Bacterial resistance to third-generation cephalosporins and colistin in the poultry industry

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PhD Thesis

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Denn Wissenschaft und Kunst gehören der Welt an, und vor ihnen verschwinden die Schranken der Nationalität.

Science and art belong to the whole world, and the barriers of nationality vanish before them.

-Johann Wolfgang von Goethe

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Summary

Antimicrobial resistance is one of the major public health threats that humans face with a significant number of annual deaths and economic losses from associated sequelae. Presence of resistance determinants in food-producing animals represents an important exposure risk since they can be transmitted to humans by direct contact or via the food chain. Among the various types of resistance already described in poultry, resistance to critically important antimicrobials, such as third-generation cephalosporins (3GCs) and colistin, is worrying due to the crucial role of these antimicrobials in severe infections encountered in health care settings. The high levels of resistance to 3GCs previously described in broilers, led to the belief that the broiler production may act as a reservoir of 3GC resistance determinants. Therefore, Chapters 2-4 aimed to investigate their presence, characteristics, transmission patterns and identify intervention measures for risk mitigation strategies. In light of the alarming emergence of mobile colistin resistance mechanisms, Chapter 5 undertook a review on their global distribution in the poultry industry and addressed the current situation and challenges of colistin resistance.

The structure of the thesis is: (i) general introduction, (ii) four main chapters of research articles either published, in press or in preparation, (iii) overall conclusion, (iv) additional information for each of the main chapters, (iv) two appendices with contribution to other research work, (v) acknowledgements, and (vi) the literature cited.

Riassunto

La resistenza agli antimicrobici è uno dei principali problemi di sanità pubblica, a causa dell'elevato numero di morti e di perdite economiche che si verificano annualmente. La presenza di determinanti di resistenza negli animali produttori di alimenti rappresenta un importante rischio per l'uomo, per la possibile trasmissione per contatto diretto o attraverso la catena alimentare. Tra i vari tipi di resistenza già descritti nelle specie avicole, la resistenza ad antimicrobici criticamente importanti, quali le cefalosporine di terza generazione (3GC) e la colistina, è particolarmente preoccupante a causa del ruolo cruciale che questi antimicrobici assumono a livello nosocomiale nel trattamento di infezioni gravi. Gli elevati livelli di resistenza alle 3GC già decritti nei broiler hanno indotto a ritenere che la filiera produttiva del pollo da carne possa fungere da *reservoir* di determinanti di resistenza nei confronti di 3GC. Pertanto, i Capitoli 2-4 riportano i risultati di studi condotti allo scopo di investigare la loro presenza, le loro caratteristiche, la loro modalità di trasmissione e di identificare misure correttive per la mitigazione del rischio. Alla luce dell'allarmante comparsa di meccanismi di trasferimento della resistenza nei confronti della colistina, il Capitolo 5 riporta una *review* sulla sua distribuzione nell'industria avicola a livello globale, soffermandosi in particolare sull'attuale situazione e sulla minaccia che essa rappresenta.
La tesi è suddivisa in: (i) introduzione generale, (ii) quattro capitoli riguardanti articoli già pubblicati, in via di pubblicazione o in preparazione, (iii) conclusione generale, (iv) informazione aggiuntiva per i principali capitoli, (iv) ringraziamenti, e (v) citazioni bibliografiche.

Chapter 1. General introduction

Global perspectives on antimicrobial resistance

"The thoughtless person playing with penicillin treatment is morally responsible for the death of the man who succumbs to infection with the penicillin-resistant organism." The confirmation of Alexander Fleming's famous prediction came soon after his discovery of penicillin, when Abraham and Chain discussed about an "enzyme able to destroy penicillin"[1] and thus provided the first evidence of antimicrobial resistance (AMR). A generally accepted definition of AMR is the ability of microorganisms to thrive upon exposure to concentrations of antimicrobials that would otherwise inhibit or kill them[2]. Presence of resistant microorganisms in permafrost samples[3] proves that AMR is not a novel phenomenon. What has changed, however, is the widespread use and abuse of antimicrobials in both human and veterinary medicine that exerts selection pressure and thus contributes to the development and proliferation of resistant microorganisms[4]. Occurrence of resistant microorganisms in food-producing animals is highly probable to represent an exposure risk for humans by either direct contact or via the food chain[5]. Today, AMR is one of the major public health threats that humans face with more than 700,000 deaths being attributed to resistant infections globally. Based on current rates, estimates predict this number to surpass cancer victims by 2050. Moreover, the economic losses from nosocomial costs and loss of productivity are vast and are expected to rise even more[6]. For these reasons, AMR is currently at the top of the political agenda[4] and efforts with an "One Health" approach to combat this global problem are undergoing[7]. This framework requires the continuous monitoring and characterisation of resistant microorganisms in food-producing animals, to identify emerging resistance determinants and estimate the risk of human exposure via the food chain[5].

Use of third-generation cephalosporins and colistin in humans and animals

Except for their pharmacological classification, antimicrobials are categorised by the World Health Organisation (WHO) based on their importance as therapeutic agents to alleviate infections in humans. In the latest document issued by WHO, third-generation cephalosporins (3GCs) and colistin, are placed at the top of the list as critically important antimicrobials (CIAs) with the highest priority due to (i) their crucial role to treat serious infections, (ii) the documented development of resistance in health care settings and (iii) the evidence of

transmission of resistance determinants from non-human sources[8]. Both antimicrobials are being used and have an important role in the treatment of animal infections as well[9]. This overlap of usage is particularly important with regard to the development and transmission of resistance between animals and humans[10]. Indications for this risk first emerged in 1969, when the Swann Report[11] committee cautioned that (sub)-therapeutic usage of antimicrobial in animals can lead to adverse effects in human health and was subsequently evidenced by *e.g.* the description of zoonotic transmission of a resistant *Salmonella* Heidelberg strain[12].

Third-generation cephalosporins

These antimicrobials belong to the *β*-lactam group of antibiotics, named after the *β*-lactam ring, which is the key element of the structure and antibacterial activity of these drugs. In comparison to earlier generations, 3GCs have an extended-spectrum as they are able to exert their antimicrobial activity against both Gram-negative and Gram-positive bacteria. Their mode of action is based on the interference with bacterial cell-wall synthesis by binding to active sites that inhibit cross-linking of the cell-wall[13]. In humans, 3GCs are used for the treatment of severe diseases such as infections by *Salmonella* and *Escherichia coli* [*e.g.* urinary tract infections (UTIs) and bloodstream infections][10]. In animals, cefquinome and ceftiofur are administered in cases of respiratory infections in ruminants (cattle, sheep and goats) and horses as well as in foot rot and mastitis in cattle[14]. Use of 3GCs in poultry is prohibited within the European Union (EU) except for their administration in day-old chicks of breeder flocks, where early mortality can lead to significant economic losses. This particular usage of 3GCs in hatcheries has been identified as the culprit for the rise of resistance in poultry production[15,16].

Colistin

This drug belongs to the polymyxin complex of the lipopeptides group of antimicrobials. It is a narrow spectrum antimicrobial, effective against fermentative (*e.g. E. coli*) and non-fermentative Gram-negative bacteria (*e.g. Pseudomonas aeruginosa*) but not Gram-positive bacteria. This limitation stems from colistin's mode of action, which is the disorganization of the outer cell membrane by binding to lipid A of the lipopolysaccharide (LPS). The latter structure is absent in Gram-positive bacteria. Colistin use was limited in humans due to its adverse

side-effects (*e.g.* nephrotoxicity), however it has re-emerged as a last-resort therapeutic option against severe infections caused by multi-drug (MDR) resistant bacteria[17]. Their primary indication in animals is the treatment of gastrointestinal (GI) infections in cattle and swine (*e.g.* post-weaning diarrhoea in piglets). Although bioavailability of colistin following oral administration is very low in poultry, its administration in cases of mild colibacillosis seems to be a common practice. In addition, colistin is used for growth-promotion in countries where this practice is allowed (prohibited in the EU)[18].

Resistance mechanisms to third-generation cephalosporins and colistin in *Enterobacteriaceae* and their presence in poultry

Bacteria can develop resistance mechanisms by mutations and/or acquisition of foreign DNA, containing resistance genes, by transformation (uptake of free DNA), transduction (incorporation of DNA from bacteriophages) or conjugation [uptake of DNA (plasmids) from other bacteria]. This mode of antimicrobial resistance development represents the acquired resistance type and is strain-specific. Conversely, the property of a genus or species to be resistant to particular antimicrobials (*e.g. Mycoplasma* spp. and *β*-lactams) is termed intrinsic or innate resistance. With regard to resistance mechanisms, they can be divided in three basic types: (i) enzymatic inactivation of antimicrobials, (ii) reduced influx or increased efflux of antimicrobials and (iii) modification or protection of antimicrobials' cellular target sites[19,20].

Resistance to third-generation cephalosporins

All the three aforementioned resistance mechanisms can be found in *Enterobacteriaceae* that are resistant to *β*-lactams, the most predominant type however is that of enzymatic degradation by *β*-lactamases. These enzymes are classified based on the similarity of their amino acid sequences (classes A to D) but also based on their spectrum of activity (groups 1 to 3)[21]. Resistance to 3GCs in *Enterobacteriaceae* is mainly mediated by extended-spectrum *β*-lactamases (ESBLs) and plasmid-mediated AmpC *β*-lactamases (pAmpCs). ESBLs belong to subgroup 2be (class A) that includes the dominant CTX-M type of enzymes as well as extended-spectrum TEM and SHV variants. AmpCs belong to group 1 (class C) with CMY, ACT, DHA and FOX being some of the representative enzymes of this group. The chromosomal mutation in the *ampc* gene promotor or

attenuator in *E. coli* (AmpC) is also included in this group. ESBLs and pAmpCs can hydrolyse cephalosporins and monobactams. An important difference is that AmpCs are not inhibited by clavulanic acid[19,21]. Further, many studies have shown that ESBL and/or pAmpC-producing *Enterobacteriaceae*, particularly *E. coli* (ESBL/pAmpC-EC) are prevalent in poultry and poultry products[22]. CTX-M-1, TEM-52, SHV-12 and CMY-2 are the predominant ESBL/pAmpC enzymes found in poultry. Therefore, given the high occurrence of such resistance determinants, the poultry production system may act as a reservoir and exposure risk for humans by either direct contact (*e.g.* farmers and veterinarians) or via the food chain (chicken meat consumers)[23].

Resistance to colistin

Mechanisms of resistance to colistin mainly consisted of chromosomal mutations that led to the binding of L-Ara4N and phosphoethanolamine (pEtN) to the lipid A of LPS and thus reduced the affinity of colistin (target modification). Therefore, the health burden from resistance to colistin was limited to sporadic clonal outbreaks of strains that acquired resistance through the aforementioned pathways[17]. However, in 2015 the first plasmid-mediated colistin resistance gene, named *mcr-1*, was described[24]. The gene encodes a pEtN transferase enzyme and induces resistance similarly to the aforementioned chromosomal mechanisms. Subsequent reports revealed the emergence of novel *mcr* genes (*mcr-2* to *mcr-9*). The predominance of *mcr* genes in food-producing animals and the excess of colistin use in livestock compared to the limited use in humans, pointed to the "animal world as the culprit of the emerging plasmid-mediated colistin resistance"[25].

Plasmids

Plasmids are autonomous DNA molecules that are able to replicate and be self-transmitted between diverse bacterial hosts belonging to the same or different species. Furthermore, mobile genetic elements such as transposons or insertion sequences can modify and enrich their gene repertoire. These characteristics make plasmids the perfect agents of AMR gene spread. Indeed, horizontal gene transfer through plasmid exchange is considered crucial in the global AMR epidemiology[26,27]. To study the epidemiology of AMR plasmids, different typing methods exist with the most common being the PCR-based replicon typing (PBRT)[28]. Epidemic AMR plasmids belong to the IncF, IncI, IncA/C,

IncN and IncH plasmid replicon types. Subgrouping of the IncI1, IncHI1, IncHI2, IncN and IncF plasmid types is possible with the plasmid multilocus sequence typing (pMLST), which targets plasmid housekeeping genes[26]. Further, the recent development of low-cost Next Generation Sequencing (NGS) technologies enabled the characterisation of the whole plasmid sequence. Both ESBL/pAmpC and *mcr* genes have been associated with several of the aforementioned plasmid types rather than specific bacterial clones, which underlines the importance of plasmids in the dissemination of these resistance determinants. A noteworthy exception is the epidemic *E. coli* clone O25b-H4-B2-ST131 that carries $bla_{\text{CTX-M-15}}$ usually on IncFII plasmids and has been associated with human UTIs worldwide[29] as well as ESBL ST648, which has been involved in human infections globally and has also disseminated in animals[30]

E. coli

E. coli is typically a commensal facultative anaerobic microorganism that inhabits the lower GI tract of mammals and birds shortly after their birth. However, several *E. coli* lineages are equipped with virulence factors that enable them to adapt to new niches and cause disease. The most successful *E. coli* virulent clones (pathotypes) that cause enteric infections are the following; enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC). Extra-intestinal pathogenic *E. coli* (ExPEC) cause UTIs, sepsis and meningitis[31]. A subset of ExPEC, the avian pathogenic *E. coli*, are significant disease agents for poultry and cause of severe economic losses in the industry. Furthermore, a link between APEC and ExPEC disease in humans has been proposed, which is worrying considering that this strains often carry AMR genes[32]. Except from the fact that *E. coli* is both a ubiquitous commensal and an opportunistic pathogen, it is also a well characterised model microorganism. For these reasons it serves as an indicator of resistance levels in AMR epidemiological studies[5]. As with plasmids, genotyping of resistant *E. coli* strains is crucial in the study of their epidemiology. Among many others, the most common typing methods include determination of phylogroups, multilocus sequence typing (MLST) and typing using whole genome sequencing (WGS) data (e.g. core-genome MLST)[33,34].

Aims of this thesis

As previously discussed, various studies have shown the presence of ESBL/pAmpC-EC in the broiler production and debated on the risks that stem from their high prevalence. In Italy, few studies have investigated this issue and none of them followed a holistic approach from breeders to slaughter, which would shed light on transmission patterns. Chapters 2, 3 and 4 addressed this problem. The aim of Chapter 2 was to establish a baseline prevalence for ESBL/pAmpC-EC in the broiler production pyramid and estimate their transmission dynamics over successive production stages. Prevalence at the isolate level (i.e. ratio of resistant to susceptible isolates in each production stage), the relationship between the resistant and generic *E. coli* population as well as the potential differences by the use of selective and non-selective media on ESBL/pAmpC-EC occurrence were the aims of Chapter 3. The aim of Chapter 4 was the high-resolution characterisation of selected ESBL/pAmpC-EC isolates by WGS, which is crucial in order to thoroughly assess their epidemiology and inform risk assessment efforts. Finally, in light of the emergence of the infamous *mcr* genes, which confer resistance to colistin, another critically important and last-resort antimicrobial, Chapter 5 undertook a global review on the current situation and challenges of colistin resistance in the poultry industry.

Chapter 2. Assessing the occurrence and transfer dynamics of ESBL/pAmpC-producing *Escherichia coli* across the broiler production pyramid

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Abstract

Extended-spectrum *β*-lactamase (ESBL)- and plasmid mediated, AmpC-type cephalosporinase (pAmpC)-producing *E. coli* (ESBL/pAmpC *E. coli*) in food-producing animals is a major public health concern. This study aimed at quantifying ESBL/pAmpC-*E. coli* occurrence and transfer in Italy's broiler production pyramid. Three production chains of an integrated broiler company were investigated. Cloacal swabs were taken from parent stock chickens and offspring broiler flocks in four fattening farms per chain. Carcasses from sampled broiler flocks were collected at slaughterhouse. Samples were processed on selective media, and *E. coli* colonies were screened for ESBL/pAmpC production. ESBL/pAmpC genes and *E. coli* phylogroups were determined by PCR and sequencing. Average pairwise overlap of ESBL/pAmpC *E. coli* gene and phylogroup occurrences between subsequent production stages was estimated using the proportional similarity index, modelling uncertainty in a Monte Carlo simulation setting. In total, 820 samples were processed, from which 513 ESBL/pAmpC *E. coli* isolates were obtained. We found a high prevalence (92.5%, 95%CI 72.1-98.3%) in day-old parent stock chicks, in which $bla_{\text{CMY-2}}$ predominated; prevalence then dropped to 20% (12.9-29.6%) at laying phase. In fattening broilers, prevalence was 69.2% (53.6-81.3%) at the start of production, 54.2% (38.9-68.6%) at slaughter time, and 61.3% (48.1-72.9%) in carcasses. Significantly decreasing and increasing trends for respectively $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-1}}$ gene occurrences were found across subsequent production stages. ESBL/pAmpC *E. coli* genetic background appeared complex and *bla*-gene/phylogroup associations indicated clonal and horizontal transmission. Modelling revealed that the average transfer of ESBL/pAmpC *E. coli* genes between subsequent production stages was 47.7% (42.3-53.4%). We concluded that ESBL/pAmpC *E. coli* in the broiler production pyramid is prevalent, with substantial transfer between subsequent production levels.

Introduction

Extended spectrum cephalosporins (ESCs) are considered critically important antimicrobials (CIAs) in both human and veterinary medicine[8,9]. Resistance to these antimicrobials is therefore a major public health concern due to the risks of therapy failure[20]. Resistance in *Enterobacteriaceae*, such as *E. coli*, caused by production of extended-spectrum *β*-lactamases (ESBLs) or AmpC *β*-lactamases (pAmpCs) and

transferability of resistance mechanisms are of particular importance, as the encoding genes (hereafter ESBL/pAmpC genes) are often located in promiscuous plasmids[19]. This property of EBSL/pAmpC genes enables their exchange between bacteria, including pathogens, and favours transmission between animals and humans[26]. Usage of ESCs in poultry has been restricted since 2012 in the European Union (EU)[35] except for occasional use in (grand)parent hatcheries[16,36]. However, numerous studies have shown high prevalence rates of ESBL/AmpC-producing *E. coli* (ESBL/pAmpC *E. coli*) in broiler flocks across Europe[37–39], even in countries with low antimicrobial use in livestock[36,40]. The presence of ESBL/pAmpC *E. coli* has been evidenced at all levels of the broiler production pyramid from breeding farms[41], hatcheries[42], fattening farms[43], slaughterhouses[44] to retail meat[45]. Whether broiler meat contaminated with ESBL/pAmpC *E. coli* represents an important source of human infections is debatable[46]. For instance, a recent comparative risk assessment estimated the exposure to ESBL/pAmpC *E. coli* through consumption of chicken meat to be lower than beef and pork[47], whereas a meta-analysis identified poultry products as a more likely source of human ESC-resistant infections compared to other food-producing animals[48]. The zoonotic transmission potential has prompted many investigations, not only on the prevalence of ESBL/pAmpC *E. coli*, but also on their transmission routes along the broiler production pyramid. A recent review[22] summarised the transfer of ESBL/pAmpC-producing bacteria in four major pathways; vertical transmission from parent to offspring, transmission in the hatchery, horizontal transmission in fattening farms, and horizontal transmission between farms or from the environment. Additionally, the authors stressed the need for more quantitative data on these transmission pathways.

In Italy, data on both prevalence and transmission dynamics of EBSL/pAmpC genes in poultry are lacking. This study aimed to establish a baseline prevalence of ESBL/pAmpC *E. coli* in the broiler production pyramid and to characterise the genetic background of resistant isolates in order to assess ESBL/pAmpC *E. coli* transfer between subsequent levels of the broiler production pyramid.

Materials and methods

Sample collection

Three production chains (chain A, B, and C) of an integrated broiler production company in Northern Italy (specifically in Veneto,

Lombardy, and Friuli-Venezia Giulia regions) were monitored from January 2017 to January 2018. In each of the 40 sampling visits, faecal samples from 20 randomly selected healthy birds were taken by cloacal swabs in the farms, and 20 carcasses were collected at the slaughterhouse. For each chain, sample collection started at the top of the production pyramid by sampling day-old Parent Stock (PS) chicks, which were the progeny of the Grandparent Stock (GPS) flock located elsewhere. Imported day-old PS chicks were sampled upon arrival to the rearing farm within one hour from delivery. Due to time constraints, samples were not collected for PS chicks of chain B. The same flock of PS chickens was sampled again during the laying period (~30-weeks-old) at the production farm (PS breeders), in which they were moved at the age of 21-weeks. Sampled breeder flocks were not combined with other flocks at the production farms. The offspring of the sampled PS breeders were tracked and sampled in four commercial fattening farms per production chain at two time points; at the age of one-day-old (broiler chicks), and around 30th day of age, within the last week prior slaughter (broilers) (S1 Table). Samples in each broiler farm were collected from the same poultry house, which contained only the progeny of the previously sampled PS breeders. Finally, 20 carcasses from the previously sampled broilers were collected at the slaughterhouse after the chilling process. All breeder flocks and all broiler flocks, except three of them, received antibiotic treatment, mainly amoxicillin for therapeutic reasons unrelated to this study (S1 Fig). Broilers from the three monitored chains were all processed in the same slaughterhouse in Veneto region. Cloacal swabs from birds were collected by veterinarians of the private company as part of the routine monitoring of the flock health status and conducted in compliance with good veterinary practices.

Isolation and detection

Cloacal swabs were directly streaked on Eosin Methylene Blue agar (Microbiol, Italy) supplemented with 1mg/L cefotaxime (CTX-EMB) and incubated at 37±0.5 °C for 20±2h. Carcasses were analysed by both a qualitative and quantitative method. For the qualitative method, carcasses were rinsed with 400mL Buffer Peptone Water (BPW), rinsates were incubated (37±0.5 °C for 20±2h24 h) and streaked on CTX-EMB (0.1mL). For the quantitative method, 1 mL of rinsate before incubation was used to produce three serial dilutions (10^{-1} to 10^{-3}). 0.1mL of rinsate (10^{0}) and dilutions were plated on CTX-EMB for

subsequent enumeration. One to two morphologically typical *E. coli* colonies on CTX-EMB (metallic green sheen) were isolated from each sample and subjected to species confirmation by combination of indole test and PCRs targeting *E. coli* housekeeping genes[49]. Confirmed *E. coli* isolates were screened for ESBL/pAmpC production by double-disk synergy test using cefotaxime (30 μg) and ceftazidime (30 μg) discs with and without clavulanic acid (10 μg) and according to CLSI guidelines[50]. Additionally, a cefoxitin disc (30 μg) was used to detect potential AmpC-producers.

