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Comparison of multidrug-resistant extraintestinal pathogenic

Escherichia coli from human, foods and animals to investigate the

possible chains of transmission

IL DOTTORE   IL REFERENTE DELLøINDIRIZZO

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Abstract

Background

Globally, antimicrobial drug resistant *Escherichia coli* is the most common etiological agent of invasive disease in humans. In Europe, increasing proportions of infections due to third generation cephalosporins (3GCs) and/or *fluoroquinolone* resistant extraintestinal pathogenic *E. coli* (ExPEC) strains are reported. It has been shown that multidrug resistant (MDR) *E. coli* can be transmitted from animals to humans and based on existing evidence, poultry is the food animal source most closely linked to human *E. coli*. However, lack of reliable data makes it difficult to assess the attributable risk of different food sources and their impact on human health.

Objectives

In the present study, our objective was to investigate the antimicrobial resistance profile, phylogenetic background and virulence factors of *E. coli* isolates from broiler chicken meat sold at retail in Palermo, Italy and to compare with the geographically and temporally matched collection of isolates from humans with infections due to this bacterium.

Materials and Methods

Isolation of MDR *E. coli* was performed during April 2013-December 2014 on a total of 250 food samples including 180 raw broiler chicken meat , 17 pork meat , 14 cow meat 19 cheese and 20 ready to eat foods samples and a total of 200 de-identified FQ-R and ESBL-producing *E. coli* isolates were collected from Ospedale Civico in Palermo , Italy. Susceptibility to a panel of nine antimicrobial agents was determined and the isolates resistant to at least three classes of antibacterial drugs were defined as MDR. PCR assays were carried out to detect extended spectrum -lactamase (ESBL), plasmid-mediated AmpC -lactamase and plasmid-mediated quinolone resistance (PMQR) genes, phylogenetic group and ExPEC-associated traits. A single nucleotide polymorphism *(*SNP*)* PCR was done to detect *E. coli* sequence type (ST)131. Enterobacterial

repetitive intergenic consensus sequence PCR (ERIC-PCR) and Raman spectroscopy were performed to analyze the relatedness among ExPEC isolates.

Results

E. coli were isolated from all of the chicken samples. However, multi-drug resistant (MDR) *E. coli* were isolated from 152 (84.4%) of the 180 chicken samples. No MDR *E. coli* isolates were found in other types of food samples. B1 was the most prevalent (114 isolates, 48.1%) followed by groups D (80 isolates, 33.7%), A (30 isolates, 12.7%) and B2 (13 isolates, 5.5%); phylogenetic group B2 was the most prevalent phylogroup among clinical isolates (136 isolates, 68.5%) followed by groups D (33 isolates, 16.8%), A (20 isolates, 10.6%) and B2 (9 isolates, 4.6%), respectively. ESBLs and AmpC -lactamases were detected by PCR in 233 (98.3%) and 29 (12.2%) of chicken meat isolates and 194 (97.5%) and 34 (17.2%) of clinical isolates, respectively. PMQR determinants were detected in 215 (90.7%) of chicken isolates and 183 (91.9%) of clinical isolates, respectively. Based on the molecular definition of ExPEC, 58 and 156 isolates from chicken meat and clinical samples were attributed with the status of ExPEC . *SNP-PCR* results confirmed that a total of 91.9% (125/136) and 69.2% (9/13) of the clinical and chicken meat isolates of *E. coli* of B2 phylogroup population were identified as ST131, respectively. Both ERIC-PCR and Raman Spectroscopy analysis showed a large heterogeneity among isolates.

Conlusion

The results of this study show an alarmingly high prevalence of MDR *E. coli* and especially ExPEC isolates from broiler chicken meat in our geographic area. The ongoing use of antimicrobial drugs in livestock should be urgently revised and stopped, particularly in the poultry sector.

Declaration

I, Arash Ghodousi, declare that this thesis represents my own work and that it has not been submitted to this or other institutions in application for a degree, diploma or any other qualifications.

Signature:

Date: 13 Janurary 2016

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List of Abbreviation and Symbols

Abbreviations	
ATCC	American Type Culture Collection
CFU	Colony forming unit
CLSI	CLSI Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleotide-tri-phosphate
ESBL	Extended-spectrum beta-lactamase
MDR	Multiple Drug Resistance
MIC	Minimal inhibitory concentration
MQ	Milli-Q
NAOH	Sodium Hydroxide
PCR	Polymerase chain reaction
ST131	*Escherichia coli* sequence type 131
TBE	Tris-borate-EDTA
TE	Tris-EDTA

Symbols	
	Alpha
	Beta
°C	Degree Celsius
g	Gram
kb	Kilo base
	Lambda
L	Liter
g	Microgram
l	Microliter
µm	Micrometer
µM	Micromolar
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
min	Minute
M	Molar
N	Normal
ng	Nanogram
nm	Nanometer
	Omega
%	Percentage
rpm	Rotation per minute
s	second
U	Unit

1.Introduction

1.1. Overview on Escherichia coli

Escherichia coli (*E. coli*) is a gram negative rod bacterium belonging to the family of *Enterobacteriaceae* . *E. coli* is part of normal flora colonizing the gastrointestinal tract of both humans and animals (1). In general, *E. coli* can benefit the hosts in a way that it maintains the normal intestinal homeostasis and stability of the luminal microbial flora. This relationship between the *E. coli* and its host is symbiotic and providing both with a number of advantages. However, *E. coli* has developed into a pathogen well adapted to its host through the loss and gain of genes. Some pathogenic *E. coli* strains cause gastrointestinal illness (intraintestinal pathogenic *E. coli*), whereas others cause extraintestinal infections (extraintestinal pathogenic *E. coli* [ExPEC]) (2).

Not only an important causative agent in human diseases, ExPEC strains are responsible for significant economic losses in animal production, particularly within the poultry industry (3), as it is one of the fastest growing industries worldwide. Recently, a lot of studies have highlighted similarities between human and avian ExPEC, particularly in their virulence genes, suggesting that poultry products could serve as a possible source of ExPEC which causes blood infections in humans (4) (Fig. 1). Moreover poultry meat exhibits the highest overall levels of *E. coli* contamination, and *E. coli* strains isolated from poultry and poultry meat are often more extensively multidrug resistant (MDR) than *E. coli* recovered from other kinds of meat (4). On the other hand, the increased poultry meat consumption worldwide could have contributed to the appearance of antibiotic (ATB) resistance in ExPEC and the emergence of ExPEC infections in humans (5). Global trades and travels also contribute to the worldwide spread of these infections (6), thus making it difficult to implement infection-control measures.

 E. coli is the leading cause of urinary tract infections (UTI), whether nosocomial or acquired in the community. It also frequently causes soft tissue (e.g., peritonitis) and central nervous system (e.g.,

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neonatal meningitis) infections. The worldwide burden of these extraintestinal infections is staggering, with hundreds of millions of people affected annually and considerable morbidity and mortality in cases of complication with bacteremia or sepsis syndrome (7). Moreover, *E. coli* pathogens, particularly those causing extraintestinal infections, have developed resistance to every class of antibiotics introduced to treat human and animal infections. The prevalence of resistance to first-line oral antibiotics, such as trimethoprim-sulfamethoxazole, amoxicillin and amoxicillin plus clavulanic acid, which are widely used to treat community-acquired *E. coli* infections, has increased steadily over time(8). The release onto the market of fluoroquinolones (FQ) and extended-spectrum cephalosporins (ESC) in the 1980s increased expectations of treatment efficacy, but these hopes have been dashed. Infact, resistance to ESC due to the production of extended-spectrum - lactamases (ESBL) by *E. coli* isolates has increased steadily over the last 20 years. There is also evidence to suggest that this increase in resistance is linked to the worldwide spread, since 2008, of a specific clone of *E. coli*, *E. coli* sequence type 131 (ST131) (3, 5, 9-12).

