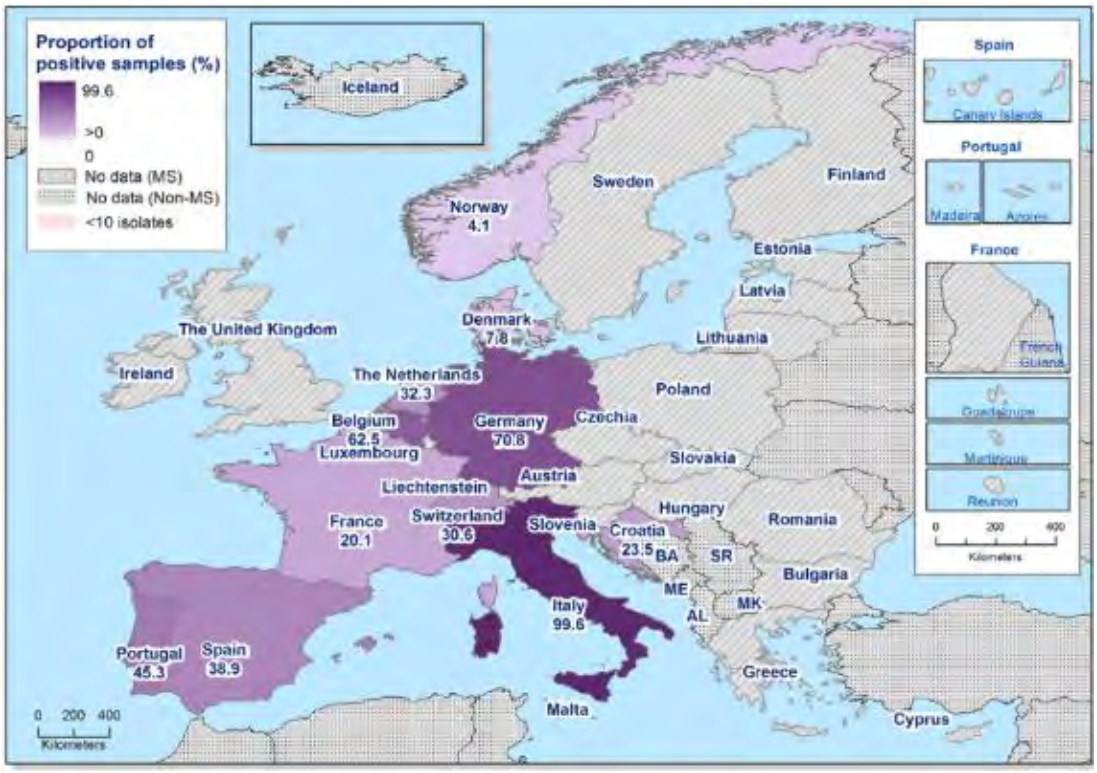


Figure 1.11. Spatial distribution of the prevalence of presumptive ESBL and/or AmpC-producing *E. coli* from bovines under 1 year of age in 2019 [143].

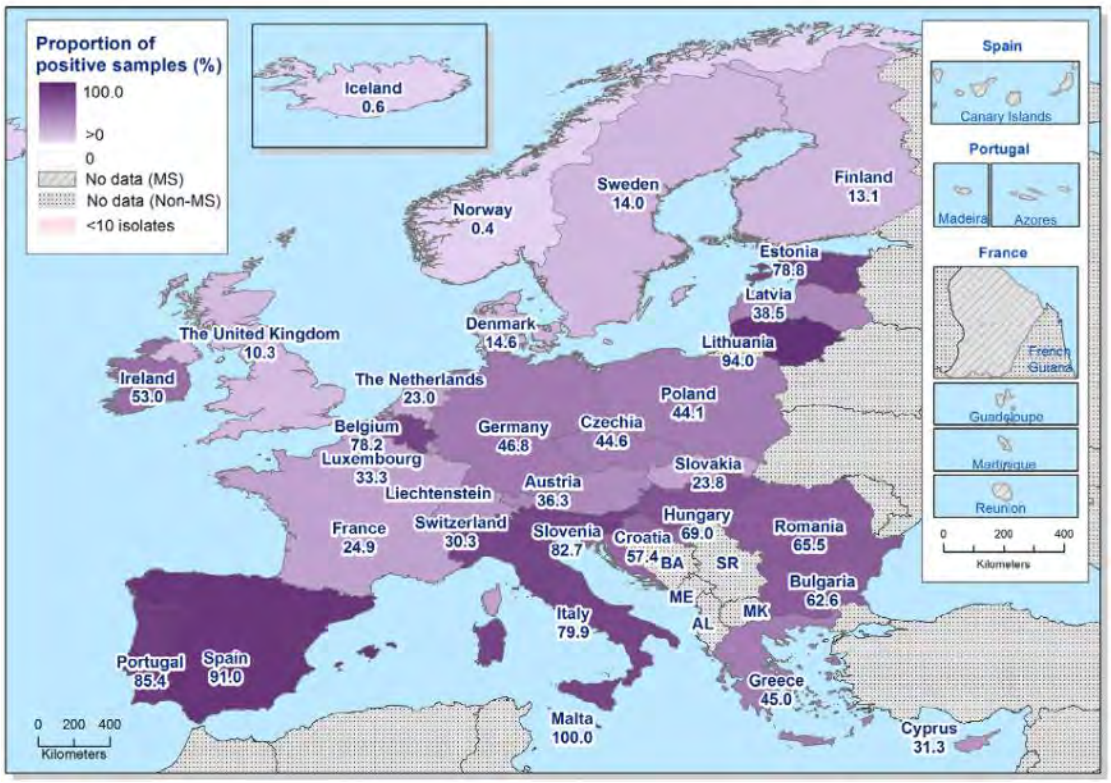


Figure 1.12. Spatial distribution of the prevalence of presumptive ESBL and/or AmpC-producing *E. coli* from broilers in 2018 [143].

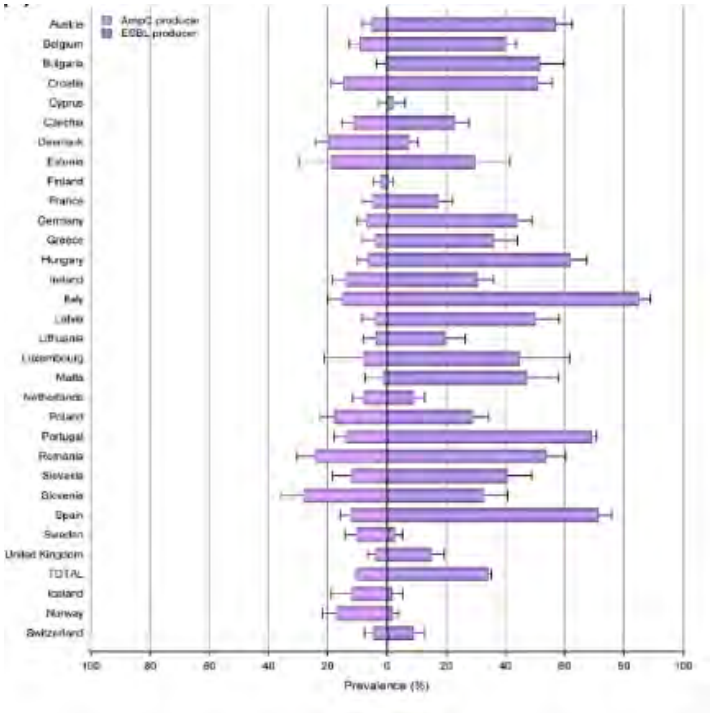


Figure 1.13. Prevalence of presumptive ESBL-producing vs. AmpC-producing *E. coli* from fattening pigs in 2019 [143].

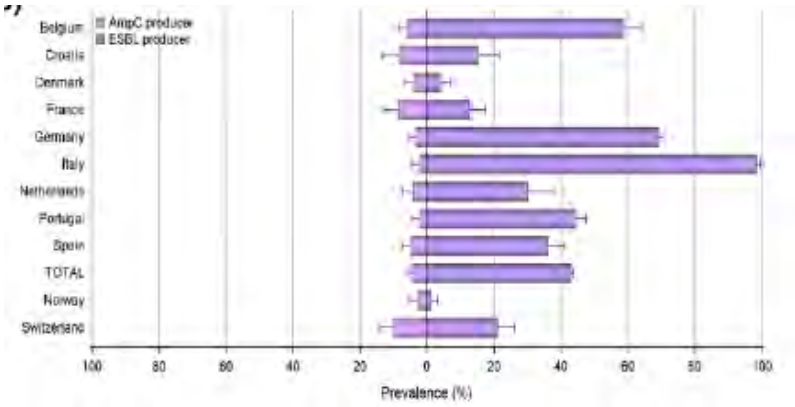


Figure 1.14. Prevalence of presumptive ESBL-producing vs. AmpC-producing *E. coli* from bovines under 1 year of age in 2019 [143].

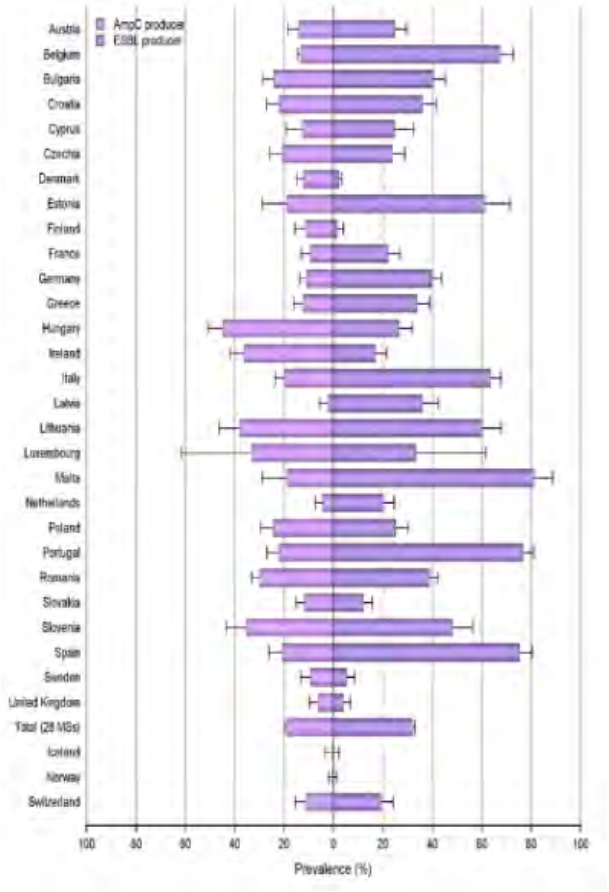


Figure 1.15. Prevalence of presumptive ESBL-producing vs. AmpC-producing *E. coli* from broilers in 2018 [143].

1.6.1. Recent findings among wild birds

The role of wild fauna and particularly of wild birds in disseminating ESBL, AmpC and CP Enterobacteriaceae has been extensively discussed [93,144–146]. Wild avian species, though not directly exposed to antibiotics, are perceived as long-term reservoirs of these microorganisms in nature and subsequently as vehicles for the diffusion of AMR. Due to their high mobility and the omnipresence of their feces in urban and rural areas, they can function as possible vectors of antibiotic resistant bacteria not only in the environment but, to some extent, also back to humans and animals, contaminating animal feed, pasture and drinking or recreational water. Interestingly, the experimental study of Sandegren et al. demonstrated the capability of mallards to carry ESBL *E. coli* for at least 29 days, a time period sufficient for them to travel far during migration, potentially carrying resistant bacteria to new areas. The same study also concluded to an extensive interindividual transmission of the resistant bacterium in a dense mallard population, a phenomenon that possibly acts as an AMR persistence mechanism [147]. Indeed, ESBL/AmpC producing bacteria have been described in migratory seabirds and birds of prey in remote areas such as in Alaska, Brazil and the Mongolian desert [148–150], which emphasizes the possibility of bird migration contributing to the dissemination of bacterial pathogens over long distances and across continents. Nevertheless, detailed analyses are needed, since the fact that wild

birds carry AMR does not itself confirm their ability to act as a contamination source for humans or other animals [151,152]. Wild birds are, moreover, deemed as sentinels and bioindicators of the environmental pollution by antibiotic resistant bacteria in a particular location [144,153]. This is mostly a result of their ability to extensively interact with humans and anthropogenic habitats and thus to reflect the characteristics of the circulating β-lactamase producers. In consequence, as wild birds could represent a major epidemiological link between natural and humanized environments, studying the presence of AMR genes among them is essential and considering their role in the dynamics of AMR mandatory [153].

Wild birds can acquire resistance determinants or resistant bacteria via direct/indirect contact with humans or environments influenced by anthropogenic activities, such as contaminated food-producing animals, hospital waste, wastewaters or soil fertilized with animal manure [154–156]. In this direction, raptors and gulls have been documented to present high colonization rates possibly due to their proximity to humanized habitats [157,158]. Additionally, carnivorous and omnivorous species are generally at the most risk of β-lactamase carriage, possibly due to the likelihood to acquire resistance via their food. For example, rodents, a species that is frequently consumed by these birds, are known to commonly carry AMR [159]. The fact that such birds seek food in cities, landfills, areas with intensive agriculture or artificial habitats including sewage treatment plants, rubbish dumps, and abattoir viscera ponds renders them more likely to carry antibiotic resistant bacteria than animals in areas with limited human footprints [160–162]. More rarely, wild birds can be subjected to care in rescue centers (e.g., in cases of injury), where transmission of β-lactamase producers or development of resistance due to the administered treatment may also occur [163]. The study of Haenni et al. (2020) outlined that these settings, where the wildlife and non-wildlife sectors interact directly, may operate as critical points in the amplification of ESBL prevalence [164]. It shall, however, be noted that the extent to which each of the abovementioned scenarios contributes to the epidemiology of AMR among wild birds is not fully clarified. The most possible transmission pathways of β-lactamase producing bacteria and resistance determinants to wildlife and their role in further disseminating them are presented in Figure 1.16.

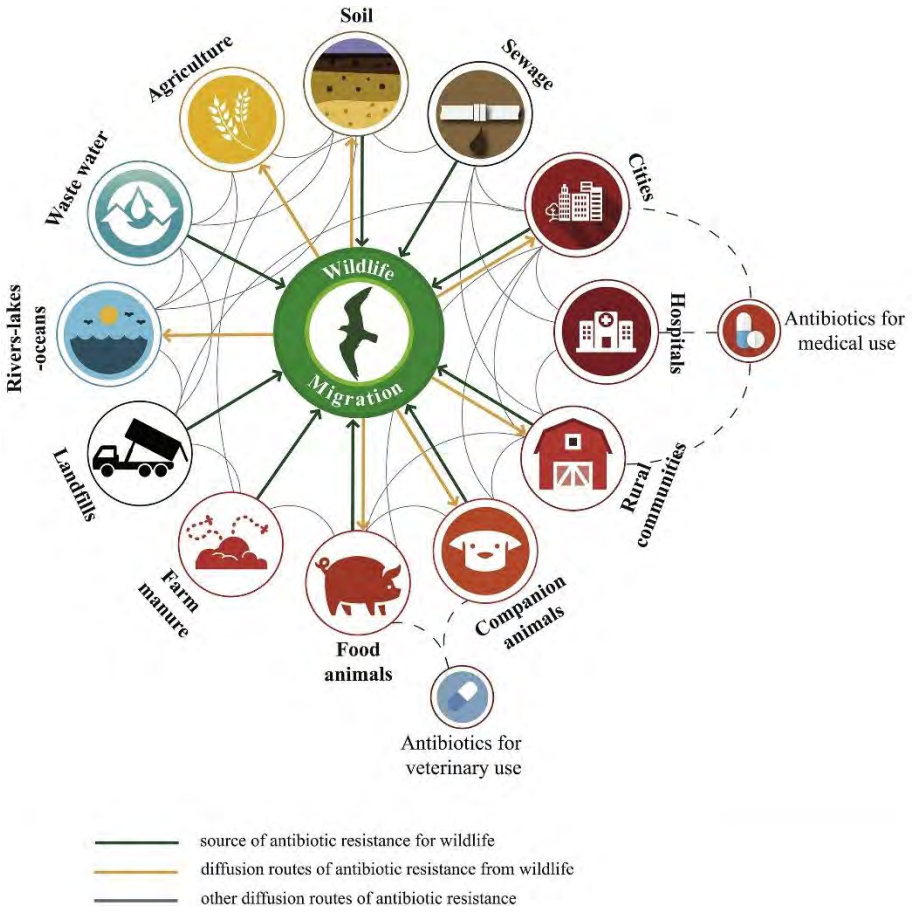


Figure 1.16. Transmission pathways of β-lactamase producing bacteria and resistance determinants to wildlife and their role in their further dissemination [160].

On the other hand, human exposure to β-lactamase producing Enterobacteriaceae harbored by wild birds might also occur and multiple pathways can be involved. The most possible route seems to be indirect, including ingestion of food for human consumption or of drinking water cross contaminated by wild animal excrements and contact via contaminated recreational water. Direct contact could also be possible, especially in cases of synanthropic birds such as gulls, during activities such as birdwatching and hunting or via the consumption of edible wild birds, especially when poorly cooked.

ESBL/AmpC

The first report of ESBL in wild animal species was published in 2006 [165], almost two decades after the first ESBL/AmpC outbreaks among humans [166,167]. This study identified *E. coli* isolates that harbored $bla_{\text{CTX-M-14}}$, $bla_{\text{TEM-52}}$, $bla_{\text{CTX-M-1}}$ and $bla_{\text{SHV-12}}$ from birds of prey sampled in Portugal [165]. Since then, rapid progress has been made in the field, with multiple publications addressing the presence, epidemiology and molecular characteristics of ESBL/AmpCs in wild birds, worldwide. ESBL/AmpC Enterobacteriaceae have been reported from several migratory and sedentary species of waterbirds, birds of prey, urban and other birds throughout the world [145] (Table 1.7). Regarding the different types of ESBL enzymes, CTX-M is the most frequently identified one, and variant CTX-M-15 seems to be the most common, as currently applies for human isolates [145,168], but opposed to food-producing animals' isolates, among which CTX-M-1 prevails [169,170]. CTX-M-1 comes

second in terms of identification frequency. These findings potentially signify a link among wild bird, human and livestock ESBL isolates. SHV-12 predominates in some studies, while TEM-52 is the preponderant TEM-type variant, though less frequently reported. CMY-2 is the most common pAmpC. The different ESBL and AmpC producing Enterobacteriaceae detected from wild birds in Europe, according to recently published studies (2014-2023) are presented in Table 1.7.

Table 1.7. Occurrence of different ESBL and AmpC producing Enterobacteriaceae among wild birds in Europe, according to recently published studies (2014-2023).

Wild Bird Species ESBL/AmpC POS [a]	Bacterial species ESBL POS	Detected ESBL genes (No.)	ESBL Sequence Type(s)	Detected AmpC genes (No.)	AmpC Sequence Type(s)	Year(s) of Sampling	Country	Reference
mallard ducks (semi-wild)	*E. coli*	*bla*$_{CTX-M-1}$ (7) *bla*$_{CTX-M-9}$ (1) *bla*$_{CTX-M-32}$ (7) *bla*$_{CTX-M-55}$ (1)	ST714, ST115, ST371, ST224, ST1276, ST3889	-	-	2020-2022	Germany	[171]
Urban Pigeons (*Columba livia*)	*E. coli*	*bla*$_{CTX-M-15}$ (3) *bla*$_{CTX-M-27}$ (3) *bla*$_{SHV-12}$ (3)	ST10, ST131, ST154, ST206, ST1488, ST2858, ST3576	-	-	2021-2022	Portugal	[172]
Mallard (*Anas platyrhynchos*) Black-headed gull (*Chroicocephalus ridibundus*) European herring gull (*Larus argentatus*) Common gull (*Larus canus*) Jackdaw (*Corvus monedula*) Eurasian coot (*Fulica atra*)	*E. coli*	*bla*$_{CTX-M-15}$ (10) *bla*$_{CTX-M}$ (19) *bla*$_{TEM}$ (25) *bla*$_{SHV}$ (6)	-	*bla*AmpC (8)	-	2017-2018	Poland	[173]
Western Rook (*Corvus frugilegus frugilegus*)	*E. coli*	*bla*$_{CTX–M–55}$ (16) *bla*$_{CTX–M–27}$ (15)	-	-	-	2016-2017	Hungary	[174]
Herring gull (*Larus argentatus*) Lesser black-backed gull (*Larus fuscus*)	*E. coli*	*bla*$_{CTX-M-15}$ (11) *bla*$_{CTX-M-1}$ (1) *bla*$_{CTX-M-14}$ (6) *bla*$_{CTX-M-27}$ (3)	-	*bla*$_{CMY-2}$ (8)	-	2011	United Kingdom	[175]

Accipiter nisus, Anas platyrhynchos domesticus, Apus apus, Athene noctua, Bubo bubo, Columba livia, Coracias garrulus, Corvus monedula, Cuculus canorus, Circaetus gallicus, Delichon urbicum, Emberiza cia, Falco tinnunculus, Garrulus glandarius, Hirundo rustica, Larus michahellis, Milvus migrans, Otus scops, Parus major, Passer domesticus, Pica pica, Ptyonoprogne rupestris, Streptopelia decaocto, Strix aluco, Sturnus vulgaris, Turdus merula, Tyto alba, Upupa epops	*E. coli*	*bla*$_{CTX–M–9}$ (68) *bla*$_{SHV-12}$ (57) *bla*$_{CTX–M–1}$ (20)	ST155, ST224, ST88, ST10, ST136, ST1246, ST162, ST1643, ST174, ST223, ST40, ST4054, ST746	-	-	2015	France	[164]
Merops apiaster, Corvus monedula, Upupa epops, Apus apus, Delichon urbicum, Bubo bubo, Hirundo rustica, Delichon urbicum, Athene noctua, Otus scops, Columba livia, Pernis apivorus, Pica pica, Merops apiaster, Oriolus oriolus	*E. cloacae*	*bla*$_{CTX–M–9}$ (50) *bla*$_{SHV-12}$ (37) *bla*$_{CTX–M–1}$ (1)	ST104, ST135, ST78	-	-			
Pica pica	*C. freundii*	*bla*$_{CTX–M–9}$ (5) *bla*$_{SHV-12}$ (5)		-	-			
Griffon vulture *(Gyps fulvus)*	*E. coli*	*bla*$_{SHV-12}$ (1)		-	-	2016	Spain	[162]
Griffon vulture *(Gyps fulvus)* Egyptian vulture *(Neophron percnopterus)*	*E. coli* *P. mirabilis*	*bla*$_{CTX-M-1}$ (7) *bla*$_{CTX-M-14}$ (5) *bla*$_{CTX-M-55}$ (3) *bla*$_{CTX-M-65}$ (3) *bla*$_{CTX-M-15}$ (1) *bla*$_{CTX-M-3}$ (1) *bla*$_{CTX-M-32}$ (1) *bla*$_{SHV-12}$ (1)	-	-	-	2016	Spain	[161]
Great spotted woodpecker *(Dendrocopos major*) Carrion crow *(Corvus corone)* *Grey heron (Ardea cinerea)* *Common blackbird (Turdus merula)*	*E. coli*	*bla*$_{CTX-M-1}$ (2) *bla*$_{CTX-M-15}$ (1) *bla*$_{CTX-M-55}$ (1) *bla*$_{CTX-M-65}$ (1)	ST540, ST205, ST1722, ST1193, ST58	-	-	2018	Switzerland	[176]
Swedish corvids (N.S.)	*E. coli*	*bla*$_{CTX-M-55}$ (1)	ST165	-	-	2017	Sweden	[177]

Common buzzard (*Buteo buteo*) Eurasian sparrowhawk (*Accipiter nisus*) Yellow-legged gull (*Larus michahellis*) Eurasian eagle-owl (*Bubo bubo*) Tawny owl (*Strix aluco*)	*E. coli*	*bla*$_{SHV-12}$ (1) *bla*$_{CTX-M-15}$ (2) *bla*$_{SHV-167}$ (1) *bla*$_{SHV-1}$ (2)	-	*bla*$_{CMY-2}$ (5)	-	2016-2017	Spain	[178]
Tawny owl (*Strix aluco*) European serin (*Serinus serinus*) Tawny owl (*Strix aluco*)	*K. pneumoniae*	*bla*$_{SHV-12}$ (1) *bla*$_{CTX-M-15}$ (1) *bla*$_{SHV-28}$ (2)	-	*bla*$_{CMY-2}$ (2)	-			
Northern goshawk (*Accipiter gentilis*) Barn owl (*Tyto alba*)	P. mirabilis	*bla*$_{SHV-28}$ (1) *bla*$_{SHV-12}$ (1)	-	*bla*$_{CMY-2}$ (1)	-			
Yellow-legged gull (*Larus michahellis*) Urban pigeon (*Columba livia f. Urbana*)	*E. coli*	*bla*$_{CTX-M-15}$ (3) *bla*$_{CTX-M-1}$ (1)	ST10, ST38, ST34, ST857	-	-	2016	France	[179]
Mallards	*E. coli* *K. pneumoniae*	*bla*$_{CTX-M-15}$ (295) *bla*$_{CTX-M-14}$-like (34) *bla*$_{CTX-M-1}$ (33) *bla*$_{CTX-M-55}$ (27) *bla*$_{CTX-M-65}$ (10) *bla*$_{CTX-M-3}$ (5)	-	-	-	2013	Sweden	[180]
N.S.	*E. coli*	*bla*$_{CTX-M}$ (31) *bla*$_{SHV}$ (29)	-	*bla*$_{CMY}$ (1)	-	2015–2017	Lithuania	[181]
White stork, common buzzard, lesser black-backed gull, black-headed gull, booted eagle, Eurasian eagle-owl, barn owl, griffon vulture, black vulture, cattle egret, black kite, mallard, European turtle-dove, Bonelli's eagle, golden eagle, white stork, Eurasian sparrowhawk, common raven, northern goshawk, common kestrel, European honey buzzard	*E. coli*	*bla*$_{CTX-M-15}$ (16) *bla*$_{CTX-M-1}$ (11) *bla*$_{CTX-M-14}$ (6) *bla*$_{CTX-M-9}$ (6) *bla*$_{SHV-12}$ (6) *bla*$_{SHV-2}$ (2)	ST23	*bla*$_{CIT}$ (13)	ST162	2015-2016	Spain	[182]
Eurasian eagle-owl, griffon vulture, lesser kestrel, mallard, crı´alo, tawny owl, great spotted woodpecker, common buzzard	*K. pneumoniae*	*bla*$_{CTX-M-15}$ (10)	-	-	-			