Molecular characterisation

ESBL/pAmpC gene groups[51] and *E. coli* phylogroups[34] were detected by multiplex PCRs for all phenotypically resistant isolates. For a selection of 119 isolates, ESBL/AmpC genes were sequenced (Macrogen, Spain) after amplification with the primers described by Dierikx *et al*.[52] to identify gene variants. This selection was done considering the variability of ESBL/pAmpC genes and *E. coli* groups per sampling (at least one isolate per phylogroup-*bla* gene combination per sampling). Moreover, isolates with an AmpC phenotype, but negative for pAmpC genes by multiplex PCR, were analysed for chromosomal mutations in the *ampC* promoter/attenuator (cAmpC) according to Haldorsen *et al*.[53].

Data analysis

Prevalence of ESBL/pAmpC *E. coli* for each production stage was calculated based upon faecal samples or carcasses being positive to ESBL/pAmpC *E. coli* (at least one isolate). Prevalence estimates and corresponding 95% confidence intervals (95% CI) were adjusted for clustering of observations at the chain and farm levels using cluster-robust standard errors. Chi-square statistic for trends in proportions was used to test the significance of trends in the relative frequencies of different ESBL/pAmpC gene groups, gene variants, and phylogroups over subsequent sampling stages across the whole broiler production pyramid. Statistical analysis was performed using STATA (StataCorp, College Station, USA).

To quantify the possible transfer of ESBL/pAmpC *E. coli* across the broiler production pyramid, the average pairwise overlap of ESBL/pAmpC genes and *E. coli* gene groups (M1), gene groups plus gene variants (M2), or gene groups plus gene variants plus phylogroups (M3), between subsequent production stages, was estimated based on

the proportional similarity index (PSI)[54], accounting for uncertainty in measurements in a Monte Carlo simulation setting. PSI values range from 0 (no similarities) to 1 (total overlap). The PSI is expressed as:

$$PSI = \left(1 - 0.5 \sum |r_{j,x,y,k} - r_{j,x,y,k+1}|\right)$$

where *r* denoted the relative frequency of ESBL/pAmpC gene group (M1), ESBL/pAmpC gene variant (as additional strata within gene group) (M2), or *E. coli* phylogroup (as additional strata within the gene variant, which in turn were strata of the respective gene group) (M3) *j* (with *j*=1,…, *J*; and *J*=7 gene groups, *J*=10 gene groups plus gene variants, and *J*=7 gene groups plus gene variants plus phylogroups), in farm *x* (with *x*=1,…, *X*; and *X*=4) of chain *y* (with *y*=1,…, *Y*; and *Y*=3) at sampling stage (*i.e.* production level) *k* (with *k*=1,…, *K*; and *K*=5). The average of the PSIs calculated over the three chains and four farms per chain gave the overall measure of overlap between sampling stages, as follows:

$$\frac{1}{n}\sum_{i=1}^{n} PSI_i = \frac{PSI_1 + PSI_2 + \ \ldots + PSI_n}{n}$$

Uncertainty was introduced in the frequencies of M1, M2 and M3 by assuming the following probability distribution:

$$\left(r_{1,x,y,k}, r_{2,x,y,k}, \ldots, 1 - \sum_{j=1}^{J-1} r_{j,x,y,k}\right) \approx Dirichlet\left(X_{1,x,y,k}, X_{2,x,y,k}, \ldots, X_{J,x,y,k}\right)$$

Where *r* is defined as above, and *X* is the number of detections of ESBL/pAmpC gene group/gene variant/phylogroup *j* in farm *x* of chain *y* at sampling stage *k*.

This analysis was performed in @RISK (Palisade Corp., USA) by setting 10,000 iterations with the Latin hypercube sampling technique and a seed of 1.

Results

Prevalence of ESBL/pAmpC *E. coli*

In total, 820 samples (*i.e.* 20 samples per chain-farm-sampling stage combination) were collected over 40 sampling visits. From these samples, 537 confirmed *E. coli* isolates from CTX-EMB were screened

for phenotypic resistance to ESCs by disk diffusion and 513 (95.5%) thereof were positive. Overall, 60.3% (95%CI 51.3-68.1%) of samples were positive for presence of ESBL/pAmpC *E. coli* (Fig 1). ESBL/pAmpC *E. coli* were recovered at all sampling events on farms (cloacal swabs) and slaughterhouse (carcasses). A high prevalence (92.5%, 95%CI 72.1-98.3%) was found in day-old PS chicks, which dropped to 20% (95%CI, 12.9-29.6%) during the laying period. In fattening broilers, prevalence was higher again (69.2%, 95%CI 53.6-81.3%) at the start of the production cycle, and decreased to 54.2% (95%CI 38.9-68.6%) in the last sampling right before slaughter (Fig 1). At the end of the production pyramid, ESBL/pAmpC *E. coli* was isolated from 61.3% (95% CI 48.1-72.9%) of carcasses (Fig 1). In 29.2% of carcasses (*n*=43 samples) positive for presence of ESBL/pAmpC *E. coli* by the qualitative method, the load was below the quantification limit (1 Log CFU/mL). In samples with countable ESBL/pAmpC *E. coli* the median load was 1.66 Log CFU/1 mL rinsing water (min 1 Log CFU/mL, max 4.2 Log CFU/mL).

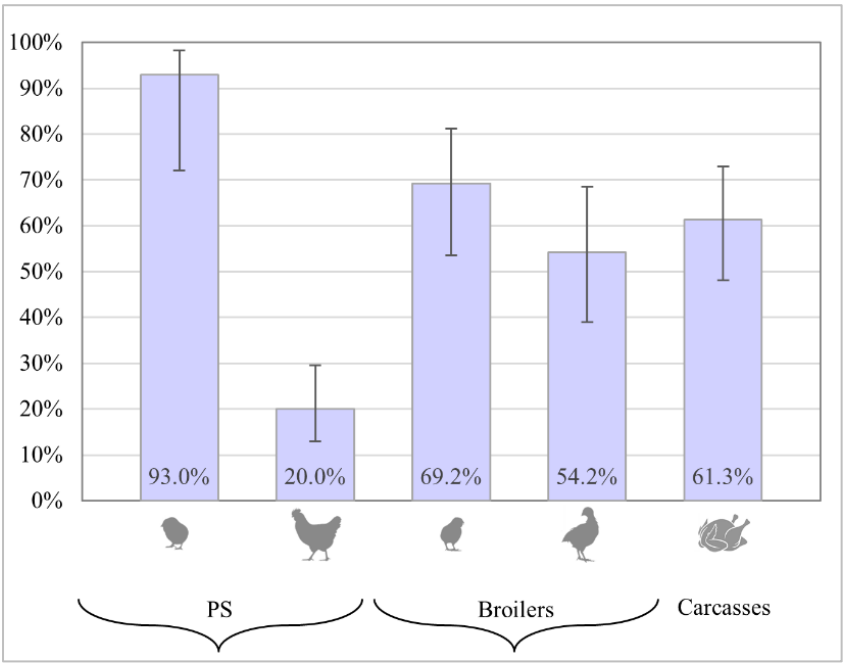

Fig 1. Prevalence of ESBL/pAmpC *E. coli* in the broiler production pyramid. From left to right: PS chicks, PS breeders, broiler chicks, broilers and carcasses. Bars represent corresponding 95% confidence intervals.

ESBL/pAmpC genes and phylogroup distribution

Out of the 513 ESC-resistant isolates, 333 (64.9%) had the ESBL and 180 (35.1%) the AmpC phenotype. By multiplex PCR, the following ESBL gene groups were identified: *bla*$_{\text{CTX-M-group-1}}$ (41.9% of all resistant isolates), *bla*$_{\text{SHV}}$ (17.6%), *bla*CTX-M-group-9 (2.9%), *bla*$_{\text{CTX-M-group-2}}$ (2.5%) and *bla*$_{\text{TEM}}$ (0.2%). All isolates with an AmpC phenotype were carrying *bla*$_{\text{CMY}}$ (25.6%), except for 18 isolates in which the following mutations were discovered in the *ampc* gene control region: −88 (C→T), −82 (A→G), −42 (C→T), −18 (G→A), −1 (C→T) and +58 (C→T). No ESBL/pAmpC genes were found for 28 ESC-resistant *E. coli*. One-hundred-eighty-three isolates with a confirmed ESBL/pAmpC gene had concurrent presence of *bla*$_{\text{TEM}}$, which upon sequencing (n=19 isolates) proved to be *bla*$_{\text{TEM-1b}}$, a broad-spectrum β-lactamase.

Sequencing of ESBL genes revealed variability in *bla*$_{\text{CTX-M-group-1}}$, which was mainly comprised of *bla*$_{\text{CTX-M-55}}$ (54.2%) and *bla*$_{\text{CTX-M-1}}$ (42.4%), whereas only one isolate (1.7%) was found for each of *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-164}}$ (Fig 2A). Two isolates had concurrent presence of more than one *bla*CTX-M-group-1 genes and thus unassignable by Sanger sequencing. In contrast, all *bla*$_{\text{CMY,}}$ *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$ belonged to the *bla*$_{\text{CMY-2}}$, *bla*$_{\text{SHV-12}}$ and *bla*$_{\text{TEM-52}}$ variants, respectively. Moreover, the *bla*$_{\text{CMY-2}}$ variant was found in all *bla*$_{\text{CMY}}$-carrying isolates.

Overall, the most prevalent gene variants were *bla*$_{\text{CTX-M-55}}$ and *bla*$_{\text{CMY-2}}$ (27% each) followed by *bla*$_{\text{CTX-M-1}}$ (21%) and *bla*$_{\text{SHV-12}}$ (14%). Other gene variants were only sporadically present. All (100%) ESBL/pAmpC *E. coli* in PS chicks carried *bla*$_{\text{CMY-2}}$ (Fig 2A). The relative frequency of this latter gene variant showed a statistically significant decreasing trend (linear slope = -0.20, SE = 0.017, $p<0.0001$) over subsequent production stages, but was present throughout the production pyramid. *bla*$_{\text{CTX-M-55}}$ appeared in PS breeders, remained present in fattening broilers where it peaked at the end of the production cycle (39.6%), and was the second most prevalent gene variant in chicken carcasses (30.7%), after *bla*$_{\text{CTX-M-1}}$ (35%). The latter was also present in broiler, but not in breeder flocks (Fig 2A). Both *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{CTX-M-55}}$ showed a significantly increasing trend (linear slope = 0.11, SE = 0.015, $p<0.0001$, and linear slope = 0.08, SE = 0.017, $p<0.0001$, respectively) from breeders to carcasses. Furthermore, *bla*$_{\text{SHV-12}}$-carrying *E. coli* were isolated from all production stages but

PS chicks, and were predominantly isolated from fattening broiler chicks (34.6%).

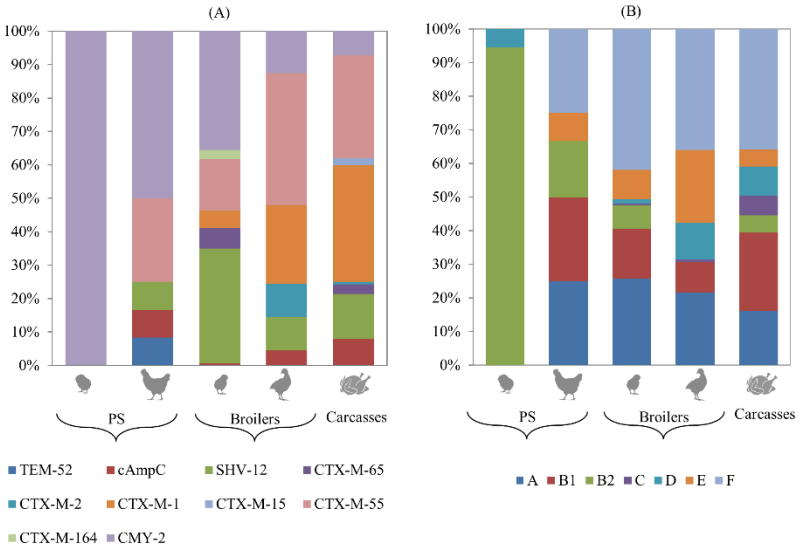

Fig 2. Distribution of ESBL/pAmpC genes (A) and *E. coli* phylogroups (B) in the broiler production pyramid. (A) cAmpC, isolates with chromosomal mutations in the *ampC* promoter/attenuator. (A) and (B) from left to right: PS chicks, PS breeders, broiler chicks, broilers and carcasses.

The most frequent *E. coli* genotype was phylogroup F (34.9%) followed by A (19.7%), B1 (15.1%) and B2 (12.1%). Phylogroups C, D, and E were each found in less than 10% of isolates. In PS chicks, phylogroup B2 predominated (94.6%), whereas in PS breeders phylogroups F, A, and B1 were equally present (25%) (Fig 2B). In subsequent production levels, phylogroup F was the most prevalent, but other phylogroups were present as well (Fig 2B). Significantly increasing and decreasing trends along the production chain were respectively found for phylogroups F (linear slope = 0.05, SE = 0.01, p=0.009) and B2 (linear slope = -0.15, SE = 0.01, p<0.0001).

ESBL/pAmpC genes were found in multiple *E. coli* phylogroups within the same production stage, but within the same sampling stage as well (Fig 3). However, phylogroup F-$bla_{\text{CTX-M-55}}$ was the most frequent (21.1%) isolate profile, especially in fattened broilers (37%) and carcasses (20%). In contrast, phylogroup A- and B2-$bla_{\text{CMY-2}}$ were the

most frequent ones in PS chicks (100%) and PS breeders (33.4%) (Fig 3).

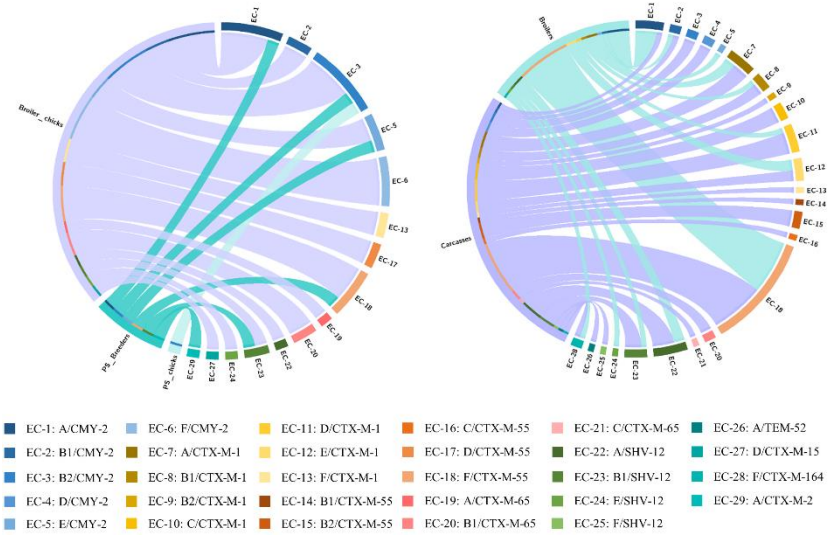

Fig 3. Detected isolates by phylogroup/*bla*-gene combination and corresponding production stages. Size of segments on the right represent the number of isolates with a specific combination. Size of segments on the left represent the number of isolates detected in different production stages. Ribbons connecting left and right segments represent the number of isolates with a specific combination found on the respective production stage. Chord diagram generated with CIRCOS[55].

ESBL/pAmpC E. coli transfer across the production pyramid

Genotyping data were used to calculate PSIs over subsequent sampling stages at three levels of increasing stratification (M1, M2, and M3). The average stepwise transfer of ESBL/pAmpC *E. coli* over the whole production pyramid was 55.5% (95%CI 48.3-61.3%) for M1, 47.7% (95%CI 42.3-53.4%) for M2, 47.2% (95%CI 44.2-50.3%) for M3. At the top of the production pyramid, 36.4 to 50.4% (depending on the level of genotyping data stratification) of ESBL/pAmpC *E. coli* found in PS breeders overlapped with (and could therefore be estimated to originate from) PS chicks (Fig 4). The overlap between fattening chicks and fattened broilers varied from 46.5 to 49.1% (M3 to M1), whereas 51.5 to 66.9% (M3 to M1) of ESBL/pAmpC *E. coli* found in carcasses were likely to originate from broilers sampled before slaughter (Fig 4). In general, the overlap of genotyping data decreased with increasing stratification of these data due to decreased chance of finding one-to-

one matches of the same genotype between two sampling stages. Moreover, the overlap increased with decreasing time elapsed between two sampling stages (Fig 4).

Discussion

This is the first study investigating the occurrence and potential transfer of ESBL/pAmpC *E. coli* across the whole broiler production pyramid in Italy. Samples predominantly originated from one Italian region, which may be considered as a potential limitation of the study concerning the representability of the samplings. However, the sampled area is among the most densely populated poultry areas in the country and thus can be considered representative of at least a large fraction of Italy's poultry production[56]. Analysis of 820 samples from three broiler production chains resulted in the identification of ESBL/pAmpC *E. coli* in all farms and batches of carcasses, with an overall prevalence of 60.3%. Day-old PS chicks showed the highest prevalence, comparable with recent findings from the Netherlands[57], but higher than those found in Norway[36]. All ESBL/pAmpC *E. coli* found in PS chicks had the AmpC phenotype, carried *bla*$_{\text{CMY-2}}$, and belonged predominantly to phylogroup B2. Therefore, a prevalent *E. coli bla*$_{\text{CMY-2}}$ lineage seems to have been introduced through the import of day-old PS chicks. However, due to the widespread occurrence of *bla*$_{\text{CMY-2}}$ in poultry, other sources of entry cannot be excluded[58]. It has been speculated that the underlying reason for high prevalence at this production level is the *in ovo* use of cephalosporins in supplying (grand)parent hatcheries, which selects for ESC-resistant *E. coli* in the gut of young hatchlings[16]. This is a problem for Nordic countries supplied by the same breeding stock, as the *bla*$_{\text{CMY-2}}$ genotype introduced at the top of the production pyramid in these countries spreads clonally to the bottom, is often the only identified ESBL/pAmpC gene[36,59,60]. Similar findings have been described in Denmark[40]. In contrast, we found a significant decrease of *bla*$_{\text{CMY-2}}$ and a gradual substitution by mainly *bla*$_{\text{CTX-M-55}}$ and *bla*$_{\text{CTX-M-1}}$ along the production pyramid, as well as the sharp reduction of prevalence in PS breeders (Fig 1). A similar decrease in the prevalence of *bla*$_{\text{CMY-2}}$-carrying *E. coli* from 95% in week 1 to 0% in week 21 has been described by Dame-Korevaar *et al*.[57] and was attributed to a selective disadvantage of *bla*$_{\text{CMY-2}}$-carrying plasmids. Although different EBSL/pAmpC genotypes were identified during the laying period, *bla*$_{\text{CMY-2}}$ was still dominant and the overlap of genotype distributions between the two sampling stages in breeders was

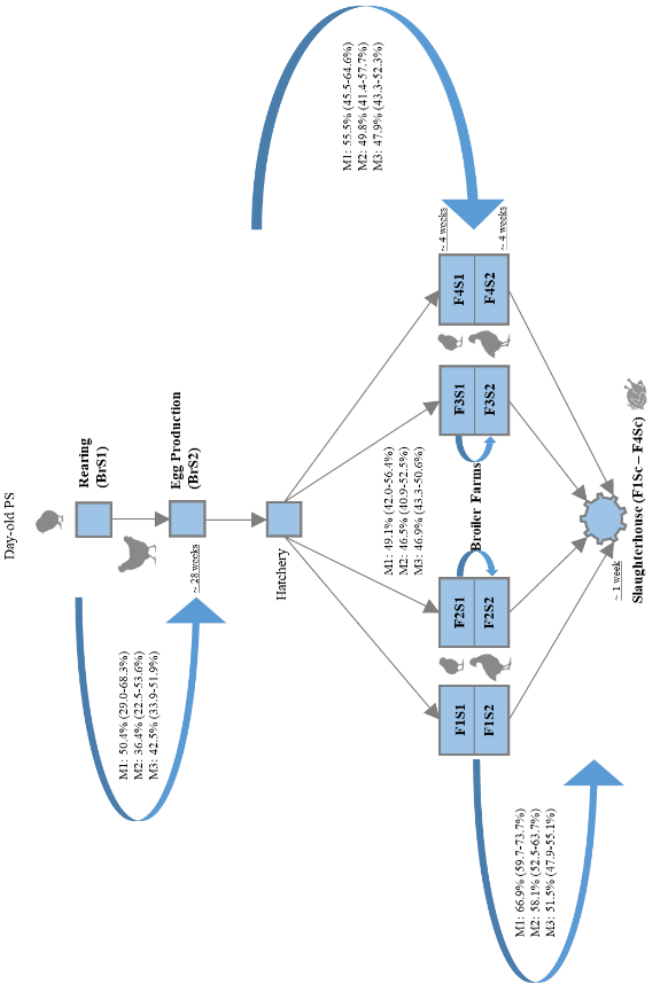

Fig. 4. Transfer of ESBL/pAmpC *E. coli* across the production pyramid. BrS1, PS chicks; BrS2, PS breeders; F1-4S1, farm 1-4 broiler chicks; F1-4S2, farm 1-4 broilers; F1-4Sc, farm 1-4 carcasses. Arrows and text boxes indicate the overlap of genotyping data in subsequent sampling stages (bold) at three levels (M1-M3) of increasing stratification. Underlined text indicates the time elapsed between sampling two consecutive stages.

substantial despite the relatively long period between them (36.4-50.4%). Persistence of *bla*$_{CMY-2}$ has been previously associated with the IncK and IncI1 plasmid types[39].