1.1.1.General characteristics of ExPEC

ExPEC strains have acquired specific virulence attributes that confer an ability to survive in different niches outside of their normal intestinal habitat in both mammals and birds. ExPEC are phylogenetically distinct from commensal and intestinal pathogenic *E. coli*. There are four main phylogenetic groups of *E. coli*: A, B1, B2, and D. ExPEC strains belong mainly to group B2, with some extend located in group D (13). Phylogroup B2 is the most predominant and the most virulent in most cases of ExPEC infections. ExPEC possess virulence factors required for extraintestinal infections (14), with some virulence factors more specific to certain ExPEC groups, such as Tsh and ColV plasmids in avian pathogenic *E. coli* (APEC), K1 capsule in neonatal meningitis *E. coli* (NMEC), and Sat and Usp in uropathogenic *E. coli* (UPEC) (Table 1). This provides strong evidence that certain genetic backgrounds are required for the acquisition and expression of certain virulence factors. ExPEC are generally very diverse, with few common virulence factors between

them (15) . These findings imply that strains utilize different factors for similar roles during various stages of the infection process (10, 16, 17). However, other virulence factors that have not yet been identified in ExPEC and hostópathogen interaction could have a significant role in the pathogenesis of these bacteria. This assortment of virulence genes is apparently made possible by a variety of genetic factors contributing to genome plasticity, including plasmids (18, 19).
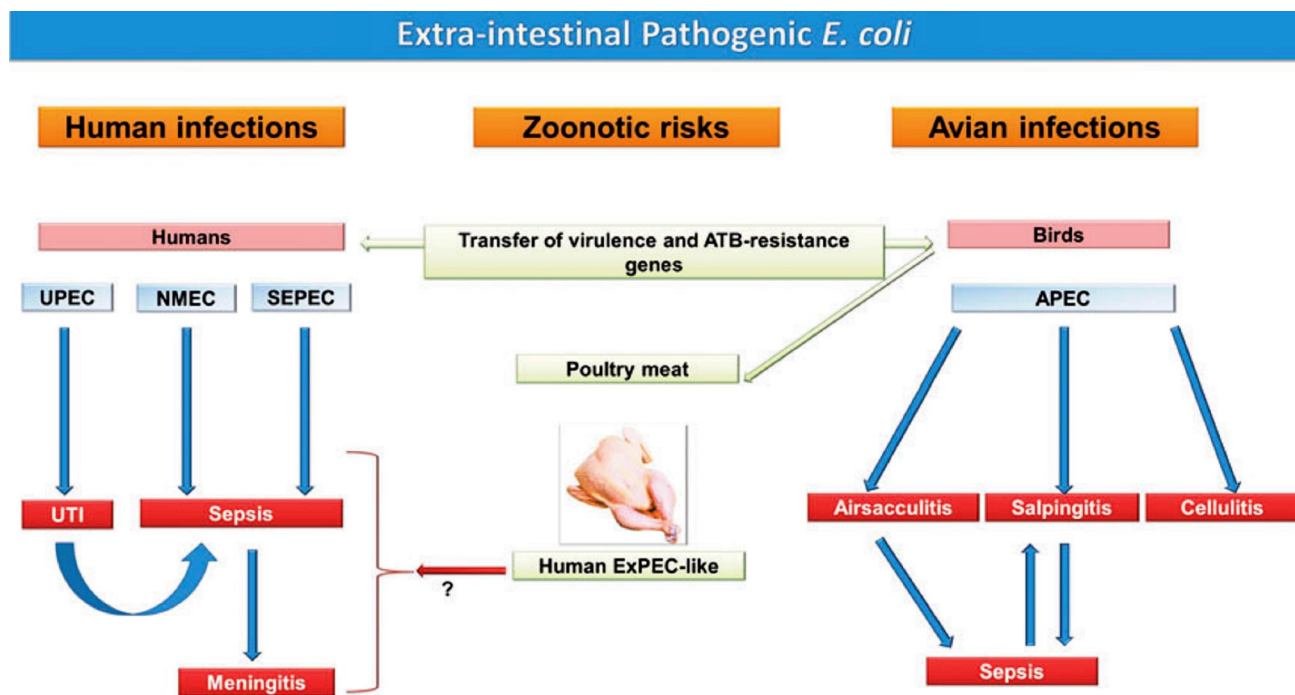
Figure 1. Avian and human extraintestinal pathogenic *Escherichia coli* (ExPEC), their infections, and zoonotic potential. The schematic diagram illustrates the major ExPEC pathotypes and their infections in humans and birds. ExPEC can cause localized infections that can become systemic (urinary tract infection [UTI] in humans and airsacculitis in birds), a systemic infection that localizes (meningitis), both a local and systemic infection (salpingitis in birds), or localized only (cellulitis in birds). The diagram also shows the potential of poultry and their products to transfer antibiotic (ATB) resistance and ExPEC to humans and cause zoonotic diseases. UPEC, uropathogenic *Escherichia coli*; NMEC, neonatal meningitis *E. coli*; SEPEC, sepsis *E. coli*; APEC, avian pathogenic *E. coli (19)*.

1.2. Biological and pathogenic characteristics of E. coli *ST131*

Following on from the initial detection of *E. coli* O25:H4, ST131 on three continents, this global clone of phylogenetic group B2 was shown, by pulsed-field gel electrophoresis (PFGE) and multiple virulence factor (VF) gene profile analyses, to consist of multiple subclones. The survival of this clone was also improved by its acquisition of various genes encoding resistance to antibiotics, including several borne on plasmids (15, 20). Many studies since 2008 have focused on these traits, in an attempt to determine the precise nature of *E. coli* ST131.

1.2.1. Phylogenetic grouping

Phylogentic analysis is revealed based on the genetic substructure of *E. coli*. Clermont *et al.* developed a simple triplex PCR based method to assign the phylogenetic groups of *E. coli* by focusing on three specific target genes (*chuA*, *yjaA* and *TSPE4.C2*) (21). The phylogenetic grouping of *E. coli* is obtained in reference to the genetic profile of these three target fragments. Phylogenetic background of *E. coli* isolates mainly originate from four phylogroups- A, B1, B2, and D. In general, phylogroups A or B1 are most likely observed in commensal *E. coli* strains. Interestingly, virulent extraintestinal *E. coli* isolates fall into mainly B2 and a minority of D phylogenetic group (21). It was proved that approximately 80-85% of phylogenetic grouping of Clermont strategy was correctly assigned (22). Therefore, this approach has been widely adopted in epidemiological research (6, 23-25). However, some *E. coli* strains, particularly A0, D1, D2 genotypes have been interpreted falsely using Clermontøs method. Hence, a modified multiplex PCR strategy has been proposed by adding an internal amplification control to improve the reliability of the results (26). Since the expansion of MLST data in recent years, more information is available for evaluation of the Clermontøs approach and understanding the genetic substructure of *E. coli*. There are now eight recognized phylogroups (A, B1, B2, C, D, E, F and Escherichia cryptic clade I) (27).

Consequently, a newly designed quadruplex PCR has been implemented. With the modification of primer sequences and newly added *arpA* gene target, phylogenetic groups C, E, F and clade I could be identified accurately. This updated quadruplex method has been confirmed to perform well with 95% *E. coli* strains accurately typed(27, 28).

1.2.2. VF-encoding genes and virotypes

Group B2 strains are known to harbor many more virulence factor (VF)-encoding genes than the other *E. coli* groups (15, 29, 30). Several studies have therefore investigated the VF gene composition of *E. coli* ST131 isolates. Key initial findings included an absence of adhesin-encoding P fimbria *pap* genes and classical group B2 cytotoxic necrotizing factor (*cnf1*) genes in intercontinental *E. coli* ST131 isolates (29). In contrast, the following VF genes have been found to be uniformly or frequently present in *E. coli* ST131 isolates: *sat* (secreted autotransporter toxin), *fimH* (type 1 fimbriae), *fyuA* (yersiniabactin receptor), *kpsM* II (group 2 capsule synthesis), *usp* (uropathogen-specific protein), *malX* (pathogenicity island marker), *iha* (adhesion siderophore receptor), *ompT* (outer membrane receptor) *iucD* (aerobactin), *iutA* (aerobactin receptor), and *tratT* (serum resistance associated) (5, 6, 29, 31, 32). All of the ST131 *E. coli* isolates investigated in these studies may be considered to be extraintestinal pathogenic *E. coli* (ExPEC), due to the presence of two (*kpsM*II and *iutA*) of the five molecular factors used to define ExPEC status (3). However, the number of VF-encoding gene profiles identified is increasing with the number of studies carried out to identify VF-encoding genes in *E. coli* ST131 isolates. These studies identified several VF-encoding genes characterizing distinct VF profiles in *E. coli* ST131 isolates, most of which can be grouped into specific PFGE clusters (5). Blanco et al. (33) referred to these VF profiles as õvirotypesö and identified a number of VF-encoding genes identifying them. Some of these genes are VF genes classically identified in non-ST131 group B2 ExPEC: *afa/draBC* (encoding Afa/Dr adhesins), *papG* (P fimbrial adhesins), and toxin genes, such as *cnf1* and *hlyA* (alpha hemolysin). The *ibeA* (invasion of brain endothelium) gene, which can be used to identify

virotype D and its subvirotypes, was previously reported in recently emerging avian O25b:H4 ST131 isolates (10). With the exception of *afa/draBC*, none of these virotype-distinguishing VF genes were found in the first strains described as ST131, even though they originated from different countries (18). This may reflect the large proportion of isolates from the recently recognized virotype C, which seems to be the most prevalent *E. coli* ST131 virotype (33, 34), in this first collection of *E. coli* ST131 isolates.