Cattle egret, common kestrel	*Enterobacter spp.*	*bla*$_{CTX-M-15}$ (1) *bla*$_{CTX-M-1}$ (1) *bla*$_{CTX-M-9}$ (1)	-	-	-			
Lesser black-backed gull	*P. mirabilis*	*bla*$_{CTX-M-9}$ (1)	-	-	-			
Yellow-legged gull (*Larus michahellis*)	*E. coli*	*bla*$_{SHV-12}$ (37) *bla*$_{CTX-M-15}$ (13) *bla*$_{CTX-M-14}$ (10) *bla*$_{CTX-M-1}$ (7) *bla*$_{SHV-2}$ (1)	-	*bla*$_{CMY-2}$ (2)		2014	Spain	[183]
Gulls (*Larus marinus, Larus argentatus, Larus canus, Croicocephalus ridibundus*)	*E. coli*	*bla*$_{CTX-M-15}$ (13) *bla*$_{CTX-M-14}$ (5) *bla*$_{CTX-M-55}$ (3) *bla*$_{SHV-12}$ (3) *bla*$_{CTX-M-1}$ (2) *bla*$_{CTX-M-32}$ (2) *bla*$_{CTX-M-27}$ (1)	ST10, ST3268, ST540, ST93, ST617, ST767, ST155, ST131, ST38, ST636, ST58, ST681	-	-	2013	Sweden	[184]
Birds of prey Bird Azores (*Sylvia atricapilla*) Owl	*E. coli*	*bla*$_{CTX-M-14}$ (3) *bla*$_{SHV-12}$ (2) *bla*$_{TEM-52}$ (2)	-	-	-	2003-2004, 2010	Portugal Spain	[185]
Ciconia ciconia, Gyps fulvus, Milvus migrans, Milvus milvus, *Tyto alba, Sturnus unicolor, Aquila chrysaetos, Cuculus canorus, L. michahellis*	*E. coli*	*bla*$_{SHV-12}$ (9) *bla*$_{CTX-M-1}$ (3) *bla*$_{CTX-M-14a}$ (2)	ST744, ST38, ST155, ST131, ST877, ST453, ST1431, ST1158, ST57, ST3778, ST156	*bla*$_{CMY-2}$ (1)	ST10	2013-2014	Spain	[186]
Mute swan, Crow, Bean goose, Sea eagle, Goshawk, Herring gull, Sparrow hawk, Pigeon, Magpie, Crow, Marsh harrier, Buzzard, Grey heron	*E. coli*	*bla*$_{CTX-M-1}$ (13) *bla*$_{CTX-M-15}$ (8)	ST131, ST410, ST405, ST617, ST88, ST1304, ST224, ST398, ST1968, ST648, ST373, ST167, ST4306, ST1670, ST1204, ST115, ST1730	*bla*$_{(LAT1-4; CMY2-7; BIL-1)}$-group (3) *bla*$_{(MOX-1,-2; CMY-1; -8-11)}$-group (1) *bla*$_{(MIR-1T; ACT-1)}$-group (1)	ST224, ST405, ST1167	2011-2014	Germany	[187]

Accipiter gentilis, Falco Linnaeus, Trichoglossus haematodus	*E. coli*	-	-	-	-	N.S.	Italy	[188]
Gull, Rook	*E. coli*	*bla*$_{SHV-12}$ (2) *bla*$_{TEM-52b}$ (1)	-	*bla*$_{CMY-2}$ (2)	-	N.S.	Czech Republic, Germany	[189]
Rook (*Corvus frugilegus*)	*E. coli*	*bla*$_{CTX-M-1}$ (39) *bla*$_{CTX-M-15}$ (25) *bla*$_{CTX-M-24}$ (4) *bla*$_{TEM-52}$ (4) *bla*$_{CTX-M-14}$ (2) *bla*$_{CTX-M-55}$ (2) *bla*$_{SHV-12}$ (2) *bla*$_{CTX-M-8}$ (1) *bla*$_{CTX-M-25}$ (1) *bla*$_{CTX-M-28}$ (1)	ST10, ST48, ST58, ST88, ST101, ST106, ST115, ST131, ST154, ST155, ST167, ST206, ST351, ST361, ST394, ST398, ST405, ST448, ST542, ST609, ST617, ST641, ST669, ST746, ST1011, ST1249, ST1141, ST1725, ST1832, ST2226, ST3014, ST3015, ST3017, ST3018, ST3019, ST3020, ST1638, ST1642	*bla*$_{CMY-2}$ (47)	ST10, ST23, ST57, ST58, ST69, ST93, ST95, ST117, ST131, ST351, ST354, ST429, ST453, ST615, ST665, ST770, ST963, ST1056, ST1167, ST1431, ST3274, ST3568, ST3778, ST4274, ST4275, ST4276	2010-2011	Czech Republic, France, Germany, Italy, Poland, Serbia, Spain, Switzerland	[190]

N.S. - not specified, [a] Wild birds' common name or scientific name is included in the table if provided in the original publication

The relatedness among human or livestock and wild bird ESBL/AmpC producers has been outlined with different findings by several researchers. For example, a well-conserved IncI1 plasmid carrying $bla_{CTX\text{-}M\text{-}1}$ was found to be widespread in Guadeloupe, occurring in various *E. coli* strains from wild animals (including birds) and humans [191]. In another study, identical microarray profiles were detected in isolates from egrets and cattle in Nigeria showing clonal relationship among them [192]. Likewise, analysis of ESBL producing *E. coli* isolates in Nicaragua revealed a shared pool of $bla_{CTX\text{-}M}$ genes between healthy humans, poultry and wild birds, as well as shared bacterial clones [193].

CPE

In 2013, a carbapenemase producing *Salmonella enterica* subsp. *enterica* serovar Corvallis was isolated from a black kite (*Milvus migrans*) in Germany, and gene $bla_{NDM\text{-}1}$ was detected on an IncA/C conjugative plasmid. This was the first ever report of a carbapenemase producing strain in wild birds. Acquisition of the gene was correlated with the migration route of the specific bird species and in particular with passage through Balkan countries [194]. Worryingly, following this, several researchers have identified CPE colonization of various different wild avian species worldwide [195–200]. Within Europe, $bla_{VIM\text{-}1}$ has been described on *E. coli* strains from yellow-legged gulls (*Larus michahellis*) both in France [201] and in Spain and in the latter report $bla_{VIM\text{-}1}$ co-occurred with $bla_{KPC\text{-}2}$ [183]. OXA-48 production has been reported in Spain on a multidrug resistant, SHV-12 producing *P. mirabilis* isolated from a barn owl (*Tyto alba*) [178], as well as from CTX-M-15 producing *E. coli*, *Enterobacter* spp. and *K. pneumoniae* isolated from common buzzards (*Buteo buteo*), a cattle egret (*Bubulcus ibis*), a Eurasian eagle-owl (*Bubo bubo*) and a lesser kestrel (*Falco naumanni*) [182]. Recently, the study of Ahlstom et al. revealed the presence of highly diverse CPE found in wild birds sampled globally [202]. In particular, yellow-legged gulls and lesser black-backed gulls (*Larus fuscus*) from Spain were found to harbor *E. coli* and *K. pneumoniae* that produced OXA-48, OXA-245, NDM-1, NDM-5, VIM-1, KPC-2 or KPC-3, while a Caspian gull (*Larus cachinnans*) from Ukraine was found to harbor an ST395 hypervirulent *K. pneumoniae* that produced NDM-1.

1.7. Molecular basis of ESBL, AmpC and CP Enterobacteriaceae spread between hosts

Due to the complexity of AMR dissemination among different hosts, several mechanisms should be evaluated when addressing the spread of ESBL, AmpC and CP Enterobacteriaceae. Outbreaks of these resistant bacteria can occur either by emerging bacterial clones or by horizontal transfer of resistance determinants between bacteria of the same and/or different species.

Clonal spread constitutes one of the major factors involved in the current prevalence of β-lactamase producing Enterobacteriaceae. The most representative example, linked to ESBL-production, is the recent, fast, global dispersal of the highly virulent, ciprofloxacin-resistant clone B2- *E. coli* O25:H4-ST131 that is associated with the CTX-M-15 pandemic [203].

Another example is the one of clonal complex CC258 *K. pneumoniae*, including ST258, ST11, ST340, ST437 and ST512, which are considered as "high risk international clones" and are associated with the spread of KPC carbapenemases [204]. The factors supporting the epidemiological success of certain clones have not been fully elucidated, however they are considered to be associated with the clones' ability to adapt to advances in antimicrobial therapy [204]. Successful clones such as ST131, ST10 and ST155 found in humans, domestic and wild animals indicate possible interspecies transmission of β-lactamase producing isolates (Table 1.7).

MGE are also important vectors for the spread of β-lactamases through HGT, given their ability to mobilize themselves and/or genes from one location to another within the cell and to be transferred from one cell to another via conjugation, transformation or, in the case of bacteriophages, transduction [205]. Plasmids act as vehicles for the carriage of other MGE and of AMR genes, transporting them within the plasmid as well as between chromosome and plasmid(s) [206]. They are highly diverse, and some seem to present a strong correlation with specific AMR families, such as the IncI plasmids which are mainly associated with ESBLs, while others even have a strong correlation with specific AMR genes, like IncA/C with $bla_{\text{NDM-1}}$ and $bla_{\text{CMY-2}}$ or IncI1 with $bla_{\text{CTX-M-1}}$ [206,207]. Interestingly, ESBLs are the most frequently described AMR enzymes encoded on plasmids [208]. Regarding other types of MGEs such as insertion sequences, transposons and integrons, their contribution in the dissemination of β-lactamases among bacteria through HGT also seems to be critical. β-lactam resistance genes have commonly been associated with complex Class-1 integrons, which contained genes such as $bla_{\text{CTX-M-2}}$, $bla_{\text{OXA-1}}$, and $bla_{\text{NDM-1}}$ [209]. Another example is that of IS*Ecp1* positive strains, which are more likely to be widely dispersed given that this genetic platform has been associated with the mobilization and improved expression of $bla_{\text{CTX-M}}$ [96].

The emergence of epidemic strains that simultaneously carry several plasmids encoding distinct ESBLs, AmpC or carbapenemases is also of concern. Crucially, some of these plasmid–bacterium associations have become particularly successful, creating "superbugs" that disseminate uncontrollably in clinical and non-clinical settings [210]. Prominent examples include *Klebsiella pneumoniae* ST11 or ST405 carrying plasmid pOXA-48 (which harbors the carbapenemase gene $bla_{\text{OXA-48}}$) and *Escherichia coli* ST131 clade C2/*H*30Rx carrying IncFII plasmids which harbors $bla_{\text{CTX-M}}$ [210]. The emergence of "superbugs" that bear a number of horizontally transferred AMR genes on plasmids and tolerate almost all antibiotics has been documented by several researchers in different ecosystems, including food-producing animals and wildlife [211,212].

PART II. WEST NILE VIRUS

1.8. West Nile Virus

1.8.1. Brief History

West Nile virus (WNV) first appeared in 1937 in Uganda, where it was isolated from the blood of a native woman as a cause of a febrile disease. In 1950 it reappeared in Egypt in humans, birds and mosquitoes. Antibodies against the virus were first detected in humans in Europe in 1958, while in 1963 the virus was found both in humans and mosquitoes. The first major epidemic in a country outside of Africa was in Romania, where in 1996 about 500 cases of encephalitis were recorded, leading to hospitalizations and 50 deaths [213]. In the USA, the virus appeared in 1999 in New York and by 2002 it had spread to almost all of the country (in 45 states). Since then, about 1.000 confirmed cases of WNV are hospitalized and about 100 deaths are recorded in the USA each year.

After Romania, the first major epidemic occurred in Greece in 2010 in the Region of Central Macedonia, causing 34 deaths and 264 cases of clinical encephalitis. The virus re-emerged in Greece in the following years with dozens of cases per year and spread almost throughout the country. WNV is nowadays detected on an annual basis worldwide. In the years 2010-2014 and 2017-2021, circulation of the virus was recorded in almost all Regions of Greece. The number of WNV reported cases of infection with and without central nervous system manifestations (CNS), and the number of deaths among cases with WNV infection in Greece during 2010-2021 is presented in Figure 1.17 [214]. The above data show that the virus has been established in various Regions of the country, as has also happened in other European countries. Therefore, the occurrence of WNV cases in Greece, as well as in other European and neighboring countries is possible and expected during subsequent periods of virus transmission.

1.8.2. Classification and Lineages

WNV belongs to the Flaviviridae family, which is further divided in the genera of flaviviruses, pestiviruses, pegiviruses and hepaciviruses. The *flavivirus* genus encompasses at least 53 species divided into 12 serologically related groups [215]. The viruses within the *flavivirus* genus can be further classified into tick-borne and mosquito-borne viruses. Mosquito-borne viruses may be divided into the encephalitic clade or Japanese encephalitis (JE) serocomplex, which contains WNV and Japanese encephalitis virus (JEV), and the non-encephalitic or hemorrhagic fever clade, which contains dengue virus (DENV) and yellow fever virus (YFV) [216].

WN viruses can be designated into nine phylogenetic lineages [217], while only lineages 1 and 2 have been associated with significant outbreaks in humans [218]. In Europe, before the emergence of lineage 2 in 2004 [219], lineage 1 provoked sporadic cases and occasional outbreaks in animals and humans. In 2004, lineage 2 was expanded from Hungary to eastern Austria and southern European countries and was simultaneously detected in Russia, extending toward Romania [217]. Since 2008, lineage 2 has caused more outbreaks in humans and animals than lineage 1 in Europe [220].

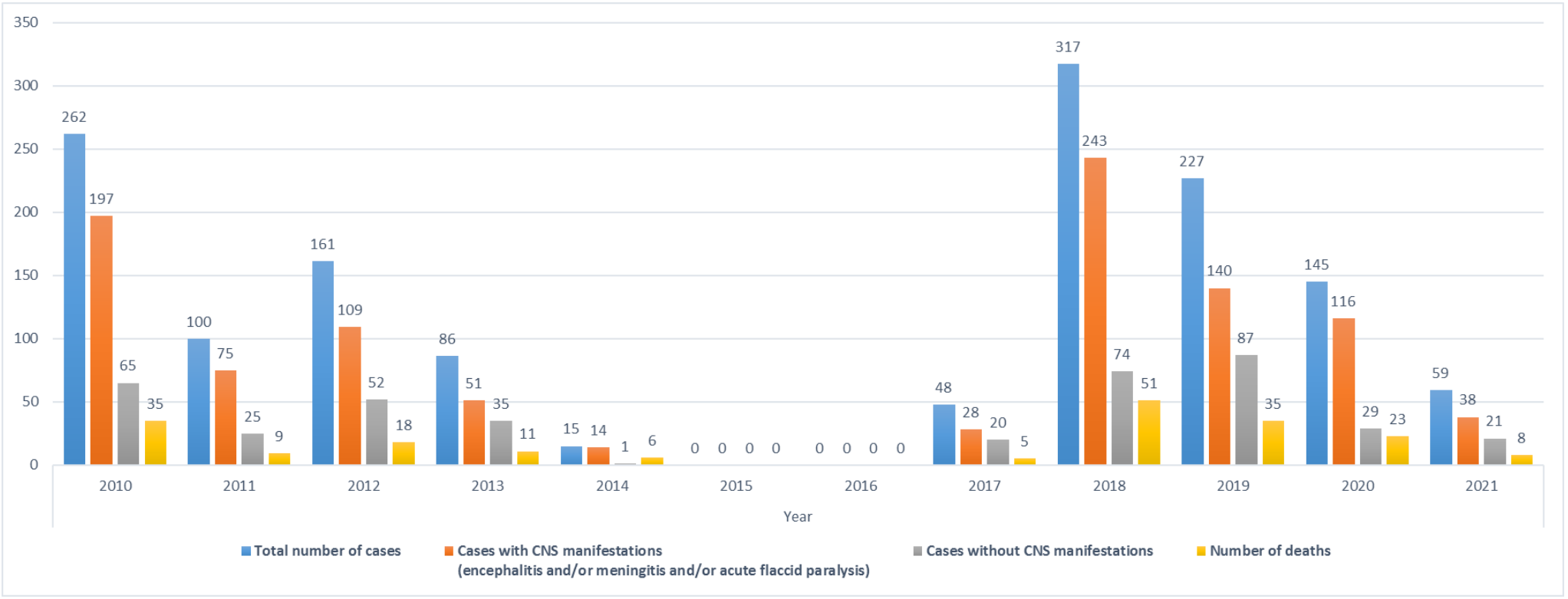


Figure 1.17. Number of reported cases of WNV infection with and without CNS central nervous system manifestations (CNS), and number of deaths among cases with WNV infection, Greece, 2010 – 2021.

1.8.3. Structure and Proteins

WNV is an enveloped, small (~50 nm in diameter), spherical, icosahedral virion containing a single-stranded, positive-sense RNA genome (+ssRNA). The genome consists of a single open reading frame of approximately 11 kb and is translated as a single polyprotein. This polyprotein is post- and co-translationally cleaved by host and viral proteases, resulting in three structural (C for capsid, E for envelope, and (pr)M for (pre)membrane) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The structural proteins are encoded at the 5′ end of the genome and are important for virus entry, fusion, egress, and encapsication of the viral genome during assembly. In particular, C protein associates with the RNA genome to form the nucleocapsid that is surrounded by a lipid bilayer. E protein that embeds in the lipid bilayer during virus assembly and is then exposed to the virion surface, is responsible for binding the cell receptor for viral entry. prM protein is also known to insert in the lipid bilayer and is thought to protect E from undergoing premature fusion upon virus release. During infection, the virus particles contains both mature and immature virions with a varying number of immature prM protein molecules. Regarding the nonstructural proteins, they have many different functions that mainly concern intracellular multiplication, virion assembly and escape from the host's immune responses. NS1 has two forms; a "cellular" form and a secreted one that is highly immunogenic and has been suggested to contribute in replication. NS5 is also necessary for viral replication. NS3 is the viral protease responsible for cleaving other nonstructural proteins from the viral polyprotein and encodes enzyme activities. NS2A, NS2B, NS4A, and NS4B inhibit one or more components of the innate immune responses against viral infection [216].

The viral proteins, structural or non-structural, and their different roles in the biology and/or the pathogenesis of WNV infections are summarized in Table 1.8. The structure of WNV virion and the viral genome are presented in Figure 1.18.

Table 1.8. WNV proteins and their function [221].

Viral Protein	Position in the Genome (Nucleotides)	Main Roles
C	97-465	RNA encapsication and uncoating Activation of apoptosis pathway and cell death
prM/M	466-741-742-966	Virion assembly Virus—host cell fusion
E	967-2469	Viral binding and entry to host cell receptors Virus particle protection Viral membrane—host cell fusion
NS1	2470-3525	Viral RNA replication Enhancement of the attachment of the virus onto the endoplasmic reticulum Virus stability Immune evasion (inhibition of complement activation)
NS2A	3526-4218	Viral RNA replication and virions assembly Immune evasion (disruption of IFN transcription)
NS2B	4219-4611	Cofactor for NS3 protease activity Virus replication and assembly
NS3	4612-6468	Serine protease (N-terminal) RNA helicase (C-terminal)
NS4A	6469-6915	Viral membrane rearrangement Inhibitor of interferon α/β host response
NS4B	6916-7680	Immune evasion (inhibitor of interferon α/β host response) Viral replication (enhancer of NS3hel activity)

NS5	768-10395	IFN-α and β antagonist Evasion of the innate immune response (IFN antagonist) Methyltransferase (N-terminal), RNA-dependent RNA polymerase (C-terminal)

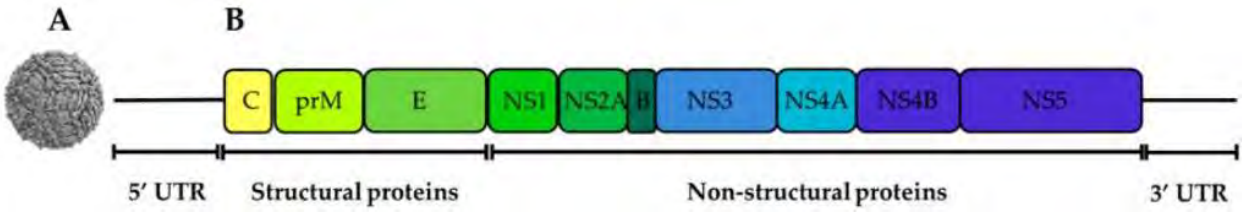


Figure 1.18. (A) The structure of WNV virion and (B) the 11 kb long viral genome represented with one ORF encoding 3 structural and 7 non-structural proteins [221].

1.8.4. Replication cycle

WNV replicates in many types of primary cell cultures and continuous cell lines from a wide variety of species (avian, mammalian, amphibian and insect). This fact suggests that highly conserved receptors and binding molecules are used or that the virus utilizes a variety of cellular proteins in different types of cells and host species [222]. The co-receptors (cellular proteins) for virus attachment, entry and fusion in the host cell remain to be fully characterized. Glycosaminoglycans are known to be used by flaviviruses as initial attachment contacts. Following glycosylation of the prM or the E protein, WN virion interacts with the DC-SIGNR dendritic cell-specific lectin and infection is efficiently promoted. However, many types of cells that do not express DC-SIGNR as well as the red blood cells can be infected by WNV. WNV infection is promoted by phosphatidylserine binding proteins of the TIM family through their interaction with virion-associated phosphatidylserine. The broader tropism observed for WNV in cell cultures may be explained by direct virus interaction with cell surface TIM proteins as well as by indirect interaction with cellular TAM proteins [222].

Cellular $\alpha_v\beta_3$ integrin and laminin-binding protein have been found to function as WNV receptors. However, the entry of WNV was shown to be independent of $\alpha_v\beta_3$ integrin or focal adhesion kinase and dependent on the lipid raft pathway. G-coupled receptor kinase (GRK) family proteins are regarded as important host factors for WNV, with GRK2 enhancing both virus entry and viral RNA synthesis [222].

WN virions enter both mammalian and mosquito cells via clathrin-mediated endocytosis. WNV entry depends on Rab5 [222]. The acidic pH of the endosome environment triggers conformational changes of the E glycoprotein. Initially, the E protein is disrupted and the E homodimers are dissociated to monomers. DII is protruded outwards and is exposed to the target membrane through its fusion loop. The fusion loops of E proteins are inserted in the outer leaflet of the cell membrane. An unstable trimer is formed after interaction of three E monomers via their fusion loops, which then becomes stable by additional interactions between the DI domains of the three E proteins. Next, DIII is believed to fold back against the trimer to form a hairpin-like configuration. These conformational changes result in the release of energy which is used to form a hemifusion intermediate by

merging the monolayers of the interacting membranes. Consequently, the nucleocapsid enters into the cytoplasm via the formed fusion pore, after its enlargement. During uncoating, the viral RNA is released by the nucleocapsid and then is translated to a polyprotein. In the endoplasmic reticulum, the NS3 serine protease and the host signal peptidase cleave this polyprotein at multiple sites. Concurrently, viral genome (ss RNA (+)) is used by the viral RNA-dependent RNA polymerase to form complementary negative sense (-) strands that are then used as templates for ss RNA (+) synthesis [223].

During virion assembly, capsid is formed from C proteins that bind together and surrounds the newly synthesized viral genome. The nucleocapsid acquires an envelope from the cellular membranes of the endoplasmic reticulum and followingly, immature virions with 60 heterotrimeric spikes from E and prM proteins are formed. These virions are transported to the trans-Golgi network (TGN) where rearrangement of E proteins occurs. The mildly acidic pH triggers the formation of 90 antiparallel homodimers of E proteins on the surface of the virion. In this acidic environment, prM is projected to the virion surface and becomes more susceptible to a furin-like serine protease. Dissociation of pr peptide from the virion happens when it is released by exocytosis to the extracellular milieu, which usually occurs 10-12 hours after cell infection. Despite this processing by furin, prM proteins still exist in many virions in the extracellular milieu and as a result prM/E heterodimers are formed. Mature, immature and partially mature virions are released from infected cells and many particles contain prM. Nevertheless, partially immature or even fully immature virions of flaviviruses can also cause infection [223].

The replication cycle of WNV is schematically represented in Figure 1.19.

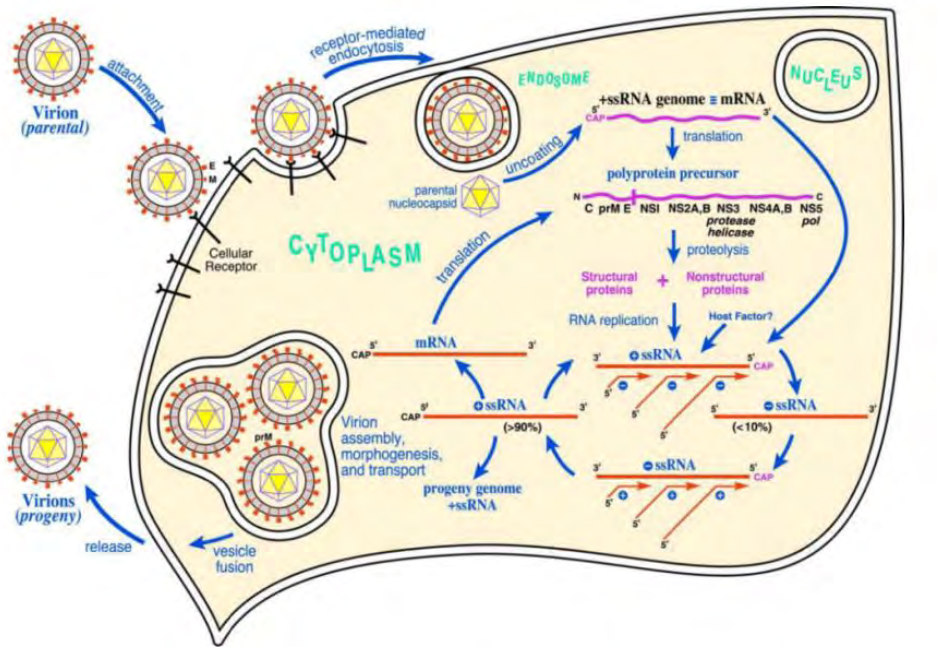


Figure 1.19. West Nile virus replication cycle [223].

1.8.5. Pathogenesis

WNV is a neurotropic virus. Its pathogenesis has been studied using animal models of infection that are thought to reflect what occurs in humans following WNV infection by a mosquito. Three distinct pathogenesis phases have been identified: the initial infection and spread (early phase), the peripheral viral amplification (visceral-organ dissemination phase) and the neuroinvasion (central nervous system (CNS) phase. Female *Culex* spp. mosquitoes acquire WNV while feeding on infected viremic birds. After a bite from an infected mosquito, WNV infects keratinocytes and skin-resident dendritic cells (DCs), including dermal DCs and Langerhans cells, which migrate to lymph nodes, resulting in a primary viremia. Followingly, the virus spreads to peripheral visceral organs such as the kidneys and spleen where a new replication cycle occurs, in epithelium cells and macrophages, respectively. Whether the virus will cross the blood-brain barrier (BBB) entering the CNS and thus causing meningoencephalitis depends on the level of viremia. It has been proposed that WNV penetrates into the CNS by changing the permeability of endothelial cells mediated by vasoactive cytokines (especially TNF-a). Other pathways of WNV entry in the CNS include infection of olfactory neurons resulting in its spread to the olfactory bulb, direct retrograde axonal transport from infected peripheral neurons or transport by infected immune cells. WNV infection of the CNS results in neuronal degeneration and neuronal loss. Mononuclear cells infiltration is observed in infected regions leading either to the containment of infection or to the destruction of infected cells and release of cytokines. Apoptosis also has an antiviral effect by inducing the death of infected cells, though it can additionally have pathological effects in non-renewing cell populations, such as neurons. Infection and injury of brain stem, hippocampal and spinal cord is observed in humans and other mammals [223,224].

