sustainability

MDPI

Article

Prevalence of Antibiotic Resistant *E. coli* Strains Isolated from Farmed Broilers and Hens in Greece, Based on Phenotypic and Molecular Analyses

Anna Xexaki [1], Dimitrios K. Papadopoulos [2], Maria V. Alvanou [2], Ioannis A. Giantsis [2,*], Konstantinos V. Papageorgiou [1], Georgios A. Delis [1], Vangelis Economou [1], Spyridon K. Kritas [1], Evangelia N. Sossidou [3] and Evanthia Petridou [1]

[1] School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[2] Department of Animal Science, Faculty of Agricultural Sciences, University of Western Macedonia, 53100 Florina, Greece
[3] Veterinary Research Institute, Hellenic Agricultural Organization—DEMETER, 57001 Thessaloniki, Greece
* Correspondence: igiants@agro.auth.gr

Abstract: The use of antimicrobials is beneficial for livestock health; however, their overuse and misuse may increase resistance to these compounds. Thus, the aim of the present study was the phenotypic and molecular examination of the presence of *Escherichia coli* antibiotic-resistant strains in broiler and laying hen farms. The resistance of *E. coli* strains was examined against various antibiotics, including several families of compounds such as penicillin class medications (ampicillin), cephalosporins (cefotaxime, cefoxitin, cefpodoxime and ceftazidime), sulfonamides (co-trimoxazole), quinolones (enrofloxacin and nalidixic acid), aminoglycosides (gentamicin), β-lactams (imipenem), aminoglycoside (streptomycin), and polymyxin (colistin). In total, 106 strains were investigated, sampled during the years 2016–2019 from 91 poultry farms, including 75 broiler farms and 16 laying hen farms, originating from three Regional Units in Greece. The examined isolates revealed the highest resistance rates to sulfamethoxazole (81.1%), nalidixic acid (73.6%), tetracyclin (70.8%), and streptomycin (70.8%). On the other hand, the resistance of the isolates to third generation cephalosporins was found to be at lower levels for ceftazidime (2.8%), ceftriaxone (3.7%) cefoxitin (4.7%), and cefotaxime (4.7%). Phenotypic tests showed that 13.6% and 10.2% of the isolates produced ESBL, while 2.7% and 1% produced AmpC b-lactamase, for broiler and laying hens, respectively. The prevalence of the *mcr-1* gene was found to be 22.7%, detected only in broiler isolates. Based on our results, *E. coli* antibiotic resistance represents a critical control point in poultry production that, apart from farm animals, may affect public health as well.

Keywords: *Escherichia coli*; antibiotics; resistance; poultry; laying hens; broiler

Citation: Xexaki, A.; Papadopoulos, D.K.; Alvanou, M.V.; Giantsis, I.A.; Papageorgiou, K.V.; Delis, G.A.; Economou, V.; Kritas, S.K.; Sossidou, E.N.; Petridou, E. Prevalence of Antibiotic Resistant *E. coli* Strains Isolated from Farmed Broilers and Hens in Greece, Based on Phenotypic and Molecular Analyses. *Sustainability* **2023**, *15*, 9421. https://doi.org/10.3390/su15129421

Academic Editors: George K. Symeon and Vassilios Dotas

Received: 7 March 2023
Revised: 7 June 2023
Accepted: 8 June 2023
Published: 12 June 2023

1. Introduction

As the use of antimicrobial substances in the primary sector is increasing, it leads to extensive human exposure to bacteria with antimicrobial resistance (AMR), indirect gene transfer among bacteria species, and the spread of antimicrobial-resistant bacteria into the environment [1]. In particular, the use of antibiotics in compound feeds has been a substantial part of poultry production, not only for infectious bacterial disease prevention, but also for the improvement of animal growth rates [2].

E. coli is a Gram-negative, facultative anaerobic, rod-shaped bacterium and a member of Enterobacteriaceae family, typically 2–3 μm long with a 0.5 μm diameter [3]. Its genome consists of a circular, double-stranded DNA molecule which is more than 1000 μm long and typically also has one or more plasmids, a number of which contain genes, approximately 4000–5000, with only 2000 of them being common among different strains [4]. Most *E. coli*

strains are harmless; however, there are some pathogenic strains that are responsible for important public health issues [5].

E. coli strains were initially classified in four major phylogenetic groups, designated as A, B1, B2, and D [6]. However, three additional phylogenetic groups were later added—namely, C, E, and F—along with one cryptic clade, I, increasing the total number to eight [7]. More recently, one extra phylogenetic group (phylogroup G) was also characterized [8]. The phylo-group F represents a sister group to phylo-group B2, while phylo-group C is closely related to phylo-group B1 [9]. The phylo-group E is now also well recognized, with its best-known member being the O157:H7 [10].

E. coli constitutes an incredibly versatile and diverse species both genetically and morphologically, which can be further subdivided into the following categories: intestinal non-pathogenic commensal isolates, intestinal pathogenic isolates, and extraintestinal pathogenic *E. coli* (ExPEC) isolates. Extraintestinal pathogenic *E. coli* (ExPEC) belongs mainly to group B2 and, to a lesser extent, to group D, while intestinal commensal symbiotic isolates mainly belong to groups A and B1 [11].

Much like with other bacterial taxa, AMR in *E. coli* is considered a main public health threat, often observed in the form of multidrug resistance [12]. The mechanism of AMR development in *E. coli* may be intrinsic or acquired, i.e., located in the bacterial chromosome or routed by other bacteria, respectively [13]. Briefly, these include the enzymatic inactivation of the drug, the modification of the drug target, the setting of limitation mechanism in drug uptake, and the activation of the efflux pump to prohibit the insertion of the antibiotic through the cell membrane.

Resistance rates are generally relatively high worldwide, reaching 50, 60%, or higher levels, depending on the antibiotic agent [14]. In Greece, rates of resistant *E. coli* strains have been detected in human patients, varying approximately between 20 and 45% for the different antibiotics [15]. Concerning farm animals, recently, Papadopoulos et al. [16] determined very high levels of multidrug resistance in swine, reaching more than 80% of the tested samples.