1.2.3. MLST and PFGE typing

E. coli ST131 isolates have been shown to have uniform housekeeping gene sequences across the seven multilocus sequence typing (MLST) loci (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) defined by Achtman (http://mlst.warwick .ac.uk/mlst/dbs/Ecoli), but some diversity has been found within the *E. coli* ST131 lineage in analyses of the eight MLST loci (*dinB*, *icdA*, *pabB*, *polB*, *putB*, *trpA*, *trpB*, and *uidA*) defined by the Pasteur Institute (http://www.pasteur.fr/recherche/genopole/PF8 /mlst/EColi.html). Matsumura et al. reported three different õPasteurö sequence types (PST) among O25b ST131 isolates (PST43, PST527, and PST568) and three among O16 ST131 isolates (PST506, PST566, and PST567) (34). Mora Gutierrez et al. found seven different PSTs in 23 *E. coli* ST131 isolates: PST43 corresponded to O25b isolates of virotypes A, B, and C; PST9, PST43, and PST527 corresponded to O25b isolates of virotype D; PST621 corresponded to O25b isolates of virotype E; and PST506, PST567, and PST625 corresponded to O16 isolates (10, 35). However, PFGE profiles displayed much higher levels of within-lineage genetic variation. This variation was noted during the initial description of clone ST131, together with the presence of ST131 isolates with similar PFGE profiles at distant locations and the presence of isolates with different profiles at the same site (33). Johnson et al. carried out PFGE profiling on a collection of 579 *E. coli* ST131 isolates obtained between 1967 and 2009 from diverse sources (humans, animals, and environmental samples) from different countries (5). This study identified 170 distinct pulsotypes accounting for between one (105 pulsotypes) and 136 (1 pulsotype, called õ968ö) isolates. There

were 65 pulsotypes containing multiple isolates (multiple-isolate pulsotypes), 12 of which contained at least six isolates, leading to their recognition as high-prevalence pulsotypes. Temporal occurrence profiles differed significantly between pulsotypes. Both multiple-isolate pulsotypes and highprevalence pulsotypes were found to be associated with more recent isolation. The 12 high-prevalence pulsotypes included three (968, 800, and 812) appearing sequentially in 1990 to 1999, 2000 to 2002, and 2005, respectively, identified as the top three most prevalent species overall and within each interval considered, from 1990 onwards. The prevalence of pulsotype 968 remained high after its initial emergence, whereas peaks in prevalence followed by a steep decline were observed for pulsotypes 800 and 812. Thus, although ST131 is highly diverse at the pulsotype level, this clonal lineage is dominated by a small number of highly prevalent pulsotypes. Spatial analysis showed that the broad geographic distribution of pulsotypes prevailed over local specific segregation patterns, indicating a pattern of widespread dispersal (pandemicity) rather than localized endemicity (3, 5, 36, 37).

1.2.4. *fimH* subtyping

All *E. coli* ST131 isolates harbor the *fimH* gene, like most other isolates of *E. coli* (38), which displays a remarkably high level of allelic diversity (11, 15, 39). The *fimH* typing region (*fim-HTR*) carries a highly diverse set of alleles that may be considered to be phylogenetically restricted (1, 7). This typing method was applied to clone ST131 isolates (1, 7, 34, 40). Adams-Sapper et al. typed 246 *E. coli* bacteremia isolates by MLST and *fimH* subtyping methods and showed that the three most frequent *fimH* types accounted for 96% of *E. coli* ST131 isolates (41). Johnson et al. explored the subclonal structure of 352 historical and recent ST131 isolates (1967 to 2011). They identified seven *fimH* types, with *fimH*30 the most frequent ($n=$ 236; 67%), followed by *fimH*22 ($n=$ 73; 21%), with *fimH*35 and *fimH*41 in joint third place (19 isolates each; 5%) (42). The diversity of *fimH* alleles in clone ST131 isolates sheds light on the molecular mechanisms

underlying clonal diversification.

1.2.5. Screening methods for detecting the *E. coli* ST131 clone and subclones

The proposed screening methods are based on the molecular diversity observed within *E. coli* and within *E. coli* ST131isolates. Once an *E. coli* isolate has been assigned to group B2 by the multiplex PCR methods developed by Clermont et al. (21, 27), other single-nucleotide polymorphism (SNP)-based methods can be used to determine whether the isolate concerned belongs to the ST131 lineage. Specific-allele PCR of the 5ø portion of the *rfb* locus can detect the most common O serogroups, including the allele specific for O25b (6). Other methods make use of so-called õST131-specificö alleles of genes used in the MLST methods designed by Achtman and the Pasteur Institute. For example, Clermont et al. described a specific-allele ST131 PCR based on SNPs of the *pabB* gene included in the Pasteur Institute MLST method (34). Johnson et al. suggested a method for *E. coli* ST131 screening based on ST131-associated SNPs (sequencing method) of the *mdh* and *gyrB* genes included in Achtmanøs MLST method (5). Weismann et al. proposed the use of an ST131-associated SNP of the *fumC* gene (Achtmanøs MLST method) for detecting clone ST131 and the use of *fimH* sequencing for the detection of subclones of ST131 *E.coli* isolates (32). This method, known as CH clonotyping, was successfully used for the direct testing of urine samples (43). Blanco et al. proposed two triplex PCRs. The first was based on the detection of O25b (O25b *rfb* allele) *E. coli* producing CTX-M-15 (encoded by the 3ø end of the *bla*CTX-M-15gene) and harboring the *afa/draBC* gene, a VF gene specific to virotype A (32). The second was based on the detection of VF genes specific for virotypes B, C, and D (*iroN*, *sat*, and *ibeA*, respectively) (24). The CH clonotyping method appears to be the most relevant of the methods described, as it can detect *E. coli* ST131 and distinguish between the two serogroups of this lineage identified to date: O25b (*fimH*30) and O16 (*fimH*41). In conclusion, various bacteriological analyses have revealed the existence of diversity within the *E. coli* ST131 lineage and have shown that subclones are characterized by combinations of bacterial traits (Figure 1).
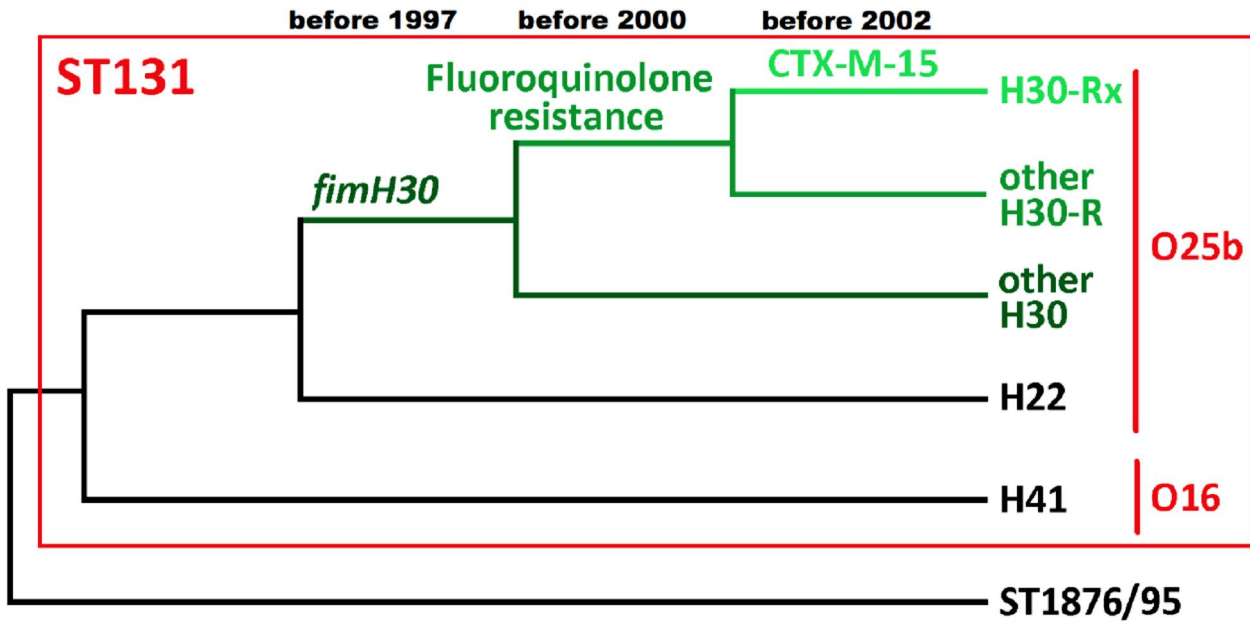
Figure 2. Schematic dendrogram of ST131 phylogeny reconstructed using whole-genome single nucleotide polymorphism analysis (1).

1.3.Antibiotic Resistance

1.3.1.Phenotypic antibiotic resistance in clinical isolates of *E. coli* ST131.

Two traits were identified as common to all the intercontinental *E. coli* ST131 isolates initially described: resistance to ESC, due to the production of ESBL CTX-M-15, and resistance to fluoroquinolones (1, 7). Subsequent studies aiming to detect *E. coli* ST131 isolates in other countries frequently identified these associated traits. However, some studies also showed a particularly high prevalence of clone ST131 among non-ESBL-producing, fluoroquinolone-resistant *E. coli* isolates (40, 44). This suggests that CTX-M enzymes may have been acquired by *E. coli* ST131 isolates that were already resistant to fluoroquinolones. One study carried out in remote northern Saskatchewan communities (Canada) showed that clone ST131 was the second most prevalent clone, after ST95, which was the most prevalent clone among the ESC- and fluoroquinolone-susceptible urine isolates studied (45).