1.8.6. Host Immune Response

The host defense against WNV infection engages both innate and adaptive immune responses. WNV activates the signaling pathways of pathogen recognition receptors including toll-like receptors (TLRs) 3 and 7 and RIG-I–like receptors (RLRs). Hence, the innate immunity is boosted and antiviral interferons (IFN-I) and proinflammatory cytokines are synthesized. Type I IFNs and IFN-stimulating genes (ISGs) participate in the control of viral infection and prevent WNV from invading into the brain. Type I and type III IFNs promote BBB integrity and decrease virus entry into the CNS. γδ T cells produce IFN-γ and promote DCs maturation and T-cell priming. Though B cells and specific antibodies help to control the spread of WNV infection, still they cannot clear it from the host. On the contrary, WNV replication can be limited by the complement system via its ability to induce a protective antibody response and by priming adaptive immune responses. WNV infection results in a long-lasting protective immunity induced by $\alpha\beta^+$ T cells. $CD4^+$ T cells sustain not only the antibody responses, but also $CD8^+$ T cell responses that eliminate viral infection. Following infection, the induction of peripheral T regulatory cells provides protection against severe disease in immunocompetent animals and humans and is thought to be essential for the generation of WNV-specific memory T cells. $CD8^+$ T-cell responses are critical in clearing the virus from tissues and preventing viral persistence [225].

1.8.7. Genetic Determinants of Virulence

Wide genetic variation within the WNV lineages is reported across the world, while the association between genetic heterogeneity and viral pathogenicity is variable. Genetic changes, such as mutations, weaken or strengthen virus pathogenicity and thereby its expansion [226].

Mutations at residues 154 to 156 of the envelope protein, which abolished the N-linked glycosylation motif (N-Y-S/T) have been proved to attenuate virus pathogenicity in mouse models. These mutations seem to alter the protein such that it cannot be recognized by oligosaccharyl-transferase, thus causing glycan loss. The N-glycosylation site is found in many virulent strains of lineage I and in lineage II strains associated with the Greek outbreak further promoting virus circulation and amplification in a mosquito-avian transmission cycle.

Virus sensitivity to high temperatures and attenuation of the neuroinvasive and neurovirulent phenotypes in mice, is caused by substitution of cysteine with serine at position 102 of the NS4B protein. It is possible that this mutation may weaken viral ability to inhibit IFN signaling. Additionally, P38G mutation in the NS4B protein has been related to stronger innate and adaptive immune responses in mice, characterized by higher levels of type I IFNs and IL-1β and stronger responses of memory and effector T cells. E249G may result in reduced in-cell viral RNA synthesis, probably by affecting the involvement of NS4B to the virus replication.

An A30P mutation in the NS2A protein can result in a reduced ability of the virus to inhibit IFN response, leading to increased levels of IFNs synthesis. Poor virus replication and non-mortality in mice were found to be related to D73H and M108K mutations.

In the NS3 protein, a H249P mutation is associated with the increased virulence of Lineage 2 strains that caused a major WNV disease outbreak during 2010-2011 in Greece. The specific mutation is considered to be related with an enhancement in RNA helicase function which favors an increase in replication rate. This way bird viremia may surpass the thresholds required for infection of many species of mosquito vectors. However, the presence of H249P has not been always sufficient to enhance virulence. Moreover, enhanced viral replication can be attributed to S365G mutation of the NS3 protein which limits the requirement of ATP for ATPase activity and allows the RNA helicase to sustain the unwinding rate of viral RNA under conditions of ATP deficiency. Substitutions of hydrophobic to hydrophilic amino acids and vice versa as well as substitutions of glycine, proline and cysteine residues are considered to have a potential effect on the secondary structure of proteins. High virulent strains have been found to possess such substitutions at NS3 (S160A and R298G), NS4A (A79T) and NS5 protein (T614P, M625R, M626R) [223].

1.8.8. Ecology

WNV is maintained in nature in a bird-mosquito cycle [14] (Figure 1.20). Birds serve as reservoirs and amplifying hosts, developing sufficient levels of viremia that permits infection of ornithophilic mosquitoes during blood meals [227]. Generally, an infected vertebrate must produce a viremia of $\geq 10^5$ pfu ml^{-1} to serve as an amplification (reservoir) host [228]. *Culex* mosquitoes are accepted as the primary global transmission vector although the virus has been isolated from mosquitoes belonging to at least 11 other genera: *Aedes*, *Aedemomyia*, *Anopheles*, *Coquilletidia*, *Culiseta*, *Deinocerites*, *Mansonia*, *Mimomyia*,

Orthopodomyia, *Psorophora* and *Uranoteania*. In Europe and Africa, the principal amplification vectors of WNV are *Cx. pipiens*, *Cx. univittatus* and *Cx. antennatus*. In Asia, members of the *Cx. vishnui* complex, such as *Cx. tritaeniorhynchus*, *Cx*. *vishnui* and *Cx. pseudovishnui*, are the major vectors, while in Australia, the principal vector is *Cx. annulirostris*. In the USA, the major amplification vectors are *Cx*. *pipiens* and *Culex restuans* in the northeast, *Culex tarsalis* in the west and *Culex quinquefasciatus* in the south [228]. Infected mosquitoes can further transmit the virus through their bites to various hosts including humans and other mammals, particularly horses [229]. Between individuals, transmission may occur by blood transfusion and organ transplantation, while few reports have suggested transmission from mother to newborn via the intrauterine route or via breast-feeding [223].

Regarding wild birds, Passeriformes Charadriiformes, Falconiformes and Strigiformes are considered the principal reservoir hosts. In contrast, Anseriformes, Columbiformes and Piciformes usually generate viremias insufficient to infect mosquitoes. Even though exposed birds most commonly do not present clinical signs, symptoms including weight loss, decreased activity, depression and neurological signs such as torticollis, opisthotonus and rhythmic side-to-side head movements as well as deaths have been reported [230,231]. The susceptibility of a bird to WNV is dependent upon numerous factors including its species and age, and the strain of the virus. Crows, jays and other members of the family *Corvidae* can display high mortality rates following WNV infection [228]. Viremic migratory birds are considered to be major long-distance dispersal agents of WNV, though the non-directional movement of resident viremic birds can also contribute to the spread of the virus [228].

Infected horses and humans are characterized as dead-end hosts since they display low-level viremia for a short period which is usually insufficient to infect mosquitoes [232–234]. Humans infected with WNV can either be asymptomatic, or develop West Nile fever or, less frequently, present severe neurologic disease [218]. Human infections follow a seasonal pattern, usually from early summer to early autumn [235]. Specific climatic conditions such as increased ambient temperatures during summer, high precipitation in late winter/early spring or during summer and summer drought are considered to favor the virus' epidemiology [236–239]. Additionally, landscape characteristics including the presence of irrigated croplands, highly fragmented forests and elevation are deemed as risk factors for WNV outbreaks [237,240]. The density of infected mosquitoes has been positively associated with the number of confirmed cases in humans [241].

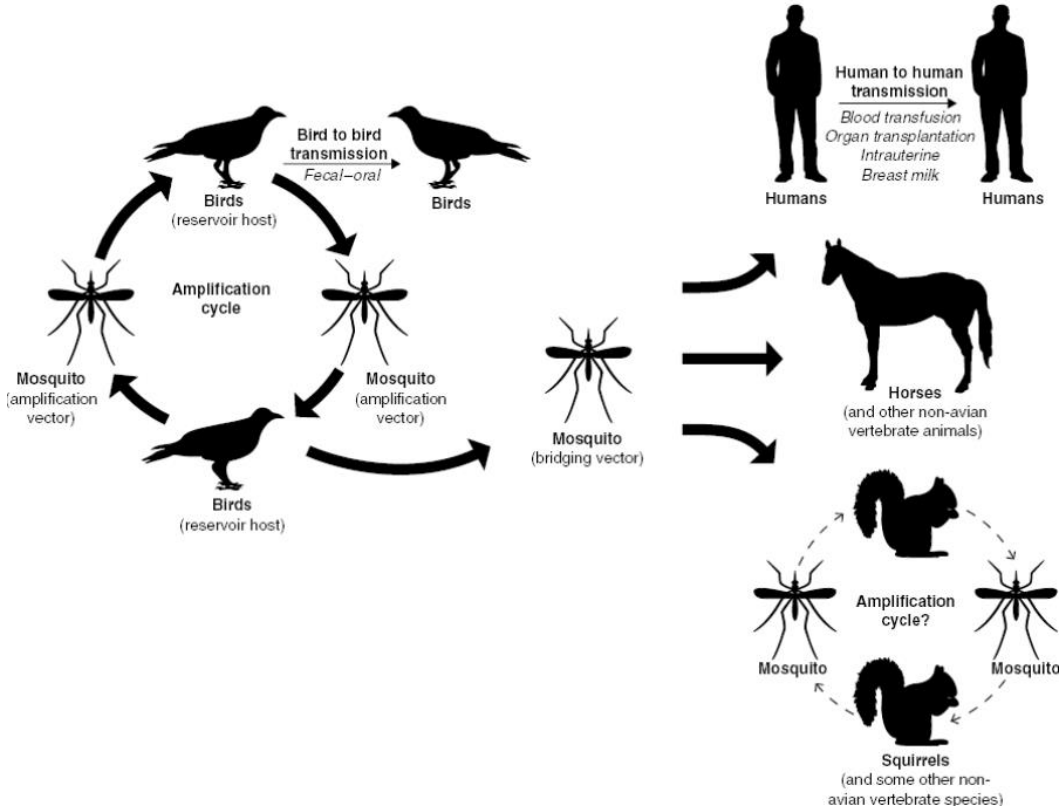


Figure 1.20. Overview of WNV transmission cycle. Thick, solid arrows signify common routes of transmission. The thin, solid arrow signifies a route of transmission that occurs infrequently. Thin, broken arrows signify proposed routes of transmission that have not been confirmed in nature [228].

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Chapter 2. Poultry and Wild Birds as a Reservoir of CMY-2 Producing *Escherichia coli*: The First Large-Scale Study in Greece

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Abstract

Resistance mediated by β-lactamases is a globally spread menace. The aim of the present study was to determine the occurrence of *Escherichia coli* producing plasmid-encoded AmpC β-lactamases (pAmpC) in animals. Fecal samples from chickens (n = 159), cattle (n = 104), pigs (n = 214), and various wild bird species (n = 168), collected from different Greek regions during 2018–2020, were screened for the presence of pAmpC-encoding genes. Thirteen *E. coli* displaying resistance to third-generation cephalosporins and a positive AmpC confirmation test were detected. bla_{CMY-2} was the sole pAmpC gene identified in 12 chickens' and 1 wild bird (Eurasian magpie) isolates and was in all cases linked to an upstream IS*Ecp1*-like element. The isolates were classified into five different sequence types: ST131, ST117, ST155, ST429, and ST1415. Four chickens' stains were assigned to ST131, while five chickens' strains and the one from the Eurasian magpie belonged to ST117. Seven pAmpC isolates co-harbored genes conferring resistance to tetracyclines (*tetM*, *tetB*, *tetC*, *tetD*), 3 carried sulfonamide resistance genes (*sul*I and *sul*II), and 10 displayed mutations in the quinolone resistance-determining regions of *gyrA* (S83L+D87N) and *parC* (S80I+E84V). This report provides evidence of pAmpC dissemination, describing for the first time the presence of CMY-2 in chickens and wild birds from Greece.

Keywords: *Escherichia coli*; AmpC β-lactamases; antimicrobial resistance; CMY-2 type; IS*Ecp1*; chickens; wild birds; livestock; Greece

2.1. Introduction

Antimicrobial resistance (AMR) is a globally emergent, constantly evolving threat affecting humans, animals, and the environment, thus today constituting one of the greatest One Health challenges. Bacterial resistance to cephalosporins is mainly mediated by the production of extended-spectrum β-lactamases (ESBL) and AmpC β-lactamases. AmpC enzymes confer resistance to β-lactams, with the exception of fourth-generation cephalosporins and carbapenems, and subsequently render this essential class of antibiotics ineffective [1,2]. The presence of an AmpC combined with loss of outer membrane porins can, notably, further mediate resistance to carbapenems [2,3]. Hence, although plasmid-encoded AmpC enzymes (pAmpC) are less prevalent than ESBL in most parts of the world, they may lead to resistance of a broader spectrum, while additionally being harder to detect [2].

The most common pAmpC β-lactamase reported in *Escherichia coli* (*E. coli*) isolates of both human and animal origin globally is CMY-2 [4]. The zoonotic potential of this resistance

determinant is illustrated by the detection of *bla*$_{CMY-2}$ on related plasmids and *E. coli* clones in various hosts [5–7]. Insertion sequences, such as ISE*cp1*, are known to play an important role in the mobilization and thus, the spread of this gene [8,9]. Among animals, poultry have been described as the most frequent *bla*$_{CMY-2}$ carrier that can also act as an important infection source for humans, especially through meat and meat products [10,11]. On the contrary, cattle and pigs are less frequently detected to harbor this gene [12]. Alarmingly, the worldwide spread of pAmpC has additionally been evidenced in wildlife and the environment [13,14]. Wild birds play an important role as vectors of AMR and have been suggested as sentinels of circulating resistance genes within a certain geographic region [15,16]. Omnivorous, synanthropic birds are more likely to carry and disseminate resistant strains due to their vicinity to human activities and their feeding habits [17]. Despite the well documented role of animals as reservoirs and spreaders of pAmpC, their ability to directly transmit resistant bacteria to humans remains debatable [10,18].

AMR constitutes a serious threat for Greek public health. According to the surveillance report of the European Centre for Disease Prevention and Control (ECDC), Greece is classified among the countries confronting AMR the most [19], while native consumption of anti-infectives for systematic use is the highest in Europe [20]. pAmpC variants of the CMY family seem to circulate among human isolates in the country [21], while there is evidence to support that this case applies for companion animal isolates as well [22,23]. In livestock and poultry, the presence of pAmpC strains has also been ascertained [12,24]. However, there is hitherto paucity of knowledge regarding the molecular characteristics of pAmpC strains isolated from farmed and wild animals, as well as their possible relationship to human hosts.

Considering the emergence of AMR and the lack of detailed data in Greece, this study aimed to evaluate the presence of pAmpC-producing *E. coli* from poultry, cattle, pigs, and wild birds, to detect the responsible pAmpC genes and to identify the *E. coli* sequence types (ST). All pAmpC-producing *E. coli* isolates that were phenotypically resistant to antimicrobials other than β-lactams, including tetracyclines, sulfonamides, and quinolones, were further tested for the respective resistance determinants.

2.2. Results

2.2.1. Detection of pAmpC Genes in *E. coli* Isolates

Among the 646 animal samples, 168 were derived from wild bird species, 104 from cattle, 214 from pigs, and the remaining 159 from chickens. A total of 13 *E. coli*, 12 from chickens (12/159, 7.5%) and 1 from a Eurasian magpie (1/168, 0.6%), was found to be resistant to third-generation cephalosporins (3GC) and had a positive pAmpC-confirmation test. Molecular screening for pAmpC encoding genes revealed that all isolates carried the CMY-2 type and no other pAmpC gene type was detected in any isolate.

All strains were positive in the PCR targeting ISE*cp1* – CMY, and sequencing analysis confirmed that *bla*$_{CMY-2}$ genes were linked to an upstream ISE*cp1*-like element.

2.2.2. Molecular Typing

Molecular typing of the 13 isolates classified them into five different STs. ST117 *E. coli* was recovered from the wild bird as well as from five chickens. Among the remaining seven chicken strains, four were assigned to ST131 and three were identified as either ST155 or ST429 or ST1415.

2.2.3. Detection of Additional Resistance Genes

According to susceptibility testing, 12 of the 13 CMY-2-positive *E. coli* strains, including the one from the wild bird, exhibited concurrent resistance to at least three classes of antibiotics. ESBL production, by phenotypic testing, was not observed for any strain. Six strains from chickens and the one from a wild bird exhibited resistance to tetracycline (TET^R). Out of the seven tetracycline-resistant strains, six carried *tetM*, while co-occurrence of *tetB*, *tetC*, and *tetD* was observed in the remaining one. Resistance to sulphonamides was expressed in two strains from chickens as well as in the one from the Eurasian magpie, which all harbored both sulI and sulII genes. Ten strains showed resistance to quinolones and fluoroquinolones (QN/FQN^R), although none carried *qnrA*, *qnrB*, or *qnrS*. Sequencing analysis of the QRDRs of *gyrA* and *parC*, performed on the resistant isolates, revealed that all strains displayed a mutation of serine-83 to leucine and a mutation of aspartic acid-87 to asparagine in *gyrA*. In addition, ST131 strains also had alterations of serine-80 to isoleucine and glutamic acid-84 to valine in the QRDR of *parC*.

The antimicrobial resistance and molecular typing results of the strains are summarized in Table 2.1.

Table 2.1. Characteristics of the plasmid-encoded AmpC β-lactamase (pAmpC)-producing *E. coli* isolates.

Isolate	Host	Sequence Type	Resistance Profile		
			Phenotype	Resistance determinants	Mutations (*gyrA*/*parC*)
C46	Chicken	ST429	AMP, AMC, TZP, CEX, CF, CEF, CFIX, CTX, CAZ, CTRX	*bla*$_{CMY-2}$	-
C70	Chicken	ST131	AMP, AMC, TZP, CEX, CF, CEF, CFIX, CTX, CAZ, CTRX, FLU	*bla*$_{CMY-2}$	S83L+D87N/S80I+E84V
C79	Chicken	ST131	AMP, AMC, TZP, CEX, CF, CEF, CFIX, CTX, CAZ, CTRX, FLU	*bla*$_{CMY-2}$	S83L+D87N/S80I+E84V
C83	Chicken	ST117	AMP, AMC, TZP, CEX, CF, CFIX, CAZ, CTRX, FLU, TET, SXT	*bla*$_{CMY-2}$, *tetM, sul*, su*l*II	S83L+D87N
C88	Chicken	ST117	AMP, AMC, TZP, CEX, CF, CFIX, CAZ, CTRX, FLU, TET	*bla*$_{CMY-2}$, *tetM*	S83L+D87N
C103	Chicken	ST117	AMP, AMC, TZP, CEX, CF, CFIX, CAZ, CTRX, FLU, TET	*bla*$_{CMY-2}$, *tetM*	S83L+D87N
C117	Chicken	ST117	AMP, AMC, TZP, CEX, CF, CFIX, CAZ, CTRX, FLU, TET	*bla*$_{CMY-2}$, *tetM*	S83L+D87N
C119	Chicken	ST117	AMP, AMC, TZP, CEX, CF, CFIX, CAZ, CTRX, FLU, TET, SXT	*bla*$_{CMY-2}$, *tetM, sul*I, *sul*II	S83L+D87N
C136	Chicken	ST131	AMP, AMC, TZP, CEX, CF, CEF, CFIX, CTX, CAZ, CTRX, FLU	*bla*$_{CMY-2}$	S83L+D87N/S80I+E84V
C138	Chicken	ST1415	AMP, AMC, TZP, CEX, CF, CEF, CFIX, CTX, CAZ, CTRX, TET	*bla*$_{CMY-2,}$ *tetB, tetC, tetD*	-
C147	Chicken	ST131	AMP, AMC, TZP, CEX, CF, CEF, CFIX, CTX, CAZ, CTRX, FLU	*bla*$_{CMY-2}$	S83L+D87N/S80I+E84V
C156	Chicken	ST155	AMP, AMC, TZP, CEX, CF, CEF, CFIX, CTX, CAZ, CTRX	*bla*$_{CMY-2}$	-

WB105	Eurasian magpie (*Pica pica*)	ST117	AMP, AMC, TZP, CEX, CF, CEF, CFIX, CTX, CAZ, CTRX, FLU, TET, SXT	*bla*$_{CMY-2}$, *tetM, sul*I, *sul*II	S83L+D87N

AMP—ampicillin, AMC—amoxicillin/clavulanic acid, TZP—piperacillin/tazobactam, CEX—cefalexin, CF—cefalotin, CEF— ceftiofur, CFIX—cefixime, CTX—cefotaxime, CAZ—ceftazidime, CTRX—ceftriaxone, FLU—flumequine, TET—tetracycline, SXT—trimethoprim/sulfamethoxazole.

2.3. Discussion

In this study, pAmpC-producing *E. coli* strains were detected in 7.5% of chickens and 0.6% of wild birds, while they were not identified in cattle and pig samples. The higher frequency of pAmpC isolates among poultry, compared to other species, was in accordance with previously published data [10,12]. Their absence in cattle and pigs was expected, considering the European Union Summary Report on Antimicrobial Resistance for the years 2017 and 2018 that described low detection among fattening pigs and zero occurrence in bovine meat from Greece [12].

To the best of our knowledge, this is the first time that CMY-2 type is identified from *E. coli* isolates of farmed chickens in Greece and *bla*$_{CMY-2}$ was the sole pAmpC gene detected, which is in agreement with previous studies [25–27]. Carriage was relatively low (7.5%), compared to recent reports from neighboring countries such as Turkey [28], Romania [29], and Italy [25]. Our finding may be indicative of CMY-2 type low occurrence in Greek poultry but, given the lack of previous screening studies, further investigations would be helpful to verify the aforementioned low prevalence. Considering the European prohibition of cephalosporins' use in poultry, the emergence of ESBL/pAmpC-producing Enterobacteriaceae may be attributed to the treatment of eggs and/or one-day-old chickens in grandparent and parent flocks, along with the current management practices [30,31]. It has been shown that broilers can maintain pAmpC *E. coli* imported to the flock via one-day old chicks or breeding animals even in the absence of selective antibiotic pressure [32,33]. This can be reflected in poultry meat, raising concern about the zoonotic capacity of pAmpC isolates.

We additionally detected a pAmpC-producing *E. coli* harbored by a Eurasian magpie (*Pica pica*) and, as far as we know, this is the first identification of CMY-2 type gene in a wild bird species from Greece. CMY-2 prevails among pAmpC *E. coli* isolates of corvids from The Czech Republic, Poland [34], Austria [16], Canada [17], and The USA [35,36], and of aquatic birds from The Netherlands [13], Spain [37], and Florida, USA [38]. We found a relatively low pAmpC carriage (0.6%) and our results are comparable with those of Alcala et al. [37] who reported 1.0% detection in Spain. Although higher pAmpC carriage has been published previously, varying from 3.4% in The Netherlands [13] to 26.9% in Florida [38], the low detection reported in our study could be attributed to the wide variety of the sampled wild bird species. Sampling and testing were performed, for screening purposes, not only in corvids and aquatic birds, but additionally in "low-risk" wild bird species, which are neither migratory nor omnivorous or aquatic-associated. Eurasian magpie is an omnivore and opportunistic scavenger, highly adapted to human environments and one of the most abundant corvids in Europe. Its diet and ecology, frequently interacting with humans and domestic animals, could explain the detection of a pAmpC-producing strain, as previously described for corvid populations [17]. Eurasian magpies are also known to form large

communal roosts outside the breeding season, which could contribute to CMY-2 persistence and dissemination by bird-to-bird transmission during winter.

ISE*cp1* was found in the upstream region of bla_{CMY-2} in all our isolates. Co-existence of ISE*cp1* with ESBL/pAmpC genes in *E. coli* strains is well documented and has been associated with their efficient capture, expression, and mobilization [39,40]. Being responsible for bla_{CMY-2} transposition to different plasmids, ISE*cp1* probably has an important role in the dissemination of this beta-lactamase and subsequently the enhancement of its zoonotic potential [41].

MLST analysis demonstrated that the CMY-2-producing *E. coli* isolates of chickens were distributed in five different STs. Four chickens' strains were assigned to ST131, a clone with a worldwide distribution that has contributed to the dissemination of the ubiquitous ESBL variant CTX-M-15, as well as other resistance genes [42,43]. This finding highlights the potential of acquired AmpC enzymes to arise as an important zoonotic issue. Further supporting this claim, we also detected bla_{CMY-2} type in a chicken *E. coli* ST155, a clone commonly reported in poultry but additionally significant for public health [44,45]. On the contrary, ST429 that was detected to express CMY-2, is a predominant avian pathogenic lineage, related only to incidental human infections [46,47]. In Greece, CMY-2-producing *E. coli* ST429 has previously been isolated from a healthy household dog [23], which could imply inter-species circulation of the clone in the country. The CMY-2 type-producing *E. coli* isolated from the Eurasian magpie (*Pica pica*) belonged to ST117, previously reported in corvids both in Europe and in Canada [17,34]. Five chickens' isolates were also assigned to this clinically important multiresistant ST, suggesting possible strain transmission among different animal hosts in the country. Detection of ST117 in poultry and a wild bird raises concern, given its frequent association to hospital-based and community-acquired human infections worldwide [48–50]. Finally, an *E. coli* of chicken origin was classified as ST1415, a rather rare ST that, to our knowledge, has not been previously related to CMY-2.

Tetracycline resistance genes were identified in 6 out of the 12 CMY-2-producing poultry isolates, as well as in the Eurasian magpie isolate. Five chickens' strains carried *tetM*, while *tetB*, *tetC*, and *tetD* were detected in the remaining one. The high frequency of tetracycline resistance among chicken pAmpC-producing isolates probably depicts the widespread use of this antibiotic in poultry husbandry all over the world [51]. Cooccurrence of bla_{CMY-2} and *tet* genes has formerly been reported in *E. coli* isolates from chicken carcasses in South Brazil [41], retail chicken meat in Canada [52], as well as in avian pathogenic *E. coli* from septicemic broilers in Egypt [53]. Additionally, the Eurasian magpie CMY-2 type-positive isolate displayed tetracycline resistance mediated by *tetM* and our finding complies with Sen et al. [35], who detected co-occurrence of *tetM* and bla_{CMY-2} in crow isolates.

Resistance to sulfonamides was detected in three strains, two from chickens and the one from the Eurasian magpie, which all harbored *sul*I and *sul*II sulfonamide resistance genes. In the past, sulfonamides were extensively used in traditional poultry production systems in order to achieve higher population densities and increased production. Overconsumption of this antimicrobial class resulted in the development of high resistance rates, reducing significantly its role in the poultry production nowadays [54,55]. As far as the Eurasian magpie isolate is concerned, resistance against chemically synthesized antibiotic classes such as sulphonamides has been reported in wild fauna, even though these antimicrobials are not expected to be widespread in the environment [56]. Co-occurrence of ESBL/pAmpC and

sulfonamide resistance determinants on the same plasmid could probably explain the latter's detection in the wild bird isolate [57].

Quinolone resistance was also reported in CMY-2 *E. coli* strains from nine chickens and the Eurasian magpie. Mutations were responsible for the QN/FQN^R phenotype and all isolates possessed the same amino acid substitution pattern in *gyrA* gene. ST131 *E. coli* possessed the S83L + D87N in *gyrA* combined with S80I + E84V in *parC*. Notably, the same mutations have been found in a collection of ST131 *E. coli* isolated from humans in Central Greece [58]. That study suggested that fluoroquinolone resistance in humans could be related to the use of these antimicrobials in the veterinary practice and the poultry production of the area. Our results verify that this specific substitutional pattern exists in *E. coli* strains of poultry origin. However, no isolate in our study co-harbored bla_{CMY-2} and plasmid mediated quinolone resistance (PMQR) genes, as has previously been described for ESBL/pAmpC-producing *E. coli* of poultry and wild bird origin [13,59,60].

2.4. Materials and Methods

2.4.1. Sample Collection

During 2018–2020, a total of 646 non duplicated fecal samples of clinically healthy animals were collected from different regions of Greece. In particular, 159 stool samples were collected from chickens, 104 from cattle, 214 from pigs, and 168 from thirty different wild bird species (Table 2.2). Samples were obtained by inserting a sterile cotton swab (Transwab® Amies, UK) into the rectum or the cloaca and gently rotating the tip against the mucosa.

Regarding sampling of different wild bird species, Larsen and Australian type traps as well as modified bird catching nets were used, located in a variety of habitats. The sampling site of each wild bird was recorded using handheld Global Positioning System (GPS) units. All wild birds were released immediately following sampling, according to the prerequisites of the Greek Legislation.