In broilers at the start of the cycle, 47.9-55.5% of the ESBL/pAmpC *E. coli* were likely to have been vertically transmitted from their parents. The impact of the hatchery on colonisation of hatchlings with ESC-resistant *E. coli* was not in the scope of this study. However, the role of the hatchery has proven to be trivial since ESBLs/pAmpCs found in the hatchery's environment (*e.g.* dust and incubators) were different than those recovered from the egg surface and hatchlings[48,61]. As Projahn *et al.*[42] showed, true vertical transmission (infection during egg formation) is rare and hatchlings seem to get colonised through a mode of pseudo-vertical transmission with contaminated eggs that enter the hatchery. Nonetheless, the finding that at least 43.3% of ESBLs/pAmpCs recovered in broiler chicks originated from their parents supports the notion of a combination of vertical transmission and environmental contamination from the farm environment, together with rapid proliferation of resistant bacteria, resulting into high prevalence (69.2%) and diversity of ESBL/pAmpC *E. coli* in young broilers[62]. The number of positive faecal samples was 15% lower in fattened broilers, which can be explained by the development of immunity to certain (resistant) *E. coli* genotypes[63] or by the fact that antimicrobials were mainly administered in the start and middle of the production cycle (S1 Fig). However, high transmission ratios and repeated shifts in phylogroups of ESBL/pAmpC *E. coli* during the fattening period, such as the ones observed in our study (Fig 3) lead to persistence of ESC-resistant *E. coli* in broiler flocks during the fattening period[63,64]. The overall transfer of EBSL/pAmpC-EC from the start to the end of the broiler production cycle was, on average, 46.5-49.1%, which indicates substantial influence of other sources, such as the environment and the effect of previously fattened flocks, on the genotype landscape of ESBL/pAmpC *E. coli*[43]. This has been documented by, *e.g.*, the finding of *bla*$_{CTX-M-2}$ being only present in fattened broilers, but not in fattening chicks (Fig 2A). The high levels of resistance found are difficult to explain since no cephalosporins were administered in the investigated flocks, but the use of amoxicillin, which was frequent in our study (S1 Fig), could have selected for ESBL/pAmpC *E. coli*[36].

At the end of the production pyramid, a large proportion of carcasses (61.3%) were found to be contaminated with ESBL/pAmpC *E. coli*, which is comparable with prevalence found in previous studies[44,65,66],

although the load of resistant *E. coli* was lower in our study. This is a worrying finding since contamination of meat with ESBL/pAmpC *E. coli* is directly linked with human exposure[22]. The contribution of chicken meat as a source of human ESBL-pAmpC-EC infections is debatable[47,48,67], however, the finding of phylogenetically related strains with the same gene and plasmid profile shared between patients and poultry meat[23,45,68,69] warrants for measures to prevent the entrance of ESBL/pAmpC *E. coli* in the lower levels of the food chain. The highest percentage of transfer of ESBL/pAmpC *E. coli* between fattened broilers and carcasses points to contamination of carcasses during processing at the slaughterhouse[65], but the time elapsed between these two sampling stages was the shortest (Fig 4). However, results at this production stage need to be interpreted with caution as cross-contamination from previously slaughtered flocks via other routes (*e.g.* scalding water, defeathering machines, transportation crates) is frequent[44,65,70].

Our study proved that the genetic background of ESBL/pAmpC *E. coli* in broilers is complex in all production stages, except perhaps for PS chicks, which isolates belonged predominantly to phylogroup B2-$bla_{\text{CMY-2}}$ (Fig 2, 3). In subsequent production stages, we found common (*e.g.* $bla_{\text{CTX-M-1}}$, $bla_{\text{SHV-12}}$) and less common (*e.g.* $bla_{\text{CTX-M-65}}$, $bla_{\text{CTX-M-2}}$) poultry-related ESBL/AmpC genes[16] to be associated with more than one phylogroup (Fig 3), even within the same farm (data not shown), suggesting that horizontal gene transfer contributes to the dissemination of resistance genes, as previously discussed[41]. In contrast, persistence of phylogroup F-$bla_{\text{CTX-M-55}}$ in significant proportions of isolates from breeders to the slaughterhouse is indicative of clonal transmission (Fig 3). An unexpected discovery was the occurrence of $bla_{\text{CTX-M-55}}$ in all production stages except for PS chicks, especially in fattened broilers where it was the most dominant gene (Fig 2A). This is an ESBL gene usually found in food-producing animals and humans in Asia[71] and has been rarely described in poultry in Europe[46,58]. Regarding *E. coli* genotypes, from PS chicks we observed a significant reduction of the predominant phylogroup B2, which was mainly substituted by group F in broilers and carcasses (Fig 2B). Both phylogroups are associated with extra-intestinal pathogenicity, as they often harbour virulence genes not found in other *E. coli* genotypes[72]. However, isolates belonging to commensal groups (A, B1, and C) were substantially present as well (37% of all isolates) (Fig 2B).

In conclusion, ESBL/pAmpC *E. coli* were detectable at all levels of the broiler production pyramid. The highest prevalence was observed in imported PS chicks, with almost all samples being positive for ESBL/pAmpC *E. coli*, particularly to $bla_{\text{CMY-2}}$. Measures to ensure ESBL-free pedigree flocks in supplying countries and avoid introduction of resistant clones by the purchase of (grand)parent stock is therefore warranted[40,46]. Based on the similarity of ESBL/pAmpC *E. coli* genotype distributions between subsequent production levels, we showed that the transfer of ESBL/pAmpC *E. coli* across the whole production pyramid is likely to be substantial, with approximately half of the genotypes found in a given production stage being likely to originate from the previous stage, and with the time between sampling stages and discriminatory power of genotyping data having an effect as well. Interventions like application of competitive exclusion mixtures, which contain live commensals from specific pathogen free (SPF) chickens, may help to control dissemination of ESBL/pAmpC *E. coli* by reducing colonisation and suppressing excretion[64]. Mitigation strategies should definitely include biosecurity and disinfection measures to avoid colonisation from environmental sources[22,43]. Prevention of cross-contamination in the slaughterhouse processing line is also crucial, as the load of ESBL/pAmpC *E. coli* on meat defines human exposure.

Chapter 3. Impact of selective and non-selective media on prevalence and genetic makeup of ESBL/pAmpC-producing *Escherichia coli* in the broiler production pyramid

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Manuscript submitted for publication

Abstract

Presence of extended-spectrum β-lactamase (ESBL)- and AmpC β-lactamase (pAmpC)-producing *E. coli* (ESBL/pAmpC-EC) in humans and animals is alarming due to the associated risks of antibiotic therapy failure. ESBL/pAmpC-EC transmission between the human and animal compartments remains controversial. Using cefotaxime-supplemented (selective) media, we recently showed high sample prevalence of ESBL/pAmpC-EC in an integrated broiler chain [i.e. Parent Stock (PS), offspring broilers and their carcasses]. Here, we used a different approach. In parallel with the selective isolation, samples were processed on non-selective media. *E. coli* isolates were tested for ESBL/pAmpC-production and those found positive were genotyped. For carcasses, total *E. coli* were enumerated. This approach enabled us to estimate prevalence at the isolate level, which mirrors ESBL/pAmpC-EC colonisation levels. We showed that although present in many animals, ESBL/pAmpC-EC were overall subdominant to the general *E. coli* population, indicating that high sample prevalence is not associated with high levels of resistance in individual hosts. This is a relevant aspect for risk assessments, especially regarding the immediate exposure of farm personnel. An exception was a particularly dominant B2/$bla_{\text{CMY-2}}$ lineage in the gut of imported PS chicks. This predominance obscured presence of latent genotypes, however bias towards particular ESBL/pAmpC-EC genotypes from the selective method or underestimation by the non-selective approach did not occur. At the slaughterhouse, we showed a link between total *E. coli* numbers and sample prevalence of ESBL/pAmpC-EC on the one hand and ESBL/pAmpC-EC numbers on carcasses on the other. Consequently, poor hygiene conditions seem to correlate with higher numbers of ESBL/pAmpC-EC, which in turn may lead to more contaminated carcasses through cross-contamination Therefore, hurdles for reducing consumers' exposure should aim at suppressing ESBL/pAmpC-EC in the broiler gut as well as controlling critical points in the processing line.

Introduction

The increased incidence of extended-spectrum β-lactamase (ESBL)- and AmpC β-lactamase (pAmpC)-producing *Enterobacteriaceae* (ESBL/pAmpC-E) in livestock and in the food chain is cause for concern due to the vital role of third-generation cephalosporins (3GCs) in infection control[8,9]. The simultaneous rise of difficult to treat

nosocomial and community-acquired infections by ESBL/pAmpC-E has supported the hypothesis that food systems with high occurrence of resistant bacteria, such as the broiler production system[37,38,41], could act as reservoirs of resistant bacteria that contribute to the public health burden[73]. This has been a point of debate with some studies providing evidence in favour[48] while others against the motion[47,73]. Nevertheless, the zoonotic transmission potential prompted the conduct of numerous investigations and the onset of monitoring programs globally over the last twenty years[22,74,75]. Early monitoring studies assessed antimicrobial resistance (AMR) at the isolate level by determining the percentage of resistant strains within a given collection[76]. The isolate level approach overlooked the role of the host and was deemed inadequate to detect emerging or latent AMR types, shifting the focus of investigations at the sample (host) level (i.e. percentage of samples colonised with resistant bacteria) with the use of antibiotic supplemented (hereafter selective) media[76,77]. However, surveillance programs, such as EFSA's annual AMR monitoring[5], combine both methods since prevalence at the isolate level provides valuable insights on AMR epidemiology and acts as a proxy for colonisation level in the studied population, especially in large-scale studies in which enumeration of resistant isolates can be labour-intensive.

We recently showed a high sample prevalence of ESBL/pAmpC-producing *E. coli* (ESBL/pAmpC-EC) in the broiler production system, with substantial transfer between subsequent production levels[78]. Here, we determined prevalence at both the sample and isolate levels using both selective and non-selective media. Data from the two methodologies were then compared with one another to assess potential differences in the obtained prevalence and genotype diversity of ESBL/pAmpC-EC in broiler production.

Materials and methods

Sample collection

Sampling was performed as previously described[78]. Briefly, three production chains of an integrated broiler production company in Northern Italy were monitored. In 40 sampling visits, faecal samples from 20 randomly selected healthy birds were taken by cloacal swabs in the farms, and 20 carcasses were collected at the slaughterhouse. We sampled Parent Stock chickens at the age of one-day (PS chicks) and at the production farms (PS breeders). PS chicks samples for one chain were not collected due to time constraints. Sampled breeders were not

combined with other flocks. The offsprings of the sampled PS breeders were sampled in four fattening farms per production chain at the start (broiler chicks) and the end (broilers) of the production cycle. Finally, 20 carcasses from the previously sampled broilers were collected at the slaughterhouse after the chilling process. All flocks except for three received antibiotic treatments for therapeutic reasons unrelated to this study.

Isolation and detection

In addition to the selective isolation with cefotaxime[78], samples were simultaneously processed on non-selective media, following the same procedure. Briefly, cloacal swabs were directly streaked on Eosin Methylene Blue agar (EMB) and incubated at 37±0.5 °C for 20±2h. Carcasses were rinsed with Buffer Peptone Water (BPW), rinsates were incubated (37±0.5 °C for 20±2h) and streaked on EMB. Rinsates and three serial dilutions (10^{-1} to 10^{-3}) were plated on EMB for subsequent enumeration. The limit of quantification (LOQ) was 1 Log CFU/mL rinsing water. At least two morphologically typical *E. coli* colonies on EMB (metallic green sheen) were isolated from each sample and screened for ESBL/pAmpC production by combination disk diffusion test using cefotaxime (30 µg) and ceftazidime (30 µg) discs with and without clavulanic acid (10 µg) and according to CLSI guidelines[50]. Additionally, a cefoxitin disc (30 µg) was used to detect potential AmpC-producers.

Molecular characterisation

ESBL/pAmpC gene groups[52] and *E. coli* phylogroups[34] were detected by multiplex PCRs for all phenotypically resistant isolates. For a selection of 33 isolates from EMB (vs 119 isolates from CTX-EMB), ESBL/AmpC genes were sequenced (Macrogen, Spain) after amplification[52] to identify gene variants (Supplementary Table 1). This selection was done considering the variability of ESBL/pAmpC and *E. coli* groups per sampling (at least one isolate per phylogroup-*bla* gene combination per sampling). Moreover, isolates with an AmpC phenotype, but negative for pAmpC genes by multiplex PCR, were analysed for chromosomal mutations in the *ampC* promoter/attenuator (cAmpC) according to Haldorsen *et al*.[53].

Data analysis

Prevalence. Prevalence of ESBL/pAmpC-EC at the sample level was calculated for each production stage based upon faecal samples or carcasses being positive to ESBL/pAmpC-EC (at least one isolate). Prevalence at the isolate level was calculated as the proportion of ESBL/pAmpC-EC isolates to the total number of isolates tested per production stage. Prevalence estimates and corresponding 95% confidence intervals (95% CI) were adjusted for clustering of observations at the chain and farm levels using cluster-robust standard errors. Statistical analysis was performed using STATA (StataCorp, College Station, USA).

Differences in genotypes. The Simpson's index (SI) of diversity[79] was used to measure the diversity of ESBL/pAmpC-EC strains in terms of gene groups, gene variants and phylogroups (of resistant strains) per sampling stage and isolation medium as the probability that two strains randomly selected from a given sampling stage and medium would belong to different gene groups, gene variants, and phylogroups, respectively. Differences in SI values between media were tested for significance using a two proportion z-test. The proportional similarity index (PSI)[54] was used to measure the overlap between media in terms of gene groups, gene variants and phylogroups. PSI values range from 0% (no similarities) to 100% (total overlap). All analyses accounted for clustering of data at the farm-chain level[78].

Contamination of carcasses. To study the correlation between the loads of total *E. coli* on EMB and putatively ESBL/AmpC growing on CTX-EMB, we followed the methodology of Reich *et al.*[66] and analysed the data with two methods: first, only samples with countable loads on CTX-EMB were included. In a second attempt, *E. coli* loads of samples negative for the quantitative but positive for the qualitative method on CTX-EMB, were arbitrarily set to 0.9 Log CFU/mL (i.e. just below LOQ) whereas for samples negative for both methods, loads were set to 0.0 Log CFU/mL. Data were not normally distributed, so the Spearman's rank correlation coefficient was used to assess correlation between total *E. coli* and ESBL/AmpC-EC. Further, to test the correlation between log-transformed concentrations and prevalence (sample level) of ESBL/AmpC-EC in carcasses, a linear regression analysis adjusted for clustering at the chain-farm level was performed.

Results

Prevalence

Isolate level. From 820 samples, 1,864 *E. coli* isolates from EMB were screened for phenotypic resistance to ESCs by disk diffusion and 146 (7.8%, 95%CI 4.3-13.9%) thereof were positive (Supplementary Table 1). The highest (71.2%, 95%CI 59.6-80.6%) and lowest (0.53%, 95%CI 0.07-3.7%) prevalence was respectively found for PS chicks and PS breeders (Fig. 1). In fattening broilers, the proportion of resistant isolates was almost double in the beginning of the cycle compared to the end, but remained low throughout the production cycle (Fig. 1). At the end of the production pyramid, 27 out of 555 (4.9%, 95%CI 3.0-7.0%) tested isolates from carcasses were phenotypically resistant to 3GCs. In contrast, CTX-EMB showed an overall high sensitivity (95.5%, 95%CI 91.8-97.6%) in detecting ESBL/pAmpC-EC as only 24 isolates out of 537 growing on selective media were not expressing phenotypic resistance. The difference in sensitivity of used media was statistically significant ($p<0.05$) both overall and at the production level.

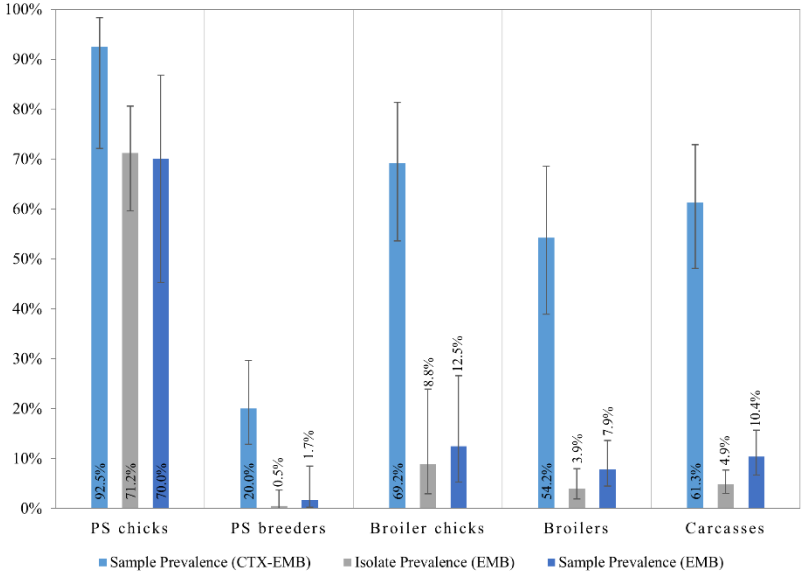

Fig. 1. Prevalence of ESBL/pAmpC-EC in the broiler production pyramid. Error bars represent corresponding 95% confidence intervals. Data labels (percentages) are placed inside and outside of their respective columns. Abbreviations: PS, Parent Stock; EMB, Eosin Methylene Blue agar; CTX-EMB, EMB supplemented with 1mg/L cefotaxime.

Sample level. There was an overall significant difference ($p<0.05$) in the estimation of sample prevalence when using selective and non-selective media. Out of 820 samples, only 12.6% (95%CI 7.9-19.3%) was found positive (at least one ESBL/pAmpC-EC recovered) with EMB whereas the prevalence for CTX-EMB was 60.3%[78]. On average, the estimated prevalence was 6.3 times lower with EMB compared to CTX-EMB when estimating prevalence at the production level (Fig. 1). For 5 out of 303 samples (4 cloacal and 1 carcass, 1.7%) negative with CTX-EMB, ESBL/pAmpC-EC were recovered in the non-selective medium. Additionally, in 14 out of 40 samplings there were no positive samples on EMB (prevalence 0%) while in all samplings at least one positive sample was found on CTX-EMB (data not shown).

Table 1. Comparison of genotype abundance and distributions between ESBL/pAmpC-EC from EMB and CTX-EMB

Production Stage	Index	Gene groups		Gene variants		Phylogroups	
		EMB	CTX-EMB	EMB	CTX-EMB	EMB	CTX-EMB
PS chicks	SI	0.0%	0.0%	0.0%	NC	**33.7%**	**10.5%**
	PSI	100%		100%		86%	
PS breeders	SI	NC	72.7%	NC	80.0%	NC	84.8%
	PSI	NC		NC		25%	
Broiler chicks	SI	71.4%	71.0%	80.6%	68.3%	68.4%	72.2%
	PSI	74%		55%		73%	
Broilers	SI	62.5%	57.0%	82.2%	76.9%	78.4%	77.1%
	PSI	73%		76%		68%	
Carcasses	SI	59.0%	51.3%	63.9%	77.5%	80.9%	78.2%
	PSI	83%		74%		89%	
Overall	SI	70.4%	69.5%	81.1%	78.5%	81.0%	78.8%
	PSI	75%		87%		77%	

Values in bold differ significantly ($p<0.05$). Abbreviations: EMB, Eosin Methylene Blue agar; CTX-EMB, EMB supplemented with 1mg/L cefotaxime; PS, parent stock;

PSI, proportional similarity index; SI, Simpson index; NC, not calculated (only one isolate from EMB).

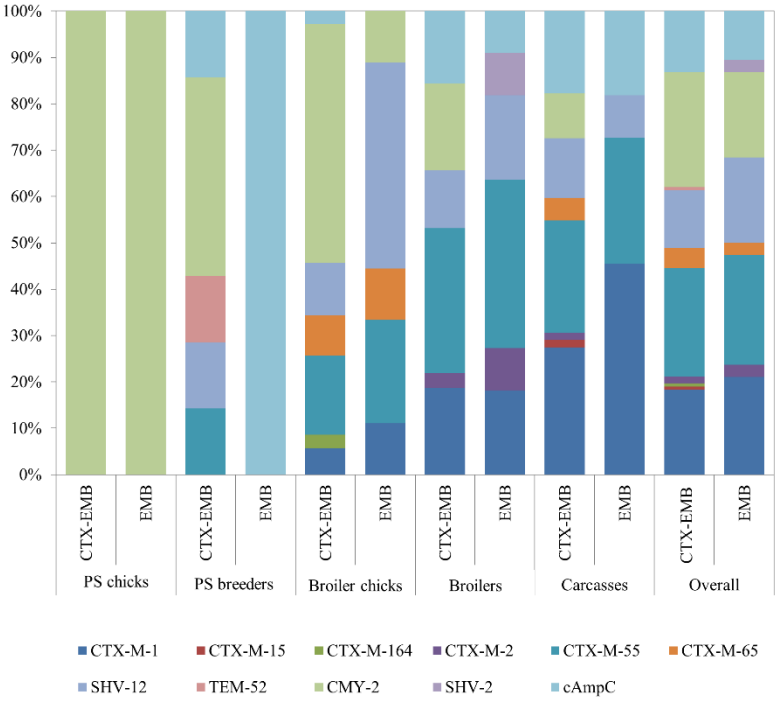

Fig. 2. Distribution of ESBL/pAmpC genes in the broiler production pyramid. Abbreviations: cAmpC, isolates with chromosomal mutations in the *ampC* promoter/attenuator; EMB, Eosin Methylene Blue agar; CTX-EMB, EMB supplemented with 1mg/L cefotaxime.