Such antibiotic-susceptible *E. coli* ST131 isolates have also been identified in the dominant fecal *E. coli* populations of healthy subjects living in the Paris area (45). It is, therefore, clear that antibiotic-susceptible *E. coli* ST131 isolates exist. A very small number of studies (29, 32)have provided information about resistance to various antibiotic families in *E. coli* ST131 isolates, comparing the results obtained with those for non- ST131 *E. coli* isolates producing or not producing ESBL. For ESBL-producing isolates, these studies showed that *E. coli* ST131 isolates were consistently more frequently resistant to amikacin than non-ST131 isolates and potentially more frequently resistant to amoxicillin-clavulanic acid, piperacillin-tazobactam, or ciprofloxacin; they were also more frequently susceptible to gentamicin or co-trimoxazole than non-ST131 isolates, depending on the country considered. For isolates that did not produce ESBL, *E. coli* ST131 isolates were systematically found to be more frequently resistant to quinolones/fluoroquinolones and to ampicillin/amoxicillin than non- ST131 isolates. Resistance to ampicillin/amoxicillin in *E. coli* isolates is widely known to be mediated principally by the production of plasmid-encoded TEM-1/-2, SHV-1, or OXA-1 enzyme. The *bla*TEM-1 and *bla*OXA-1 genes were commonly found associated with the *bla*CTX-M gene on plasmids of the IncF type. This have gone on to acquire the genes encoding the CTX-M enzymes. The *E. coli* ST131 isolates resistant to fluoroquinolones and producing IncF-mediated TEM-1 and/or OXA-1 may be the ancestors of the current CTX-M-producing *E. coli* ST131 isolates found worldwide.

1.3.2.Characterization of plasmids harbored by clinical isolates of *E. coli* ST131.

Various plasmids, differing in incompatibility groups (Inc), conjugative transfer, size, replicon types, and *bla* genes, have been characterized in *E. coli* ST131 strains of different origins. These plasmids include IncF plasmids, which have a host range limited to *Enterobacteriaceae* and are known to contribute to bacterial fitness through their virulence and antimicrobial resistance determinants (46, 47). IncF plasmids were the most common. Three IncF plasmids harbored by three epidemic *E. coli* ST131 strains (strains A, C, and D) from the United Kingdom were

completely sequenced (41, 44, 48) plasmids of the IncF family, which has a complex structure, have clearly played a major role in the dissemination of the *bla*CTX-M-15 gene expressed by *E. coli* ST131 strains. However, *E. coli* ST131 strains can harbor IncF plasmids encoding ESBL other than CTX-M-15, such as CTX-M- 14, SHV-2, and SHV-12 in particular, and they can even harbor CTX-M-15-encoding plasmids from families other than the IncF family. In particular, they can carry resistance genes on plasmids from the IncI1, IncN, and IncA/C families or on *pir*-type plasmids. The *pir*-type plasmid pJIE143, first identified in a communityacquired Australian *E. coli* ST131 isolate in 2006, has been fully sequenced and shown to be organized similarly to plasmids in the narrow-host-range IncX groups found in *Enterobacteriaceae* (32). No resistance-associated gene other than *bla*CTX-M-15 has been identified on pJIE143. CTX-M-1 enzymes may also be encoded by IncI1 (France) and IncN (Norway and Germany) plasmids, whereas CTX-M-3 (the United Kingdom) and CTX-M-65 (Germany and China) enzymes may be encoded by IncN plasmids. CMY-2, the plasmid-encoded cephalosporinase most frequently identified in *E. coli* ST131 to date, is carried by an IncI1 plasmid. Finally, although the *bla*NDM-1 gene has most frequently been detected on broad-host-range plasmids, such as IncA/C plasmids, particularly in clinical or environmental isolates from the New Delhi area (49), it has also been found on an IncFII plasmid (pGUE-NDM) in an *E. coli* ST131 isolate. This isolate was obtained in France, from a patient returning home from Darjeeling (India), where she had lived for several years without hospitalization (50). Peirano et al. reported similar findings for a patient admitted to a hospital in Chicago after hospitalization in New Delhi. However, in the New Delhi *E. coli* ST131 strain, NDM-1 was harbored by a larger IncF plasmid carrying the FIA replicon (51). The complete genome sequence of pGUE-NDM showed that *bla*NDM-1 was acquired by a plasmid resembling those previously reported to harbor *bla*CTX-M-15. These findings are particularly alarming given the success with which *E. coli* ST131 has disseminated *bla*CTX-M-15 on IncF plasmids. Finally, one *E. coli* ST131 isolate has been found to contain a plasmid (pJIE186-2) harboring only VF genes classically carried by the chromosome (52)and a second plasmid of the same incompatibility group

(IncF) harboring the classical resistance-associated genes identified in *E. coli* ST131 (*bla*CTX-M-15, *bla*OXA-1, *bla*TEM-1, *aac6ϕ-Ib-cr*, and *aac3-II*).

1.3.3.Molecular epidemiology of resistance in clinical isolates of *E. coli*.

An extensive review of studies of resistance mechanisms in *E. coli* ST131 isolates revealed that CTX-M enzymes were by far the most frequent ESBL. The most prevalent of these enzymes was CTX-M-15, which currently has a worldwide distribution. The production of other CTX-M-type enzymes in ST131 *E. coli* isolates has been documented for CTX-M-1, -2, -3, -9, -10, -14, -18, -24,-27, -28, -32, -39, -52, -55, -65, and 103 (8, 9, 16, 23, 39, 51, 53). *E. coli* ST131 isolates producing a number of these enzymes seem to be more frequent in particular countries: ST131 producing CTX-M-14 is particularly common in Canada, China, Japan, and Spain; ST131 producing CTX-M-3 is particularly frequent in the United Kingdom, and ST131 producing CTX-M-27 is common in France, Japan, and Switzerland (8, 39, 43, 45, 54-56). Other non-CTX-M ESBL Ambler class A enzymes have also been described in *E. coli* ST131 isolates. These enzymes include derivatives of the SHV family (mostly SHV-2 and SHV-12) (4, 33, 57-59) and, based on anecdotal evidence, derivatives of the TEM family (TEM-24 and -52) (13, 24). The class A carbapenemase KPC-2 has been found in *E. coli* ST131 isolates from the United States (seven isolates), France (one isolate), Ireland (one isolate), and China, where they recently caused outbreaks (3, 10, 60). Ambler class B enzymes from *E. coli* ST131 isolates have been reported in only a few studies: NDM-1 (two cases from India) (31, 60), VIM-1 (one case in Italy) (61), and IMP-8 (one case in Taiwan) (62). Ambler class C enzymes have been detected more frequently than class B enzymes in ST131 isolates. The class C enzymes detected include CMY-2 (63) and CMY-4 (64), with DHA-1 described only rarely (65). Finally, Ambler class D -lactamases, such as OXA-48, have only rarely been found in ST131 isolates (66). Two narrow-spectrum -lactamases, OXA-1 and TEM-1, have frequently been found in ST131 isolates, generally in association with CTX-M-15 enzymes, following plasmid transfer (see õCharacterization of plasmids harbored by clinical isolates of *E. coli* ST131ö above) (10, 18,