Swabs were transported under refrigeration and laboratory analysis was initiated 24–48 h from the samples' collection day.

Table 2.2. Number of samples per wild bird species included in the study.

Common Name	Scientific Name	Number of Samples
Common blackbird	*Turdus merula*	4
Common buzzard	*Buteo buteo*	5
Common pheasant	*Phasianus colchicus*	7
Common starling	*Sturnus vulgaris*)	9
Common swift	*Apus apus*	1
Common whitethroat	*Sylvia communis*	2
Common wood pigeon	*Columba palumbus*	3
Domestic Muscovy duck	*Cairina moschata domestica*	1
Domestic goose	*Anser cygnoides domesticus*	1
Eurasian collared dove	*Streptopelia decaocto*	2
Eurasian eagle-owl	*Bubo bubo*	3
European goldfinch	*Carduelis carduelis*	6
Eurasian scops owl	*Otus scops*	1
Eurasian tree sparrow	*Passer montanus*	9
Eurasian woodcock	*Scolopax rusticola*	11
Golden pheasant	*Chrysolophus pictus*	2

Great tit	*Parus major*	5
House sparrow	*Passer domesticus*	14
Lesser kestrel	*Falco naumanni*	1
Leaf warbler	*Phylloscopus spp.*	1
Little owl	*Athene noctua*	2
Long-eared owl	*Asio otus*	2
Eurasian Magpie	*Pica pica*	52
Mallard	*Anas platyrhynchos*	3
Redwing	*Turdus iliacus*	1
Rock partridge	*Alectoris graeca*	3
Sardinian warbler	*Sylvia melanocephala*	1
Short-toed snake eagle	*Circaetus gallicus*	1
Song thrush	*Turdus philomelos*	14
Yellow-legged gull	*Larus michahellis*	1

2.4.2. Isolation, Identification and Antimicrobial Susceptibility Testing of pAmpC-producing *E. coli*

For the isolation of pAmpC-producing Enterobacterales, swabs were directly streaked on ESBL selective media (CHROMID® ESBL, BioMérieux, Marcy l'Etoile, France) (a medium able to detect both ESBLs and high-level expressed AmpC cephalosporinases) and then the plates were incubated aerobically at 37 °C for 48 h in order to increase sensitivity [61]. Each morphologically different pink colony, corresponding to *E. coli* grown on the plates, was sub-cultured on MacConkey agar. Identification of the isolated bacteria and antimicrobial susceptibility testing were carried out using the automated Vitek-2 system (BioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. The antimicrobial agents tested, using the AST-GN96 card, were ampicillin, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, cefalexin, cefalotin, cefoperazone, ceftiofur, cefquinome, imipenem, gentamicin, neomycin, flumequine, enrofloxacin, marbofloxacin, tetracycline, florfenicol, polymyxin B, and trimethoprim/sulfamethoxazole. Interpretation of the antimicrobial susceptibility testing was performed automatically by the Vitek-2 software (BioMérieux, system version 8.02). Susceptibility to piperacillin/tazobactam, cefixime, cefotaxime, ceftazidime, and ceftriaxone was also tested by Etest, according to EUCAST guidelines [62].

All *E. coli* isolates that were resistant to 3GC were further tested for phenotypic AmpC production using Etest strips containing cefotetan and cefotetan plus cloxacillin (Liofilchem). Isolates that had a ratio cefotetan/cefotetan + cloxacillin ≥8 were selected for molecular detection of AmpC genes and molecular typing. Additionally, these isolates were phenotypically screened for ESBL production using Etest strips containing cefotaxime +/- clavulanic acid and ceftazidime +/- clavulanic acid (Liofilchem). An MIC ratio ≥8 or the presence of a deformed ellipse were considered indicative of ESBL production.

2.4.3. DNA Extraction of the AmpC-Producing *E. coli*

Bacterial DNA was extracted from overnight cultures of the selected isolates using the PureLinkTM Genomic DNA Mini Kit (Invitrogen, Darmstadt, Germany), according to the manufacturer's instructions for Gram-negative bacteria.

2.4.4. Molecular Confirmation of pAmpC Production and Screening of Insertion Sequence

In all isolates, simplex PCRs were performed for amplification of genes for the most common types of plasmid mediated AmpC β-lactamases using the primers described by Pérez-Pérez and Hanson [63] (Table 2.3). Post-amplification products were visualized on 2% agarose gel electrophoresis. The PCR products were purified and were analyzed by sequencing (3730xl DNA Analyzer, Applied Biosystems).

Table 2.3. Primer sequences, amplicon sizes, and optimal annealing temperatures of each simplex PCR performed for the amplification of pAmpC and other resistance genes.

Target	Primer Sequence (5'-3')	Amplicon Size (bp)	Annealing Temperature (°C)	Reference
MOX (MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11)	F: GCTGCTCAAGGAGCACAGGAT R: CACATTGACATAGGTGTGGTGC	520	55	[66]
CIT (LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1)	F: TGGCCAGAACTGACAGGCAAA R: TTTCTCCTGAACGTGGCTGGC	462	55	[66]
DHA (DHA-1, DHA-2)	F: AACTTTCACAGGTGTGCTGGGT R: CCGTACGCATACTGGCTTTGC	405	56	[66]
ACC	F: AACAGCCTCAGCAGCCGGTTA R: TTCGCCGCAATCATCCCTAGC	346	55	[66]
EBC (MIR-1T ACT-1)	F: TCGGTAAAGCCGATGTTGCGG R: CTTCCACTGCGGCTGCCAGTT	302	58	[66]
FOX (FOX-1 to FOX-5b)	F: AACATGGGGTATCAGGGAGATG R: CAAAGCGCGTAACCGGATTGG	190	55	[66]
tetA	F: GCCTTTCCTTTGGGTTCTCT R: TGTCCGACAAGTTGCATGAT	402	55	[67]
tetB	F: CACCACCAGCCAATAAAATT R: TTTATTTAAAACGATGCCCA	319	52	This study
tetC	F: TCACTGGTTAACTCAGCACG R: TCAAGTTCATTCCAACCAAT	319	52	This study
tetD	F: CTCCAATTCCCATAATTTAT R: ATCAAAATAAAGCTAATAAC	379	52	This study
tetM	F: TTATCAACGGTTTATCAGG R: CGTATATATGCAAGACG	398	57	This study
qnrA	F: AGAGGATTTCTCACGCCAGG R: CCAGGCACAGATCTTGAC	580	55	[60]
qnrB	F: GGGTATGGATATTATTGATAAAG R: CTAATCCGGCAGCACTATTA	264	55	[60]
qnrS	F: GCAAGTTCATTGAACAGGGT R: TCTAAACCGTCGAGTTCGGC	428	55	[60]
gyrA	F: TTAATGATTGCCGCCGTCGG R: TACACCGGTCAACATTGAGG	648	54	[60]
parC	F: GTGGTGCCGTTAAGCAAA R: AAACCTGTTCAGCGCCGCATT	395	55	[60]
***sul*I**	F: ACG AGA TTG TGC GGT TCT TC R: GGT TTC CGA GAT GGT GAT TG	347	55	[67]
***sul*II**	F: CCG TCT CGC TCG ACA GTT AT R: GTG TGT GCG GAT GAA GTC AG	506	55	[67]
ISEcp1 – CMY	F: AAA AAT GAT TGA AAG GTG GT R: TTT CTC CTG AAC GTG GCT GGC	546	52	[42]

The presence of ISE*cp1* insertion element upstream of the *bla*$_{CMY-2}$ was investigated by PCR, using a forward primer targeting the ISE*cp1* element and a reverse primer targeting the *bla*$_{CMY}$, as described previously [41] (Table 3).

2.4.5. Molecular Typing of Isolates

Molecular typing of isolates was based on Multilocus Sequence Typing (MLST) in which amplification of seven gene loci (adk, fumC, gyrB, icd, mdh, purA, recA) was performed by PCR (Table 3). PCR products were purified using PureLinkTM PCR Purification Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Purified products were sequenced (3730xl DNA Analyzer, Applied Biosystems) and analysis of the alleles was conducted using an online available database (https://pubmlst.org/bigsdb?db=pubmlst_ecoli_achtman_seqdef) (accessed date: 5 February 2021).

2.4.6. Molecular Detection of Additional Resistance Genes

Strains in which the presence of a pAmpC gene was confirmed and were phenotypically resistant to tetracyclines, sulfonamides, and/or quinolones were additionally tested for the respective resistance genes. In detail, genes conferring resistance to tetracycline (*tetA*, *tetB*, *tetC*, *tetD*, *tetM*), to sulfonamides (*sul*I, *sul*II), and the PMQR determinants (*qnrA*, *qnrB*, *qnrS*) were investigated by PCR. Quinolone-resistant isolates were also screened for mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* by PCR and sequencing of the amplicons was performed (3730xl DNA Analyzer, Applied Biosystems) (Table 3).

2.5. Conclusions

In this study, we investigated, for the first time, the occurrence of pAmpC-producing *E. coli* from various hosts in Greece. Chicken and wild bird strains harbored *bla*$_{CMY-2}$ type in a low prevalence, while pAmpC were not detected in cattle and pigs. ST117 and ST131 were the predominant circulating CMY-2 *E. coli* clones. Tetracycline, sulfonamide, and quinolone resistance were also identified in the CMY-2 strains, revealing the presence of *tet* genes, *sul* genes, and of mutations in the QRDRs, respectively.

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Chapter 3. ESBL-Producing *Moellerella wisconsensis*-The Contribution of Wild Birds in the Dissemination of a Zoonotic Pathogen

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Abstract

Moellerella wisconsensis is an Enterobacteriaceae with unclarified dispersion and pathogenicity. During an ongoing investigation about antimicrobial resistance in Greece, the occurrence of *M. wisconsensis* was evaluated among wild birds and humans. A total of 445 wild bird and 2000 human fecal samples were collected and screened for the presence of the organism. Subsequently, all *M. wisconsensis* strains were phenotypically and molecularly characterized regarding their antimicrobial resistance characteristics. Four *M. wisconsensis* were isolated from a common pheasant (*Phasianus colchicus*), two Eurasian magpies (*Pica pica*) and a great white-fronted goose (*Anser albifrons*). Among these four strains, the three latter presented resistance to 3rd generation cephalosporins, were phenotypically confirmed to produce ESBLs and were found to harbor $bla_{\text{CTX-M-1}}$. The three ESBL isolates additionally exhibited resistance to tetracyclines, while resistance to aminoglycosides was detected in two of them and to trimethoprim/sulfamethoxazole in one. No *Moellerella wisconsensis* strains were retrieved from the human samples tested. This is the first report that provides evidence of *M. wisconsensis* dissemination among wild birds in Greece, describing CTX-M-1 production in multidrug resistant wild birds' isolates of this bacterial species.

Keywords: *Moellerella wisconsensis*; ESBL; multidrug resistance; Enterobacteriaceae; wild birds; Greece; CTX-M-1

3.1. Introduction

The genus *Moellerella* includes a monophyletic species, *Moellerella* wisconsensis (*M. wisconsensis*), which was named after Wisconsin USA, where the majority of the earliest detected strains originated from [1]. *M. wisconsensis*, previously designated as Enteric Group 46, is a gram negative, nonmotile, facultative anaerobic and fermentative bacillus. It is a member of the Enterobacteriaceae family, presents the general characteristics of the family, and is taxonomically mostly related to *Providencia* spp. *M. wisconsensis* additionally exhibits intrinsic resistance to colistin and polymyxin B, which is considered to be a key feature for its identification [1,2].

Despite that almost forty years have passed since the first detection of *M. wisconsensis* in 1984, its exact distribution and potential pathogenicity remain widely unknown. The bacterium has been isolated from human clinical specimens and has been implicated in cases of gastroenteritis, diarrhea, cholecystitis, bacteremia, peritonitis and urinary tract infections [1–7]. Additionally, it has been retrieved from both domestic and wild animals, as well as from insects and parasites, and has been identified as the causative agent of animal disease [8–12]. However, its role in the etiology of clinical conditions has not been fully

elucidated, given the infrequent human and animal colonization. Notably, the scarcity of reported infections caused by *M. wisconsensis* has been partly ascribed to its misidentification as *Escherichia coli* (*E. coli*) or *Klebsiella pneumoniae* subsp. *ozaenae* [13]. Subsequently, *M. wisconsensis* can be regarded as a rare, though potentially underestimated, opportunistic, zoonotic pathogen with virulence that remains to be clarified.

According to the Centers for Disease Control and Prevention, Enterobacteriaceae that produce extended spectrum β-lactamases (ESBL) are classified as a serious threat for healthcare settings and the community [14]. ESBL carriage in wild birds is perceived to be a result of a spill-over phenomenon through environmental pollution with human or domestic animal strains. Wild birds can, thus, either become colonized by already resistant bacteria via their contact with human waste, sewage, and livestock manure or acquire ESBL genes horizontally from resistant isolates that occur in their environment [15]. ESBL-producing Enterobacteriaceae (ESBL-PE) seem to be particularly disseminated among aquatic associated, omnivorous and synanthropic species, while migratory wild birds are of the most concern for their further dispersal [16–18].

The aim of the present study was to describe the occurrence of *M. wisconsensis*, a rather rare Enterobacteriaceae, among fecal samples of wild birds and humans in Greece and to present its antimicrobial resistance profile.

3.2. Materials and Methods

3.2.1. Sample Collection

During an ongoing investigation regarding antimicrobial resistant bacteria in Greece, non-duplicated fecal samples were collected from 445 wild birds as well as from 2000 patients of the University Hospital of Larissa, between January 2019 and June 2021.

Wild birds were captured using Larsen and Australian type traps, as well as modified bird catching nets, sampled directly from the cloaca and immediately released, according to the prerequisites of the Greek Legislation. Alternatively, samples were collected after identifying the wild bird species, scaring off the bird, and then sampling the freshly dropped feces. Specimens were obtained using sterile cotton swabs and were placed in Amies transport medium (Transwab® Amies, Leicester, UK). Transportation was performed under refrigeration and the samples arrived in the laboratory within 48 h of their initial collection. The sampling sites of wild birds were located in a variety of habitats, including urban and suburban areas, wetlands, pastures, scrubs/meadows, forests, agroforestry formations and agricultural areas. The exact sampling position of each wild bird was recorded using handheld Global Positioning System (GPS) units (GPSMAP 62s, Garmin Ltd., Southampton, UK).

Human samples originated from the University Hospital of Larissa, a tertiary care 600-bed hospital in Thessaly region (Central Greece), which serves a population of approximately 1,000,000 inhabitants. All patients' fecal swabs were retrieved for routine cultures at the time of admission to the hospital and prior to the administration of any antibiotic therapy.

3.2.2. Isolation, Identification and Antimicrobial Resistance Phenotype of *Moellerella wisconsensis*

Swabs were directly streaked onto both MacConkey agar and ESBL selective agar (CHROMID®, BioMérieux, Marcy l'Etoile, France). One colony per plate was selected and

further processed. Identification and antimicrobial susceptibility testing of the obtained strains were performed using the Vitek-2 system (BioMérieux, Marcy l'Etoile, France) and the GN ID and AST-GN96 cards, respectively, as previously described [19]. Isolates were characterized as multidrug resistant (MDR) when they presented diminished susceptibility to at least one agent of more than three classes of antibiotics.

Bacterial DNA of all the isolates that were identified as *M. wisconsensis* was extracted from overnight cultures using the PureLinkTM Genomic DNA Mini Kit (Invitrogen, Darmstadt, Germany), according to the manufacturer's instructions for Gram-negative bacteria. Subsequently, the identity of the isolates was verified by amplification of a 760 bp fragment of the 16S rDNA via PCR [20] (Table 3.1) and sequencing of the amplicons (3730xl DNA Analyzer, Applied Biosystems, Foster City, CA, USA).

Table 3.1. Primer sequences, amplicon size and optimal annealing temperature of each simplex PCR performed in the study.

Target	Primer Sequence (5'-3')	Amplicon Size (bp)	Annealing Temperature (°C)
Moellerella wisconsensis 16S rDNA	F: CTC GTT GCG GGA CTT AAC R: ACT CCT ACG GGA GGC AGC A	760	60
bla_{CTX-M}	F: ATG TGC AGY ACC AGT AAR GTK ATG GC R: TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	60
bla_{SHV}	F: CTT TAT CGG CCC TCA CTC AA R: AGG TGC TCA TCA TGG GAA AG	327	60
bla_{TEM}	F: CGC CGC ATA CAC TAT TCT CAG AAT GA R: ACG CTC ACC GGC TCC AGA TTT AT	445	62

3.2.3. Phylogenetic Analysis

The 16S rDNA sequences of the strains obtained in the present study and of all the *M. wisconsensis* sequences available in GenBank (n = 20) were aligned by ClustalW. The phylogenetic tree was constructed with the Neighbor-Joining method [21] and the evolutionary distances were computed using the Kimura 2-parameter [22]. A bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies [23]. Analyses were conducted in MEGA 11 [24].

3.2.4. Phenotypic Evaluation and Molecular Confirmation of ESBL Production

According to the results of susceptibility testing, *M. wisconsensis* isolates that presented resistance to 3rd generation cephalosporins (cefoperazone, ceftiofur) were phenotypically screened for ESBL production by the double-disk synergy test (DDST) [25]. Isolates that presented a positive DDST were further subjected to molecular confirmation. Simplex PCRs were performed for the amplification of genes encoding the most common types of ESBLs, namely CTX-M, TEM and SHV, using the primers described by Dandachi I. et al. [26] (Table 3.1). In all the assays, sterile distilled water served as negative control, while confirmed ESBL-producing Enterobacteriaceae from our strains' collection were used as positive control. Post-amplification products were visualized on 2% agarose gel electrophoresis. The PCR products were purified and analyzed by sequencing (3730xl DNA Analyzer, Applied Biosystems).

3.3. Results

A total number of four (4/445; 0.9%) wild birds were found to be colonized with *M. wisconsensis*. The strains were identified according to their biochemical characteristics (Appendix A, Table A1). In particular, *M. wisconsensis* was isolated from the fecal samples of a common pheasant (*Phasianus colchicus*) originating from Atalanti island, two Eurasian magpies (*Pica pica*) from Lake Karla and a great white-fronted goose (*Anser albifrons*) from Lake Pamvotis. *M. wisconsensis* was not detected in any of the tested human samples.

Sequence analysis of the 16S rDNA confirmed the presence of *M. wisconsensis* in all four wild birds' samples. The four Greek isolates were aligned on a region of 675 nucleotides with 20 isolates from humans, animals and insects that had previously been deposited in GenBank and were found to present over 99.9% similarity with them. The evolutionary relationships between the 24 sequences were described by a Neighbor-Joining tree (Figure 3.1).

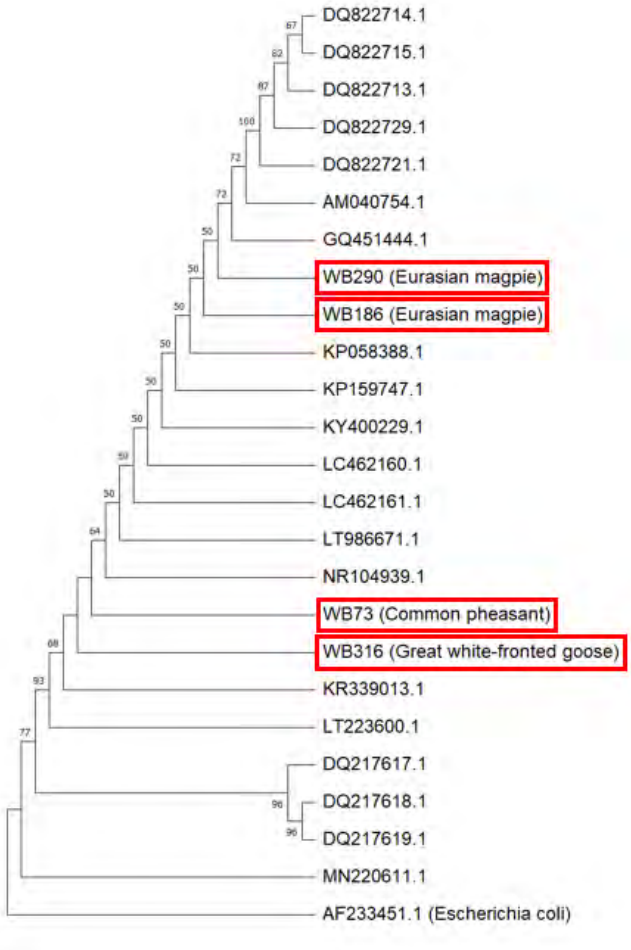

Figure 3.1. Phylogenetic tree constructed with Neighbor-Joining method by using the four 16S rDNA sequences of Greek *M. wisconsensis* isolates (WB73, WB186, WB290, WB316; in red boxes) and 20 M. wisconsensis sequences retrieved from the GenBank database. *E. coli*

strain AF233451.1 was used as the outgroup. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points; only values over 50% are indicated.

All the isolates presented resistance to polymixin B. The strain isolated from the common pheasant was obtained from MacConkey agar and was additionally resistant to cefalexin. The remaining three strains were obtained from ESBL selective agar and presented the ESBL phenotype, being resistant to penicillins (ampicillin) and 1st to 4th generation cephalosporins. These three ESBL strains were also resistant to tetracyclines, while two of them, one from a magpie and the one from goose, exhibited reduced susceptibility to aminoglycosides. The aminoglycosides resistant magpie strain was further resistant to trimethoprim/sulfamethoxazole. Molecular screening for ESBL encoding genes in the three phenotypic ESBL producers revealed that they all carried $bla_{\text{CTX-M-1}}$, while bla_{TEM} and bla_{SHV} were not detected in any isolate.

The characteristics of the four *M. wisconsensis* isolates are summarized in Table 3.2.

Table 3.2. Origin, antimicrobial resistance profile and ESBL genes of the *M. wisconsensis* isolates.

Strain ID	Wild Bird Species	Region	Regional Unit	Antimicrobial Resistance Phenotype	ESBL Genotype
WB73	Common pheasant (*Phasianus colchicus*)	Atalanti island	Fthiotida	CEX, PMB	-
WB186	Eurasian magpie (*Pica pica*)	Lake Karla	Magnesia	AMP, CEX, CF, CEP, CEF, CEQ, TET, PMB	$bla_{\text{CTX-M-1}}$
WB290	Eurasian magpie (*Pica pica*)	Lake Karla	Magnesia	AMP, CEX, CF, CEP, CEF, CEQ, GEN, TET, PMB, SXT	$bla_{\text{CTX-M-1}}$
WB316	Great white-fronted goose (*Anser albifrons*)	Lake Pamvotis	Ioannina	AMP, CEX, CF, CEP, CEF, CEQ, GEN, NEO*, TET, PMB	$bla_{\text{CTX-M-1}}$

AMP—ampicillin, CEX—cefalexin, CF—cefalotin, CEP—cefoperazone, CEF—ceftiofur, CEQ—cefquinome, GEN—gentamicin, NEO—neomycin, TET—tetracycline, PMB—polymixin B, SXT—trimethoprim/sulfamethoxazole, * intermediate resistance, "-"—the isolate did not harbor an ESBL gene.

3.4. Discussion

In the present study, we detected carriage of *M. wisconsensis* by 0.9% of the sampled wild birds and specifically by a common pheasant, two magpies and a great white-fronted goose and we describe the presence of three MDR ESBL-producing *M. wisconsensis* for the first time in Greece. Notably, the organism was not identified in the examined human samples, confirming that *M. wisconsensis* is a rare clinical isolate [2].

Even though *M. wisconsensis* is probably part of the normal gastrointestinal microbiota, its natural habitat is speculated to be associated with the environment and particularly with water [1]. Our results support this claim, since *M. wisconsensis* was detected in four wild birds' samples that lived in proximity to three different aquatic environments (Atalanti island, lake Karla, lake Pamvotis) but not in any samples collected from wild birds inhabiting other types of environments.

The isolation of *M. wisconsensis* from wild animals has sparsely been reported in previous studies. In the USA, Bangert R. and colleagues have identified fecal carriage of the bacterium by 9% of the examined captive raptors (Falconiformes and Strigiformes), which were on a diet primarily consisting of commercially prepared chicken [27]. Given the fact that these

wild birds were under captivity, it cannot be inferred whether the presence of the bacterium represented a natural colonization or was a result of their interaction with humans. In the same country, *M. wisconsensis* has also been retrieved from the oral secretions of a wild raccoon, which was suggested to be a zoonotic reservoir of the organism [10], while, in Italy, it has been isolated from fecal samples of foxes, mustelids and a wolf [28].

Three of the isolated *M. wisconsensis*, from the two magpies and the goose, presented diminished susceptibility to at least one agent of more than three antimicrobial categories and were subsequently characterized as multidrug resistant [29]. The fact that these strains harbored an ESBL gene could indicate a human or livestock to wildlife transmission of either the strain itself or of its resistance determinants. However, ESBL-producing *M. wisconsensis* was neither detected in the human samples that we examined nor has, to date, been reported from Greece in human or other animal samples. Thus, the second hypothesis seems more plausible. Nevertheless, further studies are required to fully elucidate either of the speculations. $bla_{\text{CTX-M-1}}$ was the only ESBL gene that we detected, a finding that is in agreement with data on the current molecular epidemiology of ESBL-producing Enterobacteriaceae among both domestic and wild animals worldwide [30–32]. A sole former study has described ESBL carriage in a single *M. wisconsensis* isolate that was retrieved from flies trapped in the surrounding area of a hospital in Ethiopia [33]. In that study, though, *M. wisconsensis* carried the bla_{TEM} ESBL gene (personal communication with Dr. Tufa, T.B.).

Stock I. et al. have previously detailed the natural antibiotic susceptibility of *M. wisconsensis*. According to their findings, the species presents natural sensitivity to aminoglycosides, fluoroquinolones, folate-pathway inhibitors (trimethoprim/sulfamethoxazole) and tetracyclines [13]. The resistance profile of the three MDR isolates in our study is therefore, presumably, a result of acquired antimicrobial resistance mechanisms. Genes encoding ESBLs are most commonly located on transferable plasmids, rather than the bacterial genome, which frequently also carry resistance determinants for various other antimicrobial classes, including the aforementioned ones [34]. This fact, along with the ability of Enterobacteriaceae to acquire multiple plasmids, as well as to mutate against antimicrobials could explain the reported multidrug resistance in our strains [35].

Carriage of MDR, ESBL-producing Enterobacteriaceae from magpies is probably associated with the birds synanthropic, omnivorous and scavenging behavior, as has previously been described for E. coli strains isolated from the species [19,36]. Adult magpies are sedentary, while the dispersion of juveniles is limited and does not extend beyond 30–40 km from the place of birth. Lake Karla, however, where the two ESBL *M. wisconsensis* from magpies were detected, is a site of great importance for migratory and overwintering waterbirds and foraging raptors in Greece. Subsequently, these birds could obtain the strains and further contribute to their environmental dissemination across long distances during migration. Correspondingly, the great white-fronted goose, that was also found to be colonized by a MDR ESBL-producing *M. wisconsensis*, is a migratory species that conducts long and short distance migration with a potential to diffuse both M. wisconsensis and $bla_{\text{CTX-M-1}}$. This bird most probably acquired the resistant strain or the respective resistance determinants from its habitat, since lake Pamvotis is known to be impaired by pollutants from sewage [37].