Further, *E. coli* isolates can develop multidrug resistance to various antibiotics such as β-lactams, mainly through the production of β-lactamase (ESBL) and/or plasmid-mediated AmpC β-lactamases (AmpC) [17]. ESBLs confer resistance to the majority of β-lactams but especially to third-generation cephalosporins (such as cefotaxime, ceftriaxone, and ceftazimidime) and aztreonam, though not to carbapenems and cephamycins (cefotetan and cefoxitin) [18]. Bacteria exhibiting resistance towards β-lactams were first observed in humans, but since then, an increase in the detection of ESBL/AmpC-producing *E. coli* in animals, such as pigs [19], cattle, cats, dogs [20], fish [21] horses [22], and mainly broiler chickens [19,23], has been reported.

Colistin, also known as polymyxin E, was discovered in the 1940s and is a circular, polypeptide antibiotic produced by *Paenibacillus polymyxa* var. *colistinus*, with its compounds targeting the bacterial cell membrane, as it binds to the lipopolysaccharide (LPS) component of the outer membrane of the Gram-negative bacteria [24]. During the 1970s, there was a significant reduction in the clinical use of colistin due to its side effects, while during the 1980s, it was almost completely abandoned [25]. Nowadays, colistin is widely used in intensive poultry production; thus, the emergence of plasmid-mediated enzymatic resistance is a serious concern globally. Some of the genes associated with resistance are found in the plasmid, a feature that provides them mobility [26]. Mobilized resistance to colistin is increasing globally and represents a major threat to public health. In total, nine colistin resistance genes (*mcr-1* to *mcr-9*) and the variants of these genes have been described in Enterobacteriaceae [27]. These inferences have increased the level of public health concern associated with the spread of mobile colistin resistance and pointed out the necessity of extensive screenings in Enterobacteriaceae. Poultry and livestock represent a major reservoir for colistin resistance and the transmission of resistance genes [26,28]. Similarly, resistance to other antibiotics such as quinolones are also genetically associated and have been observed when point mutations occur in specific portions of *GyrA* and

ParC, known as the quinolone resistance-determining regions (QRDR) [29]. Generally, quinolone resistance has been reported in *E. coli* isolated from retail chicken products [30]. Furthermore, resistance to quinolones has emerged following their widespread use in poultry farms, and as a result, quinolone-resistant *E. coli* isolates can be spread through poultry production [31].

Greece is a country with a traditionally intensively developed poultry sector, which has played a particular role in the national economy [32]. However, data regarding the screening of resistant *E. coli* strains in Greek poultry are rather scarce. Hence, the aim of the present study was to estimate the *E. coli* resistance rates of broiler and laying hen farms by applying phenotypic and molecular identification methodologies.

2. Materials and Methods

2.1. Sample Collection and Isolation of Bacteria

Fecal samples were collected from 75 poultry farms of broilers and 16 farms of egg-producing hens during the years 2016–2019. The samples were received via a walk-through of the broiler or the hen house unit. The premises were traversed in such a way as to obtain representative samples of the entire ward. For this purpose, each ward was divided into 9 isomeric sections, from each of which a separate feces sample was obtained from the litter in the case of broiler chickens or from the manure removal belts located under the cages in the case of egg-producing hens.

For each visit, biosecurity measures were taken, including a plastic apron and disposable plastic foot pads. The broiler farms included in the study originated from the Region of Epirus (34 out of the 75), Central Macedonia (39 out of the 75), and Attiki (12 out of the 75), while 10 and 6 of the laying hen farms originated from Central Macedonia and Epirus, respectively. The flocks included in the study had an average number of 19,000 animals, while six chambers were sampled from each farm on average. From every flock, 9 fecal samples were collected from the chamber inside the poultry house. Fecal samples from the litter material were taken with a sterile cotton swab, which was stored in Stuart transport medium and transported to the laboratory within 24 h. In total, 954 samples were collected and were pooled per unit for each farm, finally forming 106 (90 broilers and 16 egg laying hen flocks) different sample pools that were included in the analysis, whereas from the 16 broiler farms, two different flocks were sampled.

On arrival at the laboratory, the fecal pool samples were directly inoculated in brain heart infusion broth (Oxoid, Basingstoke, UK) supplemented with ampicillin (10 mg/L). After overnight enrichment at 37 °C, a full loop from the enrichment culture was streaked onto the surface of selective Tryptone Bile X-glucuronide agar plates (TBX; Oxoid, Basingstoke, UK) and incubated at 44 °C for 24 to 48 h, as recommended by the manufacturer for the selective growth of *E. coli*. Plates containing blue/green colonies were counted as presumptive *E. coli* (ISO 16649-2:2001). Oxydase and indole tests were also performed to verify the identity of the bacterial strains as *E. coli*. Briefly, moistened filter papers were utilized, on which tetramethyl-ρ-phenylenediaminedihydrochloride (Merck, Darmstadt, Germany) was dropped. Afterwards, cultured cells were inoculated on these papers, and purple violet coloration indicated positive *E. coli*. For indole tests, Tryptone plates were inoculated with pure cultures and incubated at 37 °C for 24 h. Then, Kovács reagent (Merck, Darmstadt, Germany) was added, and positive *E. coli* were identified by the formation of a reddish color. *E. coli* presence was confirmed in all 106 pools.

2.2. Examination of Phenotypic Antimicrobial Susceptibility

Antimicrobial susceptibility testing was performed using the Kirby Bauer disc diffusion method on Mueller–Hinton agar plates (Merck, Darmstadt, Germany), according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [33]. The isolates were tested for the following antibiotics: ampicillin (AMP; 10 μg), cefotaxime (CTX; 30 μg), cefoxitin (FOX; 30 μg), cefpodoxime (CPD; 10 μg), ceftazidime (CAZ; 30 mg), chloramphenicol (CHL; 30 mg), co-trimoxazole (STX; 1.25/23.75 μg), enrofloxacin (ENR; 5 μg), gentamicin (GMN;

10 μg), imipenem (IMP; 10 μg), nalidixic acid (NAL; 30 μg), streptomycin (SMN; 10 μg), and tetracycline (TET; 30 μg) (Oxoid Ltd., Basingstoke, UK). Colistin resistance was determined using the broth microdilution method, according to the European Committee on Antimicrobial Susceptibility Testing guidelines [34]. Results were interpreted with a resistance breakpoint of 2 μg/mL. Phenotypic characterization of the analyzed strains as susceptible, intermediate, or resistant was based on the breakpoint, i.e., the lowest concentration on which no bacterial growth was observed, according to [35,36].