Differences in genes and phylogroups

The comparison of resistant isolates between EMB and CTX-EMB showed no significant differences ($p>0.05$) in genotype diversity (SI), and high similarity (PSI≥75%) in their distribution frequencies (Table 1, Supplementary Table 1). At the production level, significant difference was found only for the abundance of phylogroups in PS chicks, although all resistant isolates from both media were carrying bla_{CMY-2} and thus showed no differences in terms of resistance genes (Figs. 2 and 3). In PS breeders, where prevalence was the lowest, only one resistant isolate of phylogroup A with cAmpC was recovered from EMB while five types of genes and phylogroups were recovered from

CTX-EMB, indicating an underestimation of genotype diversity by the non-selective medium (Table 1, Fig. 2). In broilers and carcasses, the abundance of genotypes was not significantly different (Table 1). For these production levels, PSI values ranged from 73-83%, 55-76%, and 73-89% for EBSL/AmpC gene groups, EBSL/AmpC gene variants and phylogroups, respectively, showing that ESBL/pAmpC-EC gene and phylogroup distributions were generally more similar than diverse between the two isolation media (Table 1).

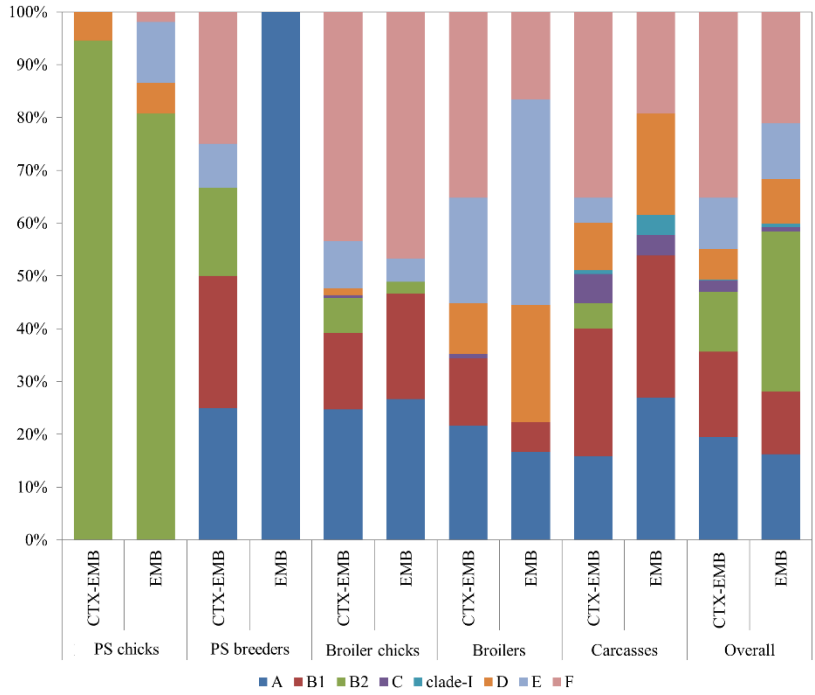

Fig. 3. Distribution of *E. coli* phylogroups in the broiler production pyramid. Abbreviations: EMB, Eosin Methylene Blue agar; CTX-EMB, EMB supplemented with 1mg/L cefotaxime.

Contamination of carcasses

The median load of putative *E. coli* counted with EMB was 3.1 Log CFU/mL (min 1 Log CFU/mL, max 5.2 Log CFU/mL) with all samples having countable *E. coli* loads. The ratio of median loads on CTX-EMB (1.66 Log CFU/mL)[78] and EMB was 3.6% indicating that ESBL/pAmpC-*E. coli* load was 1.5 log cycles lower, thus representing a minor proportion of the total *E. coli* population.

Correlation between total *E. coli* on EMB and putatively ESBL/pAmpC-*E. coli* on CTX-EMB was not significant ($p>0.05$) when all carcasses (n=240) were taken into account and values were adjusted for non-countable loads on CTX-EMB. Conversely, there was a slight but significant correlation ($p<0.05$) when only samples with countable loads on CTX-EMB were included (n=146). In addition, there was a significantly positive correlation (coefficient 0.80, 95%CI 0.30-1.2) between prevalence and concentrations on the selective medium, indicating that batches of carcasses with a higher sample prevalence also had higher concentrations of ESBL/pAmpC-EC.

Discussion

We screened a large collection of isolates from three broiler production chains with the simultaneous use of selective and non-selective media to assess the epidemiology and genetic makeup of ESBL/pAmpC-EC and gain insights on the differences of the two methodologies.

The non-selective medium has significantly underestimated occurrence of ESBL/pAmpC-EC, as sample prevalence was on average six times lower compared with the selective medium and several farms were negative for presence of ESBL/pAmpC-EC. Previous studies have shown that addition of cefotaxime in isolation media is crucial to accurately estimate ESBL/pAmpC-EC prevalence, especially when their sample levels are low[5,80]. Based on these findings, EFSA's "specific 3GC-resistance monitoring" was implemented in 2014, to supplement the non-selective "standard monitoring", which determines 3GC resistance rates of randomly selected isolates[5].

In contrast to the sample prevalence approach that detects even very low numbers in individual samples, isolate prevalence provides an estimate of the levels of resistance in the studied population[5,36,81]. Further, this method is relevant for risk assessment, since ESBL/pAmpC-EC are thought to follow random pathways in their transfer along the food chain[5]. In our study, the overall levels of resistance were low (7.8%) and in line with previous field[82] and experimental[83] studies. High proportion of resistant isolates was observed in PS chicks, with all isolates belonging to phylogroup B2 and carrying $bla_{\text{CMY-2}}$ (Figs. 2 and 3). Therefore, it is not only possible that the phylogroup B2/$bla_{\text{CMY-2}}$ lineage has been introduced in the broiler pyramid through the import of PS chicks, as previously discussed[78], but that it was predominant in the developing *E. coli* population of young hatchlings and thus more likely to disseminate in successive production

levels (Fig. 2). We subsequently observed a significant decrease of resistance rates for PS breeders with only one ESBL/pAmpC-producing isolate out of 188 tested. Unfavourable conditions for *bla*$_{CMY-2}$-carrying plasmids could justify this trend[57]. Furthermore, opposed to the high host prevalence observed with the selective approach, the levels of resistance were low for broilers and carcasses, with less than 10% of isolates being ESBL/pAmpC-producing. Nonetheless, the higher shedding densities of ESBL/pAmpC-EC in chickens compared to other animals[82,84] and the promiscuity of ESBL/pAmpC-carrying plasmids[85], justify the spread of ESBL/pAmpC-EC in individual animals and thus the high sample prevalence observed in our study. However, ESBL/pAmpC-EC seems to be a subdominant part of the *E. coli* population present in the broiler gut flora[81].

To explore whether CTX-EMB induces a selection bias over particular ESBL/pAmpC-EC genotypes or, conversely, if EMB underestimates presence of certain genotypes, we compared the two datasets. Overall, PSI and SI values showed no significant differences in the genetic makeup of ESBL/pAmpC-EC strains from the two media. In PS chicks, the predominant B2/*bla*$_{CMY-2}$ seems to have obscured presence of two additional, low occurring phylogroups that were discovered carrying *bla*$_{CMY-2}$ with EMB (Fig. 2). In PS breeders, where prevalence was the lowest, only one cAmpC isolate was recovered with EMB whereas four additional ESBL/pAmpC genes were found with CTX-EMB (Fig. 2). In broilers and carcasses, no significant differences were found between the two approaches in terms of genotype abundance and distribution (Table 1). Therefore, bias towards particular ESBL/pAmpC-EC genotypes from the selective method or underestimation by the non-selective approach did not occur.

At the slaughterhouse level, contamination of carcasses with ESBL/pAmpC-EC defines exposure rates of chicken meat consumers[86]. We showed that ESBL/pAmpC-EC were subdominant to total *E. coli*. Furthermore, the proportions found were respectively one order of magnitude lower and higher than those reported by Reich *et al.*[66] and von Tippelskirch *et al.*[44] while total *E. coli* loads were comparable. In contrast to these studies, a significant correlation between total *E. coli* and putative ESBL/pAmpC-EC was found, however we sampled the whole carcass area by rinsing and not smaller areas such the neck skin or breast. Further, our results corroborate the findings of Pacholewicz *et al.*[87], who followed the same sampling methodology, and proved that total *E. coli* can be used as a proxy of the status and fate of

ESBL/pAmpC-EC in carcasses during processing. Moreover, batches of carcasses with high sample prevalence correlated with higher concentrations of ESBL/pAmpC-EC. Hence, measures to reduce the numbers of ESBL/pAmpC-EC at the slaughterhouse could have an effect on the number of contaminated carcasses (or its parts) reaching retail level. Interventions should first aim at primary farming by reducing the numbers ESBL/pAmpC-EC in the broiler gut before slaughter[64], to decrease the unavoidable effect of contamination by faecal leakage during evisceration. Processing parameters, such as temperature and duration of scalding, can form additional control points[87]. Finally, microbiological criteria for ESBL/pAmpC-EC could help monitor the progress of such interventions[66,86] and carcasses from farms with high prevalence could be directed to abattoirs with better hygiene records[88].

Conclusion

By estimating prevalence on the isolate level, we showed that although carried by many animals, ESBL/pAmpC-EC were overall subdominant to intestinal *E. coli*. An exception to this situation was the particularly dominant B2/*bla*$_{\text{CMY-2}}$ *E. coli* in the gut of PS chicks, which obscured presence of latent genotypes. However, major differences in acquired genotypes between the selective and non-selective isolation methods were not observed. At the slaughterhouse, given the link between total and resistant *E. coli* on carcasses, improvement of hygiene and establishment of microbiological criteria can help reduce ESBL/pAmpC-EC numbers.

Chapter 4. High-resolution characterisation of ESBL/pAmpC-producing *Escherichia coli* in the broiler production pyramid

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Abstract

Presence of extended-spectrum *β*-lactamase (ESBL) or AmpC *β*-lactamase (pAmpC)-producing *E. coli* in livestock is a public health risk as their transmission to humans via the food chain is likely. The assessment of that risk requires the thorough characterisation of isolates and their underlying resistance mechanisms by the use of high-resolution methods. A high sample prevalence of ESBL/pAmpC-EC in an integrated broiler chain [*i.e.* Parent Stock (PS), offspring broilers and their carcasses] was previously shown. Here, we proceeded with the whole genome sequencing of 100 diverse ESBL/pAmpC-EC isolates from the broiler production with the aim to explore their resistance gene repertoire, characterise their plasmids, and identify transmission patterns by inferring their phylogeny. Sequenced isolates carried resistance genes that confer resistance to four antimicrobial classes, on average, in addition to cephalosporins. A complex landscape of *E. coli* genotypes was revealed, however clusters of closely related isolates with only few single-nucleotide polymorphisms (SNPs) from various stages of the production were formed. Phylogenetic comparison with publicly available genomes indicated that previously uncommon ESBL/pAmpC-EC lineages (*e.g.* ST-457) can emerge and dominate in poultry, while others (*e.g.* ST-429) contribute to the maintenance and dissemination of ESBL/pAmpC genes in the broiler sector. Furthermore, we found that the majority of isolates from diverse *E. coli* lineages shared five dominant plasmid backbones (*i.e.* IncK2, IncI1, IncX3 and IncFIB/FII) with identical ESBL/pAmpC gene insertion sites. Presence of these plasmid types has been previously reported in diverse hosts, including humans. These findings underline the importance of specific plasmid lineages in the dissemination of cephalosporin resistance genes not only within the broiler industry but also across reservoirs.

Introduction

Third-generation-cephalosporins (3GCs) are critically important antimicrobials (CIAs) in human medicine as they are one of the few available therapies in persistent infections caused by multi-drug resistant bacteria and in cases of severe *Escherichia coli* and *Salmonella* infections[8]. Their role in the treatment of food-producing animal diseases is considered crucial as well[9]. Administration of 3GCs is not authorised in poultry within the European Union[15] except for their use

in hatcheries either *in ovo* or in day-old chicks[16]. Nevertheless, several studies suggested that the broiler production system acts as a reservoir of 3GC-resistant bacteria, such as extended-spectrum *β*-lactamase (ESBL) or AmpC *β*-lactamase (pAmpC)-producing *E. coli* (hereafter ESBL/pAmpC-EC), given that their prevalence is higher compared to other animal sectors[22,46]. Further, presence of ESBL/pAmpC genes on highly transmissible plasmids, which enables their dissemination in diverse reservoirs[26], created debate on the exposure risk for consumers via the food chain[47,48]. However, the link with human exposure was supported by circumstantial evidence showing presence of identical ESBL/pAmpC genes on closely related *E. coli* inferred by the typing of a small number of genes[23,68,85]. This stresses the importance of high throughput whole genome sequencing (WGS) studies to inform quantitative microbial risk assessment (QMRA) models[89]. Such an assessment for the broiler chain would require the thorough characterisation of ESBL/pAmpC-EC and their transmission patterns across the production pyramid[22].

A high prevalence of ESBL/pAmpC-EC in an integrated broiler production chain was previously shown[78]. Here, we proceed with the WGS-assisted characterisation of a selection of ESBL/pAmpC-EC strains collected within that framework. The aims of this study were (i) to detect antimicrobial resistance genes (ARGs) other than *β*-lactamases, (ii) characterise ESBL/pAmpC gene-carrying plasmids to study their epidemiology, and (iii) infer the phylogeny of ESBL/pAmpC-EC to identify transmission events and set the isolates of this collection in the context of other publicly available genomes.

Materials and methods

Bacterial isolates

ESBL/pAmpC-EC were isolated from three production chains (A, B, and C) of an integrated broiler company[78]. Briefly, Parent Stock chickens at the age of one-day (PS chicks) and at the production farms (PS breeders) were sampled. PS offsprings were sampled in four fattening farms per chain at the start (broiler chicks) and the end (broilers) of the production cycle. Carcasses from sampled broilers were collected at the slaughterhouse after chilling. One hundred isolates were included in this study. At least one isolate per chain, production stage, ESBL/pAmpC gene and phylogroup combination was selected to fully explore the diversity of ESBL/pAmpC-EC genotypes across the

production pyramid and identify potential transmission events (Supplementary Table 1).

Whole genome sequencing and sequence reconstruction

Isolate preparation, DNA extraction and sequencing were carried out at two different sites. At the first site, DNA was isolated from 32 isolates with the Master Pure Genomic DNA-Purification Kit (Epicenter, USA), library preparation was done with the Nextera XT library kit (Illumina, USA) and sequencing on an Illumina MiSeq platform using the MiSeq v3 reagent kit (Illumina) with 2 x 300 bp paired-end reads. At the second site, DNA was isolated from 68 isolates with the Invisorb Spin Tissue Mini Kit (Invitek, Germany), library preparation was done with the Nextera XT library kit and sequencing on an Illumina HiSeqX platform with 2 x 150 bp paired-end reads at a private company (Macrogen, Korea).

Raw reads obtained by both sequencing methods were directly submitted for processing at Enterobase (https://enterobase.warwick.ac.uk/)[90]. The Enterobase backend pipeline assures high quality assemblies by including read pre-processing, trimming, assembly with SPAdes[91], post-correction and filtering (https://enterobase.readthedocs.io/). Assemblies that do not meet quality control criteria are not used for downstream analysis such as assignment of (novel) multi-locus sequence types (STs) according to the Achtman scheme[33].

In silico typing

STs were extracted from the Enterobase automated pipeline. Further, sequenced isolates were genotyped *in silico* with regard to acquired resistance genes (ResFinder 3.2)[92], plasmid replicon types (PlasmidFinder 2.0)[93] and plasmid STs (pMLST 2.0)[93], using the default settings.

Plasmid characterisation

In several isolates, the ESBL/pAmpC gene-carrying plasmid was identified by finding the gene and plasmid replicon on the same contig. To establish this association in the rest of isolates but to study the genetic context of ESBL/pAmpC genes as well, the following approach was followed; complete reference plasmids with the highest resemblance were sought by analysing contigs containing the ESBL/pAmpC gene with NCBI's BLASTn[94]. To identify all the contigs

corresponding to particular plasmids, the draft genome of each isolate and the reference plasmid were aligned with ABACAS as previously described[95]. ABACAS finds alignment positions and orders contigs according to a given reference[96]. Identification of the genetic context of ESBL/pAmpC genes was done by performing alignments of identified plasmid contigs in CLC Genomics Workbench 12.0 (Qiagen, Germany). EasyFig[97] was used to visualise aligned plasmid regions of interest.

To confirm results obtained *in silico*, transformation experiments were performed for at least one ST- ESBL/pAmpC gene case (32 isolates were transformed). Briefly, plasmid DNA was extracted with the alkaline lysis method[98], electro-competent cells were prepared according to Smith *et al.*[99] from DH5-Alpha *E. coli* cultures and transformed by electroporation in an Eppendorf Eporator (Eppendorf, Germany). Transformants with an ESBL/pAmpC-carrying plasmid were isolated on LB agar (Microbiol, Italy) containing 1 mg/L cefotaxime. The incompatibility group of plasmids transferred from the donor isolates was determined by PCR-based replicon typing (PBRT)[28].

Phylogenetic analysis

The phylogeny of sequenced isolates was inferred by conducting a single nucleotide polymorphism (SNP)-based mapping analysis. A suitable reference was selected among complete *E. coli* genomes (http://www.ncbi.nlm.nih.gov/genome/167) using refRank[100] as described previously[101]. Sequence reads, trimmed and error corrected by SPAdes, were mapped to the reference genome and a SNP tree was created with CSI phylogeny[102] using default settings. The phylogenetic analysis was extended with publicly available genomes at Enterobase, belonging to the most dominant STs of our collection and with a confirmed presence of ESBL/pAmpC genes by ResFinder. A core-genome MLST (cgMLST) tree was built at Enterobase. Isolates that showed a relatedness by clustering together in the cgMSLT tree were further analysed for SNPs with CSI phylogeny. All phylogenetic trees were imported and visualised in CLC Genomics Workbench 12.0.

Results and Discussion

Resistance genes

The most abundant gene type was that of sulphonamide resistance in 89% of isolates with *sul2* being the most prevalent and with 33% of isolates having multiple genes (Table 1). Aminoglycoside

acetyltransferases (*aac(3)*-like), nucleotidyltransferases (*aadA*-like) and phosphotransferases (*stra*, *strb*, *aph(3')*-like) were found in 87% of isolates, which predominantly carried more than one resistance mechanism. The tetracycline resistance genes *tetA* and *tetB* were respectively found in 76% and 12% of *E. coli* but only two isolates had both. Moreover, WGS data showed occurrence of *dfrA*-like genes in 45% of isolates with *dfrA14* being the most prevalent. The chloramphenicol exporter *flor* was present in 21 out of the 43 isolates that carried phenicol resistance genes while *cmlA1*, *catA1* and *catB3* were also present. Plasmid mediated quinolone resistance (PMQR) genes were identified in 20% of *E. coli*, specifically *qnrs1* (13%), *qnrs2* (4%) and *qnrB19* (2%). One isolate had presence of both *qnrA1* and *qnrS1*. In addition, quinolone resistance was mediated by chromosomal mutations in quinolone resistance determining regions (QRDR) in 49% of isolates. Isolates had an average of three different types of mutations from a total of nine observed with the most prevalent type being that of Ser83>Leu in the *gyrA* subunit (47%), followed by Ser80>Ile in the *parC* subunit (34%). Twelve isolates had combination of PMQR and QRDR. Additionally, 14 isolates harboured at least one of the macrolide resistance genes *mph*(A) and *mph*(B), which were found in twelve and two isolates, respectively. Further, the rifampicin resistance gene *ARR-3*, and *aac(6')Ib-cr*, which confers resistance to both fluoroquinolones and aminoglycosides, were found in four isolates each.

Although we did not confirm the resistances by susceptibility testing, previous studies showed high accordance between resistance phenotype and genotype[92,103] and in the near future genomics are likely to replace susceptibility testing methods[104]. Multi-drug resistance, which is frequently observed in ESBL/pAmpC-EC, is crucial for their selection and dissemination by the use of antimicrobials other than β-lactams[41].

Genomic localisation of ESBL/pAmpC genes

ESBL/pAmpC genes were generally associated with plasmids belonging to specific incompatibility groups. The most dominant association was *bla*$_{\text{CMY-2}}$-IncK2, which was found in 27% of sequenced isolates followed by *bla*$_{\text{CTX-M-55}}$-IncFIB/IncFII (25%). *bla*$_{\text{CTX-M-1}}$ was mainly found on IncI1 plasmids (13%) but three other plasmid types were also harbouring this gene (Table 1). Moreover, IncX3 (12%) and IncI1 (6%) plasmids were found carrying *bla*$_{\text{SHV-12}}$. Associations were also observed between ESBL/pAmpC genes, plasmids and STs. A particular case was that of *bla*$_{\text{CTX-M-55}}$-IncFIB/IncFII, which was

Table 1. Characteristics of 100 ESBL/pAmpC-EC isolates subjected to whole genome sequencing.