3.5. Conclusions

In conclusion, this study revealed wild birds' colonization with *M. wisconsensis* in Greece. Three out of the four isolates presented a multidrug resistant, ESBL-producing phenotype and harbored $bla_{CTX\text{-}M\text{-}1}$. Our findings underline the potential role of wild birds in both the spread of *M. wisconsensis* and the dissemination of $bla_{CTX\text{-}M\text{-}1}$.

Appendix

Table A1. Biochemical characteristics of the four *Moellerella wisconsensis* strains detected in the study.

Biochemical Reaction	WB73	WB186	WB290	WB316
Probability of correct identification	99%	99%	99%	99%
Ala-Phe-Pro-Arylamidase	-	-	-	-
Adonitol	+	+	+	+
L−Pyrrolydonyl−Arylamidase	-	-	-	-
L−Arabitol	-	-	-	-
D−Cellobiose	-	-	-	-
Beta−Galactosidase	+	+	+	+
H2S Production	-	-	-	-
Beta−N−Acetyl−Glucosaminidase	-	-	-	-
Glutamyl Arylamidase pNA	-	-	-	-
D−Glucose	+	+	+	+
Gamma−Glutamyl−Transferase	-	-	-	-
Fermentation/ Glucose	+	+	+	+
Beta−Glucosidase	-	+	(-)	+
D−Maltose	-	-	-	-
D−Mannitol	-	-	-	-
D−Mannose	+	+	+	+
Beta−Xylosidase	-	-	-	-
BETA−Alanine arylamidase pNA	-	-	-	-
L−Proline Arylamidase	-	-	-	-
Lipase	-	-	-	-
Palatinose	-	-	-	-
Tyrosine Arylamidase	+	+	+	+
Urease	-	-	-	-
D−Sorbitol	-	-	-	-
Saccharose/Sucrose	+	+	+	+
D−Tagatose	-	-	-	-
D−Trehalose	-	-	-	-
Citrate (Sodium)	+	+	+	+
Malonate	-	-	-	-
5−Keto−D−Gluconate	-	-	-	-
L−Lactate Alkalinisation	-	-	-	-
Alpha−Glucosidase	-	-	-	-
Succinate alkalinisation	+	+	+	+
Beta−N−Acetyl−Galactosaminidase	-	-	-	-
Alpha−Galactosidase	(+)	+	+	+
Phosphatase	+	(-)	(+)	+
Glycine Arylamidase	-	-	-	-
Ornithine Decarboxylase	-	-	-	-
Lysine Decarboxylase	-	-	-	-
L−Histidine assimilation	-	-	-	-
Coumarate	+	+	+	+
Beta−Glucoronidase	-	-	-	-
O/129 Resistance (comp.vibrio.)	-	+	+	-

Glu−Gly−Arg−Arylamidase	-	-	-	-
L−Malate assimilation	-	-	-	-
Ellman	-	-	-	-
L−Lactate assimilation	-	-	-	-

"+"— positive reaction, "-"— negative reaction, reactions that appear in parentheses are indicative of weak reactions that are too close to the test threshold.

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Chapter 4. Occurrence and Characteristics of ESBL- and Carbapenemase- Producing *Escherichia coli* from Wild and Feral Birds in Greece

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Abstract

Wild and feral birds are known to be involved in the maintenance and dissemination of clinically-important antimicrobial-resistant pathogens, such as extended-spectrum β-lactamase (ESBL) and carbapenemase-producing Enterobacteriaceae. The aim of our study was to evaluate the presence of ESBL- and carbapenemase-producing *Escherichia coli* among wild and feral birds from Greece and to describe their antimicrobial resistance characteristics. In this context, fecal samples of 362 birds were collected and cultured. Subsequently, the antimicrobial resistance pheno- and geno-type of all the obtained *E. coli* isolates were determined. A total of 12 multidrug-resistant (MDR), ESBL-producing *E. coli* were recovered from eight different wild bird species. Eleven of these isolates carried a $bla_{\text{CTX-M-1}}$ group gene alone or in combination with bla_{TEM} and one carried only bla_{TEM}. AmpC, fluoroquinolone, trimethoprim/sulfamethoxazole, aminoglycoside and macrolide resistance genes were also detected. Additionally, one carbapenemase-producing *E. coli* was identified, harboring bla_{NDM} along with a combination of additional resistance genes. This report describes the occurrence of ESBL- and carbapenemase-producing *E. coli* among wild avian species in Greece, emphasizing the importance of incorporating wild birds in the assessment of AMR circulation in non-clinical settings.

Keywords: ESBL; carbapenemases; $bla_{\text{CTX-M}}$; bla_{TEM}; bla_{NDM}; *Escherichia coli*; multidrug-resistance; antimicrobial resistance genes; wild birds; Greece

4.1. Introduction

Antimicrobial resistance (AMR) has recently been declared by the World Health Organization as one of the ten most important public health threats faced by humanity [1]. Among Gram-negative bacteria, the major driving force of resistance is the occurrence of β-lactamases, a family of enzymes capable of hydrolyzing the amide bond of the βlactam ring and, therefore, of rendering β-lactam antimicrobials ineffective [2]. The global establishment and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae (ESBL-PE) and of carbapenemase-producing Enterobacteriaceae (CPE) can be described as one of the most devastating pandemics of multidrug-resistant (MDR) organisms to date. Historically, MDR bacteria have affected patients in hospital settings, where factors such as exposure to antibiotics, artificial ventilation or catheterization provide an enhanced risk for acquisition [3,4]. However, over the last decades, both ESBL-PE and CPE are frequently detected in the community, as well as among domesticated and wild animals and in the environment [5,6]. The localization of ESBL- and carbapenemase-encoding genes on mobile genetic elements (MGEs) that can be efficiently disseminated between different bacteria and hosts, has

rendered ESBL-PE and CPE a One Health problem. For instance, integrons associated with plasmids contribute to the spread of AMR due to their mobility, ability to capture different resistance genes and capacity to cluster resistance genes into complex operons, which can be expressed together and jointly diffused by horizontal gene transfer [7]. In addition to antibiotic resistance genes, ESBL and carbapenemase plasmids commonly also harbor non-resistance factors, including toxin–antitoxin systems that are related to the stable inheritance of the respective resistance genes, even in the absence of selective pressure [8].

During the past few years, antibiotic-resistant bacteria from wildlife have received increasing interest from the scientific community, and the potential contribution of wild fauna as an AMR contamination source has been widely acknowledged [9]. Special attention has been given to the role of wild and feral birds, due to their wide exposure to anthropogenic environments, relative abundance and long-range movements. Subsequently, wild and feral avian species are deemed as a reservoir and vehicle for AMR dissemination [10]. Indeed, several previous studies have identified clinically relevant resistance determinants, such as the ESBL gene $bla_{\text{CTX-M-15}}$ and the carbapenemase gene bla_{KPC}, among Enterobacteriaceae isolated from wild and feral birds [11–13]. Of note, some of these isolates additionally belonged to sequence types frequently detected in human and animal clinical cases, underlining their possible interspecies transmission [14].

Regarding bacterial species, β-lactamase research has been mainly focused on *E. coli*, given its importance as a human and animal pathogen, its commensal nature and the fact that it can be easily disseminated in different ecosystems, enabling the direct comparison of resistance phenotypes in distinct hosts [9,15]. Enzymes of the CTX-M family, particularly those of group 1, are the most common ESBLs among *E. coli* of human, livestock and wild animal origin worldwide [10]. Concerning carbapenemases, Ambler class A variants of the KPC family have the most extensive global distribution among human isolates, followed by class B metallo-β lactamases (MBLs), which are mostly prevalent in Asia, and class D OXA-type genes that are commonly found in Mediterranean countries [16–18]. Correspondingly, the carbapenemase genes identified among animal strains in different countries reflect the types of carbapenemases that prevail in human isolates within these regions [15].

Greece, due to its geographic location within the eastern Mediterranean on the intersection of three continents, constitutes an important habitat for both sedentary and migratory wild birds. High ESBL-PE prevalence and endemicity of CPE among humans has been reported [19–25], however, data regarding the presence of ESBL-PE and CPE among wild birds remain scarce and no study detailing their prevalence and characteristics is available to date. In this context, the objective of the present study was to determine the occurrence and the molecular traits of ESBL- and carbapenemase-producing *E. coli* among wild and feral birds from Greece.

4.2. Materials and Methods

4.2.1. Sample Collection

Between 2019 and 2021, a total number of 362 non-duplicated fecal samples were collected from 47 different wild and feral bird species (Table 4.1), originating from 23 regional units of Greece. Samples were obtained by inserting a sterile cotton swab into recently deposited feces or directly into the cloaca of live, captured wild and feral birds. Birds were captured using Larsen-type traps, Australian-type traps or modified bird-catching nets, and immediately released after sampling, according to Greek legislation. Samples were only

collected following bird species identification. The swabs were transported in Amies medium (Transwab® Amies, Corsham, UK) under refrigeration and arrived in the laboratory within 48 h of their collection.

Table 4.1. Number of samples per wild or feral bird species collected and tested.

Family	Common Name	Scientific Name	Number of Samples
Accipitridae	Common buzzard	*Buteo buteo*	4
	Eurasian sparrowhawk	*Accipiter nisus*	1
	Short-toed snake eagle	*Circaetus gallicus*	2
	Steppe eagle	*Aquila nipalensis*	1
Anatidae	Domestic Muscovy duck	*Cairina moschata domestica*	1
	Greater white-fronted goose	*Anser albifrons*	33
	Mallard	*Anas platyrhynchos*	10
	Mute swan	*Cygnus olor*	5
Apodidae	Common swift	*Apus apus*	1
Ardeidae	Grey heron	*Ardea cinerea*	14
	Little egret	*Egretta garzetta*	8
Charadriidae	European golden plover	*Pluvialis apricaria*	1
Columbidae	Domestic pigeon	*Columba livia domestica*	40
	Eurasian collared dove	*Streptopelia decaocto*	18
Corvidae	Hooded crow	*Corvus corone cornix*	5
	Rook	*Corvus frugilegus*	4
	Western jackdaw	*Corvus monedula*	3
	Eurasian magpie	*Pica pica*	79
Emberizidae	Rock bunting	*Emberiza cia*	1
Falconidae	Common kestrel	*Falco tinnunculus*	2
Fringillidae	Common chaffinch	*Fringilla coelebs*	6
	European goldfinch	*Carduelis carduelis*	1
Hirundinidae	Barn swallow	*Hirundo rustica*	1
Laridae	Caspian gull	*Larus cachinnans*	20
	European herring gull	*Larus argentatus*	9
	Yellow-legged gull	*Larus michahellis*	8
	Mediterranean gull	*Larus melanocephalus*	2
	Sandwich tern	*Sterna sandvicensis*	1
Paridae	Great tit	*Parus major*	6
Passeridae	Eurasian tree sparrow	*Passer montanus*	9
	House sparrow	*Passer domesticus*	28
Phalacrocoracidae	Great cormorant	*Phalacrocorax carbo*	4
Phasianidae	Green peafowl	*Pavo muticus*	2
Phoenicopteridae	Greater flamingo	*Phoenicopterus roseus*	5
Phylloscopidae	Leaf warbler	*Phylloscopus spp.*	3
Rallidae	Common moorhen	*Gallinula chloropus*	2
Recurvirostridae	Black-winged stilt	*Himantopus himantopus*	2
Strigidae	Eurasian Scops owl	*Otus scops*	1
	Little owl	*Athene noctua*	2
	Long-eared owl	*Asio otus*	3
Sturnidae	Common starling	*Sturnus vulgaris*	1
Sylviidae	Common whitethroat	*Sylvia communis*	3
	Sardinian warbler	*Sylvia melanocephala*	1
Turdidae	Common blackbird	*Turdus merula*	7
	Redwing	*Turdus iliacus*	1
Tytonidae	Barn owl	*Tyto alba*	1
	Total		**362**

4.2.2. Isolation, Identification and Antimicrobial-Resistance Phenotype of ESBL- and Carbapenemase-Producing Enterobacteriaceae

Swabs were directly streaked onto both ESBL selective media (CHROMID® ESBL, BioMérieux, Marcy l'Etoile, France) and CPE selective media (CHROMID® CARBA SMART, BioMérieux), and the plates were incubated aerobically at 37 ◦C for 24–48 h. Putative *E. coli* colonies of pink to burgundy coloration were sub-cultured on MacConkey agar. Identification and antimicrobial susceptibility testing of the isolates were performed using the Vitek-2 system (BioMérieux), according to the manufacturer's instructions. The AST-GN96 card was used in order to determine the minimum inhibitory concentration (MIC) of the following antimicrobials: ampicillin, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, cefalexin, cefalotin, cefoperazone, ceftiofur, cefquinome, imipenem, gentamicin, neomycin, flumequine, enrofloxacin, marbofloxacin, tetracycline, florfenicol, polymyxin B and trimethoprim/sulfamethoxazole. Results were automatically interpreted using the Vitek-2 software (BioMérieux, system version 8.02). Isolates were considered MDR when they exhibited diminished susceptibility to at least one agent of more than three antimicrobial classes.

4.2.3. Phenotypic Confirmation of ESBL or Carbapenemase Production

E. coli isolates that presented resistance to third generation cephalosporins (cefoperazone, ceftiofur) were subjected to the double disk synergy test (DDST) for the phenotypic confirmation of ESBL production, according to EUCAST guidelines [26]. A positive result was indicated when the inhibition zones around any of the cephalosporin disks were augmented or when a "keyhole" was formed in the direction of the disk containing clavulanic acid.

Microorganisms that were resistant to imipenem were assessed for phenotypic carbapenemase production using MIC test strips containing meropenem plus phenylboronic acid and meropenem plus ethylenediaminetetraacetic acid (EDTA) (Liofilchem). Isolates that had a ratio meropenem/meropenem plus phenylboronic acid ≥8 or meropenem/meropenem plus EDTA ≥8 were considered positive for class A or class B carbapenemases, respectively.

4.2.4. Antimicrobial Resistance Genotyping of the ESBL- and Carbapenemase-Producing *E. coli*

Isolates that were found positive in the DDST or the phenotypic carbapenemase tests were characterized using the DNA microarray-based assay CarbaResist from InterArray (FZMB GmbH, Bad Langensalza, Germany). Primer and probe sequences have previously been described in detail [27]. In addition, probes for the detection of the colistin resistance gene family *mcr* were included on the present microarray (see Supplementary File S4.1). The microarray layout is presented in Supplementary File S4.2. Protocols and procedures were conducted in accordance with the manufacturer's instructions (https://www.interarray.com/Further-Genotyping-Kits, accessed on 10 May 2022). In brief, bacteria were grown overnight on Columbia blood agar, harvested and enzymatically lysed prior to DNA preparation. Genomic DNA from the bacteria was extracted using the Qiagen blood and tissue kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The DNA was used in a multiplexed primer elongation, incorporating biotin-16-dUTP. Amplicons were stringently hybridized to the microarray, washed and incubated with a

horseradishperoxidase-streptavidin conjugate. Hybridizations were detected by adding a precipitating dye. An image of the microarray was taken for further analysis.

4.3. Results

4.3.1. Occurrence and Characteristics of ESBL-Producing *E. coli*

A total of 12 *E. coli* was found to be resistant to third-generation cephalosporins and had a positive ESBL-confirmation test (DDST). The isolates were retrieved from 12 of the 362 sampled wild and feral birds (3.3%) and, in particular, from four Eurasian magpies (*Pica pica*), two Common buzzards (*Buteo buteo*), one Short-toed snake eagle (*Circaetus gallicus*), one Eurasian sparrowhawk (*Accipiter nisus*), one Steppe eagle (*Aquila nipalensis*), one Grey heron (*Ardea cinerea*), one Eurasian Scops owl (*Otus scops*) and one Common swift (*Apus apus*). These birds originated from three regional units of Greece; Korinthia and Messinia that are located in the Peloponnese region of southern Greece, and Magnesia that is located in central Greece.

The 12 ESBL isolates presented resistance to ampicillin and first- to fourth-generation cephalosporins. One exhibited an intermediate phenotype to ticarcillin/clavulanic acid, while none were resistant to carbapenems. Molecular detection of ESBL genes showed that 11 isolates carried the $bla_{\text{CTX-M-1}}$ group gene alone (n = 4) or in combination with bla_{TEM} (n = 7) and one isolate carried the bla_{TEM} alone. The AmpC gene bla_{ACT} was detected in four of the isolates and the broad spectrum β-lactamase $bla_{\text{OXA-1}}$ in only one.

All the isolates were further categorized as MDR. Reduced susceptibility to aminoglycosides was detected in one of the ESBL *E. coli* (1/12, 8.3%) which presented resistance to gentamicin, was intermediate to neomycin and harbored *aphA*. However, the remaining 11 strains were also found to possess aminoglycoside resistance genes and, in particular, *aadA2* (n = 7), *aadA4* (n = 3) and *rmtA* (n = 5).

Eleven of the isolates were additionally non-susceptible to fluoroquinolones (11/12, 91.7%), being either resistant or intermediate to both flumequine and enrofloxacin, while four of them were further resistant to marbofloxacin. The plasmid-mediated quinolone resistance gene *qnrS* was detected in all the fluoroquinolone-resistant *E. coli*.

Trimethoprim/sulfamethoxazole (11/12, 91.7%) resistance was detected in 11 isolates, which harbored a combination of *dfrA* and *sul* variants. In particular, *dfrA5* (n = 10) and *sul2* (n = 11) were the most common alleles detected, followed by *dfrA17* (n = 4) and *sul1* (n = 4), respectively.

All 12 isolates were resistant to tetracycline (12/12, 100%), whereas 11 of them harbored the macrolide resistance gene *mrx* alone (n = 2) or in combination with *mph* (n = 9).

Finally, the *intI1* gene was detected in two strains.

The antimicrobial resistance phenotypes and genotypes of the ESBL-producing *E. coli* isolates are summarized in Tables 4.2 and 4.3. An example of the microarray results is depicted in Figure 4.1.

Table 4.2. Characteristics of the ESBL- and the Carbapenemase-Producing *E. coli*.

Wild Bird Species	Regional Unit of Origin	Antimicrobial Resistance Profile												
				Genotype										
		Phenotype		ESBL Genes	Carbapenemase Genes	AmpC Genes	Other β-Lactamases Genes	Aminoglycoside Resistance Genes	PMQR	Sulfonamide Resistance Genes	Trimethoprim Resistance Genes	Macrolide Resistance Genes	Genes Associated with Mobile Genetic Elements
Magpie (*Pica pica*)	Korinthia	ESBL	AMP, CEX, CF, CFP, CEF, CEQ, FLU *, ENR *, TET, SXT	*bla*$_{CTX-M-1}$ group	-	-	-	*aadA4*	*qnrS*	*sul1, sul2*	*dfrA17*	*mph, mrx*	-
Magpie (*Pica pica*)	Korinthia	ESBL	AMP, CEX, CF, CFP, CEF, CEQ, FLU *, ENR *, TET, SXT	*bla*$_{CTX-M-1}$ group	-	*bla*$_{ACT}$	-	*aadA4, rmtA*	*qnrS*	*sul1, sul2*	*dfrA5, dfrA17*	*mph, mrx*	-
Magpie (*Pica pica*)	Korinthia	ESBL	AMP, CEX, CF, CFP, CEF, CEQ, FLU *, ENR *, TET, SXT	*bla*$_{CTX-M-1}$ group	-	*bla*$_{ACT}$	-	*aadA4, rmtA*	*qnrS*	*sul1, sul2*	*dfrA5, dfrA17*	*mph, mrx*	-
Magpie (*Pica pica*)	Korinthia	ESBL	AMP, CEX, CF, CFP, CEF, CEQ, FLU, ENR *, TET, SXT	*bla*$_{CTX-M-1}$ group	-	*bla*$_{ACT}$	-	*aadA4, rmtA*	*qnrS*	*sul1, sul2*	*dfrA5, dfrA17*	*mph, mrx*	-
Common buzzard (*Buteo buteo*)	Messenia	ESBL	AMP, CEX, CF, CFP, CEF, CEQ, FLU, ENR, MRB, TET, SXT	*bla*$_{CTX-M-1}$ group, *bla*$_{TEM}$	-	*bla*$_{ACT}$	*bla*$_{OXA-1}$	*aadA2, rmtA*	*qnrS*	*sul2*	*dfrA5*	*mph, mrx*	*intl1*
Common buzzard (*Buteo buteo*)	Messenia	ESBL	AMP, CEX, CF, CFP, CEF, CEQ, FLU, ENR *, TET, SXT	*bla*$_{CTX-M-1}$ group, *bla*$_{TEM}$	-	-	-	*aadA2*	*qnrS*	*sul2*	*dfrA5*	*mph, mrx*	-
Grey heron (*Ardea cinerea*)	Magnesia	ESBL	`AMP, TCC*, CEX, CF, CFP, CEF, CEQ, GEN, NEO *, TET	*bla*$_{CTX-M-1}$ group, *bla*$_{TEM}$	-	-	-	*aphA*	-	*sul2*	-	-	-
Short-toed snake eagle (*Circaetus gallicus*)	Messenia	ESBL	AMP, CEX, CF, CFP, CEF, CEQ, FLU, ENR, MRB, TET, SXT	*bla*$_{CTX-M-1}$ group, *bla*$_{TEM}$	-	-	-	*aadA2*	*qnrS*	*sul2*	dfrA5	*mrx*	-
Eurasian Scops owl (*Otus scops*)	Messenia	ESBL	AMP, CEX, CF, CFP, CEF, CEQ, FLU, ENR, MRB, TET, SXT	*bla*$_{CTX-M-1}$ group, *bla*$_{TEM}$	-	-	-	*aadA2*	*qnrS*	*sul2*	dfrA5	*mph, mrx*	*intl1*

Common swift (*Apus apus*)	Messenia	ESBL	AMP, CEX, CF, CFP, CEF, CEQ, FLU, ENR, MRB, TET, SXT	*bla*$_{CTX-M-1}$ *group*, *bla*$_{TEM}$	-	-	-	*aadA2*	*qnrS*	*sul2*	dfrA5	*mph, mrx*	-
Eurasian sparrowhawk (*Accipiter nisus*)	Messenia	ESBL	AMP, CEX, CF, CFP, CEF, CEQ, FLU, ENR *, TET, SXT	*bla*$_{TEM}$	-	-	-	*aadA2*	*qnrS*	*sul2*	dfrA5	*mrx*	-
Steppe eagle (*Aquila nipalensis*)	Messenia	ESBL	AMP, CEX, CF, CFP, CEF, CEQ, FLU *, ENR *, TET, SXT	*bla*$_{CTX-M-1}$ *group*, *bla*$_{TEM}$	-	-	-	*aadA2*	*qnrS*	*sul2*	*dfrA5*	*mph, mrx*	-
Caspian gull (*Larus cachinnans*)	Messenia	CPE	AMP, AMC, TCC, CEX, CF, CFP, CEF, CEQ, IMI, GEN *, FLU, ENR, MRB, SXT	*bla*$_{CTX-M-1}$ group, *bla*$_{TEM}$	*bla*$_{NDM}$	-	*bla*$_{OXA-10}$	*aadB, aadA1, aadA2, ant2Ia*	*qnrB, qnrS*	*sul1, sul2*	*dfrA5, dfrA12*	*mph, mrx*	*intI1*

AMP—ampicillin; AMC—amoxicillin/clavulanic acid; TCC—ticarcillin/clavulanic acid; CEX—cefalexin; CF—cefalotin; CFP—cefoperazone; CEF—ceftiofur; CEQ—cefquinome; IMI—imipenem; GEN—gentamicin; NEO—neomycin; FLU—flumequine; ENR—enrofloxacin; MRB—marbofloxacin; TET—tetracycline; SXT—trimethoprim/sulfamethoxazole; * intermediate resistance; PMQR—plasmid-mediated quinolone resistance genes; the isolate did not harbor genes of this category.

Table 4.3. Comparison between the microarray-based genotype and the phenotype obtained by VITEK-2 system.

Detected AMR Genotype	AMR Gene Family	Expected AMR Phenotype	Antibiotics Tested	No of Isolates Harboring the Genotype by Microarray	No of Resistant Isolates by VITEK	No of Susceptible Isolates by VITEK	Concordance (%)
bla$_{CTX-M-1}$ group	ESBL	Resistant to 4G/3G cephalosporins, other β-lactams	AMP, AMC, TCC, CEX, CF, CFP, CEF, CEQ	4	4	0	100
bla$_{CTX-M-1}$ group, *bla*$_{TEM}$	ESBL	Resistant to 4G/3G cephalosporins, other β-lactams	AMP, AMC, TCC, CEX, CF, CFP, CEF, CEQ	8	8	0	100

bla$_{TEM}$ (consensus)	ESBL	Resistant to 4G/3G cephalosporins, other β-lactams	AMP, AMC, TCC, CEX, CF, CFP, CEF, CEQ	1	1	0	100
bla$_{NDM}$	Carbapenemases	Resistant to carbapenems, 3G/4G cephalosporins, other β-lactams	IMI, AMP, AMC, TCC, CEX, CF, CFP, CEF, CEQ	1	1	0	100
bla$_{ACT}$	AmpC	Resistant to 4G/3G cephalosporins, other β-lactams	AMP, AMC, TCC, CEX, CF, CFP, CEF, CEQ	4	4	0	100
bla$_{OXA-1}$	NSBL	Resistant to other β-lactams	AMP, AMC, TCC, CEX, CF	1	1	0	100
bla$_{OXA-10}$	NSBL	Resistant to other β-lactams	AMP, AMC, TCC, CEX, CF	1	1	0	100
aadA4	Aminoglycosides	Resistant to aminoglycosides	GEN, NEO	1	0	1	0
aadA2	Aminoglycosides	Resistant to aminoglycosides	GEN, NEO	6	0	6	0
aadA2, rmtA	Aminoglycosides	Resistant to aminoglycosides	GEN, NEO	1	0	1	0
aadA4, rmtA	Aminoglycosides	Resistant to aminoglycosides	GEN, NEO	3	0	3	0
aadB, aadA1, aadA2, ant2Ia	Aminoglycosides	Resistant to aminoglycosides	GEN, NEO	1	1	0	100
aphA	Aminoglycosides	Resistant to aminoglycosides	GEN, NEO	1	1	0	100
qnrS	Quinolones	Resistant to fluoroquinolones	FLU, ENR, MRB	11	11	0	100
qnrB, qnrS	Quinolones	Resistant to fluoroquinolones	FLU, ENR, MRB	1	1	0	100

sul1, sul2, dfrA17	Sulfonamides - Trimethoprim	Resistant to sulfonamides/trimethoprim	SXT	1	1	0	100
sul1, sul2, dfrA5, dfrA12	Sulfonamides - Trimethoprim	Resistant to sulfonamides/trimethoprim	SXT	1	1	0	100
sul1, sul2, dfrA5, dfrA17	Sulfonamides - Trimethoprim	Resistant to sulfonamides/trimethoprim	SXT	3	3	0	100
sul2, dfrA5	Sulfonamides - Trimethoprim	Resistant to sulfonamides/trimethoprim	SXT	7	7	0	100
sul2	Sulfonamides - Trimethoprim	Susceptible to sulfonamides/trimethoprim	SXT	1	0	1	100
Overall concordance (mean)					**80%**		

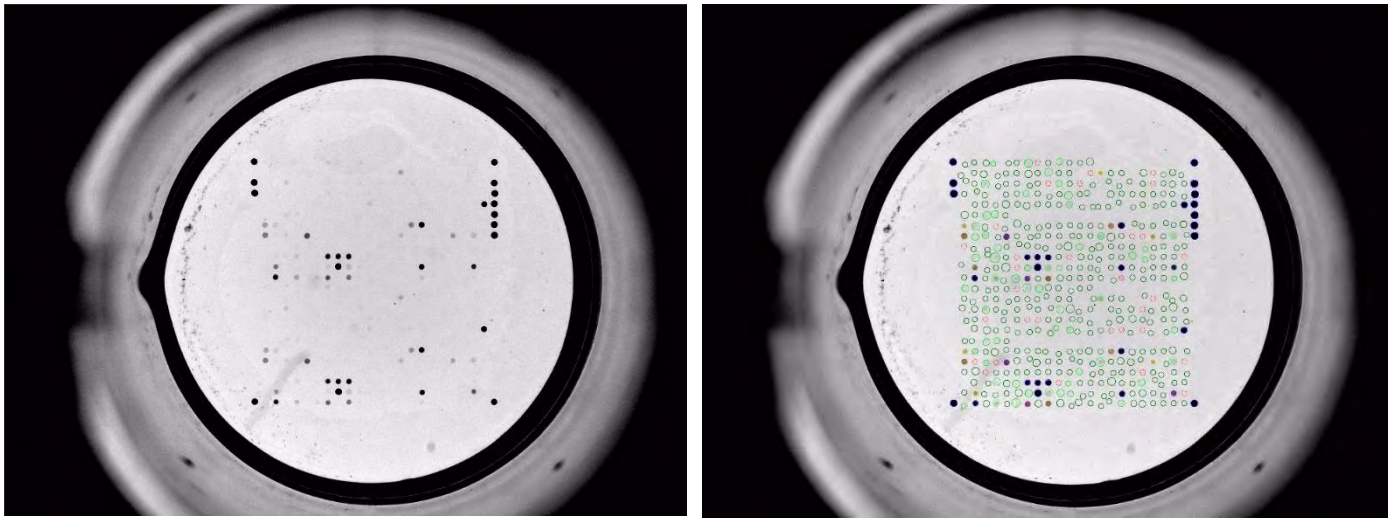

Figure 4.1. Array results for the ESBL E. coli strain obtained from a Common buzzard (left), same array with an interpretation grid for fully automated spot recognition (right).