The isolates were tested for ESBL production via a combination disk test (CDT), according to the CLSI guidelines. To perform a CDT, disks were used, including cefotaxime (30 μg), cefotaxime/clavulanic acid (30/10 μg), ceftazidime (30 μg), and ceftazidime/clavulanic acid (30/10 μg). The test was performed on Mueller–Hinton agar using a 0.5 McFarland inoculum, followed by incubation at 37 °C for 18 h. An increase in the diameter of the inhibition zone $\geq$ 5 mm in the presence of clavulanic acid is indicative of ESBL production.

The screening of strains for the production of AmpC β-lactamases was based on a resistance or reduced susceptibility to cefoxitin or imipenem, which acts as an inducer of antibiotic resistance. Cefotaxime (30 μg) and ceftazidime (30 μg) were placed around a strain and at a distance of 25 mm from the center of the disk. The test is positive when the repulsion of the edge of the zone of inhibition in cefotaxime or ceftazidime is induced on the side of the disc towards the inducer, according to Dunne and Hardin [36].

2.3. E. coli Phylogeny

Genomic DNA was extracted from 106 cultures using the NucleoSpin Microbial DNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The concentration and purity of the eluted DNA were determined using a Q5000 microvolume spectrophotometer (Quawell, Thmorgan, Beijing, China). To assign the phylogeny of *E. coli* strains, all isolates were subjected to a polymerase chain reaction (PCR), targeting the *chuA* and *yjaA* genes, which can reliably identify the phylogenetic group of the *E. coli* strain, according to the methodology developed by Clermont et al. [34]. PCRs were performed in reactions with a total volume of 20 μL, containing 10 μL FastGene Taq 2X Ready Mix (NIPPON Genetics, Tokyo, Japan), 0.3 pmoL of each forward and reverse primer (Table 1), and distilled water up to the total volume. The conditions of each reaction were 95 °C for 3 min, 95 °C for 30 s, annealing temperature (Table 1) for 40 s, 72 °C for 45 s, and a final extension step at 72 °C for 5 min. The amplified products were examined by electrophoresis in agarose gel stained with ethidium bromide and photographed using a photo documentation system.

2.4. Molecular Investigation of Antimicrobial Resistance Genes and Phylogeny

The molecular investigation of resistance was characterized by targeting the colistin resistance genes *mcr-1* and *mcr-2*, the ESBL resistant *blaTEM* gene, the tetracycline resistant tet(X) gene, and the quinolone resistance *qnrA* gene. PCRs were performed as described in Section 2.3 using the primer pairs *mcr-1F–mcr-1R*, *mcr-2F–mcr-2R*, *blaTEM-F–blaTEM-R*, *tet(X)-F–tet(X)-R*, and *qnrA-F–qnrA-R*, as described in Yuan et al. [37], and the annealing temperatures in Table 1. In samples considered positive, the amplified gene fragment proceeded to purification. After purification of the PCR products using the commercial NucleoSpin Gel and PCR Clean up kit (Macherey-Nagel, Düren, Germany), the purified products were bidirectionally sequenced by applying the Sanger methodology in a Prism 3730XL automatic capillary sequencer from the company CeMIA (Larissa, Greece), using both forward and reverse primers.

2.5. Statistical Analysis

Statistical analysis of the data was carried out using the software package SPSS 23.0 (IBM Corporation, Armonk, NY, USA), using the inferential statistics method. A χ^2 test was applied to compare the phenotypic and molecular findings. All hypothesis testing was conducted at a significance level of $\alpha = 0.05$ ($p < 0.05$).

Table 1. Primers used for the amplification of the target genes.

Primer	Sequence (5′-3′)	Target Gene	Expected Band Size	Annealing Temperature	Reference
mcr-1F	AGTCCGTTTGTTCTTGTGGC	*Colistin resistance gene 1*	320 bp	55 °C	[38]
mcr-1F	AGATCCTTGGTCTCGGCTTG				
mcr-2F	CAAGTGTGTTGGTCGCAGTT	*Colistin resistance gene 2*	715 bp	55 °C	[38]
mcr-2R	TCTAGCCCGACAAGCATACC				
blaTEM-F	CATTTCCGTGTCGCCCTTATTC	*Carbapenem resistance gene*	800 bp	60 °C	[39]
blaTEM-R	CGTTCATCCATAGTTGCCTGAC				
tet(X)-F	GGAAACCGGCTAATGGCAT	*Tetracycline resistance genes*	230 bp	55 °C	[40]
tet(X)-R	AATCCTACAAATGACAACGTCG				
qnrA-F	AGAGGATTTCTCACGCCAGG	*Quinolones resistance gene*	580 bp	54 °C	[41]
qnrA-R	TGCCAGGCACAGATCTTGAC				
ChuA.1	GACGAACCAACGGTCAGGAT	*chuA*	279 bp	55 °C	[34]
ChuA.2	TGCCGCCAGTACCAAAGACA				
YjaA.1	TGAAGTGTCAGGAGACGCTG	*yjaA*	211 bp	55 °C	[34]
YjaA.2	ATGGAGAATGCGTTCCTCAAC				
TspE4C2.1	GAGTAATGTCGGGGCATTCA	fragment TSPE4.C2	152 bp	55 °C	[34]
TspE4C2.2	CGCGCCAACAAAGTATTACG				

3. Results

3.1. Antimicrobial Susceptibility

From the 106 sample pools investigated, all of the obtained *E. coli* strains showed resistance to at least one antimicrobial substance. Specifically, 76.4% of the strains showed resistance to at least one of the examined β-lactam antibiotics, while 89.7% showed resistance to at least one quinolone.