66, 67). Another resistance gene, *aac(6')-Ib-cr*, which confers resistance to both aminoglycosides (amikacin and tobramycin) and ciprofloxacin, has frequently been detected in association with CTX-M-15 enzymes (23, 43, 57, 65). Only a few studies in Spain and Portugal have reported the detection of *qnr* determinants another plasmid-mediated mechanism of quinolone resistance in ST131 isolates (24, 68, 69). The main mechanism reported to confer resistance to fluoroquinolones in *E. coli* ST131 isolates is amino acid substitutions within the quinolone resistance-determining regions (QRDR) of GyrA and ParC, the targets of quinolones/fluoroquinolones (2, 29, 32, 36). These reports clearly indicate that all the resistance mechanisms acquired by *E. coli* ST131 isolates to date, with the exception of fluoroquinolone resistance, are plasmid mediated. However, one or several copies of the *bla*CTX-M-15 gene have been detected on the chromosome (2, 29, 32, 36, 39, 59, 70, 71). Some strains with chromosomally encoded CTX-M-15 harbor an IncFII-type plasmid, but without the *bla*CTX-M-15 gene. This suggests that the *bla*CTX-M-15 gene may have been transferred from the plasmid to the chromosome (12).
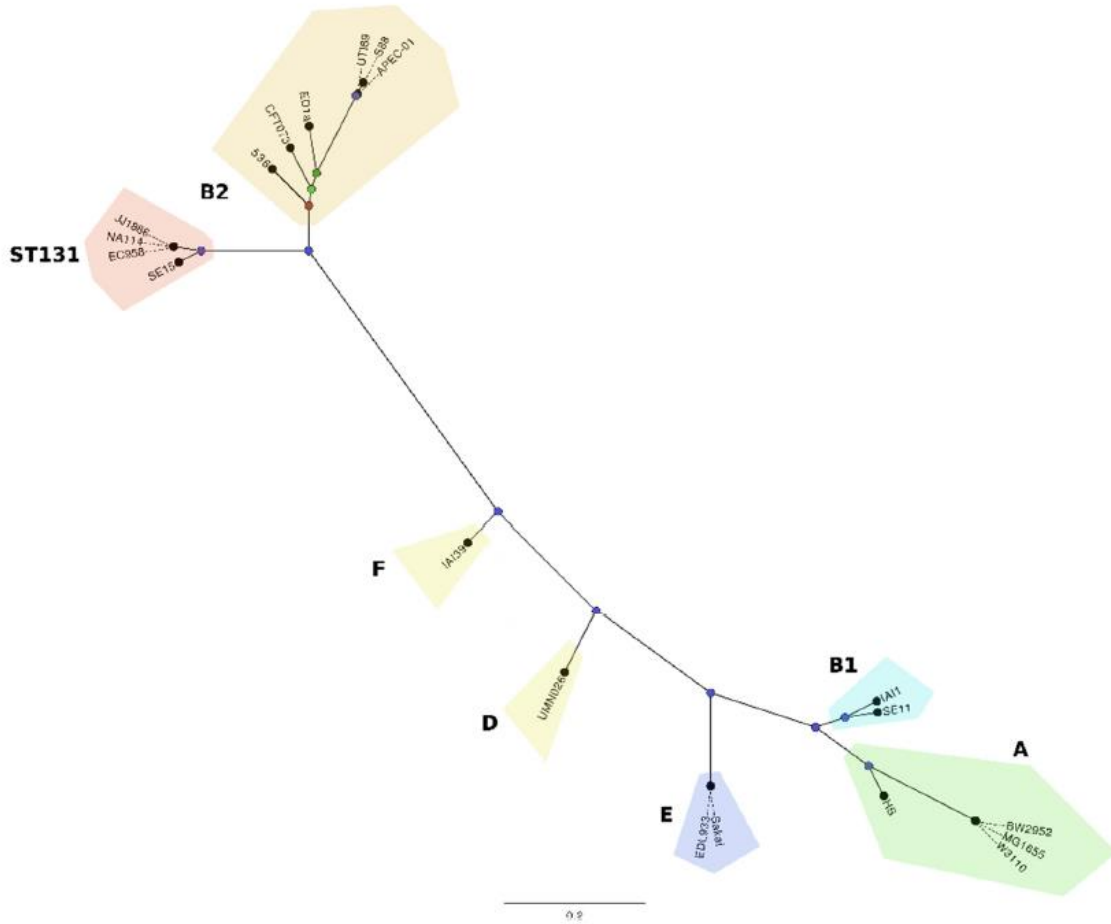
Figure 2. Maximum likelihood phylogenetic comparison of ST131 strains EC958, JJ1886, NA114 (clade C) and SE15 (clade A), and 16 representative strains from other *E. coli* phylogroups. The phylogenetic relationships were inferred with the use of 70,777 SNPs identified between the genomes of the 20 *E. coli* strains and 1000 bootstrap replicates. The major *E. coli* phylogroups are coloured as follows; group B2-ST131: (red); group B2 non-ST131: APEC-01, S88, 536, UTI89, CFT073, ED1A (orange); group D: UMN026, IAI39 (yellow); group A: BW2952, MG1655, W3110, HS (green); group B1: SE11, IAI1 (aquamarine); group E: O157 EDL933, O157 Sakai (blue). Nodes are coloured according to bootstrap support for branching at that node: 1000 (blue), 858 (dark green), 770 (light green),659 (red). The Figure is adapted from Forde *et al*. 2014 (72).

1.4.Pathogenic Characteristics

1.4.1.Infection spectrum.

E. coli ST131 strains cause community- and hospital-acquired UTI (cystitis and pyelonephritis) and bacteremia worldwide (11, 61, 73). They have also been reported to cause other types of infection: intra-abdominal and soft tissue infections, meningitis, osteoarticular infection, myositis , epididymo-orchitis, and septic shock (11, 61, 73). This spectrum of infections, typical of ExPEC, has increased the degree of concern about ST131, which is already considered a major potential problem due to its multidrug resistance.

1.4.2.Transmissibility.

ST131 transmission has essentially been documented between members of the same household and between family members and pets (dogs and cats in particular) (74). Ender et al. provided strong evidence of the transmission of a CTX-M-15-producing ST131 isolate resistant to gentamicin, trimethoprim- sulfamethoxazole, and fluoroquinolones between a father and his daughter (75). The father was admitted to a hospital for pyelonephritis due to a clone ST131 strain, where he was visited by his adult daughter. She used his bathroom during the visit and subsequently developed emphysematous pyelonephritis, renal abscess, bacteremia, and septic shock due to the same ST131 strain, which appeared to be particularly virulent. However, the VF gene profile of this strain identified it as a classical virotype A strain. Johnson et al. provided novel evidence of the within household transmission of an ST131 strain between an infected patient (an 8-month-old girl with an osteoarticular infection) and another previously healthy member of the same family (the girløs mother). The same ST131 strain was detected in the digestive tracts of both patients (76), but it remains unclear in which direction the infection was transmitted. In this case, the ST131 strain was a fluoroquinolone-resistant strain that did not produce an ESBL.

1.4.3. Pathogenesis.

Various studies have investigated the virulence potential of *E. coli* ST131.

(i) Biofilm production and metabolic potential. Very few studies have investigated the biofilm production and metabolic potential of *E. coli* ST131 isolates, but the results of these studies are concordant. Clermont et al. found that the two ST131 strains studied produced a biofilm after 48 h, and Kudinha et al. found that the prevalence of isolates producing biofilms was greater among ST131 *E. coli* isolates than among non-ST131 clinical isolates (77).

(ii) Adhesion and colonization abilities. Martinez-Medina et al., who characterized the similarity and divergence of adherent invasive *E. coli* (AIEC) and ExPEC strains, found that one of the 12 ExPEC ST131 strains tested had an AIEC phenotype and displayed 50% similarity to two other ST131 AEIC strains (78). The VF genes harbored by these three strains were different, suggesting that ST131 AIEC strains, like other AIEC strains, have virulence-specific features that can currently be detected only phenotypically. These features include an ability to adhere to and invade intestinal epithelial cells and an ability to survive and replicate within macrophages.

1.5. Genomics of E.coli *ST131*

The entire genome sequences of 13 *E. coli* ST131 strains are currently available (48). In 2011, Avasthi et al. (79) published the chromosome sequence of strain NA114, a typical uropathogenic *E. coli* ST131 isolate from the city of Pune in Western India (80), and the sequence of the single 3.5-kb plasmid harbored by this strain. The NA114 chromosome was 4,935,666 bp long, with a GC content of 51.16% and a coding percentage of 88.4%. It had 4,875 protein-encoding sequences, 67 tRNAs, and three rRNA genes. The authors limited their comments on this genome essentially to confirmation of the presence of the virulence- associated genes classically identified by PCR in *E. coli* ST131 and the detection of several genes rarely identified in *E. coli* ST131 isolates (*cnf1*, *sfa*, and *aer*) and an intact polyketide synthetase island. Andersen et al. (48)recently reported the complete genome of strain JJ1886, a uropathogenic strain (81) considered representative of the

epidemic and highly virulent CTX-M-15-producing *H*30-Rx subclone of *E. coli* ST131. This complete genome corresponds to a 5,129,938-bp chromosome with a GC content of 50.8%, 5,086 protein-encoding sequences, 88 tRNAs, and 22 rRNA genes plus five plasmids of 1.6, 5.2, 5.6, 56, and 110 kb in size. Again, few analytical data have been reported for this genome sequence. However, the authors indicated that only the largest plasmid (110 kb) carried genes for antibiotic resistance. The *bla*CTX-M-15 gene was found to have been integrated into the strain JJ1886 chromosome by the insertion of an incomplete Tn*3* element into a lambda-like prophage. Further analyses are required to clarify the differences and similarities between strains NA114 and JJ1886, but the data already available indicate some differences between these strains.

1.6.Epidemiology of E. coli STI131

1.6.1.Global Dissemination of *E. coli* ST131

Following the initial identification of *E. coli* ST131 in 2008 in a limited number of countries on three continentsô North America (Canada) Europe (France, Portugal, Spain, Switzerland), and Asia (India, South Korea, Kuwait, and Lebanon) (16, 17)ô this clone was successfully detected in many other countries on these three continents and on the two remaining continents, Africa and Oceania (Figure 3).

Figure 3. Global dissemination of *Escherichia coli* ST131 clone (2013). Articles which mentioned for the first time the presence of clone ST131 in a given country or a given location are cited. Red stars indicate isolates producing ESBL enzymes, and blue stars indicate fluoroquinolone-resistant, non-ESBL-producing isolates (11).

Overall, depending on the country considered, *E. coli* ST131 has accounted for 12 to 27% of all *E. coli* isolates causing infections in the general population over the last 10 years. Among UTI *E. coli* isolates, the prevalence of clone ST131 varied with age: <10% in children, 13% in women of reproductive age, and >20% in elderly patients. Banerjee et al. showed, with unselected isolates, that *E .coli* ST131 predominated in adults over the age of 50 years and that the prevalence of infections with this clonal group increased with age (11). If only ESBL-producing or fluoroquinolone-resistant *E. coli* isolates were considered, the prevalence of clone ST131 was generally much higher, varying from 20 to 66% among ESBL producers and from 10 to 72% among fluoroquinolone-resistant isolates, depending on the country, type of infection, and subpopulation considered.