4.3.2. Occurrence and Characteristics of Carbapenemase-Producing *E. coli*

Among the 362 samples tested, only one, originating from a Caspian gull (*Larus cachinnans*), yielded a positive culture on the CPE selective media (1/362, 0.3%). The isolated *E. coli* strain was resistant to imipenem and was phenotypically detected to produce MBL in the meropenem-EDTA test.

This isolate was MDR, also being resistant to the other tested β-lactams, as well as to gentamicin, flumequine, enrofloxacin, marbofloxacin and trimethoprim/sulfamethoxazole, while it was susceptible to neomycin and tetracycline.

AMR genotyping revealed that the isolate harbored the carbapenemase gene *bla*NDM, as well as genes encoding for other β-lactamases (*bla*CTX-M-1/15, *bla*TEM, *bla*OXA-10). Additionally, genes associated with aminoglycoside (*aadB*, *aadA1*, *aadA2*, *ant2Ia*), quinolone (*qnrB*, *qnrS*), sulfonamide (*sul1*, *sul2*), trimethoprim (*dfrA5*, *dfrA12*) and macrolide (*mph*, *mrx*) resistance were detected. The isolate also carried the *intI1* gene.

The antimicrobial resistance phenotype and genotype of the carbapenemase-producing *E. coli* is presented in Tables 4.2 and 4.3.

4.4. Discussion

The present study describes the occurrence and the molecular characteristics of ESBL and carbapenemase-producing *E. coli* recovered from wild and feral birds' feces in Greece. The incidence of ESBL-producing *E. coli* was rather low (3.3%), which is in accordance with reports from Italy (4/103, 3.9%) [28], Sweden (3/100, 3%) [29], Brazil (5/204, 2.4%) [30] and Alaska (3/76, 3.9%) [31]. The presence of carbapenemase-producing *E. coli* was even lower (0.3%) and comparable to that identified in recent research from other countries, including Spain [32].

However, our results differ significantly from former studies that have reported higher ESBL detection rates in Chile (67/124, 54%) [33], Spain (68/132, 51.5%) [34], Pakistan (26/150, 17.3%) [35] and The Netherlands (51/414, 12.3%) [36], as well as comparatively elevated CPE detection rates in Tunisia (2/150, 1.3%) [12], Algeria (3/32, 9.4%) [37], France (22/158,

13.9%) [38] and Australia (120/504, 23.8%) [39]. This discrepancy could be attributed either to a lower prevalence of such resistant bacteria in the region or to the fact that many prior studies have only been focused on "target" wild bird species, i.e., migratory, omnivorous and aquatic birds. Nevertheless, the systematic review conducted by Chung et al. (2018) has highlighted that, based on the birds' migratory status and habitat type, no significant differences in antibiotic resistance rates are observed [40]. Subsequently, in this study, we evaluated ESBL and CPE carriage in several species, including not only high-risk ones, for screening purposes.

ESBL-producing *E. coli* was retrieved from eight different wild bird species. Four species; Common buzzard, Short-toed snake eagle, Steppe eagle and Eurasian sparrow hawk, are raptors and two; Steppe eagle and Eurasian magpie, can be considered omnivores and scavengers. Their diet, which is commonly in direct contact with anthropogenic environments and agricultural animals and waste, constitutes a major potential route for ESBL acquisition [41]. Another alternative pathway is the environment inhabited by the aforementioned species, as also applies to the Grey heron, a waterbird. Aquatic associated species have been documented to be important ESBL hosts as a result of human activities in their habitat [36,42]. Of note, ESBL strains were also detected in samples from a Eurasian Scops owl and a Common swift, two insectivorous birds that live in urbanized areas in vicinity to humans, possibly underlining the importance of human-derived environmental contamination in the dissemination of AMR. Nevertheless, a potential transmission via insects, such as houseflies, cannot be ruled out either [43,44]. Four of the birds that were found to be colonized by ESBL *E. coli* (Short-toed snake eagle, Steppe eagle, Grey heron, Common swift) are also migratory and could thus act as dispersion vectors across long distances [45].

Molecular characterization of the ESBL isolates revealed a dominance of $bla_{\text{CTX-M-1}}$ group, which is consistent with the majority of published studies on wild avian species to date [12,32,46–51]. *E. coli* producing CTX-M group 1 β-lactamases, especially CTX-M-1 and CTX-M-15, has been described as an alarming cause of human infections globally, while variant CTX-M-1 further prevails among food-producing animals [52–54]. Notably, we also detected the acquired AmpC gene bla_{ACT} in four of the isolates, but not $bla_{\text{CMY-2}}$, even though prior studies worldwide as well as in Greece have described the latter as the most dominant plasmid encoded AmpC beta-lactamase gene in Enterobacteriaceae of wild bird origin [55–57]. Co-occurrence of bla_{ACT} and $bla_{\text{CTX-M-1}}$ group has previously been reported in an *Escherichia fergusonii* isolated from a Cattle egret in Tunisia [12].

Of special interest is the fact that a carbapenemase-producing, bla_{NDM} positive *E. coli* was identified in the fecal sample of a Caspian gull. The species is one of the most numerous in Greece with a wide range of different feeding habitats. This particular bird was sampled in Porto Lagos, a coastal area in eastern Macedonia, within a wide wetland complex that includes a lagoon, lakes and numerous islands. This site is part of the Ramsar Convention and is regarded as a location of considerable ecological value for breeding and wintering waterbirds and raptors [58]. In Porto Lagos, Caspian gull individuals are either resident or may originate from several other regions, such as the Black Sea, having undertaken a lengthy migration, especially in their first calendar year [59]. Noteworthily, another NDM producing strain, namely a NDM-1 *Klebsiella pneumoniae* recovered from the feces of a Caspian gull, has recently been reported from the Azov-Black Sea in Ukraine [60]. Therefore, we could hypothesize that this resistance gene has been imported to Greece via migratory birds or

alternatively that it circulates in the region due to human contamination. In most parts of the world, NDM-type MBLs are sporadically described among humans [3], however, studies in Greece during the past three years have outlined a wide dissemination and establishment of NDM-producing Enterobacteriaceae [61,62]. Given the fact that Caspian gulls frequently feed on anthropogenic food sources such as trawler discards and refuse dumps or nearby livestock-associated facilities such as slaughterhouses, uptake of the resistant strain or the resistance determinants during feeding could also be possible [63]. NDM-producing *E. coli* has also been recovered from other gull species and, in particular, from Yellow-legged gulls (*Larus michahellis*) in Spain, Lesser black-backed gulls (*Larus fuscus*) in Spain and Franklin's gulls (*Larus pipixcan*) in Chile [60]. Additionally, Black kites (*Milvus migrans*) have been found to harbor $bla_{\text{NDM-5}}$ *E. coli* in Pakistan and $bla_{\text{NDM-1}}$ *Salmonella enterica* subsp. *enterica* serovar Corvallis in Germany, the latter being the first ever description of a carbapenemase producer isolated from wild animals [60,64]. Here we report, for the first time in Greece, the detection of a carbapenemase-producing organism from a wild bird, which was identified as NDM positive *E. coli*.

Although our study focused on β-lactamase mediated resistance (ESBLs and carbapenemases), we additionally identified high rates of non-wild-type susceptibility to almost all the antibiotic classes tested, including tetracyclines, sulfonamides, trimethoprim and quinolones. Microarray analysis confirmed the presence of resistance genes against several antibiotics, with variants *sul2*, *dfrA5*, *qnrS*, *mrx* and *aadA2* being the most frequently detected resistance determinants for sulfonamides, trimethoprim, quinolones, macrolides and aminoglycosides, respectively. The detection of multiple AMR genes in all the isolates probably indicates the wide dissemination of AMR determinants in the environment and could be attributed to the presence and cohabitation of transferable plasmids [17,65]. Moreover, two ESBL- and the carbapenemase-producing isolates carried *intI1*, a gene encoding an element known to play a crucial role in the recruitment, spread and expression of resistance genes [66]. Numerous earlier studies have shown an association of both $bla_{\text{CTX-M}}$ and bla_{NDM} with the variable region of class 1 integrons in *E. coli* isolates of human and animal origin [67–70]. Among wild animals, *intI1* presence is related to their close contact with humans, farm animals and pets [71]. However, previous research has presented a higher incidence of *intI1* among ESBL producers isolated from wildlife varying between 72.4% and 100% [46,50,72], a discrepancy that requires further investigation.

4.5. Conclusions

This study provides evidence of wild birds' colonization with ESBL- and carbapenemase producing *E. coli* in Greece. The circulation of $bla_{\text{CTX-M-1}}$ group, bla_{TEM} and bla_{NDM}, along with genes conferring resistance to five classes of non β-lactam antimicrobials (fluoroquinolones, trimethoprim, sulfonamides, aminoglycosides, macrolides), poses a serious threat for the spread of MDR bacteria. Thus, wild birds should be regarded as a reservoir, vehicle and indicator of AMR in the environment. Considering that ESBL-PE and CPE detection rates among humans in Greece remain high, regular surveillance studies are required to fully unveil the extent of wild birds' role in the circulation of such pathogens.

Supplementary Material

Supplementary Table S4.1. Genes detected by the CarbaResist DNA microarray-based assay.

Target gene	Gene function	Accession number
gad	glutamate decarboxylase of *Escherichia coli*	AE014075.1
ihfA	integration host factor subunit alpha	U00096.3
dnaE	DNA polymerase III subunit alpha	U00096.3
basC	acinetobactin biosynthesis protein of *Acinetobacter baumannii*	AY571146.1
efp	elongation factor P of *Acinetobacter baumannii*	CP001172.1
pld	phospholipase D of *Acinetobacter baumannii*	CP000521.1
cfa	colicin five activity protein of *Citrobacter freundii* and *Citrobacter braakii*	U09771.1
ecfX	extracytoplasmic function sigma factor of *Pseudomonas aeruginosa* [LavenirR-JocktaneD-2007]	DQ996558.1
invA	invasin A, highly specific for genus *Salmonella*	CP000026.1
ipaH9.8	invasion plasmid antigen	AF047365.1
khe	klebsolysin of *Klebsiella pneumoniae*	AF293352.1
lacY	lactose permease; the *lacY* gene is missing in all *Shigella spp*.	U00096.2
rrs	16S rRNA	U00096.3
*bla*BIC	carbapenemase, class A beta-lactamase	GQ260093.1
*bla*DIM	carbapenemase, class B metallo beta-lactamase	KC004136.2
*bla*GES	carbapenemase, class A beta-lactamase	AY219651.1
*bla*GIM	carbapenemase, class B metallo beta-lactamase	consensus
*bla*GOB	carbapenemase, class B metallo beta-lactamase	consensus
*bla*IMI-3 (NmcA)	carbapenemase, class A beta-lactamase associated with imipenem resistance	AY780889.1
*bla*IMI-R	regulator of *bla*IMI-3 (NMC-A)	Z21956.1
*bla*IMP	carbapenemase, class B metallo beta-lactamase	consensus
*bla*IMP25 (*bla*SIM-1)	carbapenemase, class B metallo beta-lactamase (synonym: *bla*SIM)	EU686387.1
*bla*IMP35	carbapenemase, class B metallo beta-lactamase	JQ432564.1
*bla*IND	carbapenemase, class B metallo beta-lactamase of Chryseobacterium	consensus
*bla*KHM	carbapenemase, class B metallo beta-lactamase	consensus
*bla*KPC	carbepenemase, class A serin beta-lactamase	consensus
*bla*NDM	carbapenemase, class B metallo beta-lactamase (New Dehli metallo beta-lactamase)	consensus
*bla*PAM-1	carbapenemase, subclass B3 metallo beta-lactamase (Pseudomonas alcaligenes metallo-betalactamase)	AB858498.1
*bla*SFH-1	carbapenemase, class B metallo beta-lactamase	AF197943.1
*bla*SMB-1	carbapenemase, class B metallo beta-lactamase	AB636283.1
*bla*SME	carbapenemase, class A beta-lactamase	consensus
*bla*SPM-1	carbapenemase, class B metallo beta-lactamase	AY341249.1
*bla*VIM	carbapenemase, class B metallo beta-lactamase	consensus
*bla*VIM-2	carbapenemase, class B metallo beta-lactamase	AF191564.1
*bla*VIM-7	carbapenemase, class B metallo beta-lactamase	AJ536835.1
*bla*OXA-23-like	carbapenemase, class D beta-lactamase	AJ132105.1
*bla*OXA-40-like	carbapenemase, class D beta-lactamase	AF509241.1
*bla*OXA-48-like	carbapenemase, class D beta-lactamase	AY236073.2
*bla*OXA-51-like	carbapenemase, class D beta-lactamase	CP000863.1
ISABa1 to *bla*OXA-51	Insertion sequence ABa1 is adjacent to *bla*OXA-51-like gene. This combination mediated carbapenem resistant in Acinetobacter baumannii isolates	CP001921.1
no ISABa1 to *bla*OXA-51	Insertion sequence ABa1 is not adjacent to *bla*OXA-51-like gene. This combination does not mediate carbapenem resistant in Acinetobacter baumannii isolates	CU459141.1
*bla*OXA-54	carbapenemase, class D beta-lactamase	AY500137.1
*bla*OXA-55	carbapenemase, class D beta-lactamase	AY343493.1
*bla*OXA-58	carbapenemase, class D beta-lactamase	AY665723.1
*bla*OXA-134/235/284	carbapenemase *bla*OXA-134 or -235 or -284, class D beta-lactamase	AYHO01000005.1
*bla*OXA-143/182/253/255	carbapenemase *bla*OXA-40-like, class D beta-lactamase	GQ861437.1
*bla*OXA-181/232	carbapenemase *bla*OXA-48-like, class D beta-lactamase	CP000469.1

Target gene	Gene function	Accession number
*bla*OXA-214	carbapenemase, class D beta-lactamase	JN861783.1
*bla*OXA-279	carbapenemase, class D beta-lactamase	APOK01000044.1
*bla*OXA-292	carbapenemase, class D beta-lactamase	APRH01000012.1
*bla*CME	extended spectrum beta-lactamase, class A	AF033200.1
*bla*CTX-M1/15	extended spectrum beta-lactamase, class A	X92506.1, HQ202266.1
*bla*CTX-M2	extended spectrum beta-lactamase, class A	AF286192.1
*bla*CTX-M8	extended spectrum beta-lactamase, class A	AY750914.2
*bla*CTX-M9	extended spectrum beta-lactamase, class A	FQ482074.1
*bla*MOX-CMY9	extended-spectrum beta-lactamase precursor, class C	AF381617.1
*bla*PER-1	extended-spectrum beta-lactamase, class A beta-lactamase PER-1	Z21957.1
*bla*PER-2	extended-spectrum beta-lactamase, class A beta-lactamase PER-2	X93314.1
*bla*SHV	class A beta-lactamase - consensus sequence for *bla*SHV genes, including extended-spectrum beta-lactamases	consensus
*bla*TEM	class A beta-lactamase - consensus sequence for *bla*TEM genes, including extended-spectrum beta-lactamases	consensus
*bla*VEB	extended-spectrum beta-lactamase, class A	consensus
*bla*OXA-18	extended spectrum beta-lactamase, class D	EU503121.1
*bla*OXA-45	extended spectrum beta-lactamase, class D	AJ519683.1
*bla*OXA-1	narrow spectrum beta-lactamase, class D	AY458016.1
*bla*OXA-9	narrow spectrum beta-lactamase, class D	M55547.1
*bla*OXA-2	consensus probe for extended and narrow spectrum class D beta-lactamases belonging to group *bla*OXA-2	consensus
*bla*OXA-10	consensus probe for extended and narrow spectrum class D beta-lactamases belonging to group *bla*OXA-10	consensus
*bla*OXA-60	narrow spectrum beta-lactamase, class D	AF525303.2
*bla*MIR	extended spectrum beta-lactamase, class C beta-lactamase	M37839.2
*bla*ACC	AmpC beta-lactamase	EF554600.1
*bla*ACT	AmpC beta-lactamase	U58495.2
*bla*CMY	AmpC beta-lactamase, consensus sequence	consensus
*bla*DHA	AmpC beta-lactamase	EF406115.1
*bla*FOX	AmpC beta-lactamase	consensus
*bla*MOX	AmpC beta-lactamase	consensus
aac(3')	3-N-aminoglycoside acetyltransferase; associated with resistance to astromicin; gentamicin; sisomicin	consensus
aac(3')-Ia	3-N-aminoglycoside acetyltransferase; associated with resistance to astromicin; gentamicin; sisomicin	U90945.1
aac(3')-Ib	3-N-aminoglycoside acetyltransferase; associated with resistance to astromicin; gentamicin; sisomicin	KJ679408.1
aac(3')-Ic	3-N-aminoglycoside acetyltransferase; associated with resistance to astromicin; gentamicin; sisomicin	AJ511268.1
aac(3')-Ie	3-N-aminoglycoside acetyltransferase; associated with resistance to astromicin; gentamicin; sisomicin	AY458224.1
aac(3')-IVa	3-N-aminoglycoside acetyltransferase; associated with resistance to apramycin; dibekacin; gentamicin; netilmicin; sisomicin; tobramycin	EU784152.1
aac(6')	aminoglycoside 6'-N-acetyltransferase, associated with resistance to amikacin; dibekacin; isepamicin; netilmicin; sisomicin; tobramycin	consensus
aac(6')-31	aminoglycoside 6'-N-acetyltransferase; associated with resistance to streptomycin, spectinomycin	AJ640197.1
aac(6')-Ib	aminoglycoside 6'-N-acetyltransferase; associated with resistance to streptomycin, spectinomycin	M21682.1
aac(6')-II	aminoglycoside 6'-N-acetyltransferase; associated with resistance to streptomycin, spectinomycin	EF127959.1
aac(6')-IIa	aminoglycoside 6'-N-acetyltransferase; associated with resistance to streptomycin, spectinomycin	EU912537.1
aac(6')-IIc	aminoglycoside 6'-N-acetyltransferase; associated with resistance to streptomycin, spectinomycin	EU855788.1
aac-aph	6'-aminoglycoside-N-acetyltransferase/2''-aminoglycoside phosphotransferase; associated with resistance to gentamycin	AE017171.1
aadA1	aminoglycoside adenyltransferase; associated with resistance to streptomycin, spectinomycin	EU704128.1
aadA2	aminoglycoside adenyltransferase; associated with resistance to streptomycin, spectinomycin	EU704128.1
aadA4	aminoglycoside adenyltransferase; associated with resistance to streptomycin, spectinomycin	Z50802.3
aadB	2''-aminoglycoside nucleotidyltransferase	L06418.4
ant2	aminoglycoside (2") adenylyltransferase; associated with resistance to dibekacin; gentamicin; kanamycin; sisomicin; tobramycin	L06418.4
aphA	aminoglycoside 3'-phosphotransferase; kanamycin resistance protein	AY260546.3

Target gene	Gene function	Accession number
armA	16S rRNA methylase, associated with aminoglycoside resistance	AB117519.1
grm	16S rRNA methylase, associated with gentamicin resistance	M55521.1
npmA	16S rRNA methylase, associated with aminoglycoside resistance	AB261016.1
rmtA	16S rRNA methylase, associated with aminoglycoside resistance	AB083212.2
rmtB	16S rRNA methylase, associated with aminoglycoside resistance	DQ345788.1
rmtC	16S rRNA methylase, associated with aminoglycoside resistance	AB194779.2
rmtD	16S rRNA methylase, associated with aminoglycoside resistance	DQ914960.2
mph	macrolide 2'-phosphotransferase	consensus
mrx	member of macrolide inactivation gene cluster *mphA – mrx - mphR*	consensus
qepA	QepA - fluoroquinolone/quinolone efflux pump	AM886293.1
qnrA1	quinolone or fluoroquinolone resistance protein	AY931018.1
qnrB	quinolone or fluoroquinolone resistance protein	AB281054.1
qnrC	quinolone or fluoroquinolone resistance protein	EU917444.1
qnrD	quinolone or fluoroquinolone resistance protein	FJ228229.1
qnrS	quinolone or fluoroquinolone resistance protein	AM234722.1
sul1	dihydropteroate synthetase type 1	AJ698325.1
sul2	dihydropteroate synthetase type 2	DQ464881.1
sul3	dihydropteroate synthetase type 3	AJ459418.2
dfrA1	dihydrofolate reductase type 1	AJ884723.1
dfrA5	dihydrofolate reductase type 5	AB188269.1
dfrA7	dihydrofolate reductase type 7	AB161450.1, AM237806.1
dfrA12	dihydrofolate reductase type 12	AB154407.1
dfrA13	dihydrofolate reductase type 13 (synonym A21)	Z50802.3
dfrA14	dihydrofolate reductase type 14	AJ313522.1
dfrA15	dihydrofolate reductase type 15	Z83311.1
dfrA17	dihydrofolate reductase type 17	AF169041.1
dfrA19	dihydrofolate reductase type 19	AJ310778.1
intI1	class 1 integron integrase	AY260546.3
intI2	class 2 integron integrase	AY183453.1
intI3	class 3 integron integrase	EF469602.1
tnpISEcp1	transposase for the transposon ISEcp1	AB543698.1
oqxA	OqxA - membran fusion protein, component of RND-type multidrug efflux pump, associated with olaquindox resistance	EU370913.1
oqxB	OqxB - integral membrane protein, component of RND-type multidrug efflux pump, associated with olaquindox resistance	EU370913.1
higA	higA is the antitoxin of the translation-dependent mRNA interferase toxin higB	U43847.1
higB	Ectopic expression of higB causes inhibition of cell growth which is alleviated by co-expression of higA	U43847.1
splA	splA is the antitoxin of the translation-dependent mRNA interferase toxin splT	EU294228.1
splT	Ectopic expression of splT causes inhibition of cell growth which is alleviated by co-expression of splA	EU294228.1
mcr-1 / mcr-2	mcr-1 and mcr-2, phosphoethanolamine transferase associated with resistance to colistin and polymyxin-type antibiotics	KP347127.1
mcr-3	mcr-3, phosphoethanolamine transferase associated with resistance to colistin and polymyxin-type antibiotics	KY924928.1
mcr-4	mcr-4, phosphoethanolamine transferase associated with resistance to colistin and polymyxin-type antibiotics	MF543359.1.1
mcr-5	mcr-5, phosphoethanolamine transferase associated with resistance to colistin and polymyxin-type antibiotics	NG_055658.1
mcr-6	mcr-6, phosphoethanolamine transferase associated with resistance to colistin and polymyxin-type antibiotics	NG_055781.1
mcr-7	mcr-7, phosphoethanolamine transferase associated with resistance to colistin and polymyxin-type antibiotics	NG_056413.1
mcr-8	mcr-8, phosphoethanolamine transferase associated with resistance to colistin and polymyxin-type antibiotics	NG_061399.1
mcr-9	mcr-9, phosphoethanolamine transferase associated with resistance to colistin and polymyxin-type antibiotics	MK070339.1

Supplementary Figure S4.2. CarbaResist microarray layout.

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Chapter 5. Presence of West Nile virus (WNV) Lineage 2 among wild birds in the Regions of Peloponnese and Western Greece

Abstract

West Nile virus (WNV), a zoonotic mosquito-borne virus, has recently caused human outbreaks in Europe, including Greece. Its transmission cycle in nature includes wild birds as amplifying hosts and ornithophilic mosquito vectors. The aim of this study was to assess WNV circulation among wild birds from two regions of Greece, Peloponnese and Western Greece, during 2022. To this end, a total of 511 birds belonging to 37 different species were sampled and molecularly screened. WNV RNA was detected from February to November in a total of 71 wild birds of 9 species originating from both investigated regions. The first eight positive samples were sequenced on a part of NS3 and according to the phylogenetic analysis, they belonged to the evolutionary lineage 2 and presented similarity to previous outbreak-causing Greek strains (Argolis 2017, Macedonia 2010 and 2012). It was more likely to identify a PCR positive bird as the population density and the distance from water sources decreased. The present report provides evidence of WNV occurrence in both Peloponnese and Western Greece during 2022, highlighting the need of avian species surveillance to be conducted annually and throughout the year. Magpies are proposed as sentinels for WNV monitoring.

Keywords: West Nile Virus, wild birds, Greece

5.1. Introduction

West Nile virus (WNV) is a zoonotic flavivirus that has evolved to be one of the most widespread arboviruses in the world [1]. It is transmitted via ornithophilic mosquitoes, mainly of the *Culex* genus, particularly to avian species, but also to mammalian hosts [2]. WNV was first described in Uganda in 1937 [3]. Since then, sporadic cases and several outbreaks were reported in Africa, the Middle East, Europe, and Asia [4]. Introduction of the virus in the United States was reported in 1999 and was characterized by significant avian morbidity (mainly *Corvidae* spp.) in the area of New York, followed by morbidity in humans. Over a decade, WNV has spread in all continents except Antarctica [5]. In the last few years, WNV cases have been observed particularly in Southern Europe, often associated with the major flyways of migratory birds [6]. Annual late-summer outbreaks of WNV occur regularly in European countries that border the Mediterranean Sea and the virus is endemic in some regions [7].