The highest rates of resistance were observed in sulfamethoxazole, followed by nalidixic acid, tetracycline, piperacillin, streptomycin, and enrofloxacin. In contrast to the high rates of resistance to quinolones, resistance to third-generation cephalosporins was particularly low for aztreonam, ceftazidime, ceftriaxone, cefoxitin, and cefotaxime (Table 2). No strain showed resistance to imipenem. Eleven strains (10.3%) were resistant to colistin (MIC > 2 μg/L).

Table 2. Microbial resistance results of *E. coli* strains isolated from poultry farms.

Antimicrobial	Disk Composition	Thresholds (mm)		Resistant Strains–Broilers	Resistant Strains–Egg-Laying Hens	Resistant Strains–Total
		Sensitive	Resistant			
Sulfamethoxazole	23.75 μg	≥15	≤11	83 (91.1%)	3 (18.8%)	86 (81.1%)
Nalidixic acid	30 μg	≥19	≤13	71 (78%)	7 (43.8%)	78 (73.6%)
Tetracycline	30 μg	≥15	≤11	68 (74.7%)	7 (43.8%)	75 (70.8%)
Piperacillin	100 μg	≥21	≤17	55 (60.4%)	12 (75%)	67 (63.2%)
Streptomycin	10 μg	≥15	≤11	69 (75.8%)	6 (3.8%)	75 (70.8%)
Enrofloxacin	5 μg	≥21	≤15	54 (59.3%)	10 (6.3%)	64 (60.3%)
Aztreonam	30 μg	≥21	≤17	1 (1.1%)	-	1 (1%)
Ceftazidime	10 μg	≥21	≤17	3 (3.3%)	-	3 (2.8%)
Ceftriaxone	30 μg	≥23	≤19	4 (4.4%)	-	4 (3.7%)
Cefoxitin	30 μg	≥18	≤14	5 (5.5%)	-	5 (4.7%)
Cefotaxime	30 μg	≥26	≤22	5 (5.5%)	-	5 (4.7%)
Imipenem	10 μg	≥23	≤19	-	-	0

3.2. Phenotypic Tests for the Detection of ESBLs and AmpC-β-Lactamases

We detected phenotypic evidence for both ESBL and AmpC β-lactamase production. In particular, phenotypic tests revealed that 13.6% and 10.2% of the isolates produced ESBL, while 2.7% and 1% produced AmpC b-lactamase, for broiler and laying hens, respectively.

3.3. Molecular Identification

All of the *E. coli* strains belonged to the B1 phylogenetic group. The *mcr-1* gene was detected in 22 out of the 106 isolates (Figure 1), all originating from broilers. The results were confirmed using Sanger sequencing, with the haplotype derived in complete homology with the reference sequence with the GenBank accession number OM839890,

which was then used as a positive control. As far as the *mcr-2* gene is concerned, although a band was amplified after the PCR reaction, it failed to be confirmed after sequencing for both poultry groups. No other bands were amplified in any of the remaining examined genes. No statistical association was observed between the phenotypic and molecular data.

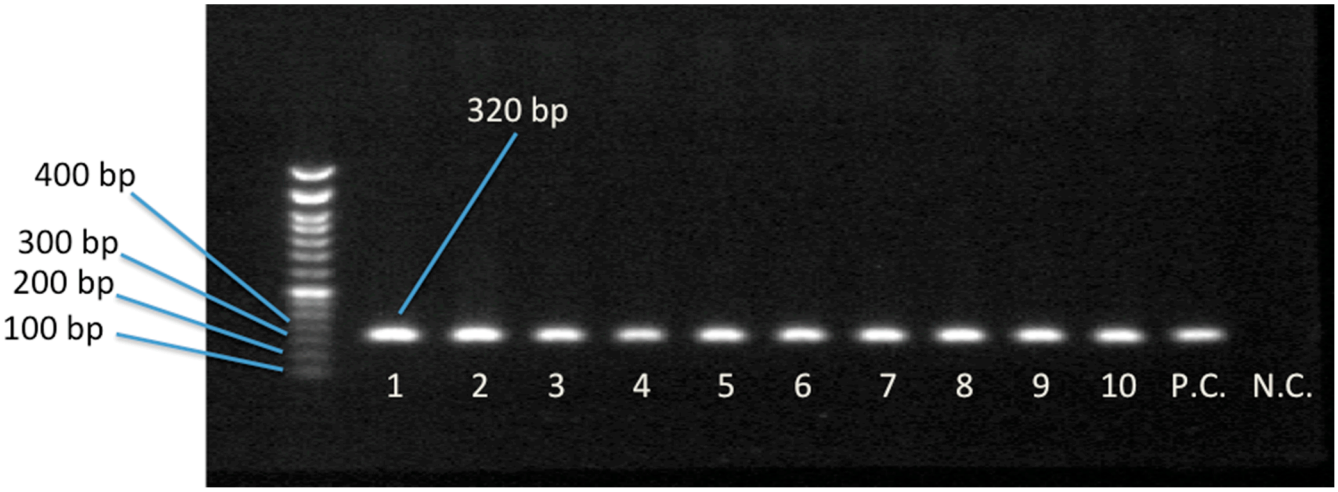

Figure 1. The PCR products of the 320 bp fragment after amplification with the *mcr-1*F–*mcr-1*R primer pair. Lanes 1–10: samples; P.C.: positive control, N.C.: negative control (no template DNA in the PCR).

4. Discussion

The animal production sector, and in particular poultry production, represents one potential source of multidrug-resistant bacteria, which possess plasmid-mediated resistance genes [42]. Under this prism, to the best of our knowledge, this is the first systematic study to assess the prevalence and the patterns of antimicrobial resistance in broilers and laying hens in Greece.

Increased rates of *E. coli*-resistant strains to quinolones isolated from farm animals have been previously reported [43], in line with the high resistance percentage to quinolones among strains both from broilers and laying hens detected in the current study. The above observations are in accordance with a report from the European Food Safety Authority (EFSA) for 2016 on antimicrobial resistance to microbial indicators in humans, food, and animals [44]. According to the EFSA, data collected from broilers among 30 countries on non-pathogenic *Escherichia coli* strains indicated higher resistance rates to quinolones, while resistance percentages to third-generation cephalosporins and colistin were lower. In laying hens, the same pattern was observed but with lower percentages, except for ceftazidime, cefotaxime, cefpodoxime, colistin, and sulfamethoxazole. Furthermore, other studies on poultry showed resistance levels to quinolone antibiotics ranging from 53% to 73% in the Czech Republic [45,46]. Nevertheless, the fact that, occasionally, resistance is only phenotypically observed, as was the case in the present study, indicates a phenotypic plasticity, in the sense that under environmental pressure such as the presence of antibiotics in microorganisms, resistant phenotypes are occasionally produced by non-resistant genotypes.