1.6.2.Molecular epidemiology of *E. coli* ST131 and *E. coli* ST131 subclones.

The relationships between virotypes and phenotypic, genotypic, epidemiological, or clinical traits have been established (9). All isolates of virotypes A and B and 63% of those of virotype C produced CTX-M-15, whereas none of the virotype D isolateswere found to produce this enzyme (*P*<0.001 for all comparisons with virotype D). In contrast, virotype D isolates produced group CTX-M-9 enzymes, SHV-12, and CTX-M-32. Ciprofloxacin resistance was significantly associated with virotype A, B, and C isolates. A cross analysis of pulsotypes and virotypes revealed four major clusters, which were largely virotype specific. All virotype A, B, and C isolates considered contained *fimH*30 and an ISL*3*-like transposase in the *fimB* gene, whereas all the virotype D isolates studied had a *fimH*22 gene and no ISL*3*-like transposase in the *fimB* gene. The associations of the four virotypes with demographic data and with the acquisition and type of infection have also been explored. Virotype B has been shown to be significantly associated with older patients and a lower likelihood of symptomatic infections, specifically for urinary tract infection, whereas virotype C was significantly associated with a higher likelihood of symptomatic infection. Virotype D was significantly associated with younger patients and community-acquired infections. Virotypes A and B displayed a significantly stronger association with nursing home residents than did virotypesCand D. Various teams have applied similar epidemiological approaches to ST131 subclones *H*30, *H*30-R and *H*30-Rx (15, 29, 33, 80).

1.7.Food Animals and Antimicrobials: Impacts on Human Health

For many decades, antibiotic resistance has been recognized as a global health problem. It has now been escalated by major world health organizations to one of the top health challenges facing the 21st century (3, 4, 35, 59, 74). Some of its causes are widely accepted, for example, the overuse and inappropriate use of antibiotics for nonbacterial infections such as colds and other viral infections and inadequate antibiotic stewardship in the clinical arena (59). But the relationship of drug-

resistant bacteria in people to antibiotic use in food animals continues to be debated (4, 13, 24, 35, 59, 68, 82).

1.7.1. Zoonotic Potential of ExPEC.

In recent years, much attention has been directed toward controlling zoonotic infections, which remain a major worldwide health concern.Meat and eggs are known to be a source of human pathogens such as Campylobacter, Listeria, and Salmonella, which frequently leads to a food recall of the suspected contaminated products (83). Recent studies on the zoonotic risk of ExPEC have prompted the Centers for Disease Control and Prevention to release information reports to caution the public on the zoonotic potential of ExPEC and their eventual transmission through chicken meat (23, 55, 59). The zoonotic risk of APEC isolates was initially related to the fact that some human and avian ExPEC have similar phylogenic backgrounds and share some virulence genes (4). The sequencing of the genome of the APEC strain O1:K1:H7 revealed that it is highly similar to human UPEC and NMEC (37). Interestingly, a comparison of a large number of ExPEC from human and chicken diseases for their phylogenetic background and the presence of virulence-associated genes has shown that although most isolates fall into genetically distinct pathotype groups (APEC, NMEC, and UPEC), with distinguishable characteristics, the study identified a genotyping cluster that includes ExPEC with overlapping traits and was considered potentially zoonotic (9). The role of poultry as a source of human ExPEC (Fig. 1) is suggested by multiple epidemiological studies that reveal the presence of avian ExPEC in both the intestines of healthy poultry and poultry meat from retail markets, strains that are often genetically similar to those found to be responsible for human infections (4). Johnson et al. (25) demonstrated that 92% (180/195) of poultry meat samples tested were contaminated with E. coli, with 46% (83/180) of strains having virulence factors associated with ExPEC and 15.6% (28/180) identified as UPEC. However, according to recent reports, not all ExPEC strains have zoonotic potential. A subset of ExPEC strains from specific

clonal groups, including ST95 and ST23, could have a broad host range distribution and cause diseases in both humans and chickens (10, 84).

The claims that ExPEC have zoonotic potential are reinforced by the experimental evidence on the ability of human ExPEC to cause diseases in chicken models for colibacillosis and avian ExPEC to cause infections in animal models of human infections (52, 84).

Zhao et al. (52) have determined that UPEC and APEC strains sharing the same virulence gene profiles caused lesions of colibacillosis in chickens and showed the same tendency of gene expression, including iron acquisition, in a murine model of human UTI. The correlation between E. coli UTI in humans and poultry meat consumption is strongly reinforced by recent investigations that have shown that *E. coli* B2 isolated from meat and intestines of healthy chickens are able to cause infection in a murine model of human UTI (85). Moreover, B2 *E. coli* fromUTI patients, poultrymeat, and healthy chickens exhibiting high virulence genotypes were clonally related and were virulent in a mouse model of UTI (86). The zoonotic risk of ExPEC appears to be mainly related to their large plasmids. Growing evidence shows that APEC plasmids could be a source of virulence genes for other ExPEC strains (68, 87). Studies have shown that UPEC and APEC isolates have certain genes in common that are associated with large transmissible plasmids of APEC (88). Some virulence genes associated with APEC plasmids (aerobactin, salmochelin, and sit operons) also occur on plasmids of UPEC (89). Additionally, APEC and NMEC have virulence genes of ColV plasmids in common (90), and APEC plasmids in E. coli can contribute to the pathogenicity of urinary infection in mice and meningitis in rats (6).

1.7.2. ExPEC Infections in Poultry: Impact on Poultry Industry.

Bacterial infections due to APEC, a subgroup of ExPEC (Fig. 1), are responsible for significant, worldwide economic losses for the poultry farms (91), which is considered one of the most important industries in many countries, including the United States, Brazil, and China. APEC

strains cause multiple systemic infections in birds, commonly referred to as avian colibacillosis (Fig. 1). While the intestines and the environment serve as reservoirs for APEC (75), the clinical outcome of APEC infection in birds depends on the bacterial strain, the host, the route of infection, and predisposing environmental factors. Similar to most other pathogens, APEC strains take advantage of host weaknesses to cause infections in chickens, turkeys, and other avian species. APEC infection can lead to septicemia, fibrinous lesions of internal organs (airsacculitis, pericarditis, perihepatitis), and death. APEC strains also cause local infections in birds, such as cellulitis, salpingitis, synovitis, and omphalitis (40). The main clinical signs associated with most of these infections are depression, fever, yellowish or greenish droppings, and lesions of internal organs. *E. coli* infections lead to a 1610% mortality rate in chickens, with even higher mortality rates in broilers (23, 92-94)and commercial organic chickens (95).

1.7.3 Animal- and Food-Borne *E. coli* ST131.

There have been very few reports on *E. coli* ST131 from either animals (healthy or sick) or foods. Prospective, targeted cohort studies are rare in veterinary medicine, so many of the published data were obtained in passive surveys or with opportunistic, nonrepresentative sampling methods. The prevalence of animal colonization (intestinal carriage) or infection with ST131 *E. coli* isolates, regardless of their susceptibility/resistance status for ESC and/or fluoroquinolones (FQ), therefore remains unclear (86, 96). In such a context, it is difficult to assess the contribution of animal *E. coli* ST131 to the global expansion of *E. coli* ST131 in humans (including the differential expansion of certain ST131 subclones).

1.7.4. *E. coli* ST131 in food animals and foodstuffs.

Only a few studies have investigated the presence of *E. coli* ST131 in food animals. One FQ-susceptible, non-ESBL-producing *E. coli* ST131 isolate was identified among 101 (0.9%) *E. coli* isolates from healthy chickens and turkeys in Italy (97). Schink et al. (98) identified one *E. coli*

ST131 isolate among 22 ESBL producers in a collection of 1,378 *E. coli* isolates from various animals (mostly pigs, poultry, and cattle). A single *E. coli* ST131 isolate was found in a pig with a gastrointestinal infection. This *E. coli* ST131 isolate harbored an IncN plasmid encoding CTX-M-1 (98). *E. coli* ST131 isolates producing CTX-M-9 have occasionally been recovered from poultry feces. In some of these instances, the animal isolates have presented a certain similarity to human ST131 isolates (99). Mora et al. (35) reported on CTX-M-9-producing *E. coli* ST131 among poultry *E. coli* isolates collected in different countries during different time periods. Three (0.2%) of the 1,601 *E. coli* isolates collected from diseased poultry in Spain, France, and Belgium between 1991 and 2001 belonged to clonal group ST131, none produced ESBL, and two were resistant to quinolones. One (1.8%) of the 57 fecal *E. coli* isolates collected from healthy chickens in Spain in 2003 was an FQ-susceptible, CTX-M-9-producing *E. coli* ST131 isolate. Finally, seven (1.5%) of the 463 *E. coli* isolates collected from diseased chickens in Spain between 2007 and 2009 were *E. coli* ST131, and two isolates produced CTX-M-9 and were susceptible to FQ (35). *E. coli* ST131 was also found in a pig in Denmark (100). In contrast, no ST131 isolates were identified by Wu et al. among 39 clinical isolates of *E. coli* from cattle, sheep, chicken, and pigs (101). Randall et al. also identified no such isolates among 388 broiler chicken cecal samples and 442 turkey rectal swab samples (102). *E. coli* ST131 was not found in cases of mastitis in cattle or in veal calves in the Netherlands (103) and France (104). The O25b PCR excluded the presence of *E. coli* ST131 from 896 commensal *E. coli* isolates from 326 pigs, 316 chickens, 88 cattle, 58 ducks, 22 geese, 61 pigeons, and 25 partridges in China (105). *E. coli* ST131 also appears to be very rare in foodstuffs of animal origin (11).