The host range for WNV is wide and includes over 300 species of birds, mammals, reptiles, and amphibians [8,9]. Wild birds are considered the main reservoir of the virus, which is maintained in nature in an enzootic bird-mosquito cycle [10]. The replication of WNV varies among hosts and is significantly higher in birds compared to other species [8]. Some bird species may have high levels of viremia for extended time without presenting clinical signs, therefore could contribute to the maintenance of the virus in nature as well as to its geographical expansion during annual migrations. Nevertheless, some species of raptors, jays and crows, are highly susceptible and can develop severe or fatal encephalitis [11]. Clinical infection in susceptible, incidental, dead-end hosts (humans and horses) is usually

asymptomatic, but may also result in mild febrile disease with flu-like symptoms, or occasionally in neuroinvasive disease, manifested with neurological deficits (ataxia, weakness, mental disturbances) and possibly death [12].

In Greece, the first report regarding detection of WNV antibodies in wild birds (*Pica pica*, *Corvus cornix*) dates back to 2009 in Central Macedonia [13]. Seropositive birds (*Pica pica*, *Corvus cornix*, *Streptopelia turtur*) were also identified in the same area during 2010-2011, while molecular detection of WNV lineage 2 was performed from a magpie near the village Trilofos in 2010 [13,14]. Positive avian sera (*Pica pica*, *Streptopelia turtur*) were additionally reported in 2011 in the regional units of Serres, Thessaloniki, Trikala, Larissa and Karditsa [15]. In 2017, Eurasian magpies with neurologic signs were identified in Argolida regional unit of Peloponnese region and subsequently WNV was molecularly detected [16]. Recently (2020), presence of the virus was reported on ten different wild bird species (*Pica pica, Passer domesticus, Parus major, Passer hispaniolensis, Garrulus glandarius, Corvus monedula, Ardea purpurea, Athene noctua, Strix aluco, Curruca communis*) originating from Peloponnese [17]. Human WNV infections as well as deaths were initially reported in the country on 2010 and by 2021 a total of 1420 cases including 201 fatalities were recorded from different regions [18].

Considering the established presence of WNV in Greece and the acknowledged value of wild bird surveillance systems in evaluating virus' circulation and supporting vector control decisions, the present study aimed to assess the occurrence of WNV in a sample of birds from two neighboring regions of Greece, namely Peloponnese and Western Greece, during the year 2022. In addition, the association of environmental parameters with the presence or absence of WNV in wild birds was investigated.

5.2. Materials and Methods

5.2.1 Study area

The present study was conducted in two distinct neighboring administrative entities of Greece, region of Peloponnese and region of Western Greece.

Region of Peloponnese includes the regional units Argolis, Arcadia, Laconia, Corinthia and Messenia, while region of Western Greece comprises of the regional units Achaia, Ilia and Aitoloakarnania. Wetland habitats of global interest for wild birds' breeding and wintering, with significant numbers of waterfowl and raptors are located in both regions. Additionally, other categories of habitats, as forest-agroforest areas, of their mountainous and semi-mountainous areas act as important ecosystems for avifauna. Given the presence of large numbers of wild birds on an annual basis (wintering-breeding) in the regions' natural habitats and considering that wild birds are the WNV reservoir in nature, a risk for viral transmission in these areas arises. This could contribute to the beginning of a new transmission cycle (birds-mosquitoes-birds), which may include humans and other mammals as final hosts.

5.2.2. Collection of biological material from wild birds

In the regions of Peloponnese and Western Greece, observations of wild bird species were conducted during 2022 regarding both resident species that could be a WNV reservoir and migratory species that could contribute to the entry of new virus strains in the regions.

Point-count stations, line-transects and direct count stations were used as recording techniques for their monitoring.

Sampling of wild birds was performed in diverse ecological niches of natural ecosystems, suburban and urban areas. Small cage traps (20 cm x 30 cm x 20 cm), Larsen Traps (60 cm x 50 cm x 50 cm), Multi Catch Larsen Trap (160 cm x 200 cm x 150 cm), groundnets and mistnets were used for capturing. To maximize the number of collection sites, portable traps were moved periodically to different locations and habitat types. The sampling procedure included collection of blood samples, and/or oropharyngeal swabs and/or fecal swabs (Copan). Upon the end of specimen collection, birds were released into their natural habitats according to the prerequisites of the Greek Legislation. Furthermore, environmental fecal samples were obtained by inserting a sterile cotton swab into recently deposited feces (n=21). Fresh bird carcasses (n=18) found alongside the road network during the field work were collected and shipped to the laboratory. Tissue samples (i.e., brain, heart, liver, spleen, kidney) were acquired by performing necropsies and stored immediately at -70^{o}C until molecular screening. During the 2022 official hunting season, hunter harvested tissue samples were additionally shipped to the laboratory from the Greek Hunting Federation of Peloponnese region. Coordinates of all sampling sites were recorded by Global Positioning System (GPS) units.

Specimens were collected from a total of 511 birds belonging to 37 different species. Specifically, 418 wild birds were sampled from Peloponnese region from January to November of 2022 and 93 from Western Greece from September to November of 2022. The sampling sites of both regions are depicted in Figure 5.1. Birds were characterized according to their migratory status as residents, passage migrants, summer visitors (breeding), partial migrants (breeding), non-breeding visitors and winter visitors.

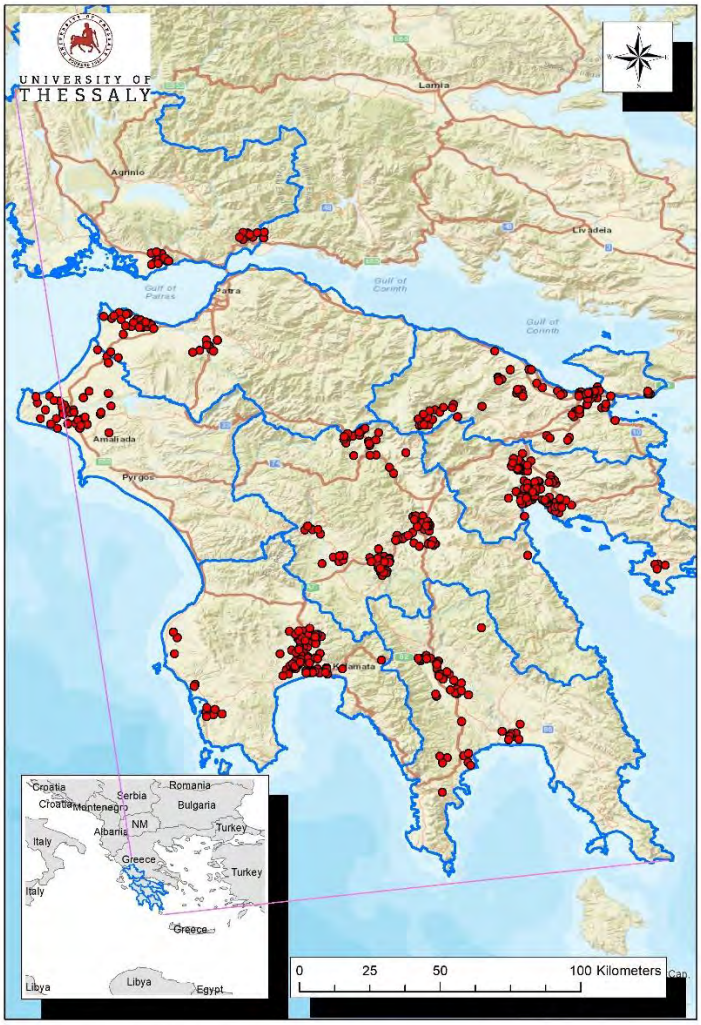

Figure 5.1. Map of Peloponnese and Western Greece regions. Sampling sites of wild birds are depicted with red dots. The boundaries of the regional units (Peloponnese region: Argolis, Arcadia, Laconia, Corinthia and Messenia, Western Greece region: Achaia, Ilia and Aitoloakarnania) are outlined with blue color.

5.2.3. Molecular detection of WNV in wild birds

Extraction of viral RNA was performed from blood samples, oropharyngeal swabs and fecal swabs (QIAGEN - QIAamp Viral RNA Mini kit) as well as from 20 mg of brain tissue samples and pooled tissue samples (kidney, heart, liver) (QIAGEN RNeasy Mini kit), according to the manufacturers' instruction. Particularly regarding swabs, prior to the extraction process they were immersed in 500 μl of PCR grade water, were shaken and squeezed to the sides of the tube to extract the liquid.

Hexaprimers were used for Reverse transcription (RT) by utilizing a commercial cDNA synthesis kit (SuperScript™ First-Strand Synthesis System for RT-PCR). A 423 bp fragment of the NS3 region of WNV lineage 2 was amplified by performing nested PCR [19]. PCR products of the second round were visualized by electrophoresis on 2% agarose gel and a 100 bp DNA marker was used to determine their amplicon sizes. The presence of WNV was confirmed on the first eight PCR positive samples by performing Sanger sequencing (3730xl DNA Analyzer,

Applied Biosystems, Foster City, CA, USA) using the primers WN-NS3up2 and WN-NS3do2 of the second PCR round [19].

5.2.5. Phylogenetic Analysis

Sequence alignment was performed by using ClustalW and was based on 398 bases of the NS3 region of 57 WNV strains and one Yellow fever virus (YFV) strain. Specifically, the analysis encompassed eight WNV lineage 2 strains, isolated from Peloponnese Region in 2022 and 49 WNV lineage 1, 2, 3, 4, 5, 7 and 8 strains, retrieved from GenBank database, while YFV (DQ235229.1) was used as an outgroup. The phylogenetic tree was constructed with the Neighbor-Joining algorithm [20] and the evolutionary distances were computed using the LogDet (Tamura-Kumar) method [21]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.87). A bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies [22]. Analysis was conducted with MEGA11 software [23].

5.2.6. Environmental variables

Environmental variables (Table 5.1) comprised climatic conditions, topography, land uses and human activities. Climate indices were derived from the WorldClim version 1.4. [24], digital elevation model was extracted from CGIAR-CSI GeoPortal [25] and hydrological data were retrieved from HydroSHEDS [26]. Human population density and 44 categories of land uses (Table S5.1) were downloaded from the European Environmental Agency [27,28]. The variable distance from livestock farms (sheep, goats, cattle) was generated for this study from the Geodata base of Laboratory of Microbiology and Parasitology, Veterinary Faculty, University of Thessaly. Eight environmental layers were created for the analysis by using ArcGIS 10·1 GIS software (ESRI, Redlands, CA, USA) and subsequently, the environmental variables were associated with the presence or absence of WNV RNA in wild birds. Data sets were converted to a common projection map extent and resolution prior to use in the modelling program.

Table 5.1. Environmental variables.

Environmental variable	Code
Annual mean temperature (°C)	clima1
Maximum temperature of warmest month (°C)	clima2
Heat stress index[1]	HI
Altitude (m)	dem
Distance from water collections and hydrographic network (m)	waterdis
Distance from livestock farms (sheep, goats, cattle, m)	farmdis
Land uses (44 classes)	landcorine
Human population density (people/km^2)	popden

[1]Heat Stress index: Number of days that experienced 'strong heat stress' (UTCI between 32 and 38°C) during June, July and August 2022 (Data source: ERA5-HEAT. Credit: C3S/ECMWF).

5.2.7. Statistical Analysis

Statistical analysis was performed using Stata 17 (StataCorp. 2021. Stata: Release 17. Statistical Software. College Station, TX: StataCorp LLC) and evaluated for significance at the 5% level. Descriptive statistics of collected data were performed. The evaluation of the potential association between detection (presence or absence) of WNV RNA in collected samples with the recorded environmental variables was performed with the use of a logistic regression model. Presence or absence of WNV RNA was the dependent variable, while the parameters: (i) annual mean temperature (^{o}C), (ii) maximum temperature of warmest month (^{o}C), (iii) heat stress index, (iv) altitude (m), (v) distance from water collections and hydrographic network (m), (vi) distance from livestock farms (sheep, goats, cattle, m), (vii) land uses , and (viii) human population density (people/km^2), were the independent ones. All independent variables were initially screened one by one in univariate logistic regression models. During this process, a significance level of 0.25 was applied as a screening criterion, since a more traditional level (such as $p < 0.05$) could fail to identify variables known to be important [29]. Subsequently, factors with $p < 0.25$ were offered simultaneously to a full model, successively reduced by backwards elimination [30] until only significant ($p < 0.05$) variables remained. Two-factor interactions between the remaining variables were created and tested for significance, by offering them one at a time to the model. Lastly, previously excluded variables were re-offered one-by-one to the final model, to ensure that variables that could significantly add to the model were not omitted.

5.3. Results

5.3.1. Molecular Detection of WNV in Wild Birds

During 2022, a total of 418 wild birds belonging to 39 different species from Peloponnese region and 93 wild birds from three different species from Western Greece region were molecularly screened for the presence of WNV. Viral RNA was detected in 71 wild birds of 9 species (Table 5.2, Figure S5.1).

Particularly, virus presence in Peloponnese region was demonstrated in 52 birds of 9 different species: magpie (n=37), song thrush (n=6), hooded crow (n=2), common starling (n=2), common blackbird (n=1), Eurasian jay (n=1), black-winged stilt (n=1), great tit (n=1) and house sparrow (n=1). Positive samples originated from all five regional units of the region (Argolis, Arcadia, Laconia, Corinthia, Messenia) and were obtained during the months February-April, July and September-November. Sanger sequencing was performed on the eight first strains of the virus obtained from magpies (n=4), song thrushes (n=3) and a common starling (n=1) that were detected as early as the end of February from regional units of Arcadia, Corinthia and Messenia (Table S5.2).

Regarding Western Greece region, sampled species consisted of magpies, song thrushes and common starling. Nineteen magpies were found positive throughout the sampling period (September to November) and originated from all three regional units of the region (Achaia n=5, Ilia n= 9 and Aitoloakarnania n=5).

The virus was neither detected in the birds found dead on the road network (n=18) nor in the environmental fecal samples (n=21).

The results of WNV detection are summarized in Table 5.2. The sampling sites where positive and negative wild birds occurred are depicted in Figure 5.2.

Table 5.2. Tested and WNV positive wild bird species from Peloponnese and Western Greece regions during 2022.

Scientific Name	Common Name	Status [a]	Regional Units of Peloponnese Region					Regional Units of Western Greece Region		
			Argolis	Arcadia	Corinthia	Laconia	Messenia	Achaia	Ilia	Aitoloakarnania
Anas (Mareca) penelope	Eurasian wigeon	WV, PM	0/1	-	-	-	-	-	-	-
Anas platyrhynchos	Mallard	WV, r	0/1	-	-	-	-	-	-	-
Ardea cinerea	Grey heron	R, PM	0/2 [E]	-	-	-	-	-	-	-
Ardeola ralloides	Squacco heron	SV, PM	0/1[E]	-	-	-	-	-	-	-
Buteo buteo	Common buzzard	R, WV	0/1[D]	0/1[D]	-	-	-	-	-	-
Calidris spp.	-	PM, WV	0/3[E]	-	-	-	-	-	-	-
Carduelis carduelis	European goldfinch	R, wv	0/1	-	-	-	0/2	-	-	-
Cettia cetti	Cetti's warbler	R	-	-	-	-	0/2	-	-	-
Charadius dubius	Little ringed plover	SV, PM	0/3[E]	-	-	-	-	-	-	-
Columba livia	Rock dove	R	0/1	-	-	-	0/4+1[D]	-	-	-
Corvus cornix	Hooded crow	R	0/2	0/5	0/11	1/17	1/7	-	-	-
Corvus (Coloeus) monedula	Western jackdaw	R	-	0/6	-	-	-	-	-	-
Egretta garzetta	Little egret	PM, R	0/3[E]	-	-	-	-	-	-	-
Erithacus rubecula	European robin	WV, r	0/2	0/1+1[D]	0/1	-	0/1	-	-	-
Fringilla coelebs	Common chaffinch	R, WV	-	0/1[D]	-	-	0/1[D]	-	-	-
Gallinago gallinago	Common snipe	WV, PM	0/1	-	-	-	-	-	-	-
Garrulus glandarius	Eurasian jay	R	0/6	0/3	1/1	-	0/3	-	-	-
Himantopus himantopus	Black-winged stilt	PM, SV	1/6	-	-	-	-	-	-	-
Hirundo rustica	Barn swallow	SV, PM	-	-	0/1[D]	-	-	-	-	-
Induna (Iduna) pallida	Eastern olivaceous warbler	SV	-	-	-	0/3	0/4	-	-	-
Lanius collurio	Red-backed shrike	SV, PM	-	0/1	-	-	-	-	-	-
Larus michahellis	Yellow-legged gull	R	0/2[E]+1[D]	-	-	-	-	-	-	-
Larus (Chroicocephalus) ridibundus	Black-headed gull	WV, r	0/5[E]	-	0/3	-	-	-	-	-
Numerius (Numenius) arquata	Eurasian curlew	WV, PM	0/1[E]	-	-	-	-	-	-	-
Otus scops	Eurasian scops owl	PLM	0/1[D]	-	0/1	-	-	-	-	-
Parus major	Great tit	R	-	-	-	1/4	0/3	-	-	-
Passer domesticus	House sparrow	R	0/4	0/7	0/13+1[D]	0/2	1/6	-	-	-
Phalacrocorax carbo	Great cormorant	WV, r	0/1[E]	-	-	-	-	-	-	-

Pica pica	Magpie	R	9/47+2[D]	15/58+2[D]	4/36	0/4	9/37	5/22+1[D]	9/38	5/14
Scolopax rusticola	Eurasian woodcock	WV, r	-	-	0/2	0/1	-	-	-	-
Streptopelia decaocto	Eurasian collared dove	R	-	-	0/8	-	-	-	-	-
Strix aluco	Tawny owl	R	-	-	0/2[D]	-	-	-	-	-
Sturnus vulgaris	Common starling	WV, R	-	0/1	1/1	-	1/3	0/8	-	0/7
Sylvia (Curruca) communis	Common whitethroat	SV, PM	-	0/1	-	-	-	-	-	-
Sylvia (Curruca) melanocephala	Sardinian warbler	R	-	-	0/2	0/1	0/2	-	-	-
Turdus merula	Common blackbird	R, WV	-	-	0/1	1/4	0/1	-	-	-
Turdus philomelos	Song thrush	WV, r	2/8	0/4	3/10	0/8+1[D]	1/4	-	-	0/3

R - resident, PM - Passage Migrant, SV - Summer visitor (breeding), PLM - Partial migrant (breeding), WV - Winter visitor

- no samples were tested

[a] Capital letters denote that the species is common in this category while small letters that it is rare [31,32]

D - dead wild birds

E – environmental samples

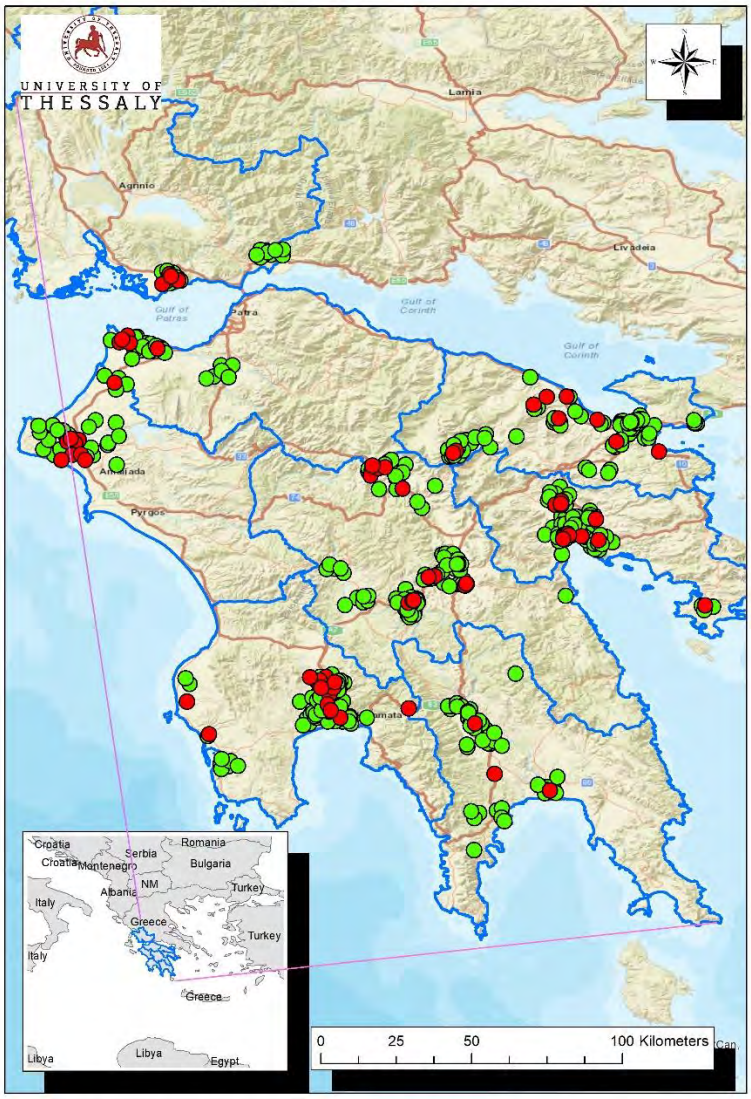

Figure 5.2. Map of the study area. Locations of WNV positive and negative wild birds are depicted with red and green dots, respectively. The boundaries of the regional units (Peloponnese region: Argolis, Arcadia, Laconia, Corinthia and Messenia, Western Greece region: Achaia, Ilia and Aitoloakarnania) are outlined with blue color.

5.3.2. Phylogenetic analysis

Part of NS3 of the first eight WNV strains was recovered from eight different birds sampled from the geographical areas of Arcadia (n=3), Corinthia (n=2), and Messenia (n=3) of Peloponnese region during February 2022. According to the phylogenetic tree that was constructed (Figure 3), these strains belong to the evolutionary lineage 2 and form a group together, displaying 98.86-100% nucleotide similarity. They show high resemblance (98.46-100%) to those that circulated in Argolis in 2017 and in Macedonia in 2010 and 2012, as well as to Belgian, Italian, Serbian, Bulgarian and Hungarian stains. Peloponnese wild birds' strains were phylogenetically closer to Greek strains that circulated in Central and Northern

Greece prior to 2020 (97.9-99.4%), than to the ones obtained from the same areas after 2020 (97.63-99.7%), as the latter formed a separate group.

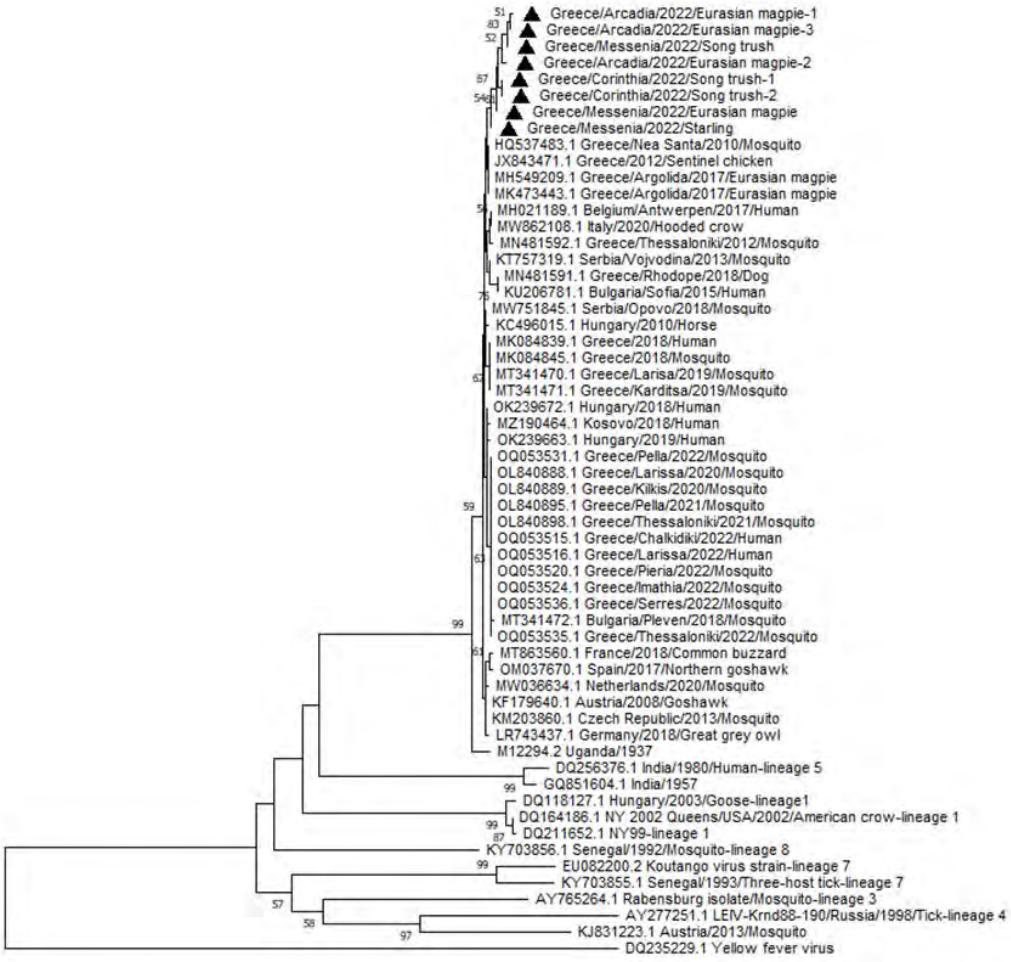

Figure 5.3. Phylogenetic tree depicting the relationships of eight WNV lineage 2 strains, isolated from Peloponnese Region in 2022 (black triangles) and 49 WNV strains retrieved from GenBank database. YFV strain DQ235229.1 was used as an outgroup. The evolutionary history was inferred using the Neighbor-Joining method and distances were computed using the LogDet (Tamura-Kumar) method. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.87). Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points; only values over 50% are indicated. This analysis involved 58 nucleotide sequences and there was a total of 398 positions in the final dataset.

5.3.3 Statistical Analysis

After the screening process, two parameters, namely, human population density and distance from water collections and the hydrographic network, were eligible for inclusion in the full model. The final model comprised the aforementioned two variables that were significant and retained. Their interaction was not significant (p = 0.453).

For one unit increase of population density it was 0.996 (OR: 0.996, 95% CI: 0.992; 0.9999, p=0.047) times less likely to obtain a positive PCR result for WNV in bird samples. Practically, one unit increase of population density resulted in a 0.4% (95% CI: 0.01; 0.8) decrease in the odds of obtaining a positive WNV PCR result. Additionally, for one unit increase of distance

from water collections and hydrographic network, it was 0.998 (OR: 0.988, 95% CI: 0.997; 0.999, p<0.001) times less likely to get positive WNV PCR results in bird samples. Essentially, one unit increase of distance from water sources resulted in a 0.2% (95% CI: 0.1; 0.3) decrease in the odds of detecting WNV RNA.

5.4. Discussion

The present study was conducted in the context of an active WNV surveillance program in Peloponnese and Western Greece regions of Greece during 2022, focusing on the detection of WNV in wild birds. To this end, 511 wild birds were molecularly screened for the presence of WNV, and the virus was detected in 71.

WNV presence in Peloponnese was evident not only in migratory-winter visitor birds (song thrush, common starling, black-winged stilt), but also in sedentary birds (magpies, hooded crow, house sparrow, Eurasian jay, great tit). The engagement of resident birds is considered necessary for the maintenance and the spread of the virus to adjacent regions [33,34]. The strains that were detected in Peloponnese in February 2022 and were subjected to sequencing, belonged to the evolutionary lineage 2. According to the phylogenetic analysis, they displayed great similarity with those that circulated in Argolis in 2017 and in Macedonia in 2010 and 2012, which were responsible for three major outbreaks [16,35,36]. Hence, our findings indicate potential maintenance of the virus in Peloponnese over the years. Based on the phylogenetic analysis, our strains formed a separate group from the ones that circulated in Central and Northern Greece during 2022, suggesting a possible different origin or evolution [37]. Nevertheless, sequencing was performed in a part of NS3 WNV genome, thus analysis on a larger area of the genetic material of the strains would provide more reliable information about their relationship.