Data concerning laying hen poultry farms from Belgium, Germany, Italy, and Switzerland revealed lower resistance rates to quinolones, third-generation cephalosporins, and colistin than in the present study. More specifically, resistance to ciprofloxacin and nalidixic acid were 10.4% and 10.7, respectively; to cefpodoxime, 4.9%; and to colistin, 0.9% [47]. Similarly, resistance to quinolones was estimated at a level of 16.7%, while no resistance was found to colistin or cefpodoxime [48]. In Spain, resistance to ciprofloxacin and nalidixate were found to be 4.6% and 3%, respectively, while no resistance was revealed to colistin, ceftazidime, or cefotaxime [49]. The observed differences in antimicrobial resistance rates between broilers and laying hens may be due to the different antimicrobial substances used in each case.

Similarly, ESBL production from *E. coli* strains has been referred as a recent problem in poultry [50–52]. Although it is not very clear whether ESBL production from *E. coli* represents a major problem to the poultry sector, it might be a direct threat to public health. Keeping this in mind, the illustration of *E. coli* strains as producing ESBL/AmpC β-lactamases in the poultry sector in Greece is of high importance for β-lactam resistance screening.

In our study, none of the examined samples showed resistance to imipenem, which is most likely due to the prohibition of carbapenems in animals in Europe. A literature review covering the years 1980 to 2017 revealed that *E. coli* resistance to carbapenems remains low (<1%) among the European countries, while higher resistance rates are observed to carbapenems in Asian countries and Algeria, with the percentage resistance reaching 26% [53]. A study concerning laying hens (n = 276) in Germany showed an imipenem resistance of 1.8% [54]. These results are generally in agreement with our findings.

Furthermore, in the present study, the *mcr-1* gene was found to be present in broilers' *E. coli* isolates. Twenty-two samples (20.8%) from our survey tested positive. Colistin is an antimicrobial substance widely used in veterinary medicine to treat infections of the digestive tract, particularly in poultry and pigs. In 2015, the plasmid-mediated resistance gene *mcr-1* was first reported in poultry in China [55]. Since then, there has been an increase in research worldwide that has detected this specific gene in animals, in humans, and in foods of animal origin [56–60]. Thus far, it has mainly been detected in *E. coli* strains, as well as in other enterobacteroids belonging in the genera *Salmonella*, *Shigella*, *Klebsiella*, and *Enterobacter* [61]. Our results reveal that *E. coli* strains carrying the *mcr-1* gene are circulating in poultry farms in Greece, threatening the use of colistin as a last-resort antibiotic and highlighting the need for the surveillance of antibiotic resistance. Despite the limitation of our study regarding the detection of only one resistance gene, the fact that colistin is one of the main antibiotics for the treatment of serious human infections makes these results worth mentioning. The monitoring, collection, and presentation of all relevant information on the *mcr* gene at a global scale are of major importance in order to prevent public health threats and apply proper measures.

5. Conclusions

In conclusion, the occurrence of resistance to antimicrobials in *E. coli* in Greek poultry constitutes an issue of high importance. Antibiotic resistance to several antimicrobial substances was observed in high prevalence in *E. coli* strains. Concerning one of these substances, resistance was also reflected with the identification of a resistance gene. Although phenotypic resistance rates were high for various antibiotic compounds, the most noteworthy finding of the present study, from a public health point of view, is probably the identification of the *mcr-1* gene, which provides resistance to colistin. The circulation of this gene in Greek poultry poses risks for the transmission of resistance to other animals or to humans, as well. These results raise significant concerns regarding the use of antibiotics in Greek poultry, which traditionally plays an important role in the primary production sector. Based on these results, the proper use of antibiotics should be applied by the farm owners and operators.