1.8. Epidemiological Study of ExPECs

Bacterial genomes are generally considered to be streamlined, and yet numerous families of short (30ó150 bp) interspersed repetitive sequences have been described in bacteria. Little is known about the origins, evolution, mode of generation, or possible function of these elements. Most families are

restricted to single species or very closely related species, while many other species appear to have no such elements. This suggests that if these repeats have any functions they have been acquired recently, may not apply to all members of the family, and are unlikely to concern fundamental aspects of bacterial growth, survival, and replication. Thus, while some repetitive sequences have been reported to act as binding sites for a variety of proteins, including DNA polymerase and DNA gyrase (64), this may be incidental. Most short bacterial repetitive sequences are imperfect palindromes, with the potential to form secondary structures, which may enhance mRNA stability(106). Alternatively, most repetitive elements may be nonfunctional junk.

1.8.1. Enterobacterial repetitive intergenic consensus sequences

Enterobacterial repetitive intergenic consensus (ERIC) sequences, also described as intergenic repetitive units, differ from most other bacterial repeats in being distributed across a wider range of species. ERIC sequences were first described in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium , and other members of the Enterobacteriaceae, as well as *Vibrio cholerae* (107, 108). The ERIC sequence is an imperfect palindrome of 127 bp (figure 4). In addition, shorter sequences produced by internal deletions have also been described, as well as longer sequences due to insertions of about 70 bp at specific internal sites. ERIC sequences have been found only in intergenic regions, apparently only within transcribed regions. The number of copies of the ERIC sequence varies among species: it was initially estimated by extrapolation that there may be about 30 copies in *E. coli* K-12 and perhaps 150 *S. enterica* Typhimurium LT2, while the genome sequence of *Photorhabdus luminescens*has been reported to contain over 700 copies (109).

Figure 4. The ERIC sequence. The 127-bp sequence is shown as a hairpin; lines (and colons) connect bases in the two arms complementary in DNA (and in RNA) (109).

1.8.2. Raman Spectroscopy.

The control of outbreaks is essential both in hospitals and other healthcare practices as well as in the community. Frequently, molecular typing methods are used to establish the clonal relationships between isolates and confirm the clinical or epidemiological data, thus supporting the decisions in an outbreak situation. A suitable typing technique should have complete typability, be timely, cost-effective, reproducible, and have the correct discriminatory power (110). In a putative outbreak situation the latter is a crucial determinant of a typing method because over-discrimination will result in missed relationships while under-discrimination results in clustering of potentially unrelated strains and thus unnecessary interventions. However, some bacterial species are highly clonal and most routine typing techniques lack the discriminatory power to allow for reliable typing (111, 112), for example, the molecular typing of *Escherichia coli* is for this reason notoriously difficult. Raman spectroscopy of bacterial samples is usually performed by a modified light microscopic device (Figure 5). The sample consists of a dried suspension from a bacterial culture and is illuminated with laser light. This will generate a Raman signal that can easily be separated from the laser light by an optical filter that only allows the Raman spectra to pass. These filtered signals are captured by a simple camera device and images are usually stored and analyzed on a small personal computer. As Raman spectra of bacteria are representations of their overall molecular composition (both nucleic and fatty acids, proteins, and carbohydrates) they can be used as highly specific spectroscopic fingerprints of the total cell content. Since Raman spectroscopy typing is based on the analysis of the total bacterial composition (and not only part of its DNA composition), it may therefore provide sufficient discriminatory capability for typing of closely-related microbes such as *E. coli*. However, a systematic review of literature only showed results for typing of E. coli using Raman spectroscopy in a preliminary retrospective study in a research-based setting (110-112).
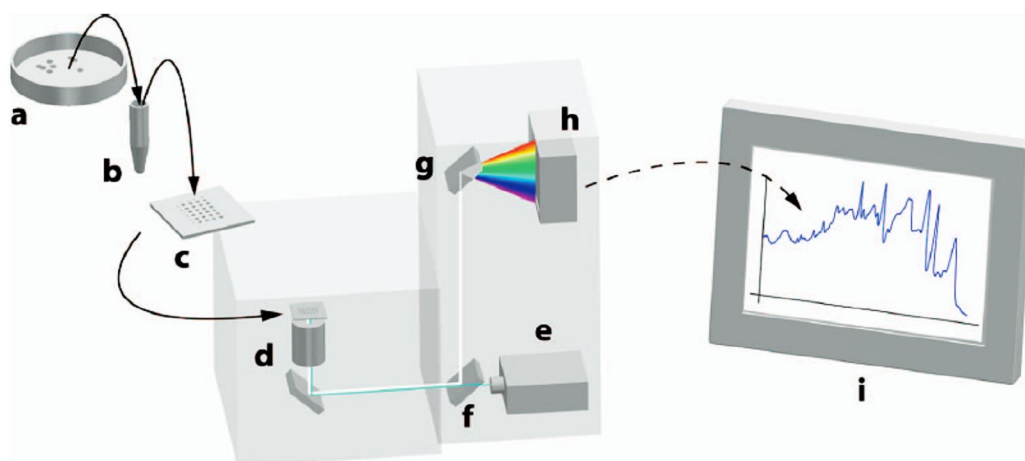
Figure 5. Overview of Raman procedure and spectrometer. Biomass from a bacterial culture (a) on TSA medium is collected using a 1- l inoculation loop and suspended in 5 l of demineralized water (b). After a brief centrifugation step to remove air bubbles, the wet pellet is transferred onto a fused silica slide (c), where it is allowed to dry (a typical slide holds 24 samples). The slide with the dried biomass is placed in the measurement stage (d), where the samples are illuminated with laser light (e). The Raman signal generated is collected along the same optical path and separated from the laser light using an optical filter (f) that only reflects light of a higher wavelength than the laser. The laser light is passed through. The wavelength of the Raman signal is dispersed on an optical grating (g) and collected using a near-infrared-optimized charge-coupled device detector (h). The Raman spectra are gathered, stored, and analyzed on a personal computer (i) (110).

1.9. Aims of the research

The first aim of this study was to characterize of ExPEC isolates recovered from retail meat, particularly chicken and fresh broiler chicken meat, broiler chickens and to compare with clinical isolates. The second and most important aim of the research was to determine whether transmission is primarily human to human through food or whether an animal reservoir can be involved. In the case of human-to human transmission through food, *E. coli* strains from humans will be introduced during the meat preparation process by food handlers. In the case of an animal reservoir, *E. coli* would derive from the cecal content of the animal itself, and contamination could occur more likely during the slaughtering process. On the basis of previous findings, we hypothesize that a food animal reservoir could exists for ExPEC that cause UTIs in humans and that chicken could be the

primary source. To evaluate this hypothesis, we will analyze isolates from animals entering the food chain. *E. coli* isolates recovered from the cecal contents of slaughtered food animals (beef cattle, chickens, and pigs) will be compared with geographically and temporally matched collection of isolates from humans with UTIs.

2.Materials and Methods

2.1. Bacterial isolates

2.1.1. Isolates from broiler chicken meat.

A total of 250 food samples including 180 raw broiler chicken meat , 17 pork meat , 14 cow meat 19 cheese and 20 ready to eat foods samples were bought from a range of grocery stores in Palermo, Italy, during April 2013-December 2014. Retail chicken samples were stored at 4°C and processed no later than 96 hours after purchase. A 5-g portion of each specimen was added to 45 ml of modified Trypticase soy broth (mTSB), containing 20 mg/L of novobiocin (Sigma Chemical Co., St. Louis, Mo.). The enrichment cultures were incubated at 37°C for 18 to 24 h and then subcultured onto MacConkey agar. When *E. coli*ólike growth was present, one typical, well-isolated colony was streaked for isolation onto a BAP. The BAP(s) were incubated at 35°C for 24 hours in ambient air and examined for purity. *E. coli* presumptively identified as *E. coli* by indole, citrate, methyl red and Voges-Proskauer tests. To confirm identification, PCR amplifications of *trp*A gene, as a species specific gene, for *E. coli* was performed.