WNV was detected for the first time among magpies in Western Greece region, an area neighboring to Peloponnese, from September to November. Considering that WNV surveillance on wild birds was implemented for the first time in this region and was initiated with a delay, sampling was focused on magpies which are a sedentary species that has been shown to present susceptibility to the virus. Additionally, magpies live in proximity to humans in a variety of habitats and are regarded as an ideal species to be used as sentinels for the surveillance and early detection of WNV both in endemic and non-endemic areas [38]. It shall be noted that in recent years (2019-2020), WNV has not been detected neither among humans nor in entomological surveillance studies on female *Cx. pipiens* s.l. mosquitoes that were conducted in Western Greece [18,39]. Thus, our results should be evaluated in subsequent periods of potential virus transmission for prevention of human cases.

In the present study, WNV positive birds were detected in Peloponnese as early as February and until November and in Western Greece up to November. According to the literature, WNV is usually detected in wild birds between July and September [40], one to two months before human cases, that are most commonly reported during July-October and peaking between mid-August and mid-September [41]. Subsequently, our findings could indicate a seasonal change in the ecology of the virus which could be attributed to a variety of factors. Specific environmental parameters such as increased ambient temperatures during summer, high precipitation in late winter/early spring or during summer and summer drought as well as the presence of competent mosquito vectors have previously been associated with an increase in the virus' circulation [42–45]. Another potential scenario is that the virus could

have spread southwards during the autumn migration of birds from Northern Europe to wintering areas in Africa with resting stops in Greece. This hypothesis has previously been supported with the detection of WNV antibodies in young migratory turtle doves born in northern Europe, on their arrival in Greek territory [15]. Furthermore, long-term persistence of WNV in tissues of infected animals may result in the infection of birds by prey ingestion even months after the end of the mosquito season, providing a mechanism of overwintering [46].

To get a better understanding on virus' ecology, the locations of positive and negative birds for WNV RNA were associated with eight environmental variables. Two parameters, namely human population density and distance from water collections and hydrographic network, were statistically significant. It was more likely to identify a PCR positive bird as the population density decreased. This was expected considering that WNV is maintained in nature in a mosquito-bird cycle and wild birds mainly inhabit rural ecosystems, while their presence in the vicinity of humans is mainly driven by their need to feed. Additionally, a decrease of the distance from water sources resulted in an increase of the odds for WNV detection among the tested wild birds. Wetlands are the most important ecosystems for enzootic transmission of the virus due to the presence of both resident and migratory bird as well as of competent mosquito vectors, particularly *Cx*. *pipiens* [47]. Overall, water bodies are considered to favor the activity of mosquitoes and their capacity to complete different life cycle stages [48]. As such, short distances from water bodies have been shown to be associated with more WNV human and animal cases, mainly due to increased mosquito exposure [49].

During 2022, 286 human cases of WNV were recorded in five regions of Greece, while the virus was also identified in human and mosquito samples [18,37]. In our study, WNV was molecularly detected in wild bird species, and especially in magpies in both Peloponnese and Western Greece. Given that positive birds were identified as early as in the late February and until as late as in November, it is suggested that surveillance of avian species shall be imposed throughout the year. Ideally, magpies could be used as sentinels for WNV monitoring, since identification of virus spread among them could lead in the implementation of preventive measures that would hamper disease outbreaks in humans.

Supplementary Materials

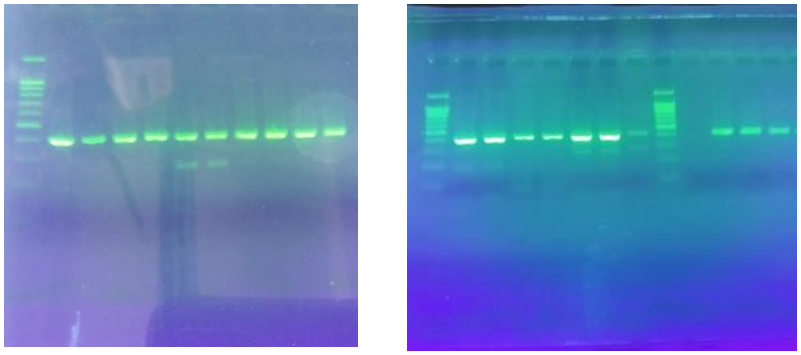

Figure S5.1. Gel electrophoresis of WNV positive strains.

Supplementary Table S5.1. Categories of Land Uses.

Categories of Land Uses			Code
1st Level	**2nd Level**	**3rd Level**	
Artificial surfaces	Urban fabric	Continuous urban fabric	1
		Discontinuous urban fabric	2
	Industrial, commercial and transport units	Industrial or commercial units	3
		Road and rail networks and associated land	4
		Port areas	5
		Airports	6
	Mine, dump and construction sites	Mineral extraction sites	7
		Dump sites	8
		Construction sites	9
	Artificial, non-agricultural vegetated areas	Green urban areas	10
		Sport and leisure facilities	11
Agricultural areas	Arable land	Non-irrigated arable land	12
		Permanently irrigated land	13
		Rice fields	14
	Permanent crops	Vineyards	15
		Fruit trees and berry plantations	16
		Olive groves	17
	Pastures	Pastures	18
	Heterogeneous agricultural areas	Annual crops associated with permanent crops	19
		Complex cultivation patterns	20
		Land principally occupied by agriculture, with significant areas of natural vegetation	21
		Agro-forestry areas	22
Forest and semi natural areas	Forests	Broad-leaved forest	23
		Coniferous forest	24

		Mixed forest	25
	Scrub and/or herbaceous vegetation associations	Natural grasslands	26
		Moors and heathland	27
		Sclerophyllous vegetation	28
		Transitional woodland-shrub	29
	Open spaces with little or no vegetation	Beaches, dunes, sands	30
		Bare rocks	31
		Sparsely vegetated areas	32
		Burnt areas	33
		Glaciers and perpetual snow	34
Wetlands	Inland wetlands	Inland marshes	35
		Peat bogs	36
	Maritime wetlands	Salt marshes	37
		Salines	38
		Intertidal flats	39
Water bodies	Inland waters	Water courses	40
		Water bodies	41
	Marine waters	Coastal lagoons	42
		Estuaries	43
		Sea and ocean	44

Supplementary Table S5.2. Nucleotide sequences of the first eight first PCR positive samples.

Wild Bird Species	**Origin**	**Sequence**

Song thrush	Souli, Corinthia	CAAAAGATTGAGGACGGCTGTACTGGCACCCACCAGGGTCGTCGCTGCTGAGATGTCTGA GGCCCTGAGAGGACTTCCCATCCGGTACCAAACCTCAGCAGTGCCCAGAGAGCACAGTGG AAATGAGATCGTTGATGTCATGTGCCATGCCACTCTCACACACAGGCTGATGTCTCCACA CAGAGTCCCCAATTACAATCTGTTCATAATGGATGAAGCCCATTTCACGGATCCAGCGAG CATCGCAGCCAGAGGATACATAGCAACCAAGGTTGAATTGGGCGAAGCCGCCGCGATTTT CATGACGGCAACGCCACCCGGGACTTCTGACCCCTTTCCAGAGTCCAA
Song thrush	Souli, Corinthia	AAAGATTGAGGACGGCTGTACTGGCACCCACCAGGGTCGTCGCTGCTGAGATGTCTGAGG CCCTGAGAGGACTTCCCATCCGGTACCAAACCTCAGCAGTGCCCAGAGAGCACAGTGGAA ATGAGATCGTTGATGTCATGTGCCATGCCACTCTCACACACAGGCTGATGTCTCCACACA GAGTCCCCAATTACAATCTGTTCATAATGGATGAAGCCCATTTCACGGATCCAGCGAGCA TCGCAGCCAGAGGATACATAGCAACCAAGGTTGAATTGGGCGAASCCGCCGCGATTTTCA TGACGGCAACGCCACCCGGGACTTCTGACCCCTTTCCAGAGTCCA
Magpie	Agios Vasileios, Messenia	AAAGATTGAGGACGGCTGTACTGGCACCCACCAGGGTCGTCGCTGCTGAGATGTCTGAGG CCCTGAGAGGACTTCCCATCCGGTACCAAACCTCAGCAGTGCCCAGAGAGCACAGTGGAA ATGAGATCGTTGATGTCATGTGCCATGCCACTCTCACACACAGGCTGATGTCTCCACACA GAGTCCCCAATTACAATCTGTTCATAATGGATGAAGCCCATTTCACGGATCCAGCGAGCA TCGCAGCCAGAGGATACATAGCAACCAAGGTTGAATTGGGCGAAGCCGCCGCGATTTTCA TGACGGCAACGCCACCCGGGACTTCTGACCCCTTTCCAGAGTCCAA
Common starling	Valtos, Messenia	AAGATTGAGGACGGCTGTACTGGCACCCACCAGGGTCGTCGCTGCTGAGATGTCTGAGGC CCTGAGAGGACTTCCCATCCGGTACCAAACCTCAGCAGTGCCCAGAGAGCACAGTGGAAA TGAGATCGTTGATGTCATGTGCCATGCCACTCTCACACACAGGCTGATGTCTCCACACAG AGTCCCCAATTACAATCTGTTCATAATGGATGAAGCCCATTTCACGGATCCAGCGAGCAT CGCAGCCAGAGGATACATAGCAACCAAGGTTGAATTGGGCGAAGCCGCCGCGATTTTCAT GACGGCAACGCCACCCGGGACTTCTGACCCCTTTCCAGAGTCCA

Songthrush	Mazia, Messenia	ACAAAAGATTGAGGACGGCTGTACKGGCACCCACCAGGGTCGTCGCTGCTGAGATGTCTG AGGCCCTGAGAGGACTTCCCATCCGGTACCAAACCTCAGCAGTGCCCAGAGAGCACAGTG GAAATGAGATCGTTGATGTCATGTGCCATGCCACTCTCACACACAGGCTGATGTCTCCAC ACAGAGTCCCCAATTACAATCTGTTCATAATGGATGAAGCCCATTTCACGGATCCAGCGA GCATCGCAGCCAGAGGATACATAGCAACCAAGGTTGAATTGGGCGAAGCCGCCGCGATTT TCATGACGGCAACGCCACCCGGGACTTCTGACCCCTTTCCAGAGTCCA
Magpie	Tegea, Arcadia	CCGGAATGRCATTGGACTCTGGAAGGGGTCAGAAGTCCCGGGTGGCGTTGCCGTCATGAAAATCGCGGCGGCTTCGCCCA ATTCAACCTTGGTTGCTATGTATCCTCTGGCTGCGATGCTCGCTGGATCCGTGAAATGGGCTTCATCCATTATGAACAGA TTGTAATTGGGGACTCTGTGTGGAGACATCAGCCTGTGTGTGAGAGTGGCATGGCACATGACATCAACGATCTCATTTCC ACTGTGCTCTCTGGGCACTGCTGAGGTTTGGTACCGGATGGGAAGTCCTCTCAGGGCCTCAGACATCTCAGCAGCGACGA CCCTGGTGGGTGCCAGTACAGCCGTCCTCAATCTTTTGTTGATGGCCTCCTTGATGATTTGGGGGGGTARKMWTGCCAA
Magpie	Tegea, Arcadia	CCGGAKGRCTTGGACTCTGGAAGGGGTCAGAAGTCCCGGGTGGCGTTGCCGTCATGAAAATCGCGGCGGCWTCGCCCAAT TCAACCTTGGTTGCTATGTATCCTCTGGCTGCGATGCTCGCTGGATCCGTGAAATGGGCTTCATCCATTATGAACAGATT GTAATTGGGGACTCTGTGTGGAGACATCAGCCTGTGTGTGAGAGTGGCATGGCACATGACATCAACGATCTCATTTCCAC TGTGCTCTCTGGGCACTGCTGAGGTTTGGTACCGGATGGGAAGTCCTCTCAGGGCCTCAGACATCTCAGCAGCGACGACC CTGGTGGGTGCCAGTACAGCCGTCCTCAATCTTTTGTTGATGGCCTCCTTGATGATTTGGGGAAGTTATCTTGCA
Magpie	Tegea, Arcadia	TARRMATCAMMAAAAAGATTGAGGACGGCTGTACTGGCACCCACCAGGGTCGTCGCTGCTGAGATGTCTGAGGCCCTGAG AGGACTTCCCATCCGGTACCAAACCTCAGCAGTGCCCAGAGAGCACAGTGGAAATGAGATCGTTGATGTCATGTGCCATG CCACTCTCACACACAGGCTGATGTCTCCACACAGAGTCCCCAATTACAATCTGTTCATAATGGATGAAGCCCATTTCACG GATCCAGCRAGCATCGCAGCCAGAGGATACATAGCAACCAAGGTTGAATTGGGCRAAGCCGCCGCGATTTTCATGACGGC AACGCCACCCGGGACTTCTGACCCCTTTCCAGAGTCCAATGCTCCTATCTCGGACATGCAAACARAGGGCCCARACAAA

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General Conclusions

The studies conducted in this PhD thesis led to the following conclusions:

I. Wild birds in Greece carry β-lactamase producing Enterobacteriaceae of public health importance.
   - pAmpC dissemination was evidenced with the presence of CMY-2 *E. coli* in chickens and a Eurasian magpie. The isolated strains were MDR, presenting resistance to tetracyclines, sulfonamides and quinolones and harboring *tet* genes, *sul* genes and mutations in the QRDRs, respectively. Despite the rather low pAmpC occurrence among the sampled birds, clinically relevant clones were identified; ST117 and ST131 being the predominant, further enhancing concerns on a One Health perspective.
   - Colonization of wild birds by a rather rare but possibly zoonotic bacterium, *M. wisconsensis*, was demonstrated. Fecal carriage of *M. wisconsensis* was detected in a common pheasant, two Eurasian magpies and a great white-fronted goose. The strains isolated from the two latter species were MDR, ESBL-producing and harbored $bla_{\text{CTX-M-1}}$. These findings underline the potential role of wild birds in both the spread of *M. wisconsensis* and the dissemination of $bla_{\text{CTX-M-1}}$.
   - MDR, ESBL-producing *E. coli* was recovered from eight different wild bird species: Eurasian magpie, common buzzard, short-toed snake eagle, Eurasian sparrowhawk, steppe eagle, grey heron, Eurasian Scops owl and Common swift. Genes $bla_{\text{CTX-M-1}}$ group and bla_{TEM} were detected along with AmpC, fluoroquinolone, trimethoprim/sulfamethoxazole, aminoglycoside and macrolide resistance determinants. The importance of incorporating wild birds in the assessment of ESBL circulation in non-clinical settings is highlighted.
   - One MDR carbapenemase-producing *E. coli* that harbored bla_{NDM} was identified from a Caspian gull. Given the critical nature of carbapenems for human medicine, the increasing global prevalence of CPE among animals and the potential for cross-species transfer, identification of this strain in a wild bird pinpoints the imperative need for the application of an integrated surveillance system for CPE.

II. WNV circulated among both migratory and sedentary wild birds in Peloponnese and Western Greece regions, during 2022
   - Viral RNA was detected as early as February in wild birds originating from Peloponnese and up until November in both regions. This finding could imply a change in the ecology of the virus which could possibly be attributed to climatic conditions favoring its survival. Alternatively, the virus could have spread southwards during the autumn migration of birds from Northern Europe to wintering areas in Africa with resting stops in Greece.
   - WNV was identified for the first time among magpies in Western Greece region, an area neighboring to Peloponnese. Its presence should be monitored in subsequent periods of potential virus transmission for prevention of human cases.

- The association of environmental parameters with the locations of WNV positive and negative wild birds provided information about the virus' ecology. It was more likely to identify a WNV PCR positive bird as the population density and the distance from water sources decreased.

The aforementioned findings demonstrate that wild birds in Greece are implicated in the epidemiology of important human and animal pathogens. The circulation of AmpC, ESBL and carbapenemase genes along with genes conferring resistance to several non β-lactam antimicrobial classes (fluoroquinolones, trimethoprim, sulfonamides, aminoglycosides, tetracyclines, macrolides) in Enterobacteriaceae of wildlife origin poses a serious threat for the spread of MDR bacteria. WNV remains an established zoonotic disease in Greece, with a constantly evolving ecology that shall be kept under surveillance. Wild birds should be regarded as a reservoir, vehicle and indicator of these agents, thus a multisectoral, One Health approach is necessary to address such complex health threats. Progress towards global health security requires a greater focus on the interface between humans, animals and the environment and a strong collaboration between the human health and animal health sectors.

UNIVERSITY OF THESSALY

SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY SCIENCE

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

DETECTION OF PATHOGENS TRANSMITTED BETWEEN WILD BIRDS AND DOMESTIC ANIMALS OR HUMANS

Summary

This thesis aimed to investigate the occurrence of selected zoonotic pathogens among wild birds in Greece, focusing on Extended Spectrum β-Lactamase (ESBL), AmpC and carbapenemase producing Enterobacteriaceae, as well as on West Nile Virus (WNV).

Plasmid encoded AmpC (pAmpC) presence was evaluated among *Escherichia coli* (*E. coli*) isolates from fecal samples of poultry, cattle, pigs, and wild birds. The responsible pAmpC genes and sequence types (ST) of the detected strains were determined, while all pAmpC-producing *E. coli* that were phenotypically resistant to antimicrobials other than β-lactams were further tested for the respective resistance determinants. Thirteen pAmpC *E. coli* were identified from twelve chickens and one Eurasian magpie (*Pica pica*), which all harbored $bla_{\text{CMY-2}}$ linked to an upstream ISE*cp1*-like element. The isolates were classified into five different sequence types, with ST131 and ST117 being the most common. Seven pAmpC isolates co-harbored genes conferring resistance to tetracyclines (*tetM*, *tetB*, *tetC*, *tetD*), three carried sulfonamide resistance genes (*sul*I and *sul*II), and ten displayed mutations in the quinolone resistance-determining regions of gyrA (S83L+D87N) and parC (S80I+E84V).

The occurrence of *Moellerella wisconsensis* (*M. wisconsensis*), a rather rare Enterobacteriaceae, among fecal samples of wild birds and humans in Greece was also investigated. The isolates were subsequently phenotypically and molecularly characterized regarding their antimicrobial resistance characteristics. Four *M. wisconsensis* were recovered from a common pheasant (*Phasianus colchicus*), two Eurasian magpies (*Pica pica*) and a great white-fronted goose (*Anser albifrons*). Among these four strains, the three latter exhibited an ESBL phenotype and were found to produce CTX-M-1. No *Moellerella wisconsensis* strains were retrieved from the human samples tested.

Followingly, the occurrence and the molecular characteristics of ESBL- and carbapenemase-producing *E. coli* recovered from wild and feral birds was assessed. Twelve multidrug-

resistant (MDR), ESBL-producing *E. coli* were detected from eight different wild bird species and carried a $bla_{\text{CTX-M-1}}$ group gene and/or a bla_{TEM}. AmpC, fluoroquinolone, trimethoprim/sulfamethoxazole, aminoglycoside and macrolide resistance genes were also detected. Moreover, one carbapenemase-producing *E. coli* was identified from a Caspian gull (*Larus cachinnans*), harboring bla_{NDM} along with a combination of additional resistance genes.

Lastly, the occurrence of WNV in wild birds from two neighboring regions of Greece, namely Peloponnese and Western Greece, during the year 2022 was determined. WNV RNA was detected from February to November in a total of 71 wild birds of 9 species originating from both investigated regions. Selected strains were sequenced and were found to belong to the evolutionary lineage 2, presenting genetic similarity to previous outbreak-causing Greek strains (Argolis 2017, Macedonia 2010 and 2012). The locations of positive and negative birds for WNV RNA were associated with environmental variables, leading to conclusions regarding the virus' ecology.

This thesis provides insight into the role of wild birds in the epidemiology of important zoonotic antimicrobial resistant bacterial and viral agents. The importance of incorporating wild birds in the assessment of the circulation of AMR Enterobacteriaceae in non-clinical settings is emphasized. The need of WNV avian species surveillance to be conducted annually and throughout the year is also highlighted.

ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΚΤΗΝΙΑΤΡΙΚΗΣ

ΕΡΓΑΣΤΗΡΙΟ ΜΙΚΡΟΒΙΟΛΟΓΙΑΣ ΚΑΙ ΠΑΡΑΣΙΤΟΛΟΓΙΑΣ

ΑΝΙΧΝΕΥΣΗ ΠΑΘΟΓΟΝΩΝ ΠΟΥ ΜΕΤΑΔΙΔΟΝΤΑΙ ΜΕΤΑΞΥ ΑΓΡΙΩΝ ΠΤΗΝΩΝ ΚΑΙ ΖΩΩΝ Ή ΑΝΘΡΩΠΩΝ

Περίληψη

Η παρούσα διατριβή είχε στόχο τη διερεύνηση της παρουσίας επιλεγμένων ζωονοτικών παθογόνων σε άγρια πτηνά στην Ελλάδα, εστιάζοντας στην ανίχνευση Εντεροβακτηριοειδών που παράγουν Εκτεταμένου φάσματος β-λακταμάσες (Extended Spectrum β-Lactamase, ESBL), AmpC κεφαλοσπορινάσες και καρβαπενεμάσες, καθώς και στον ιό του Δυτικού Νείλου (ΙΔΝ).

Η παρουσία πλασμιδιακών AmpC (pAmpC) ενζύμων αξιολογήθηκε μεταξύ στελεχών *Escherichia coli* (*E. coli*) που απομονώθηκαν από δείγματα κοπράνων ορνίθων, βοοειδών, χοίρων και άγριων πτηνών. Προσδιορίστηκαν τα υπεύθυνα pAmpC γονίδια και τα sequence types (ST) των ανιχνευθέντων στελεχών, ενώ όλες οι pAmpC *E. coli* που ήταν φαινοτυπικά ανθεκτικές σε αντιμικροβιακά πέραν των β-λακταμών εξετάστηκαν περαιτέρω για την ανίχνευση των αντίστοιχων γονιδίων αντοχής. Δεκατρείς pAmpC *E. coli* ταυτοποιήθηκαν από δώδεκα δείγματα ορνίθων και ένα δείγμα καρακάξας (*Pica pica*), και όλες έφεραν το γονίδιο bla_{CMY-2} συνδεδεμένο με στοιχείο IS*Ecp1* προς τα πάνω. Τα στελέχη ταξινομήθηκαν σε πέντε διαφορετικά ST, με τα ST131 και ST117 να είναι τα πιο κοινά. Επτά pAmpC στελέχη επίσης έφεραν γονίδια που προσδίδουν αντοχή στις τετρακυκλίνες (*tetM*, *tetB*, *tetC*, *tetD*), τρία έφεραν γονίδια αντοχής στις σουλφοναμίδες (*sul*I και *sul*II) και δέκα παρουσίαζαν μεταλλάξεις που προσδίδουν αντοχή στις κινολόνες στις quinolone resistance-determining regions των γονιδίων gyrA (S83L+D87N) και parC (S80I+E84V).

Διερευνήθηκε, επίσης, η παρουσία του βακτηρίου *Moellerella wisconsensis* (*M. wisconsensis*), ενός μάλλον σπάνιου Εντεροβακτηριοειδούς, μεταξύ δειγμάτων κοπράνων άγριων πτηνών και ανθρώπων στην Ελλάδα. Τα στελέχη που απομονώθηκαν στη συνέχεια χαρακτηρίστηκαν φαινοτυπικά και μοριακά ως προς τα χαρακτηριστικά που τους προσδίδουν αντιμικροβιακή αντοχή. Τέσσερις *M. wisconsensis* ανακτήθηκαν από ένα δείγμα φασιανού (*Phasianus colchicus*), δύο δείγματα καρακαξών (*Pica pica*) και ένα δείγμα ασπρομέτωπης χήνας (*Anser albifrons*). Μεταξύ των τεσσάρων αυτών στελεχών, τα τρία τελευταία εμφάνιζαν ESBL φαινότυπο και παρήγαγαν CTX-M-1. Δεν απομονώθηκαν στελέχη *Moellerella wisconsensis* από τα ανθρώπινα δείγματα που εξετάστηκαν.

Ακολούθως, αξιολογήθηκε η παρουσία στελεχών *E. coli* που παράγουν ESBL και καρβαπενεμάσες σε άγρια πτηνά και προσδιορίστηκαν τα μοριακά χαρακτηριστικά τους. Δώδεκα πολυανθεκτικές *E. coli* που παρήγαγαν ESBL ανιχνεύθηκαν από οκτώ διαφορετικά είδη άγριων πτηνών και έφεραν γονίδιο της ομάδας $bla_{CTX\text{-}M\text{-}1}$ και/ή γονίδιο bla_{TEM}. Ανιχνεύθηκαν επίσης γονίδια AmpC, καθώς και γονίδια που προσδίδουν αντοχή στις φθοροκινολόνες, την τριμεθοπρίμη/σουλφαμεθοξαζόλη, τις αμινογλυκοσίδες και τα μακρολίδια. Επιπλέον, μια *E. coli* που παρήγαγε καρβαπενεμάση ανιχνεύθηκε σε δείγμα από γλάρο της Κασπίας (*Larus cachinnans*), και έφερε το γονίδιο bla_{NDM} καθώς και συνδυασμό λοιπών γονιδίων αντιμικροβιακής αντοχής.

Στο τελευταίο κεφάλαιο, εκτιμήθηκε η εμφάνιση του ΙΔΝ σε άγρια πτηνά από δύο γειτονικές περιφέρειες της Ελλάδας, την Πελοπόννησο και τη Δυτική Ελλάδα, κατά τη διάρκεια του έτους 2022. RNA του ΙΔΝ ανιχνεύθηκε από τον Φεβρουάριο έως τον Νοέμβριο σε συνολικά 71 άγρια πτηνά 9 ειδών που προέρχονται και από τις δύο περιοχές που ερευνήθηκαν. Επιλεγμένα στελέχη αλληλουχήθηκαν και βρέθηκαν να ανήκουν στην εξελικτική γενιά 2, παρουσιάζοντας γενετική ομοιότητα με προηγούμενα ελληνικά στελέχη που προκάλεσαν επιδημία (Αργολίδα 2017, Μακεδονία 2010 και 2012). Οι γεωγραφικές θέσεις των θετικών και αρνητικών αγρίων πτηνών για RNA του ΙΔΝ συσχετίστηκαν με περιβαλλοντικές μεταβλητές, οδηγώντας σε συμπεράσματα αναφορικά με την οικολογία του ιού.

Η διατριβή αυτή παρέχει πληροφορίες σχετικά με το ρόλο των άγριων πτηνών στην επιδημιολογία σημαντικών, ζωονοτικών, βακτηρίων και ιών. Τονίζεται η σημασία της ενσωμάτωσης των άγριων πτηνών στην αξιολόγηση της κυκλοφορίας Εντεροβακτηριοειδών που παρουσιάζουν αντιμικροβιακή αντοχή σε μη κλινικά περιβάλλοντα. Υπογραμμίζεται επίσης η ανάγκη επιτήρησης του ΙΔΝ στα άγρια πτηνά, η οποία προτείνεται να διεξάγεται ετησίως και καθ' όλη τη διάρκεια του έτους.