Author Contributions: Conceptualization, E.P., E.N.S. and S.K.K.; methodology, A.X. and D.K.P.; software, M.V.A.; validation, G.A.D. and V.E.; formal analysis, K.V.P.; investigation, A.X. and I.A.G.; resources, E.P., E.N.S. and S.K.K.; data curation, M.V.A.; writing—original draft preparation, A.X. and M.V.A.; writing—review and editing, I.A.G., E.P. and E.N.S.; visualization, K.V.P.; supervision, E.P.; project administration, S.K.K.; funding acquisition, S.K.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Acknowledgments: This work is dedicated to the memory of Georgios Filiousis.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Silbergeld, E.K.; Graham, J.; Price, L.B. Industrial Food Animal Production, Antimicrobial Resistance, and Human Health. *Annu. Rev. Public Health* **2008**, *29*, 151–169. [CrossRef] [PubMed]
2. Dawadi, P.; Bista, S.; Bista, S. Prevalence of Colistin-Resistant *Escherichia coli* from Poultry in South Asian Developing Countries. *Vet. Med. Int.* **2021**, *2021*, 6398838. [CrossRef] [PubMed]
3. Skerman, V.B.D.; Sneath, P.H.A.; McGowan, V. Approved Lists of Bacterial Names. *Int. J. Syst. Evol. Microbiol.* **1980**, *30*, 225–420. [CrossRef]
4. Lukjancenko, O.; Wassenaar, T.M.; Ussery, D.W. Comparison of 61 Sequenced *Escherichia coli* Genomes. *Microb. Ecol.* **2010**, *60*, 708–720. [CrossRef] [PubMed]
5. World Health Organization. *E. coli*. 2018. Available online: http://www.who.int/mediacentre/factsheets/fs125/en/ (accessed on 7 December 2022).
6. Herzer, P.J.; Inouye, S.; Inouye, M.; Whittam, T.S. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. *J. Bacteriol.* **1990**, *172*, 6175–6181. [CrossRef] [PubMed]
7. Clermont, O.; Christenson, J.K.; Denamur, E.; Gordon, D.M. The Clermont *Escherichia coli* phylo-typing method revisited: Improvement of specificity and detection of new phylo-groups. *Environ. Microbiol. Rep.* **2013**, *5*, 58–65. [CrossRef]
8. Clermont, O.; Dixit, O.V.; Vangchhia, B.; Condamine, B.; Dion, S.; Bridier-Nahmias, A.; Denamur, E.; Gordon, D. Characterization and rapid identification of phylogroup G in *Escherichia coli*, a lineage with high virulence and antibiotic re-sistance potential. *Environ. Microbiol.* **2019**, *21*, 3107–3117. [CrossRef]
9. Clermont, O.; Olier, M.; Hoede, C.; Diancourt, L.; Brisse, S.; Keroudean, M.; Glodt, J.; Picard, B.; Oswald, E.; Denamur, E. Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. *Infect. Genet. Evol.* **2011**, *11*, 654–662. [CrossRef]
10. Tenaillon, O.; Skurnik, D.; Picard, B.; Denamur, E. The population genetics of commensal *Escherichia coli*. *Nat. Rev. Microbiol.* **2010**, *8*, 207–217. [CrossRef]
11. Pitout, J.D.D. Extraintestinal Pathogenic *Escherichia coli*: A Combination of Virulence with Antibiotic Resistance. *Front. Microbiol.* **2012**, *3*, 9. [CrossRef]
12. Abdelwahab, G.E.; Ishag, H.Z.A.; Al Hammadi, Z.M.; Al Yammahi, S.M.S.; Mohd Yusof, M.F.B.; Al Yassi, M.S.Y.; Al Mansoori, A.M.A.; Al Hamadi, F.H.A.; Al Hamadi, I.A.S.; Hosani, M.A.A.A.; et al. Antibiotics Re-sistance in *Escherichia coli* Isolated from Livestock in the Emirate of Abu Dhabi, UAE, 2014–2019. *Int. J. Microbiol.* **2022**, *2022*, 3411560. [CrossRef] [PubMed]
13. Arbab, S.; Ullah, H.; Wang, W.; Zhang, J. Antimicrobial drug resistance against *Escherichia coli* and its harmful effect on animal health. *Vet. Med. Sci.* **2022**, *8*, 1780–1786. [CrossRef] [PubMed]
14. Daneman, N.; Fridman, D.; Johnstone, J.; Langford, B.J.; Lee, S.M.; MacFadden, D.M.; Mponponsuo, K.; Patel, S.N.; Schwartz, K.L.; Brown, K.A. Antimicrobial resistance and mortality following *E. coli* bacteremia. *Eclinicalmedicine* **2023**, *56*, 101781. [CrossRef]
15. Falagas, M.E.; Polemis, M.; Alexiou, V.G.; Marini-Mastrogiannaki, A.; Kremastinou, J.; Vatopoulos, A.C. Antimicro-bial resistance of *Esherichia coli* urinary isolates from primary care patients in Greece. *Med. Sci. Monit.* **2008**, *14*, CR75. [PubMed]
16. Papadopoulos, D.; Papadopoulos, T.; Papageorgiou, K.; Sergelidis, D.; Adamopoulou, M.; Kritas, S.K.; Petridou, E. Antimicrobial resistance rates in commensal *Escherichia coli* isolates from healthy pigs in Greek swine farms. *J. Hell. Vet. Med. Soc.* **2021**, *72*, 2909–2916.
17. Laube, H.; Friese, A.; von Salviati, C.; Guerra, B.; Käsbohrer, A.; Kreienbrock, L.; Roesler, U. Longitudinal Monitoring of Extended-Spectrum-Beta-Lactamase/AmpC-Producing *Escherichia coli* at German Broiler Chicken Fattening Farms. *Appl. Environ. Microbiol.* **2013**, *79*, 4815–4820. [CrossRef]
18. Cantón, R.; González-Alba, J.M.; Galán, J.C. CTX-M Enzymes: Origin and Diffusion. *Front. Microbiol.* **2012**, *3*, 110. [CrossRef]
19. Blanc, V.; Mesa, R.; Saco, M.; Lavilla, S.; Prats, G.; Miró, E.; Navarro, F.; Cortés, P.; Llagostera, M. ESBL- and plasmidic class C β-lactamase-producing *E. coli* strains isolated from poultry, pig and rabbit farms. *Vet. Microbiol.* **2006**, *118*, 299–304. [CrossRef]
20. Ewers, C.; Bethe, A.; Semmler, T.; Guenther, S.; Wieler, L. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: A global perspective. *Clin. Microbiol. Infect.* **2012**, *18*, 646–655. [CrossRef]
21. Jiang, H.-X.; Tang, D.; Liu, Y.-H.; Zhang, X.-H.; Zeng, Z.-L.; Xu, L.; Hawkey, P.M. Prevalence and characteristics of -lactamase and plasmid-mediated quinolone resistance genes in *Escherichia coli* isolated from farmed fish in China. *J. Antimicrob. Chemother.* **2012**, *67*, 2350–2353. [CrossRef]
22. Dierikx, C.M.; Van Duijkeren, E.; Schoormans, A.H.W.; Van Essen-Zandbergen, A.; Veldman, K.; Kant, A.; Huijsdens, X.W.; Van Der Zwaluw, K.; Wagenaar, J.A.; Mevius, D.J. Occurrence and characteristics of extended-spectrum-β-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses. *J. Antimicrob. Chemother.* **2012**, *67*, 1368–1374. [CrossRef] [PubMed]