ESBL/ pAmpC gene	Plasmid Replicon type [No. of isolates][a]	pMLST/RST[b]	MLST [No. of isolates]	Other resistance genes[c, d]
*bla*CMY-2	IncB/O/K/Z [27][a]	-	ST-9298 [1]	*aac(3)-VIa*, *aadA1*, *tetA*, *sul1*
			ST-429 [7]	***aac(3)-VIa****, ***aadA1****, *tetA*, ***sul1****
			ST-155 [5]	*bla*TEM-1b, *aac(3)-VIa*, *aadA1*, *tetA*, *sul1*
			ST-10 [3]	*bla*TEM-1b, *aac(3)-VIa*, *aadA1*, *dfrA1*, *tetA*, *sul1*
			ST-140 [2]	***aac(3)-VIa****, ***aadA1****, *tetA*, ***sul1****
			ST-2485 [2]	*aac(3)-VIa*, *aadA1*, *tetA*, *sul1*
			ST-371 [2]	***aac(3)-VIa***, ***aadA1***, *tetA*, ***sul1***
			ST-38 [2]	*aac(3)-VIa*, *aadA1*, aph(3')-Ic, *strA*, *strB*, *tetB*, *sul1*, *sul2*
			ST-1163 [1]	*aac(3)-VIa*, *aadA1*, *sul1*
			ST-373 [1]	-
			ST-9340 [1]	*bla*TEM-1b, *aac(3)-VIa*, *aadA1*, *tetA*, *sul1*, *dfrA1*
	IncA/C2 [3]	pST3	ST-355 [2]	*aac(3)-VIa*, *aadA1*, *strA*, *strB*, *floR*, *tetA*, *sul1*, *sul2*
			ST-88 [1]	*bla*TEM-1b, *aac(3)-VIa*, *aadA1*, *aadA2*, *strA*, *strB*, *cmlA1*, *floR*, *tetA*, *sul1*, *sul2*, *sul3*
*bla*CTX-M-55	IncFIB/IncFII [25]	F18:A-:B1	ST-457 [25]	***bla**TEM-1b**, aac(3)-IIa, *aadA1*, ***aph(3')-Ia****, *strA*, *strB*, *floR*, ***tetA****, ***sul2****, *sul3*, ***dfrA14****
*bla*CTX-M-1	IncI1 [13]	pST3[b]	ST-115 [3]	***aadA5****, ***tetA****, ***sul2****, ***dfrA17****
			ST-117 [3]	***tetA***, ***sul2***
			ST-155 [2]	*bla*TEM-1b, *aadA5*, *aph(3')-Ia*, *strA*, *strB*, *catA1*, ***tetA***, *tetB*, ***sul2***, *dfrA17*
			ST-453 [2]	*bla*TEM-1b, *strA*, *strB*, ***tetA***, ***sul2***, *dfrA14*
			ST-23 [1]	*bla*TEM-1b, *aadA1*, ***tetA***, ***sul2***, *sul3*

(*continued on next page*)

Table 1 (continued)

ESBL/ pAmpC gene	Plasmid Replicon type [No. of isolates][a]	pMLST/RST[b]	MLST [No. of isolates]	Other resistance genes[c, d]
			ST-2485 [1]	*bla*TEM-1b, ***tetA***, ***sul2***
			ST-753 [1]	*bla*TEM-1b, ***tetA***, *qnrS1*, ***sul2***
	IncI2 [2]	-	ST-38 [2]	*aadA1*, *strA*, *strB*, *tetA*, *mph*(B), *sul1*, *sul2*, *dfrA1*
	IncFIB/IncFII [1]	Unknown	ST-4937 [1]	*aadA1*, *strA*, *strB*, *catA1*, *tetA*, *mph*(A), *sul1*, *dfrA1*
	IncHI2 [1]	Unknown	ST-3107 [1]	aac(3)-IId, *tetA*, *sul2*, *dfrA14*, *dfrA17*
*bla*SHV-12	IncX3 [12]	-	ST-744 [8]	*bla*TEM-1b, *aadA5*, *aph(3')-Ia*, *strA*, *strB*, *catA1*, *tetB*, ***qnrS1***, *mph*(A), *sul1*, *sul2*, *dfrA17*
			ST-1629 [3]	*tetA*, ***qnrS1***, *sul1*
			ST-746 [1]	*cml*, *qnrA1*, ***qnrS1***, *sul1*, *dfrA5*
	IncI1 [6]	pST26	ST-155 [3]	*aadA1*, *aadA2*, *cmlA1*, *tetA*, *sul3*
		pST95	ST-117 [1]	*aadA1*, *aadA2*, *cmlA1*, *tetA*, *sul3*
		pST3	ST-1303 [1]	*bla*TEM-1b, *tetA*, *qnrB19*
		pST26	ST-4512 [1]	*aadA1*, *aadA2*, *cmlA1*, *tetA*, *sul3*
*bla*CTX-M-65	Chromosome [4]	-	ST-2179 [4]	*bla*OXA-1, *bla*TEM-1a, *bla*TEM-1b, *aadA1*, *aadA2*, *aadA5*, *strA*, *strB*, *catB3*, *cmlA1*, *tetA*, *qnrS2*, *mph*(A), *sul1*, *sul2*, *sul3*, *dfrA17*, *ARR-3*, *aac(6')Ib-c*
*bla*TEM-52b	IncX1 [2]	-	ST-388 [1]	*qnrB19*
			ST-695 [1]	-
*bla*CTX-M-2	IncQ1 [2]	-	ST-4980 [2]	*bla*TEM-1b, *aadA1*, *strA*, *strB*, *tetA*, *sul1*, *sul2*, *dfrA1*
*bla*CTX-M-15	IncY [1]	-	ST-69 [1]	*bla*TEM-1b, *strA*, *strB*, *tetA*, *qnrS1*, *sul2*
*bla*SHV-2	Untypable [1]	-	ST-69 [1]	*aadA1*, *aadA2*, *cmlA1*, *tetA*, *sul3*

[a] The IncB/O and IncK replicons were identified by PBRT. Further analysis indicated that these plasmids belong to the novel incompatibility group IncK2[103]

[b] Four isolates had truncated *ardA* genes in their assemblies but the other four alleles (*pilL*2, *repI*12, *sogS*1 and *trbA*4) were those of pST3. One isolate had also a truncated *ardA* gene and a novel *sogS* allele, differing from *sogS*1 by the mutation 24C > 24T. One isolate represented a novel IncI1 pST, closely related to pST3 (arda1 > arda7).

[c] Underlined resistance genes indicate less than 100% ascosciation with the corresponding *E. coli* ST.

[d] Resistance genes in bold were found on the ESBL/pAmpC gene-carrying plasmids after analysis with ABACAS. The following plasmid types, found on 76% of isolates, were recontructed: *bla*CMY-2-IncB/O/K/Z, *bla*CTX-M-55-IncFIB/IncFII, *bla*CTX-M-1- IncI1, *bla*SHV-12-IncX3. Resistance genes with marked with * indicate less than 100% ascosciation with the corresponding *E. coli* ST and plasmid type.

exclusively found in ST-457 isolates. In contrast, *bla*$_{CMY-2}$-IncK2 was found in various STs although predominantly in ST-429 (Table 1). Only five STs were associated with more than one gene-plasmid type. The genetic context the most dominant plasmid types, identified in 76% of sequenced isolates, is described below.

IncK2 plasmids carrying bla$_{CMY-}$

In 27 *E. coli* genomes, *bla*$_{CMY-2}$ was associated with the novel incompatibility group IncK2[105]. A genetic region of ~12 kb including *bla*$_{CMY-2}$ was highly conserved in all plasmids and it was almost identical (99.81 - 100% identity) to that of plasmid pDV45 (Accession no. KR905384.1). In this region, the mobile element IS*Ecp1-bla*$_{CMY-2}$-*blc-sugE* was integrated inside the *tra* locus between the *traU* and *traT* genes in all cases (Fig 1A). The two imperfect inverted repeats (IV-L: TGACGGTGATCCT; IV-R: AGCATCTCCGTCA) described by Seiffert *et al.*[105] were found flanking the mobile element in all analysed plasmids. The highly conserved cluster of IncK2 plasmids has disseminated in diverse *E. coli* of various reservoirs[101,106], but they seem to be dominant in broilers and broiler meat[69,107,108]. In our study, IncK2 plasmids have been found in 11 different STs in isolates recovered from different stages of the production pyramid (Table 1, Fig 2). It is noteworthy that plasmids with almost identical IncK2 backbones have been identified in *E. coli* isolated from hospitalised humans[69,105]. A recent study reports an IncK2 plasmid harbouring *mcr-1*, *bla*$_{TEM-1}$ and *sul2* but not *bla*$_{CMY-2}$ in *E. coli* from retail chicken meat[109]. ResFinder analysis on IncK2 plasmid contigs showed that *bla*$_{CMY-2}$ was the only resistance mechanism with the exception of 4 plasmids that carried *aac(3)-VIa*, *aadA1* and *sul1* (Table 1).

IncI1 plasmids carrying bla$_{CTX-M-1}$

All *bla*$_{CTX-M-1}$-carrying IncI1 plasmids belonged to pST3 or to closely related variants (Table 1). The genetic region containing *bla*$_{CTX-M-1}$ (~12kb) was highly conserved amongst all plasmids (99.96% identity) and it was compared to the reference IncI1 plasmid R64 (Accession no. AP005147) (Fig 1B). In all isolates, *bla*$_{CTX-M-1}$ was associated to the *ISEcp1* transposase. The mobile element *ISEcp1-bla*$_{CTX-M-1}$ was integrated in the shufflon region, between the *rci* shufflon-specific recombinase and the *pilV* genes, which represent a hotspot for DNA rearrangements[110]. *rci* binds to specific recombination sites (*sfx* repeats) and causes rearrangement of shufflon segments, which leads to variable

arrangements of the shufflon region even within the same plasmid preparation[111] in what seems to be a non-random process[112]. It is therefore highly probable that the observed re-arrangements in the integration site of *bla*$_{\text{CTX-M-1}}$ (Fig 1B) do not represent multiple insertion events but rather recombination events due to *rci* activity. The insertion of the module *ISEcp1-bla*$_{\text{CTX-M-1}}$ into the shufflon region in IncI1 plasmids seems to be common for those belonging to pST3, as previous studies reporting this plasmid type from various sources have shown[110,113–115]. A recent study reported identical *bla*$_{\text{CTX-M-1}}$-carrying IncI1 pST3 plasmids from chicken, chicken meat and bloodstream infections in humans[116]. Furthermore, additional resistance genes, mainly *tetA* and *sul2* (Table 1), were found and their co-localisation with *bla*$_{\text{CTX-M-1}}$ has been described before[108,109,116].

IncX3 plasmids carrying bla$_{SHV-12}$

In all cases, the genetic context of *bla*$_{\text{SHV-12}}$ comprised of two opposite oriented *IS*26 transposases, with *qnrS1* being inserted upstream of the left-most *IS26* element (Fig 1C). The most similar plasmids identified by BLASTn analysis of the ~11 kb region, which includes also the quinolone resistance determinant *qnrS1*, are those reported in 2018 by Liakopoulos *et al.*[117] (*e.g.* Accession no. KX618704, 99.82 - 100% identity). Similar IncX3 plasmids with the same *bla*$_{\text{SHV-12}}$ surrounding region have been isolated from different countries and sources, including humans[117,118]. In the Netherlands, IncX3 plasmids emerged as a dominant *bla*$_{\text{SHV-12}}$ propagator and gradually displaced IncI1 plasmids[117]. Also in our study, *bla*$_{\text{SHV-12}}$ was found more frequently on IncX3 compared to IncI1 plasmids (Table 1). Except for *bla*$_{\text{SHV-12}}$ and *qnrS1*, no additional resistance genes were co-located on IncX3 plasmids.

IncFIB/FII plasmids carrying bla$_{CTX-M-55}$

bla$_{\text{CTX-M-55}}$ was found on IncFIB/IncFII plasmids of the replicon sequence type (RST) F18:A-:B1 carried by *E. coli* ST-457. BLASTn analysis of a ~28kb region including *bla*$_{\text{CTX-M-55}}$ returned homologous (99-100% identity) regions from *bla*$_{\text{CTX-M-55}}$-carrying F18:A-:B1 plasmids isolated from human clinical samples in the United States (Accession no. CP041997.1, KX276657.1, CP029748.1) and wastewater in Canada (MK878525.1). The human related plasmids carried *mcr* genes. In their study, McGann *et al.*[119] focused on the description of the surrounding region of the *mcr-1* gene in plasmid

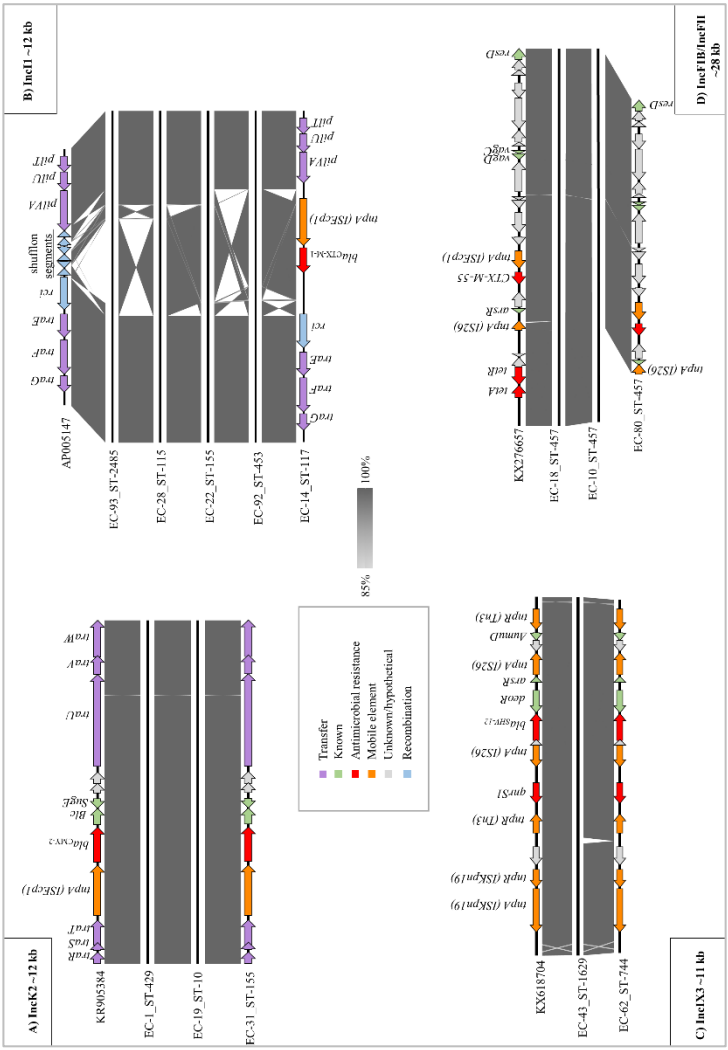

Fig 1. Linear comparison of (A) IncK2, (B) IncI1, (C) IncX3 and (D) IncFIB/IncFII plasmids carrying bla_{CMY-2}, $bla_{CTX-M-1}$, bla_{SHV-12} and $bla_{CTX-M-55}$, respectively. The open reading are represented with arrows, with the arrowhead indicating the direction of transcription. Their role in transfer, recombination, antimicrobial resistance, their association to mobile genetic elements and known or unknown functions are colour-coded. Areas shaded in grey indicate nucleotide identity.

pMR0516mcr (KX276657.1) isolated from a clinical ST-457 isolate. Here, we show that $bla_{CTX-M-55}$ is flanked by two IS elements, namely *ISEcp1* directly downstream and *IS26* upstream of $bla_{CTX-M-55}$ (Fig 1D). In addition, the tetracycline resistance gene *tetA* and the *tetR* repressor

(*tetAR*) were found upstream of *IS26*, except for five isolates in which *tetAR* was absent (Fig 1D). Other co-located resistance genes (*e.g* $bla_{\text{TEM-1b}}$, *dfrA14*, *sul2*) on pMR0516mcr were also found in the majority of $bla_{\text{CTX-M-55}}$-carrying IncFIB/FII plasmids of this study (Table 1).

MLST

In-silico typing revealed a diversity of *E. coli* genotypes by assigning the 100 sequenced isolates in 31 unique STs. Two novel STs were found with one isolate each. ST-9298 was isolated from a breeder and is closely related to ST-429 as they differ by only one SNP in the *icd* allele (*icd*13 > *icd*1072: 109T > 109A). ST-9340 was recovered from day-old broiler chick and is closely related to ST-46 (gyrB1 > gyrB812: 336C > 336T). Both novel STs were carrying $bla_{\text{CMY-2}}$ on IncK2 plasmids. Overall, ST-457 (25% of isolates) and ST-155 (10%) were the most dominant STs followed by ST-744 (8%) and ST-429 (7%) (Table 1). The majority of the remaining STs had one (14 STs), two (6 STs) or three (4 STs) isolates assigned to them with exception of ST-2179, ST-117 and ST-38, which had four isolates each. The diversity of STs increased along the production pyramid with two and four unique STs in respectively PS chicks and PS breeders, 12 and 14 STs in broiler chicks and fattened broilers whereas 19 STs were found at the slaughterhouse level (Fig 2).

SNP analysis

Genomes were mapped against the refRank selected complete genome of *E. coli* K12 (Accession no. NC_000913.3). All isolates covered at least 72% of the reference genome translating to a core genome alignment of 3.34 Mbp. Compared to the seven alleles of MLST typing, the SNP-based mapping analysis achieved a much higher resolution. As expected, isolates clustered primarily in accordance to their ST and subsequently on their ST clonal complex (Cplx) (Fig 2). The largest cluster was formed by 15 isolates belonging to six STs of the ST10 Cplx. Two isolates belonging to ST-373 (ST168 Cplx) and ST-4937 grouped together with ST-3107 (ST-10 Cplx), with which they share 3-4 MLST alleles. The novel ST-9298 was placed closely but not inside the core ST-429 cluster. Further, SNP analysis enabled discrimination of groups within the STs (Fig 2). Distinctive subgroups were formed according to the ESBL-pAmpC gene content in cases where an ST was associated with more than one ESBL-pAmpC gene (*e.g.* ST-155, ST-

38, and ST-2485). In addition, subgroups were formed according to the production stage and chain of sequenced isolates (*e.g.* ST-155, ST-457). ST-457 was the most widespread ST as it was present in all three chains and in various stages of the production pyramid. Two distinct clusters were formed with a mean pairwise difference of 1,704 SNPs (Supplementary Fig 1). The first cluster had very closely related isolates (mean pairwise difference: four SNPs) from the broiler chicks, fattened broilers and carcasses of one chain A flock. In the same cluster, isolates

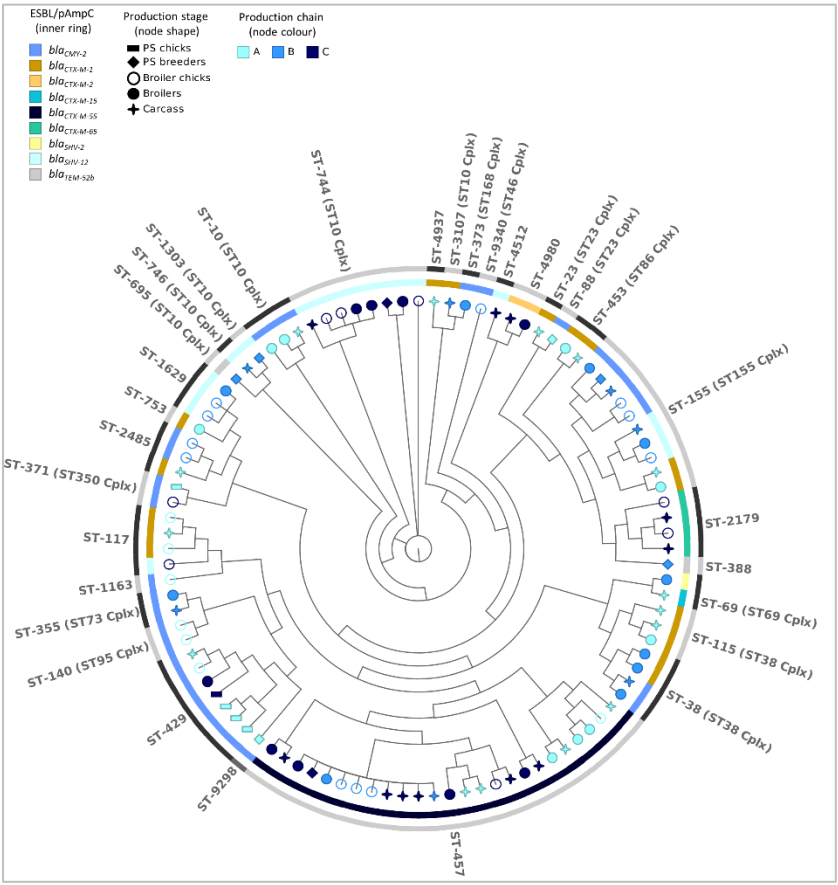

Fig 2. Phylogenetic analysis of 100 sequenced isolates. The SNP tree was created with CSI phylogeny. Alternating dark/light grey outer rings represent the different MLST clusters. Color-coded inner rings and node colours respectively represent the ESBL/pAmpC genes and production chains of isolates. The node shape denotes the production stage of isolates.

from two distantly located farms and their carcasses were also found. The second cluster was formed by ST-457 strains differing by only two SNPs, on average, isolated from broilers and carcasses of different broiler farms from all three production chains and one isolate from the breeder flock of chain B (Supplementary Fig 1). Given that in previous reports epidemiologically linked isolates had 0-23 SNPs[101], our results indicate a clonal expansion of this ST-457 cluster in the majority of the studied broiler production system. ST-457 is a rare ST in Europe[119]. A *bla*$_{\text{KPC-3}}$-*bla*$_{\text{CTX-M-55}}$-carrying strain isolated from a bloodstream infection in Italy[120] is amongst the few reported isolates. Other studies show concurrent presence of *mcr-1* and *bla*$_{\text{CTX-M-55}}$ on ST-457 and association with human disease[106,119]. Only 15 out of 22 ST-457 genomes at Enterobase had ESBL/pAmpC genes but none of them carried *bla*$_{\text{CTX-M-55}}$ or was isolated from poultry. In the cgMLST tree, a set of our isolates clustered closely with human isolates carrying *bla*$_{\text{CTX-M-1}}$ from the Netherlands (n = 2, unpublished) and one *bla*$_{\text{CMY-2}}$ isolate from Germany[101]. SNP analysis showed that these isolates differed on average by 77 SNPs whereas an average pairwise difference of 1,976 SNPs was found when all isolates were compared (Supplementary Fig 2).

Isolates of ST-155 formed three clusters according to their ESBL/pAmpC content. The within-cluster SNP differences pointed to a clonal dissemination of this ST (Supplementary Fig 1). On the cluster formed by *bla*$_{\text{CMY-2}}$-carrying isolates, closely related strains (mean pairwise difference: two SNPs) were isolated from the breeder flock (BrS2) and subsequently from broiler chicks (F1S1), fattened broilers (F1S2) and carcasses (F1Sc) of the same flock. cgMLST analysis showed that ESBL/pAmpC isolates of ST-155 predominate in poultry, although human isolates have been reported as well. The isolates of this study clustered separately, forming groups of ESBL/pAmpC-EC of poultry source from various countries (Supplementary Fig 2).

On average, five pairwise SNPs were found for all ST-744-*bla*$_{\text{SHV-12}}$ *E. coli*, which were isolated from breeders, broilers and carcasses of chain C (Supplementary Fig 1). A prevalence of ESBL/pAmpC ST-744 *E. coli* was indicated by the analysis with isolates available at Enterobase. None of the isolates available at the moment (n = 79) were carrying *bla*$_{\text{SHV-12}}$ or have been isolated from poultry. Our ST-744 isolates clustered with human isolates carrying *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-1}}$ and *bla*$_{\text{CTX-M-14}}$ whereas those of animal origin were more distantly located on the cgMLST tree (Supplementary Fig 2).

Furthermore, the isolates of ST-429/ST-9280 were the only ones to be isolated from day-old imported PS chicks and subsequently from broilers (two isolates) and a carcass of chain A and C (Supplementary Fig 1). cgMLST analysis of ST-429 isolates revealed a large cluster of *bla*$_{CMY-2}$-carrying *E. coli* of predominantly poultry origin. These isolates differed by only 24 SNPs on average (Supplementary Fig 2). They were mainly isolated from various stages of the broiler production, including isolates from breeders and poultry meat. In contrast, the only isolate of human source was carrying *bla*$_{TEM52c}$ and had 2,439 SNPs on average with the isolates that formed the large, poultry-related cluster. Analysis of all ST-429-*bla*$_{CMY-2}$ isolates with plasmidFinder revealed presence of the IncK/B/O plasmid replicons. Plasmid contig identification with ABACAS using plasmid pDV45 as reference, showed that *bla*$_{CMY-2}$ was carried by IncK2 plasmids presence of the *ISEcp1-bla*$_{CMY-2}$*-blc-sugE* element (data not shown). Our results demonstrate the dissemination of a highly conserved ST-429 lineage carrying *bla*$_{CMY-2}$ on IncK2 plasmids in the poultry production of at least five European countries.

In summary, our WGS approach revealed a complex landscape of *E. coli* genotypes. Sequenced isolates had, on average, resistance genes to four antimicrobial classes in addition to β-lactams. The co-localisation of several of these resistance genes on ESBL/pAmpC gene-carrying plasmids might explain the persistence of these resistance determinants even in the absence of cephalosporin use in poultry[113]. Further analysis demonstrated that 76% of sequenced isolates shared five dominant plasmid types that harboured ESBL/pAmpC genes. The finding of diverse STs from various stages of the production sharing closely related plasmid backbones with identical ESBL/pAmpC gene insertion sites, is a strong indication that horizontal gene transfer plays a crucial role in the dissemination of cephalosporin resistance determinants in the broiler production[22]. Contribution by the clonal expansion of several successful *E. coli* lineages was also observed and has been discussed before[59]. The identification of phylogenetically related isolates in distantly located farms but in subsequent production levels of the same chain as well, calls for increased biosecurity measures to prevent transmission via the movement of trucks, personnel and equipment[22].

We have previously discussed about a potential introduction of *bla*$_{CMY-2}$ isolates belonging to phylogroup B2 through the import of day-old PS chicks[78]. Here, we show that at least a part of these isolates belong to ST-429, a highly conserved *bla*$_{CMY-2}$ lineage, which is prevalent in the

European poultry production. Among others, a closely related isolate was recovered from a breeder flock in Germany. These findings warrant for eradication of ESBL/pAmpC-EC in pedigree flocks to avoid their spread through the interconnected breeder stock supply chains[22,40,59]. This can be achieved by the complete cessation of ceftiofur administration in these flocks[121]. Further comparison with public data proved that occurrence of ESBL/pAmpC-EC in poultry is a dynamic phenomenon since uncommon STs and ESBL/pAmpC genes in the European context, such as ST-457 and $bla_{\text{CTX-M-55}}$[45,77], can emerge and dominate in poultry. The phylogenetic comparison with publicly available genomes did not enable elucidation of transmission events between reservoirs since isolates were not epidemiologically linked. However, the closer relationship of ST-457 and ST-744 isolates with those of human origin compared to those from livestock, raises questions about their ability to colonise the human gut upon ingestion, transmit their resistance determinants and/or exert their virulence potential. Using a gut model, Anjum *et al.*[123] showed that both human and poultry meat isolated *E. coli* were able to adapt and transmit their AMR plasmids to other *Enterobacteriaceae* in the human gut. Nevertheless, recent studies have shown that the dissemination of ESBL/pAmpC genes between animals and humans is predominantly mediated by specific plasmid lineages and not by the expansion of several successful *E. coli* clones[85,124]. This hypothesis is further supported by the discovery of closely related plasmid backbones with identical ESBL/pAmpC gene insertion sites between the isolates of this study and those of human source[105,116,117,119].

Our results provide valuable insights on the characteristics and transmission patterns of ESBL/pAmpC-EC in the broiler production. In addition, they add to the growing body of high throughput data that will inform and facilitate the conduct of thorough QMRAs aiming to estimate the risk of human exposure to resistance determinants via the (broiler) food chain and identify intervention measures with the maximum mitigation effect.

Chapter 5. A review on the current situation and challenges of colistin resistance in poultry production

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Abstract

Colistin has been re-assessed as a critically important antimicrobial in humans due to its efficacy against multi-drug resistant Gram-negative bacteria, in particular *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*. The recent discovery of mobile colistin resistance determinants in humans and animals has brought concerns regarding the future of this antimicrobial. In this paper, we aim to highlight the current challenges with colistin resistant bacteria and to summarise reliable global data on colistin resistance in poultry production. In addition, we present and compare data from a screening for colistin resistance carried out on a collection of clinical *Escherichia coli* isolated from poultry in Italy. In Europe, resistance rates for *Salmonella* and *E. coli* are in general low with sporadic incidence of high colistin resistance levels. Absence of resistance or very low rates have been recorded in countries where colistin is either not employed (*e.g.* Norway) or used in minimal amounts (*e.g.* Denmark) in food-producing animals. In large poultry meat producing countries, such as China and Brazil, the widespread use of colistin has resulted in the dissemination of resistance determinants in diverse bacterial species. Worryingly, these bacteria are often co-resistant to other critically important antimicrobials, such as extended-spectrum cephalosporins. The data gap for many countries and for zoonotic bacteria, the role of the "phantom resistome" and the circulation of *mcr*-carriers expressing resistance phenotypes close or below the current ECOFF values, should be considered in future investigations. The importance of poultry as a cheap protein source and the global effort to mitigate colistin resistance and preserve this essential antimicrobial require a thorough re-assessment of colistin use in poultry.

Introduction

Colistin or polymyxin E is a decapeptide antimicrobial discovered shortly after the Second World War. Although efficient against human *Enterobacteriaceae* infections, it was soon replaced due to its systemic toxicity. Today, the emergence of multidrug-resistant (MDR) bacteria, which pose a severe risk for public health and are responsible for severe infections and deaths, have triggered the resurgence of colistin in the arsenal of clinicians as a last-resort antimicrobial against these "superbugs"[17]. In veterinary medicine, colistin has been widely administered for prevention, treatment, metaphylaxis and growth promotion[125,126]. Until recently, resistance mechanisms were considered

to be limited to chromosomal mutations only. However, in 2015 a transferable plasmid-mediated gene, which could rapidly spread between bacterial species and hosts, has raised worldwide concern[24]. Epidemiological data and the widespread use of colistin in animal husbandry have led to the belief that transmissible colistin resistance has emerged and is being transferred from animals to humans[25]. Among food-producing animals, a multitude of investigations has demonstrated a notable prevalence of colistin resistance in poultry worldwide, especially in Asia[18,127,128].

This review aims to provide an update on the current situation and challenges regarding colistin resistance in poultry production. To this end, relevant scientific articles were sought using the keywords colistin, resistance, resistant, *mcr*, chicken, broiler, layer hens, poultry or meat in Scopus (www.scopus.com) and PubMed (www.ncbi.nlm.nih.gov/pubmed). Additionally, national reports on antimicrobial resistance (AMR) were sought using web search engines. Searches were performed from December 2017 to mid-March 2018. Studies not published in English or not including any relevant data for poultry or poultry meat were excluded. Due to the unreliability of routine antimicrobial susceptibility testing (AST) methods (*i.e.* disk-diffusion and Etest strips)[17,129], only studies using the dilution methods were considered. All included studies used AST standards of CLSI or EUCAST except for two (Supplementary Table S1). As an adjunct to this review, we present the results from a study carried out on a selection of clinical *Escherichia coli* strains isolated from poultry in Italy.

Structure, mode of action and antimicrobial spectrum of colistin

Polymyxin B and colistin (or polymyxin E) comprise the polymyxin complex in the cyclic lipopeptides group of antimicrobials. Polymyxin, a decapeptide attached to a fatty acid side chain, was firstly isolated in 1947 as a fermentation product of *Bacillus polymyxa*[13]. Polymyxin B and colistin differ by only one amino acid in position 6 of the peptide ring and because the latter molecule is administered as colistin methanesulfonate, an inactive form of the drug (prodrug). Both compounds have a similar mode of action and spectrum of activity[17,130]. Colistin competitively displaces divalent cations of membrane lipids by binding to lipid A of the lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria. This electrostatic interaction between the cationic colistin and the negatively charged LPS leads to

disorganisation of the outer cell membrane, leakage of bacterial contents and cell death[131].

Due to the nature of its antibacterial mode of action, colistin is inactive against Gram-positive bacteria, Gram-negative cocci and *Mycoplasma* spp. Furthermore, *Proteus* spp., *Serratia* spp., and *Burkholderia* spp. are inherently resistant to this drug. Therefore, colistin is a narrow spectrum antimicrobial targeting fermentative (*Escherichia coli, Klebsiella* spp., *Salmonella* spp.) and non-fermentative (*Acinetobacter baumannii, Pseudomonas aeruginosa*) Gram-negative bacteria[17].

Chromosomal and plasmid-mediated colistin resistance mechanisms

Chromosomal resistance pathways mainly involve the linkage of the cationic groups 4-amino-4-deoxy-l-arabinose (L-Ara4N) and phosphoethanolamine (pEtN) to the lipid A molecule of the LPS[131]. The addition of these molecules is regulated by a large group of genes and operons, such as the two component systems PhoPQ and PmrAB, which control the synthesis, and the *pmrC-pmrE* genes, which code for the proteins responsible for the addition of L-Ara4N and pEtN to the LPS[130,132]. This mechanism leads to a qualitative modification of the LPS and thus reduces the affinity of polymyxins to the bacterial surface[127]. Although this pathway results in a significant increase of the minimum inhibitory concentration (MIC) in the mutant strain (*e.g.* 20–30-fold MIC increase for *S. enterica*)[131], the risk of colistin resistance was considered low for two reasons. First, the mutation mechanisms proved to be unstable *in vitro*. Second, the resistant strains could only disseminate vertically (clonally)[125,133]. Therefore, the resistance spread potential remained low.

In November 2015, a Chinese research group reported the emergence of a transferable plasmid-mediated resistance gene, named *mcr-1*, shifting colistin resistance from a contained problem to a global issue. The 1626-bp gene encoding for a PEtN transferase and inducing resistance similarly to the chromosomal mechanisms, was isolated from animals, humans and meat in China[24]. Subsequent retrospective analysis demonstrated that *mcr-1* was circulating since the 1980s with the earliest isolates originating from chickens[134]. The earliest isolate in Europe was carried by diarrheic veal calf in France in 2005[135]. Additional investigations revealed a global dissemination of *mcr-1* in diverse bacterial species and hosts covering many countries in Europe, Asia, Africa and the Americas[128,132]. The broad distribution of the gene in veterinary isolates, the co-existence with β-lactamases usually found

in animals (*e.g.* CMY-2) and the widespread use of colistin in veterinary medicine compared to human medicine are the reasons why the "animal world" has been blamed as a reservoir of resistant bacteria and primary source of transmission to humans[25,125,136]. Recent publications have reported the continuous emergence of novel *mcr* genes[137–140] and allelic variants[141,142], which are difficult to identify in routine monitoring. Furthermore, *mcr* genes have been found on diverse plasmid backbones (IncI2, IncHI2, IncX4 and pHNSHP45) with high *in vitro* transfer rates and often harboured together with other resistance determinants, such as β-lactamases[125].

Susceptibility testing challenges

Testing for susceptibility to polymyxins can be challenging since these large cationic molecules are poorly diffused into media and adhere to common plastic labware[128]. Moreover, variation in the MIC values between colistin and polymyxin B for the same strain[17] and the existence of less susceptible subpopulations (heteroresistance) in some species[131] complicate laboratory investigations. In addition, susceptibility differences at the serovar level exist for *Salmonella* spp., as increased MICs, probably related with the LPS structure (O-antigen), have been described for *S.* Enteritidis and *S.* Dublin[143]. So far, both CLSI and EUCAST have recommended the use of broth microdilution (BMD) in cation-adjusted Muller Hilton Broth (CAMHB) for susceptibility testing of colistin[144]. Agar dilution has also proven to be a reliable method, highly correlated with BMD, and employed by many studies[17,145]. On the other hand, routine susceptibility testing methods, such as disk-diffusion and Etest strips, are now considered unreliable as they result in considerably lower detection rates of resistance compared to dilution methods[17,129,146]. To note, clinical breakpoints for veterinary AST are not available yet, therefore epidemiological cut-off (ECOFF) values are used for the classification of veterinary isolates in wild type (without acquired resistance) and non-wild type (with acquired resistance). Additionally, *mcr-1*-positive strains usually express low to moderate resistance to colistin (0.5 to 32 μg/ml) and are often found with MIC values below the current ECOFF values[127]. These strains are classified as wild type and can evade standard epidemiological studies, which usually analyse only non-wild type strains.

Use of colistin in human medicine

After its discovery, colistin was used in human medicine for the treatment of infections caused by Gram-negative bacteria. However, due to severe side effects such as neuro- and nephrotoxicity, more potent and less toxic drugs (*e.g.* quinolones, β-lactams) have replaced colistin during the 1970s. Over the next 20 years, administration of colistin was limited to topical and ophthalmic use, while systemic use was restricted to the treatment of secondary infections in patients affected by cystic fibrosis[17]. However, during the last two decades, the situation has dramatically changed. The emergence of multidrug-resistant (MDR), extremely drug-resistant (XDR) or even pan-drug-resistant (PDR) bacteria[127] is responsible for the death of 700,000 people annually and is estimated to surpass cancer victims by 2050[6,18]. In a world close to the "post-antibiotic era", colistin has resurged as a last-resort drug in the arsenal against resistant bacteria such as carbapenemase-producers, *A. baumannii* and *P. aeruginosa*. For this reason, the World Health Organization (WHO) has included colistin in the list of critically important antimicrobials for humans with the highest priority[147]. For animals, colistin is considered a highly important antimicrobial agent for its use against Gram-negative enteric infections[9].

Use of colistin in veterinary medicine and in poultry production

Colistin has been extensively used in veterinary medicine for prophylaxis, metaphylaxis, treatment of bacterial infections and growth promotion. In the EU, little is known about the historical use of this drug in animal husbandry, but its first application dates back to the 1950s[136]. Nowadays, colistin-containing products are authorised at the national level. Regarding sales, polymyxins are the 5th most sold veterinary antimicrobials for food-producing animals with wide variability in sales among Member States[148]. On the other side of the Atlantic, the United States Food and Drug Administration (FDA) and the Public Health Agency (PHA) of Canada have never licenced a colistin-containing product for oral use in livestock. There is, however, a documented off-label use allowed by regulation loopholes[18]. At the global level, China is by far the leading colistin producer (17.5 million tonnes) and at the same time the largest consumer absorbing over 90% of its production[24]. Colistin is often added to feed at low doses and used as a growth promoter in countries where this practice is allowed[149].

The main indication of colistin is the treatment of gastrointestinal (GI) infections caused by non-invasive, susceptible *Enterobacteriaceae* in pigs, cattle, small ruminants and poultry[125]. Colistin products are chiefly administered in group treatments through the oral route via feed or, more often, via drinking water. The 495 tonnes of polymyxins consumed in 2013 in Europe were predominantly administered orally to swine and poultry[18]. In France, for example, exposure of pigs to colistin accounted for one-third of the total exposure to antimicrobials and the corresponding proportion was even higher for poultry[149]. However, there is a notable lack of detailed consumption data for each food-producing animal category, and currently only aggregated data are available[148]. Nevertheless, in the last Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) report an attempt to infer antimicrobial consumption data at the animal species level using sales estimates as proxy, revealed a significantly positive association between consumption of polymyxins and resistance in indicator *E. coli* of poultry and pig origin[150].

Colistin is predominantly employed in pig and cattle production to control enteric infections caused by *E. coli* and *Salmonella* (*e.g.* post-weaning diarrhoea in piglets) or for metaphylaxis[126,136]. For poultry, there are no relevant indications other than colibacillosis given that for *Salmonella* infections antimicrobial use is prohibited[151]. Indeed, several publications mention that the main indication of colistin in poultry is the treatment of mild colibacillosis[136,149,152]. Nonetheless, the bioavailability of colistin following oral administration in poultry has proven to be very low since it is not well absorbed from the GI tract (*e.g.* there is no withdrawal time for eggs after oral administration of colistin)[153,154]. Therefore, the attained blood and tissue levels are inadequate to treat common signs of colibacillosis in poultry, *i.e.* localised (*e.g.* omphalitis) or systemic infection (*i.e.* colisepticemia)[155]. Although there is anecdotal evidence that polymyxins can be used in mild colibacillosis if administered for at least seven days and at higher doses, other drugs such as sulphonamides, tetracyclines and penicillins are certainly more appropriate[151]. Additionally, primary diarrheal disease due to *E. coli*, against which colistin would be effective, is considered rare in poultry[155].

A turning point in the history of colistin use in veterinary medicine has been a referral of the United Kingdom to EMA in 2009 posing public and animal health concerns, originating from differences in posology and withdrawal periods of colistin used in food-producing animals via

drinking water. The Committee for Medicinal Products for Veterinary Use (CVMP) recommended removing GI infections caused by *Salmonella* spp. from the Summary of Product Characteristics (SPCs) on the basis of a negative risk-benefit balance, due to the interference with national *Salmonella* control programmes[156]. Further amendments in 2014 and 2016 removed all indications for prophylaxis and restricted colistin use to the treatment of enteric infections caused by susceptible non-invasive *E. coli* only. Furthermore, use of colistin in combination with other antimicrobials was banned[125].

Detection of mcr-1 *and* mcr-2 *in clinical* E. coli *isolated from poultry in Italy*

A total of 229 non-duplicate[56], clinical *E. coli* strains collected between 2008 and 2012, were screened to detect the presence of the *mcr-1* and *mcr-2* genes. Specifically, these strains were isolated from tissues and organs of birds showing signs suggestive of colibacillosis and were previously analysed for resistance to quinolones, extended-spectrum cephalosporins (ESC) and class 1 and 2 integrons[56,157]. In detail, strains were isolated from turkeys (n = 109), broilers (n = 98), and layer hens (n = 22). To detect the presence of *mcr-1* and *mcr-2* genes, PCRs and sequencing were carried out according to Y.-Y. Liu *et al*.[24] and Xavier *et al*.[137], respectively. Additionally, for strains with a confirmed presence of *mcr* genes, the MIC was determined using the BMD method in CAMHB according to CLSI-EUCAST recommendations[144], and EUCAST ECOFF values were used for interpretation of MICs[158].

Out of 229 strains, 29 (12.6%) were positive for *mcr-1* gene, and none was found positive for *mcr-2*. A higher frequency of *mcr-1*-carrying *E. coli* in turkeys (22.9%) compared to broilers (2%) and layer hens (9%) was found (Table 1). Sequence analysis of all strains showed a sequence identity of 100% compared to the original *mcr-1* sequence (NCBI accession number: NG_050417). Most strains (n = 19) had MIC values of 4 mg/L and were classified as non-wild type according to EUCAST. The remaining strains (n = 10) had MIC values of ≤2 mg/L, corresponding to the wild type phenotype. Among the 29 *mcr-1* positive strains, 16 strains had additional phenotypic and genotypic resistances; 12 were resistant to aminoglycosides and trimethoprim (*aadA1-dfrA1* genes in class 1 and 2 integrons), three to ESC and penicillins ($bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-1}}$) and one to quinolones (*qnrB19*). Additionally, three isolates were MDR.

Colistin resistance and mcr *genes in bacterial isolates from poultry*

In total, 62 studies providing colistin resistance data for Europe (*n* = 30), Asia (*n* = 23), South America (*n* = 4), North America (*n* = 1), Africa (*n* = 2) and two studies with data from various countries are presented in Table 1, including the results of our study. The most represented country is China (*n* = 14), followed by Japan (*n* = 5), Denmark and Portugal (*n* = 4, each), whereas for each of Brazil, Italy, the Netherlands, Norway and Switzerland, three studies have been published. For Europe, data from ten different countries are included in addition to three European reports with data for multiple countries. Overall, data have been collected from 1999 to 2016 except for two retrospective studies analysing isolates from the 1980s (Table 1). However, the majority of studies (*n* = 44) have been published between 2016 and 2017 following the discovery of *mcr-1* in November 2015. To note, until 2014 colistin was not included in the panel of routine AMR monitoring in Europe and other countries as well, which explains the relative scarcity of publications from previous years[81,128].

Salmonella spp.

In the first European AMR monitoring report including colistin resistance data, contamination with colistin-resistant (CST-R) *Salmonella* was higher in turkey meat (29.6%) compared to chicken meat (5.5%), although prevalence was higher for broiler flocks. Additionally, notably high percentages were found for layer hens (10.5%)[81]. In the most recent report, a reduction of CST-R *Salmonella* for all production types, especially for turkey meat (0.7%), has been described[5]. An earlier European study on chickens at slaughter reported 21 out of 49 *Salmonella* resistant to colistin[159]. In Portugal and Italy, the prevalence of CST-R *Salmonella* was high and low, respectively[160,161]. In Asia, studies focusing on *Salmonella* are scarce but indicate low levels of colistin resistance in chickens and meat thereof (Table 1). In the single study from South America, Vinueza-Burgos *et al.*,[162] reported a moderate isolation rate (16.1%) in chickens at slaughter. Regarding other poultry productions, Chiou *et al*.[163] found 0% and 2.3% CST-R *Salmonella* in diseased ducks and geese, respectively, in Taiwan.