23. Dierikx, C.; van Essen-Zandbergen, A.; Veldman, K.; Smith, H.; Mevius, D. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. *Vet. Microbiol.* **2010**, *145*, 273–278. [CrossRef]
24. Hancock, R.E.W.; Chapple, D.S. Peptide Antibiotics. *Antimicrob. Agents Chemother.* **1999**, *43*, 1317–1323. [CrossRef]
25. Biswas, S.; Brunel, J.-M.; Dubus, J.-C.; Reynaud-Gaubert, M.; Rolain, J.-M. Colistin: An update on the antibiotic of the 21st century. Expert Rev. *Anti-Infect. Ther.* **2012**, *10*, 917–934. [CrossRef]
26. Zhang, J.; Chen, L.; Wang, J.; Yassin, A.K.; Butaye, P.; Kelly, P.; Gong, J.; Guo, W.; Li, J.; Li, M.; et al. Molecular detection of colistin resistance genes (mcr-1, mcr-2 and mcr-3) in nasal/oropharyngeal and anal/cloacal swabs from pigs and poultry. *Sci. Rep.* **2018**, *8*, 3705. [CrossRef] [PubMed]
27. Borowiak, M.; Baumann, B.; Fischer, J.; Thomas, K.; Deneke, C.; Hammerl, J.A.; Szabo, I.; Malorny, B. Development of a Novel mcr-6 to mcr-9 Multiplex PCR and Assessment of mcr-1 to mcr-9 Occurrence in Colistin-Resistant *Salmonella enterica* Isolates From Environment, Feed, Animals and Food (2011–2018) in Germany. *Front. Microbiol.* **2020**, *11*, 80. [CrossRef] [PubMed]
28. Hoelzer, K.; Wong, N.; Thomas, J.; Talkington, K.; Jungman, E.; Coukell, A. Antimicrobial drug use in food-producing animals and associated human health risks: What, and how strong, is the evidence? *BMC Vet. Res.* **2017**, *13*, 211. [CrossRef]
29. Ruiz, J. Mechanisms of resistance to quinolones: Target alterations, decreased accumulation and DNA gyrase protection. *J. Antimicrob. Chemother.* **2003**, *51*, 1109–1117. [CrossRef]
30. Johnson, J.R.; Kuskowski, M.A.; Smith, K.; O'bryan, T.T.; Tatini, S. Antimicrobial-Resistant and Extraintestinal Pathogenic *Escherichia coliin* Retail Foods. *J. Infect. Dis.* **2005**, *191*, 1040–1049. [CrossRef]
31. Seo, K.; Lee, Y. Prevalence and characterization of plasmid-mediated quinolone resistance determinants qnr and aac (6')-Ib-cr in ciprofloxacin-resistant *Escherichia coli* isolates from commercial layer in Korea. *J. Microbiol. Biotechnol.* **2020**, *30*, 1180–1183. [CrossRef]
32. Dotas, V.; Gourdouvelis, D.; Hatzizisis, L.; Kaimakamis, I.; Mitsopoulos, I.; Symeon, G. Typology, Structural Char-acterization and Sustainability of Integrated Broiler Farming System in Epirus, Greece. *Sustainability* **2021**, *13*, 13084. [CrossRef]
33. CLSI. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, 11th ed.; Approved Standard; Clinical and Laboratory Standards Institute (CLSI): Wayne, PA, USA, 2012.
34. Clermont, O.; Bonacorsi, S.; Bingen, E. Rapid and Simple Determination of the *Escherichia coli* Phylogenetic Group. *Appl. Environ. Microbiol.* **2000**, *66*, 4555–4558. [CrossRef] [PubMed]
35. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Di-ameters. Version 6.0. 2016. Available online: http://www.eucast.org (accessed on 7 December 2022).
36. Dunne, W.M.; Hardin, D.J. Use of several inducer and substrate antibiotic combinations in a disk approximation assay format to screen for AmpC induction in patient isolates of *Pseudomonas aeruginosa*, *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. *J. Clin. Microbiol.* **2005**, *43*, 5945–5949. [CrossRef]
37. Yuan, J.; Wang, X.; Shi, D.; Ge, Q.; Song, X.; Hu, W.; Wei, D.; Ge, C.; Li, X.; Hu, C. Extensive antimicrobial resistance and plasmid-carrying resistance genes in mcr-1-positive *E. coli* sampled in swine, in Guangxi, South China. *BMC Vet. Res.* **2021**, *17*, 86. [CrossRef] [PubMed]
38. Rebelo, A.R.; Bortolaia, V.; Kjeldgaard, J.S.; Pedersen, S.K.; Leekitcharoenphon, P.; Hansen, I.M.; Guerra, B.; Malorny, B.; Borowiak, M.; Hammerl, J.A.; et al. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5 for surveillance purposes. *Eurosurveillance* **2018**, *23*, 17-00672. [CrossRef]
39. Dallenne, C.; Da Costa, A.; Decre, D.; Favier, C.; Arlet, G. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. *J. Antimicrob. Chemother.* **2010**, *65*, 490–495. [CrossRef]
40. He, T.; Wang, R.; Liu, D.; Walsh, T.R.; Zhang, R.; Lv, Y.; Ke, Y.; Ji, Q.; Wei, R.; Liu, Z.; et al. Emergence of plasmid-mediated high-level tigecycline resistance genes in animals and humans. *Nat. Microbiol.* **2019**, *4*, 1450–1456. [CrossRef]
41. Cattoir, V.; Poirel, L.; Rotimi, V.; Soussy, C.-J.; Nordmann, P. Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. *J. Antimicrob. Chemother.* **2007**, *60*, 394–397. [CrossRef]
42. Leverstein-van Hall, M.A.; Dierikx, C.M.; Cohen Stuart, J.; Voets, G.M.; van den Munckhof, M.P.; van Essen-Zandbergen, A.; Platteel, T.; Fluit, A.C.; van de Sande-Bruinsma, N.; Scharinga, J.; et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin. Microbiol. Infect.* **2011**, *17*, 873–880. [CrossRef]
43. Webber, M.; Piddock, L.J. Quinolone resistance in *Escherichia coli*. *Vet. Res.* **2001**, *32*, 275–284. [CrossRef]
44. EFSA (European Food Safety Authority); ECDC (European Centre for Disease Prevention and Control). The European Union summary report on antimicrobia resistance in zoonotic and indicator bacteria from humans, animals and food in 2016. *EFSA J.* **2018**, *16*, 5182–5270.
45. Skočková, A.; Koláčková, I.; Bogdanovičová, K.; Karpíšková, R. Characteristic and antimicrobial resistance in *Escherichia coli* from retail meats purchased in the Czech Republic. *Food Control.* **2015**, *47*, 401–406. [CrossRef]
46. Hricová, K.; Röderová, M.; Pudová, V.; Hanulík, V.; Halová, D.; Julínková, P.; Dolejská, M.; Papoušek, I.; Bardoň, J. Quinolone-resistant *Escherichia coli* in Poultry Farming. *Central Eur. J. Public Health* **2017**, *25*, 163–167. [CrossRef]
47. VAN Hoorebeke, S.; VAN Immerseel, F.; Berge, A.C.; Persoons, D.; Schulz, J.; Hartung, J.; Harisberger, M.; Regula, G.; Barco, L.; Ricci, A.; et al. Antimicrobial resistance of *Escherichia coli* and *Enterococcus faecalisin* housed laying-hen flocks in Europe. *Epidemiol. Infect.* **2010**, *139*, 1610–1620. [CrossRef] [PubMed]
48. Harisberger, M.; Gobeli, S.; Hoop, R.; Dewulf, J.; Perreten, V.; Regula, G. Antimicrobial Resistance in Swiss Laying Hens, Prevalence and Risk Factors. *Zoonoses Public Health* **2011**, *58*, 377–387. [CrossRef] [PubMed]