S. Enteritidis was predominant among CST-R *Salmonella* strains when serotype information was provided (Supplementary Table S1). For example, 100% of CST-R *Salmonella* were *S.* Enteritidis in the studies of de Jong *et al*.[159] and Figueiredo *et al*.[161]. Similar results were found in the two European summary reports[5,81]. Specifically for the earliest

report[81], where higher resistance rates were reported, 72% of CST-R *Salmonella* from broilers and 80% from laying hens were *S.* Enteritidis. *mcr-1* was identified only in *S.* Typhimurium[163,164].

Commensal and pathogenic E. coli

Colistin resistance in indicator *E. coli* from poultry was the predominant type of study. Results between the two European AMR monitoring reports for colistin resistance in indicator *E. coli* isolated from broiler and turkey flocks are comparable. For both monitoring years (2014 and 2016) prevalence was generally low for chickens and turkeys but with three to seven times higher percentages for turkeys (Table 1). Also, the last report has shown low colistin resistance rates for chicken meat (3.9%) but moderate for turkey meat (10.1%). Data from the rest of European studies on colistin resistance in indicator *E. coli* for chicken, turkey, layer hen flocks and poultry meat ranged from very low levels in Nordic countries (≤1%) to low levels (≤10%) in the rest of Europe (Table 1). Exceptions are the moderate prevalence found in turkey flocks and meat in Germany[165], Italy[166] Switzerland[167], and the high prevalence found in Portugal[168]. In our study, the only European study focusing on *mcr-1* and *mcr-2* prevalence on clinical *E. coli* isolates, 22.9% of turkey isolates were *mcr-1* positive whereas the corresponding rate for chickens and layer hens was roughly 10-times and 2-times lower, respectively. To note, several of these *mcr-1* positive isolates were found with wild type phenotype MICs. Data from other poultry production types (*e.g.* ducks, geese) are not available in Europe. In Asia, the prevalence of CST-R indicator *E. coli* in poultry was higher compared to Europe. In China, resistant isolates in broilers were found in low percentages except for the study of P. Zhang *et al.*[169] who found a prevalence of 14%. The retrospective study of Shen *et al.*[134] showed that the *mcr-1* gene was circulating in CST-R *E. coli* in chickens since the 1980s, and revealed a rapid increase of resistant isolates from 2009 to 2014, a finding corroborated by Huang *et al.*[170]. Data from Japan and South Korea indicated a low prevalence of CST-R commensal *E. coli* in chickens; however, the number of studies is limited. Asian studies on clinical *E. coli* in chickens report low colistin resistance rates with the exception of the extremely high prevalence (73.1%) reported by B.-T. Liu *et al.*[171] in China. In the single study on poultry species other than chickens, Yassin *et al.*[172] discovered a 6.8% prevalence of CST-R clinical *E. coli* isolated from diseased ducks. Two studies on *E. coli* in Brazil represented South America. Lentz *et al.*[173] discovered 10 *mcr-1*

positive out of 343 isolates expressing very low MICs (0.25-2 mg/L), whereas a moderate prevalence of the gene was found in chicken meat[174]. Limited studies from Africa showed a low prevalence of clinical and commensal *E. coli* in South African and Tunisian broilers, respectively (Table 1). Furthermore, the retrospective investigation of Barbieri *et al.*[152] discovered low prevalence of *mcr-1* in clinical *E. coli* and absence of the gene in their commensal counterparts, in an international collection of poultry-derived isolates.

Other bacteria

Several studies have focused on the prevalence of CST-R and *mcr-1*-carrying *Enterobacteriaceae*. High to extremely high percentages of *mcr-1*-positive *Enterobacteriaceae* have been reported for the Chinese broiler production (Table 1). Moreover, Y. Wang *et al.*[175] coined the term "phantom resistome" after the discovery of a substantially higher rate of *mcr-1* positive samples when analysed with direct sampling testing, compared to the isolation of *Enterobacteriaceae* expressing the resistance phenotype. High prevalence of CST-R *Enterobacteriaceae* has been reported in chickens and meat thereof in Brazil, whereas the corresponding European percentages were significantly lower (Table 1). In the single study from the USA, no *mcr-1* positive *Enterobacteriaceae* were recovered from chicken and turkey flocks[176]. Colistin resistance in *K. pneumoniae* was investigated in two studies from China in which a high prevalence (23.4%) was found for chicken at slaughter, while the corresponding percentage for chicken meat was 0% (Table 1). One study from Portugal reported alarming levels of colistin resistance in *Acinetobacter* spp. isolated from chicken and turkey meat, including two strains of the nosocomial pathogen *A. baumannii*[177]. Furthermore, three studies analysed the presence of *mcr* genes with direct sample testing (total DNA). In a concurrent study in Algeria and France, low prevalence and absence of *mcr-1*, respectively, was found in chickens[178]. In contrast, a Chinese study on chicken, duck and goose flocks not only reported high prevalence of the *mcr-1* gene, but also identified for the first time the presence of all novel *mcr* genes (*mcr-1* to *mcr-5*) in the examined poultry species[179,180].

Additional findings in the reviewed studies

Data from the reviewed studies demonstrate that *mcr-1* circulates at high rates among CST-R isolates in poultry. However, since *mcr-1* is not always discovered in phenotypically resistant isolates, novel *mcr-*

like genes or chromosomal pathways may be involved. Moreover, *mcr-1* was harboured on diverse plasmid backbones (IncHI2, IncP, IncFIB, IncI1, IncX4), mainly IncI2 (Table 1). These plasmids have been shown to have high *in vitro* transfer rates and the ability to transfer between *Enterobacteriaceae* through conjugation[125,181]. Additionally, *mcr-1*-carrying bacteria were found expressing colistin resistance ranging from very low (0.25-2 mg/L) to low-moderate MICs (2-8 mg/L), with only a few exceptions (Table 1). A significant number of studies reported co-resistance to other critically important antimicrobials such as ESC (n =17), quinolones (*n* = 14) and carbapenems (n = 4), among other antimicrobials. Frequently, CST-R isolates possess resistance determinants to a total of three or more antimicrobial classes, which classifies them as MDR. Detailed information on additional resistances and resistance genes discovered in the CST-R isolates of the reviewed studies can be found in the Supplementary Table S1.

Discussion and Conclusion

In Europe, colistin resistance and *mcr* prevalence in poultry remain low, especially in Nordic countries where colistin is either not employed in veterinary practice or used in minimal amounts. The sporadic frequency of higher detection rates may be related to greater colistin use in some EU Member States. Although a significant association between colistin use and resistance in poultry and swine has already been suggested from consumption estimates[150], collection of detailed consumption data for poultry is crucial to confirm this association and to evaluate the ongoing reduction measures on colistin use in animals. In our study, an overall prevalence of 12.7% was found for *mcr-1*, suggesting that the gene was circulating in the Italian poultry population before its discovery in 2015. Moreover, our study revealed a higher prevalence of *mcr-1* positive *E. coli* in turkeys compared to broilers and layer hens, although some isolates were classified as wild type according to the current EUCAST ECOFF value. Nevertheless, recent studies carried out in Italy and Europe, have revealed high isolation rates of CST-R *E. coli* in turkeys and meat thereof compared to other poultry production types and prompted for a thorough investigation to understand the underlying reasons of this phenomenon[5,81,165,166,168]. Colistin resistance in *Salmonella* spp., especially in chickens, is often related with the intrinsically resistant *S.* Enteritidis, while for other serotypes resistance is probably mediated by *mcr* genes[81,159,161]. In China and Brazil, two of the largest poultry meat producers globally, the widespread use of

colistin in poultry for treatment and growth promotion practices has resulted in an increased selective pressure and high prevalence of colistin resistance in diverse bacterial species. In light of this worrying trend, regulations have been implemented to reduce overall administration of colistin and to ban its growth promoting usage[24,182]. Further investigations to gain in-depth insight into the epidemiology and prevalence of colistin resistance in poultry are warranted, especially in countries where data are still scarce or lacking. Additionally, the data gap on zoonotic pathogens such as extra-intestinal pathogenic *E. coli* (ExPEC), *K. pneumoniae* and *Salmonella* spp. should be addressed.

Reviewed data suggest that although *mcr-1* is highly prevalent among CST-R poultry-derived bacteria, this resistance determinant is not the only implicated mechanism. Chromosomal mutations and other *mcr* genes seem to contribute as well. The recent discovery of all novel *mcr* genes in poultry[179,180] and the development of a multiplex PCR protocol for their fast detection[183] prompt for surveillance of all *mcr* genes in future investigations. Furthermore, the higher prevalence of *mcr*-like genes when all *Enterobacteriaceae* are considered and the role of the "phantom resistome" should not be overlooked[175]. A notable finding of our study is the occurrence of *mcr-1*-positive *E. coli* expressing low MICs, occasionally below the current ECOFF value. Similar observations have been reported in the reviewed studies; *mcr-1*-carrying poultry isolates showed MIC values usually close to the limit of susceptibility and as low as 0.25 mg/L[173]. The occurrence of bacteria with low resistance phenotypes has been detected in other veterinary isolates[127]. These microorganisms can evade routine monitoring and disseminate undetected leading to an underestimation of the prevalence of *mcr* genes[184]. Hence, analysis of all bacterial isolates for the presence of *mcr*-like genes, rather than screening phenotypically-resistant isolates only, should be considered in future studies.

The *mcr-1*-positive *E. coli* strains of our collection were previously found resistant to various antimicrobials, including quinolones and ESC. Several studies report CST-R isolates with MDR phenotypes and genotypes, which usually include critically important antimicrobials[175,177,184]. The circulation of MDR bacteria in the poultry production chain is a worrying phenomenon. Thus, potential risks to one of the world most essential protein sources should be thoroughly evaluated. The public health significance of this problem is even more critical since several studies have suggested an association between

poultry meat and the occurrence of persistent human ExPEC infections[32,185].

A global One Health effort to preserve the efficacy of colistin is undergoing. In Europe, Member States are called to achieve a 65% reduction of colistin sales by 2020[125]. Veterinarians can now administrate colistin against susceptible, non-invasive *E. coli* only. Given the increasing colistin resistance trends in poultry, the lack of relevant indications other than *E. coli* and the poor efficiency of this antimicrobial against colibacillosis, colistin use in poultry should be thoroughly re-assessed.

Table 1. Colistin resistance and *mcr* prevalence in microorganisms isolated from poultry.

Species[a]	Country	Data Period	Source[b]	% CST-R (no. CST-R isolates/no. isolates tested) [MIC range of CST-R isolates] {breakpoint applied}[c]	% *mcr* (no. *mcr-1* isolates/no. of isolates tested) [replicon type of plasmid harbouring the *mcr* gene][d]	References
Various	Algeria	2015	CS	-	Samples positive for *mcr-1*: 1.2% (6/503) resulting in 3 *mcr-1+ E. coli*	Chabou *et al.*, 2016
ENT	Brazil	2003-2015	CF, CM	CF, 40.4% (113/280); CM, 52.4% (22/42) [8-16] {2}	CF, 5.0% (14/280), CM, 0% (0/42)	Fernandes *et al.*, 2016
EC	Brazil	2015	CS	[*mcr-1*+: 0.25-2] {2}	2.9% (10/343)	Lentz *et al.*, 2016
EC	Brazil	2016	CM	[*mcr-1*+: 2-8] {2}	19.5% (8/41) [IncX4]	Monte *et al.*, 2017
EC	China	2015	CD	73.1% (57/78) [*mcr-1*+: 4-16] {2}	67.9% (53/78) [IncI2]	B.-T. Liu *et al.*, 2017
KP	China	2013-2014	CM	0% (0/26) {2}	-	Guo *et al.*, 2016
EC	China	2007-2014	CF, CS	CF, 2.4% (28/1150); CS, 9.5% (31/325) {4}	-	Huang *et al.*, 2017
Various	China	2014-2015	CF, DF, GF	-	|*mcr-1*|: CF, 31.8% (477/1498); DF, 34.6% (45/130); GF, 71.6% (78/109) |*mcr-2*|: CF, 5.5% (82/1498); DF, 2.3% (3/130); GF, 5.5% (6/109) |*mcr-3*|: CF, 5.2% (78/1498); DF, 13.8% (18/130); GF, 11.9% (13/109) |*mcr-4*|: CF, 17.2% (257/1498); DF, 15.4% (20/130); GF, 49.5% (54/109) |*mcr-5*|: CF, 9.9% (148/1498); DF, 7.7% (10/130); GF, 17.4% (19/109)	J. Zhang *et al.*, 2018; L. Chen *et al.*, 2018
ENT	China	2015-2016	CF, CM	-	Samples positive for *mcr-1 Enterobacteriaceae*: CF, 63% (113/180); 67.4% (29/43) [IncX4]	K. Chen *et al.*, 2017
S	China	2007-2015	CF	8.9% (22/246) [*mcr-1*+: 16] {8}	22.7% (5/22) [IncHI2]	Li *et al.*, 2016
EC	China	2008-2015	CF	14% (1063/7568) {2}	-	P. Zhang *et al.*, 2017
EC	China	1970-2014	CF	-	6.5% (104/1611)	Shen *et al.*, 2016
EC	China	2013	CS	8.3% (10/121) [4-16] {2}	8.3% (10/121) [IncI2]	X. Wang *et al.*, 2017
KP	China	2013	CS	23.4% (11/47) [4-128] {2}	0% (0/47)	X. Wang *et al.*, 2017
ENT	China	2014-2015	B, CF, CS, CM	100% of *mcr-1*+ isolates	B, 75% (45/60); CF, 49.7% (164/330); CS, 44% (22/50); CM, 25% (12/48)	Y. Wang *et al.*, 2017
EC	China	2011-2014	CM	100% of *mcr-1*+ isolates {2}	11.8% (35/297)	Y.-Y. Liu *et al.*, 2016
EC	China	2010-2015	CD	100% of *mcr-1*+ isolates [4-16]	5.1% (58/1136) [IncI2]	Yang *et al.*, 2017
EC	China	2004-2012	CD, DD	[*mcr-1*+: 4-8 mg/L] {4}	CD, 3.2% (13/404); DD, 6.8% (3/44)	Yassin *et al.*, 2017
EC	Denmark	2014-2016	CF, CM	CF, 0% (0/520); CM, 0.4% (3/746) {2}	-	DANMAP, 2014, 2015, 2016
EC	Denmark	2012-2014	CM	-	1.3% (5/380) [IncI2]	Hasman *et al.*, 2015
S	Ecuador	2013-2014	CS	16.1% (10/62) [2-4] {2}	0% (0/10)	Vinueza-Burgos *et al.*, 2016
S	Europe	2003-2005	CS	42.9% (21/49) [4-8] {4}	-	de Jong *et al.*, 2012
EC	Europe	2003-2005	CS	0% (0/518)		de Jong *et al.*, 2012

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Table 1 (continued)

Species[a]	Country	Data Period	Source[b]	% CST-R (no. CST-R isolates/no. isolates tested) [MIC range of CST-R isolates] {breakpoint applied}[c]	% *mcr* (no. *mcr-1* isolates/no. of isolates tested) [replicon type of plasmid harbouring the *mcr* gene][d]	References
S	Europe	2014	CM, TM, CF, TF, LH	CM, 5.5% (37/673); TM 29.6% (67/226); CF, 8.3% (137/1656); TF, 1.8% (13/726); LH, 10.5% (83/792) {2}	-	EFSA/ECDC, 2016
EC	Europe	2014	CF, TF	CF, 0.9% (454/5045); TF, 7.4% (123/1663) {2}	-	EFSA/ECDC, 2016
S	Europe	2016	CM, TM, CF, TF, LH	CM, 1.4% (11/763); TM, 0.7% (21/295); CF, 2.2% (38/1717); TF, 0.3% (2/663); LH, 5.8% (71/1216) {2}	-	EFSA/ECDC, 2018
EC	Europe	2016	CF, CM, TF, TM	CF, 1.9% (90/4729); CM, 3.9% (18/466); TF, 6.1% (105/1714); TM, 10.1% (48/473) [4- ≥16] {2}	-	EFSA/ECDC, 2018
EC	Europe	2002-2014	CS	1.5% (53/3581) {2}	83% (44/53)	El Garch *et al.*, 2018
Various	France	2015	CF	-	Samples positive for *mcr-1*: 0% (0/330)	Chabou *et al.*, 2016
EC	France	2011-2014	CF, TF	CF ,1.1% (7/621); TF, 5.9% (14/239) {2}	CF, 100% (7/7); TF, 100% (14/14)	Perrin-Guyomard *et al.*, 2016
EC	Germany	2010-2014	LH, CF, CS, CC, CM, TF, TS, TM, eggs	LH, 1.3% (24/1795); CF, 5.3% (39/738); CS, 8.5% (43/503); CC, 11.2% (26/233); CM, 5.3% (31/580); TF, 17.5% (117/669); TS, 9.2% (73/790); TM, 9.2% (62/676); eggs, 2.2% (2/90) {2}	LH, 0.2% (3/1795); CF, 5.0% (37/738); CS, 7.6% (38/503); CC, 10.3% (26/233); CM, 4.3% (25/580); TF, 16.4% (110/669); TS, 7.8% (62/790); TM, 8.4% (57/676); eggs, 0% (0/90)	Irrgang *et al.*, 2016
EC	Germany	2013	CF	6.8% (30/438) {2}	-	Schwaiger *et al.*, 2013
EC	Italy	2014	TS	19.33% (58/300) [4-32] {4}	100% (58/58)	Battisti, 2016
S	Italy	2012-2015	CF, CM	CF, 5.3% (13/243); CM, 1.0% (1/93) [*mcr-1*+: 8-16] {2}	CF, 15.4% (2/13); CM, 0% (0/1)	Carnevali *et al.*, 2016
EC	**Italy**	**2008-2012**	**TD, CD, LHD**	**[*mcr-1*+: <1-4]**	**TD, 22.9% (25/109); CD, 2% (2/98); LHD, 9% (2/22)**	**This study**
EC	Japan	2008-2011	CF	0% (0/25)	-	JVARM, 2013
EC	Japan	2000-2014	CF	-	0.7% (14/2017)	Kawanishi *et al.*, 2017
EC	Japan	1999	CF	2.3% (7/304) {1.56}	-	Kijima-Tanaka, 2003
EC	Japan	2015	CM	5.8% (9/154) [*mcr-1*+: 8-16] {2}	88.9% (8/9) [IncX4]	Nishino *et al.*, 2017
EC	Japan	2015-2016	CM	100% of *mcr-1*+ [8] {2}	1.4% (1/70) [IncI2]	Ohsaki *et al.*, 2017
EC	Netherlands	2009-2010	B, CF	0% 0/52 {2}	-	Dierikx *et al.*, 2013
EC	Netherlands	2010-2015	CF, TF, LH, CM, TM	CF, 0.7% (16/2226); TF, 0.5% (1/192); LH, 0% (0/190); CM, 2.8% (52/1860); TM, 11.4% (23/201) {2}	CF, 62.5% (10/16); TF, 100% (1/1); LH, 0% (0/0); CM, 76.9% (40/52); TM, 87% (20/23)	MARAN, 2016
S	Netherlands	2010-2015	CF, TF	CF, 4.3% (53/1227); TF, 6.2% (2/32) {2}	CF, 20.8% (11/53); TF, 100% (2/2)	MARAN, 2016
ENT	Netherlands	2015	CM	100% of *mcr-1*+ isolates [4-16] {2}	15.9% (34/214) samples positive	Schrauwen *et al.*, 2017

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Table 1 (continued)

Species[a]	Country	Data Period	Source[b]	% CST-R (no. CST-R isolates/no. isolates tested) [MIC range of CST-R isolates] {breakpoint applied}[c]	% *mcr* (no. *mcr-1* isolates/no. of isolates tested) [replicon type of plasmid harbouring the *mcr* gene][d]	References
EC	Norway	2013-2016	LH, TF, TM, CF	LH, 0.5% (9/186); TF, 7.2% (19/265); TM, 0% (0/154); CF, 0% (0/386) {2}	-	NORM-VET, 2013, 2014, 2016
A	Portugal	2013-2014	CM, TM	CM, 42.3% (22/52); TM, 40% (16/40) {4}	-	Carvalheira *et al.*, 2017
S	Portugal	2011-2012	poultry	20.7% (23/111) {2}	4.3% (1/23)	Figueiredo *et al.*, 2015, 2016
EC	Portugal	2014	CS, TS	CS, 3.0% (6/202); TS, 27% (50/185) [8-16] {2}	2 out of 3 CST-R a- ESBL+ strains	Manageiro *et al.*, 2017
EC	South Africa	2008-2015	CF	5.9% (302/5134) {4}	19 out of 20 CST-R that were screened from 2015 period [IncI2]	Perreten *et al.*, 2016
EC	South Korea	2005-2015	CC, CF, CD	CC, 0.8% (11/1308); CF, 1.2% (21/1687); CD, 5.7% (11/193) {2}	CC, 0.4% (5/1308); CF, 0.3% (5/1687); CD, 0% (0/193)	Lim *et al.*, 2016
EC	Spain	2014	TF	1.8% (3/170) [4] {2}	100% (3/3)	Quesada *et al.*, 2016
EC	Sweden	2012-2016	CF, TF, LH, CM	CF, 0% (0/372); TF, 0% (0/144); LH, 0% (0/61); CM, 1% (1/92) {2}	-	Swedres-Svarm, 2014, 2016
ENT	Switzerland	2016	CS	0% (0/1000) {4}	-	Buess *et al.*, 2017
EC	Switzerland	2014-2016	CM	-	1.1% (6/536)	FOPH/FFVO, 2016
EC	Switzerland	2016	CM, TM	samples positive for CST-R *E. coli*: CM, 6.5% (7/108); TM, 25% (5/20) [4-8] {4}	100% of CST-R *E. coli*	Zurfluh *et al.*, 2016
S	Taiwan	2011-2014	CD, TD, DD, GD	CD, 3.8% (17/450); TD, 0% (0/170); DD, 0% (0/206); GD, 2.3% (2/88) {4}	CD, 11.8% (2/17); GD, (0/2) [IncX4]	Chiou *et al.*, 2017
EC	Tunisia	2013	CF	4.2% (2/48)[8] {2}	100% (2/2)	Maamar *et al.*, 2018
ENT	USA	2016	CF, TF	-	CF, 0% (0/238); TF, 0% (0/125)	Meinersmann *et al.*, 2016
EC	various	1980-2015	CF, CD	100% of *mcr-1*+ isolates [4-16] {2}	APEC, 1.22% (12/980); AFEC, 0% (0/220) [IncI2, IncI1, IncFIB]	Barbieri *et al.*, 2017
S	Vietnam	2009	CM	0% (0/52) {8}	-	Ellerbroek *et al.*, 2010
S	Vietnam	2012-2013	CF	6.3% (3/48) {2}	-	Lettini *et al.*, 2016

[a]*ENT*, *Enterobacteriaceae*; *EC*, *E. coli*; *EC*, *clinical E. coli; KP*, *Klebsiella pneumoniae*; *S*, *Salmonella* spp.; *Various*, total DNA of samples analysed for *mcr* presence (direct sampling testing).
[b]B, breeder chickens; CF, chickens at farm; CS, chickens at slaughter; CD, diseased chickens at farm; CM, chicken meat; CC, chicken carcass; TF, turkeys at farm; TS, turkeys at slaughter; TM, turkey meat; TD, diseased turkeys; LH, laying hens; LHD, diseased laying hens; DD, diseased ducks; GD, diseased geese.
[c,d]-, not determined data

Chapter 6. Overall conclusion

Considering the public health risk associated with the presence of extended-spectrum *β*-lactamase (ESBL)- and AmpC *β*-lactamase (pAmpC)-producing *E. coli* (ESBL/pAmpC-EC) in food-producing animals, Chapters 2-4 of this thesis aimed to assess the presence, characteristics and transmission pathways of ESBL/pAmpC-EC in an integrated broiler production system.