49. Moreno, M.A.; García-Soto, S.; Hernández, M.; Bárcena, C.; Rodríguez-Lázaro, D.; Ugarte-Ruíz, M.; Domínguez, L. Day-old chicks are a source of antimicrobial resistant bacteria for laying hen farms. *Vet. Microbiol.* **2019**, *230*, 221–227. [CrossRef] [PubMed]
50. Tan, H.S.; Yan, P.; Agustie, H.A.; Loh, H.S.; Rayamajhi, N.; Fang, C.M. Characterisation of ESBL/AmpC-Producing Enterobacteriaceae isolated from poultry farms in Peninsular Malaysia. *Lett. Appl. Microbiol.* **2023**, *76*, ovac044.
51. Falgenhauer, L.; Imirzalioglu, C.; Oppong, K.; Akenten, C.W.; Hogan, B.; Krumkamp, R.; Poppert, S.; Levermann, V.; Schwengers, O.; Sarpong, N.; et al. Detection and Characterization of ESBL-Producing *Escherichia coli* From Humans and Poultry in Ghana. *Front. Microbiol.* **2019**, *9*, 3358. [CrossRef]
52. Chai, M.H.; Sukiman, M.Z.; Jasmy, N.; Zulkifly, N.A.; Yusof, N.A.S.M.; Mohamad, N.M.; Ariffin, S.M.Z.; Ghazali, M.F. Molecular Detection and Antibiogram of ESBL-Producing and Carbapenem-Resistant *Escherichia coli* from Rabbit, Swine, and Poultry in Malaysia. *Trop. Anim. Sci. J.* **2022**, *45*, 16–23. [CrossRef]
53. Köck, R.; Daniels-Haardt, I.; Becker, K.; Mellmann, A.; Friedrich, A.W.; Mevius, D.; Schwarz, S.; Jurke, A. Carbapenem-resistant Enterobacteriaceae in wildlife, food-producing, and companion animals: A systematic review. *Clin. Microbiol. Infect.* **2018**, *24*, 1241–1250. [CrossRef]
54. Schwaiger, K.; Schmied, E.-M.V.; Bauer, J. Comparative Analysis of Antibiotic Resistance Characteristics of Gram-negative Bacteria Isolated from Laying Hens and Eggs in Conventional and Organic Keeping Systems in Bavaria, Germany. *Zoonoses Public Health* **2008**, *55*, 331–341. [CrossRef]
55. Liu, Y.-Y.; Wang, Y.; Walsh, T.R.; Yi, L.-X.; Zhang, R.; Spencer, J.; Doi, Y.; Tian, G.; Dong, B.; Huang, X.; et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. *Lancet Infect. Dis.* **2016**, *16*, 161–168. [CrossRef] [PubMed]
56. Quesada, A.; Ugarte-Ruiz, M.; Iglesias, M.R.; Porrero, M.C.; Martínez, R.; Florez-Cuadrado, D.; Campos, M.J.; García, M.; Píriz, S.; Sáez, J.L.; et al. Detection of plasmid mediated colistin resistance (MCR-1) in *Escherichia coli* and *Salmonella enterica* isolated from poultry and swine in Spain. *Res. Vet. Sci.* **2016**, *105*, 134–135. [CrossRef] [PubMed]
57. Anjum, M.F.; Duggett, N.A.; AbuOun, M.; Randall, L.; Nunez-Garcia, J.; Ellis, R.J.; Rogers, J.; Horton, R.; Brena, C.; Williamson, S.; et al. Colistin resistance in *Salmonella* and *Escherichia coli* isolates from a pig farm in Great Britain. *J. Antimicrob. Chemother.* **2016**, *71*, 2306–2313. [CrossRef] [PubMed]
58. Cannatelli, A.; Giani, T.; Antonelli, A.; Principe, L.; Luzzaro, F.; Rossolini, G.M. First Detection of the mcr-1 Colistin Resistance Gene in *Escherichia coli* in Italy. *Antimicrob. Agents Chemother.* **2016**, *60*, 3257–3258. [CrossRef]
59. Zurfluh, K.; Klumpp, J.; Nüesch-Inderbinen, M.; Stephan, R. Full-Length Nucleotide Sequences of mcr-1 -Harboring Plasmids Isolated from Extended-Spectrum-β-Lactamase-Producing *Escherichia coli* Isolates of Different Origins. *Antimicrob. Agents Chemother.* **2016**, *60*, 5589–5591. [CrossRef]
60. Yang, Y.-Q.; Li, Y.-X.; Song, T.; Yang, Y.-X.; Jiang, W.; Zhang, A.-Y.; Guo, X.-Y.; Liu, B.-H.; Wang, Y.-X.; Lei, C.-W.; et al. Colistin Resistance Gene mcr-1 and Its Variant in *Escherichia coli* Isolates from Chickens in China. *Antimicrob. Agents Chemother.* **2017**, *61*, e01204-16. [CrossRef]
61. Schwarz, S.; Johnson, A.P. Transferable resistance to colistin: A new but old threat. *J. Antimicrob. Chemother.* **2016**, *71*, 2066–2070. [CrossRef]