In chapter 2, a high sample prevalence in the greater part of the production pyramid and a substantial transfer of ESBL/pAmpC-EC over subsequent production stages was shown. The high percentage of positive samples and genotype uniformity at the top of the pyramid pointed to an introduction of resistant clones through the import of day-old breeders. Chapter 3 demonstrated that although present in many animals, ESBL/pAmpC-EC were overall subdominant to the general *E. coli* population. Therefore, a high sample prevalence seems to not be associated with high levels of resistance in individual hosts, which is an informative aspect for the exposure part of a risk assessment model. Furthermore, a link between the loads ESBL/pAmpC-EC and generic *E. coli* on carcasses was suggested. In chapter 4, an extended resistance gene repertoire, conferring resistance to multiple antimicrobial classes, was shown for the majority of sequenced isolates. Successful *E. coli* lineages were able to clonally expand within and across production chains and in various stages of the production pyramid. It was demonstrated that novel ESBL/pAmpC-EC lineages can emerge and dominate in broilers, while others assist in the propagation of ESBL/pAmpC genes in the industry. Nonetheless, the crucial role of plasmids in the dissemination of cephalosporin resistance genes within the broiler industry but also across reservoirs was revealed.

Based on these findings, several intervention measures were proposed. Ensuring ESBL-free pedigree flocks by *e.g.* the complete cessation of ceftiofur use, is of paramount importance to avoid the introduction and subsequent dissemination of ESBL/pAmpC-EC from the top of the pyramid to multiple broiler production systems. The discovery of virtually identical isolates in distantly located farms further supports this argument. However, given the significant transfer over subsequent production stages identified in Chapter 2, at least part of the ESBL/pAmpC-EC dissemination may be explained by transmission events within the studied integrated production system, which stresses the importance of strict biosecurity measures. Such measures should definitely address the movement of trucks, personnel and equipment,

which can be shared between farms in an integrated company. Although subdominant to the total *E. coli* population, competitive exclusion may further suppress ESBL/pAmpC-EC and thus control their dissemination and reduce the effect of carcass contamination through faecal leakage. Interventions at the slaughterhouse are crucial since the load of ESBL/pAmpC-EC on carcasses defines the exposure of chicken meat consumers. The link between resistant and generic *E. coli* loads suggests that an overall improvement of hygiene could further reduce ESBL/pAmpC-EC numbers. Critical control points such as temperature and duration of scalding should be closely monitored and the establishment of relevant microbiological criteria can assure compliance. Finally, given the crucial role of plasmids in AMR spread across sectors and reservoirs, methods that may inhibit their dissemination must be further developed and tested *in vivo*[215].

Chapter 5 aimed to describe the current situation and challenges of resistance to another critically important antimicrobial, colistin. A widespread presence of resistance, usually conferred by *mcr* genes, was revealed. Levels are generally lower in Europe, where colistin is moderately used, and higher in large poultry meat producers (*e.g.* China and Brazil), where colistin is largely administered for both therapy and growth-promotion. Pitfalls such as the general data gap, the role of latent resistance-carriers and the silent dissemination of "susceptible" *mcr*-carriers, were identified. Finally, given the crucial role of colistin as last-resort antimicrobial in humans and the low bioavailability of this antimicrobial in poultry, the necessity to use colistin in the poultry industry was questioned.

Overall this thesis has generated a large amount of data, but a crucial question remains unanswered; what is the public health risk deriving from the presence of the aforementioned resistance determinants in the poultry industry? Before answering, it is important to note that although human-to-human transmission is the primary source of ESBL/pAmpC-EC carriage, consumption of chicken meat and non-occupational contact with chickens have been also identified as important risk-factors[216] The answer to this complex question cannot be the output of one thesis but rather the compilation of public data and their integration in a quantitative microbial risk assessment (QMRA) with a farm-to-fork approach. The output of this research work could, therefore, be informative for QMRA efforts. For example, the AMR profile of strains, the context of AMR genes and the potential pathogenicity associated with discovered genotypes are important for risk

identification and characterisation. Further, the probability of identified hazards to be transmitted from one step of the production to the other and reach the processing level, as well as the dynamics of resistant *E. coli* in the chicken gut, which affect faecal shedding probabilities, are informative to the exposure assessment step. Nevertheless, the complexity of QMRAs warrants the collection of more high-throughput, multi-omics data, which will reduce the uncertainty of models in estimating the probability of transmission and identify intervention measures across animal production chains with the maximum risk mitigation effect[88].

Additional information

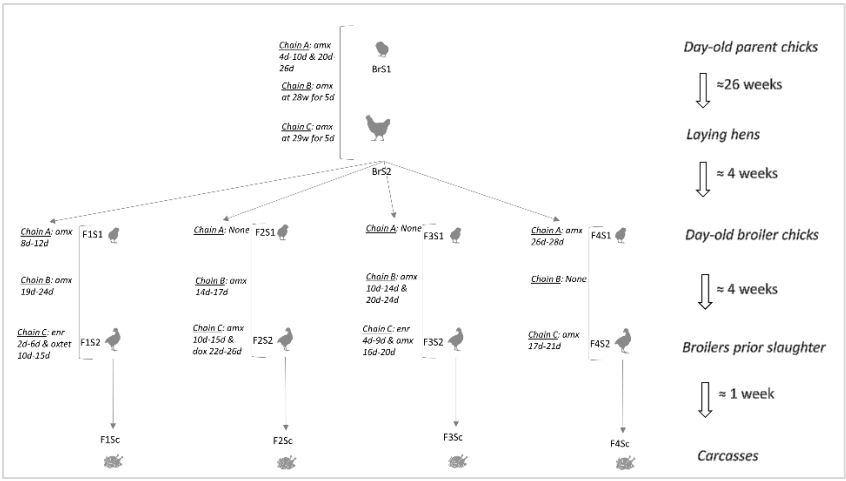
Chapter 2

S1 Table. Information on the three sampled broiler production chains:

Chain	Production stage [a]	Date of sampling	Age at sampling [b]
A	PS chicks - PS breeders	05/01/2017 - 04/07/2017	1d - 26w
	F1_broiler chicks - F1_broilers - F1_carcasses	21/07/2017 - 23/08/2017 - 31/08/2017	1d - 33d
	F2_broiler chicks - F2_broilers - F2_carcasses	28/07/2017 - 29/08/2017 - 04/09/2017	1d - 32d
	F3_broiler chicks - F3_broilers - F3_carcasses	03/08/2017 - 06/09/2017 - 07/08/2017	1d - 35d
	F4_broiler chicks - F4_broilers - F4_carcasses	16/08/2017 - 14/09/2017 - 18/09/2017	2d - 33d
B	PS breeders	10/01/2017	29w
	F1_broiler chicks - F1_broilers - F1_carcasses	08/02/2017 - 06/03/2017 - 16/03/2017	1d - 28d
	F2_broiler chicks - F2_broilers - F2_carcasses	08/02/2017 - 06/03/2017 - 16/03/2017	1d - 28d
	F3_broiler chicks - F3_broilers - F3_carcasses	22/02/2017 -20/03/2017 - 28/03/2017	2d -28d
	F4_broiler chicks - F4_broilers - F4_carcasses	22/02/2017 - 20/03/2017 - 29/03/2017	2d -28d
C	PS chicks - PS breeders	19/04/2017 -15/11/2017	1d - 30w
	F1_broiler chicks - F1_broilers - F1_carcasses	05/12/2017 - 03/01/2018 - 09/01/2018	1d - 28d
	F2_broiler chicks - F2_broilers - F2_carcasses	12/12/2017 - 09/01/2018 - 17/01/2018	1d - 27d
	F3_broiler chicks - F3_broilers - F3_carcasses	18/12/2017 - 16/01/2018 - 24/01/2018	1d - 30d
	F4_broiler chicks - F4_broilers - F4_carcasses	19/12/2017 - 16/01/2018 - 24/01/2018	1d - 29d

[a] F1-F4, Farm 1-4

[b] w, weeks; d, days



S1 Fig. Hierarchical structure of sampled farms. BrS1, PS chicks; BrS2, PS breeders; F1-4S1, farm 1-4 broiler chicks; F1-4S2, farm 1-4 broilers; F1-4Sc, farm 1-4 carcasses. amx, amoxicillin; enr, enrofloxacin; oxtet, oxytetracycline; dox, doxycycline. Samples were not collected from PS chicks of chain B

S1 File. Raw data file**.** Excel file containing the raw data of this study. Available at: https://doi.org/10.1371/journal.pone.0217174

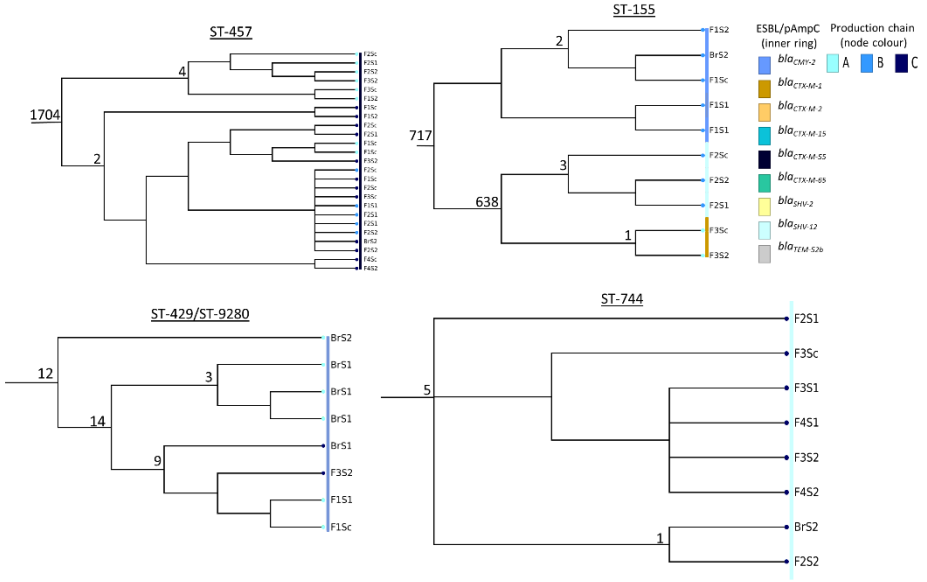
Chapter 3

Supplementary Table 1. Isolate information used for data analysis. This file containing extended tabular data is available upon request.

Chapter 4

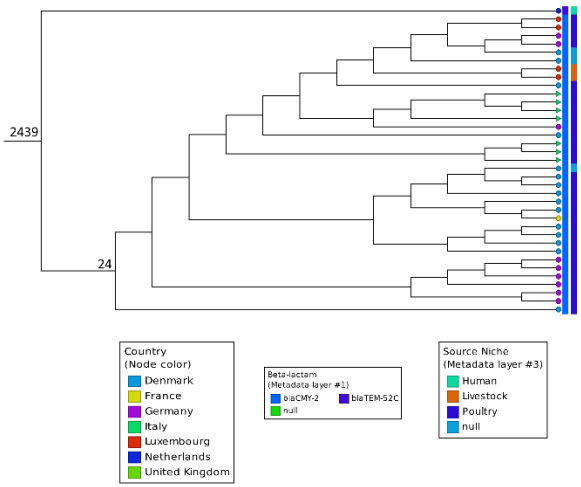
Supplementary Table 1. Information on ESBL/pAmpC isolates included in this study

Chain		Production stage		ESBL/pAmpC gene		*E. coli* phylogroup		Sample type	
A	37%	PS chicks	5%	*bla*$_{SHV-2}$	1%	A	21%	cloacal	62%
B	32%	PS breeders	8%	*bla*$_{CTX-M-15}$	1%	B1	17%	carcass	33%
C	31%	Broiler chicks	26%	*bla*$_{CTX-M-2}$	2%	B2	12%	tissue	5%
		Broilers	28%	*bla*$_{TEM-52b}$	2%	C	2%		
		Carcasses	33%	*bla*$_{CTX-M-65}$	4%	D	9%		
				bla$_{CTX-M-1}$	17%	E	9%		
				bla$_{SHV-12}$	18%	F	30%		
				bla$_{CTX-M-55}$	25%				
				bla$_{CMY-2}$	30%				

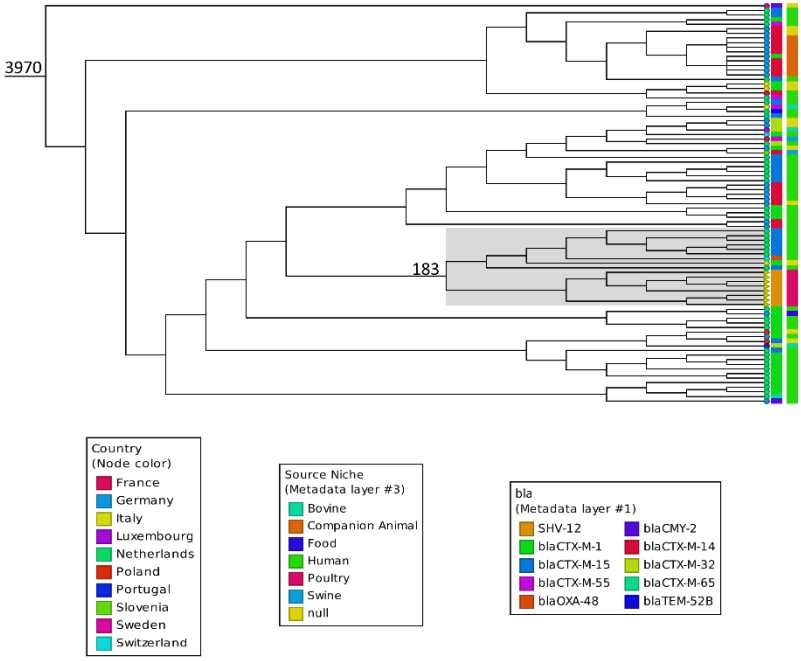


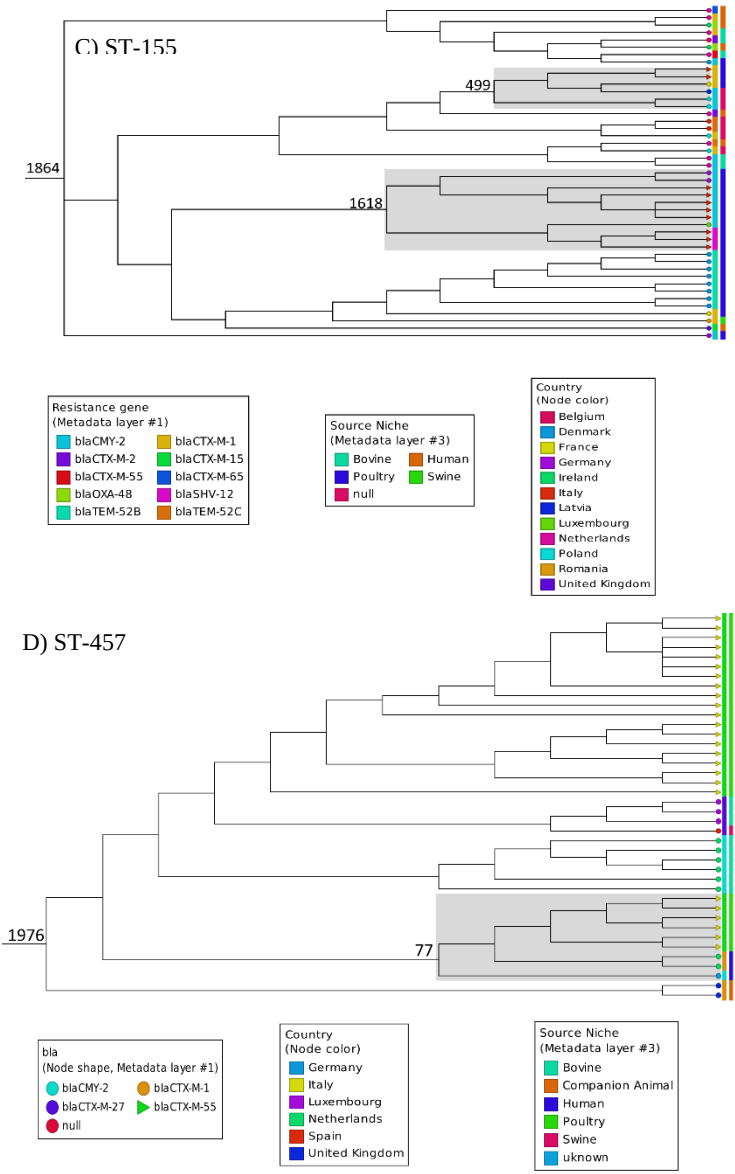
Supplementary Fig 1. Phylogenetic analysis of the most prevalent Sequence Types (STs) of this study. SNP analysis was done with CSI phylogeny. Colour-coded nodes and ribbons respectively represent the production chains and ESBL/pAmpC genes of isolates. Numbers before nodes indicate the SNPs between isolates belonging to the corresponding subtree. Text next to the nodes denotes the farm of isolates with the following coding system: BrS1, PS chicks; BrS2, PS breeders; F1-4S1, farm 1-4 broiler chicks; F1-4S2, farm 1-4 broilers; F1-4Sc, farm 1-4 carcasses.

A) ST-429



B) ST-744





Supplementary Fig 2. Phylogenetic analysis of (A) ST-429, (B) ST-744, (C) ST-155 and (D) ST-457 ESBL/pAmpC-EC isolated from various sources in Europe. Source niche, ESBL/pAmpC genes and country of origin are colour-coded according to the legend. The isolates of this study have a triangular node shape. Numbers before nodes indicate the SNPs between isolates belonging to the corresponding subtree.

Chapter 5

Supplementary Table S1. Detailed information on additional resistances and resistance genes discovered in the CST-R isolates of the reviewed studies. Available at: https://doi.org/10.1080/03079457.2018.1524573

Appendices: Contribution to other studies

Appendix 1. Occurrence and diversity of *Campylobacter* species in captive chelonians

Authors: Carlotta De Luca, Gregorio Iraola, Ilias Apostolakos, Elena Boetto, Alessandra Piccirillo

Manuscript submitted for publication

Abstract
The aim of this study was to assess the occurrence and diversity of *Campylobacter* species in chelonians. From July 2016 to September 2017, a total of 452 individuals from a large variety of tortoises (*n* = 366) and turtles/terrapins (*n* = 86) kept in private collections and breeding centres, wildlife rescue centres, zoos, pet shops, and veterinary clinics from Northern Italy, were sampled and subjected to microbiological examination. *Campylobacter* genus and species confirmation was performed by single and multiplex PCRs. Out of 452 samples, five (1.1%) tested positive: three for *C. iguaniorum* (two *Testudo graeca* and one *Testudo hermanni hermanni*), one for *C. fetus* subsp. *testudinum* (*Stigmochelys pardalis*) and one for *C. geochelonis* (*Testudo hermanni hermanni*). This study suggests that *Campylobacter* spp. are not common in chelonians but a variety of species can be detected in these hosts, including those potentially pathogenic for humans. Further studies are needed to understand the epidemiology and the pathogenic potential for both animals and humans of reptile-associated *Campylobacter* spp.

Appendix 2. Rapid detection and quantification of plasmid-mediated colistin resistance genes (*mcr*-1 to *mcr*-5) by Real-Time PCR in bacterial and environmental samples

Manuscript submitted for publication

Authors: Roberta Tolosi, Ilias Apostolakos, Lisa Carraro, Guido Grilli, Petra Cagnardi, Alessandra Piccirillo

Abstract
Colistin is an antimicrobial commonly used in animals and has recently emerged as a last-resort treatment in humans. Previous studies showed that resistance can be induced by plasmid-mediated *mcr* genes, representing a major threat for public health as these genes could be easily disseminated through horizontal gene transfer (HGT). The aim of this study was to validate a rapid method to detect and quantify plasmid-mediated colistin resistance genes (*mcr-1* to *mcr-5*) by real-time PCR in diverse matrices. We developed two new SYBR Green real-time PCR assays for *mcr-4* and *mcr-5* and optimized previously published protocols for detection and quantification of *mcr-1* to *mcr-3*. The assays could detect all five mcr genes with the lower limit of 102 copy numbers, proving to be the linear up to the limit of detection. The assays enabled the detection of colistin resistance genes not only in bacterial isolates, but also in complex samples (i.e. manure and agricultural soil), electing them as reliable tools for the detection of mcr genes directly from the environment. Our method represents a set of sensitive, rapid and effective assays that enable the detection and quantification of colistin resistance in surveillance studies.

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