2.1.2.Clinical isolates of *E. coli*

During April 2013-December2014, a total of 200 de-identified FQ-R and ESBL-producing *E. coli* isolates were collected from Ospedale Civico, Palermo , Italy. The clinical isolates were collected mainly from patients with UTI, Septicemia and surgical site infections caused by this organism. The UTI isolates were collected from patients included in the study only if they will show typical symptoms of UTI such as dysuria, increased urinary frequency or urgency, pyuria, and hematuria, delivered a mid-stream urinary sample taken after washing the external urethral meatus with sterile saline, and the urine sample is positive for leucocytes by the dipstick test and reveale at least $\times 10^{3}$ CFU/ml of a typical urinary pathogen (96).

2.2. Antimicrobial susceptibility and ESBL/AmpC status

Susceptibility to nine antimicrobial agents including amoxicillin-clavulanic acid (20-10 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), gentamicin (10 µg), imipenem (10 µg), sulfamethoxazole-trimethoprim (25 µg) and tetracycline (30 µg) was determined by disk diffusion and interpreted according with the EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines (http://www.eucast.org/clinical_breakpoints/). Intermediately resistant isolates were categorized as resistant. Production of ESBLs was determined by the double disk synergy test (DDST) phenotypic confirmatory test, and AmpC expression was evaluated by the three-dimensional test with cefoxitin disk. For the purposes of the study, isolates exhibiting resistance to at least three antibacterial drug classes (amoxicillin-clavulanic acid, third generation cephalosporins [3GCs]/cefepime, carbapenems, fluoroquinolones, aminoglycosides, sulfamethoxazole-trimethoprim and tetracycline) were defined as MDR.

2.3. Phylogenetic grouping by multiplex polymerase chain reaction

Isolates were assigned to phylogenetic groups according to the method of Clermont et al. (21). Isolates were assigned to one of four groups (A, B1, B2, or D) based on their possession of two genes (chuA and yjaA) and a DNA fragment (TSPE4.C2), as determined by PCR. Amplifications were performed as previously described (Clermont et al., 2000). Samples were subjected to horizontal gel electrophoresis in 2% agarose, and the sizes of the amplicons were determined by comparison to the 100bp DNA. Positive and negative bacterial controls for each phylogenetic group were included in the analysis.

The figure below shows dichotomous decision tree to determine the phylogenetic group of an *E. coli* strain by using the results of PCR amplification of the *chuA* and *yjaA* genes and DNA fragment TSPE4.C2.
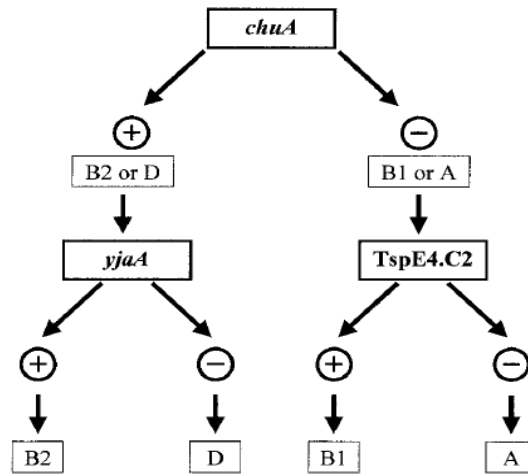
2.5. Multiplex PCR assay for ESBLs and AmpC detection

Three multiplex PCRs were used in this study: a bla_{TEM}/bla_{SHV}/$bla_{OXA\text{-}1\text{-}like}$ multiplex PCR; a $bla_{CTX\text{-}M}$ multiplex PCR including groups 1, 2 and 9; and a plasmid-mediated AmpC -lactamase gene multiplex PCR including six groups based on percentage of similarity (28). Total DNA (1 mL) was subjected to each multiplex PCR in a 25 mL reaction mixture containing 1X PCR buffer (10 mM TrisóHCl, pH 8.3/50 mM KCl/1.5 mM MgCl2), 200 mM concentration of each deoxynucleotide triphosphate, a variable concentration of specific-group primers (Table 1) and 1 U of Taq polymerase. Amplification was carried out as follows: initial denaturation at 94°C for 10 min; 30 cycles of 94°C for 40 s, 60°C for 40 s and 72°C for 1 min; and a final elongation step at 72°C for 7 min.

Table 1. Group-specific primers used for the assays

PCR name	β-Lactamase(s) targeted	Primer name	Sequence (5'–3')	Length (bases)	Annealing position[a]	Amplicon size (bp)	Primer concentration (pmol/μL)
Multiplex I TEM, SHV and OXA-1-like	TEM variants including TEM-1 and TEM-2	MultiTSO-T_for	CATTTCCGTGTCGCCCTTATTC	22	13–34	800	0.4
		MultiTSO-T_rev	CGTTCATCCATAGTTGCCTGAC	22	812–791		0.4
	SHV variants including SHV-1	MultiTSO-S_for	AGCCGCTTGAGCAAATTAAAC	21	71–91	713	0.4
		MultiTSO-S_rev	ATCCCGCAGATAAATCACCAC	21	783–763		0.4
	OXA-1, OXA-4 and OXA-30	MultiTSO-O_for	GGCACCAGATTCAACTTTCAAG	22	201–222	564	0.4
		MultiTSO-O_rev	GACCCCAAGTTTCCTGTAAGTG	22	764–743		0.4
Multiplex II CTX-M group 1, group 2 and group 9	variants of CTX-M group 1 including CTX-M-1, CTX-M-3 and CTX-M-15	MultiCTXMGp1_for	TTAGGAARTGTGCCGCTGYA[b]	20	61–80	688	0.4
		MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT[b]	21	748–728		0.2
	variants of CTX-M group 2 including CTX-M-2	MultiCTXMGp2_for	CGTTAACGGCACGATGAC	18	345–362	404	0.2
		MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT[b]	21	748–728		0.2
	variants of CTX-M group 9 including CTX-M-9 and CTX-M-14	MultiCTXMGp9_for	TCAAGCCTGCCGATCTGGT	19	299–317	561	0.4
		MultiCTXMGp9_rev	TGATTCTCGCCGCTGAAG	18	859–842		0.4
Multiplex III ACC, FOX, MOX, DHA, CIT and EBC	ACC-1 and ACC-2	MultiCaseACC_for	CACCTCCAGCGACTTGTTAC	20	744–763	346	0.2
		MultiCaseACC_rev	GTTAGCCAGCATCACGATCC	20	1089–1070		0.2
	FOX-1 to FOX-5	MultiCaseFOX_for	CTACAGTGCGGGTGGTTT	18	396–413	162	0.5
		MultiCaseFOX_rev	CTATTTGCGGCCAGGTGA	18	557–540		0.5
	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11 and CMY-19	MultiCaseMOX_for	GCAACAACGACAATCCATCCT	21	3–23	895	0.2
		MultiCaseMOX_rev	GGGATAGGCGTAACTCTCCCAA	22	900–879		0.2
	DHA-1 and DHA-2	MultiCaseDHA_for	TGATGGCACAGCAGGATATTC	21	113–133	997	0.5
		MultiCaseDHA_rev	GCTTTGACTCTTTCGGTATTCG	22	1109–1088		0.5
	LAT-1 to LAT-3, BIL-1, CMY-2 to CMY-7, CMY-12 to CMY-18 and CMY-21 to CMY-23	MultiCaseCIT_for	CGAAGAGGCAATGACCAGAC	20	570–589	538	0.2
		MultiCaseCIT_rev	ACGGACAGGGTTAGGATAGY[b]	20	1107–1088		0.2
	ACT-1 and MIR-1	MultiCaseEBC_for	CGGTAAAGCCGATGTTGCG	19	189–207	683	0.2
		MultiCaseEBC_rev	AGCCTAACCCCTGATACA	18	871–854		0.2

2.6. Detection of plasmid-mediated quinolone resistance (PMQR) determinants

Screening of the six PMQR determinants was carried out by two sets of multiplex PCR amplification, one for *qnrA*, *qnrB*, *qnrC*, and *qnrS* and the other for *aac(6∅)-Ib* and *qepA* (Table 2). In each multiplex PCR, all of the primers were added to the template DNA (1 mL) in a 25 mL reaction mixture containing 1X PCR buffer (10 mM TrisóHCl, pH 8.3/50 mM KCl/1.5 mM MgCl2), 200 mM concentration of each deoxynucleotide triphosphate. Clinical isolates that had previously been confirmed to carry the *qnr* genes, *aac(6∅)-Ib*, and *aac(6∅)-Ib-cr* by DNA sequencing were used as positive controls. Positive and negative controls were included in each PCR. PCR conditions were: 5 min of denaturation at 95°C; 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 50 s; and then 10 min of elongation at 72°C. Amplification products were identified by their sizes after electrophoresis on 1.8% agarose gels at 100 V for 60 min and staining with ethidium bromide. Positive results for *qnr* genes were confirmed by direct sequencing of PCR products. (113).

Table 2. Primer sequences used to detect PQMR

Gene	Primer	Sequence (5′→3′)	Size of PCR-amplified product (bp)
qnrA	qnrAF	ATTTCTCACGCCAGGATTTG	516
	qnrAR	GATCGGCAAAGGTTAGGTCA	
qnrB	qnrBF	GATCGTGAAAGCCAGAAAGG	476
	qnrBR2	ATGAGCAACGATGCCTGGTA	
qnrC	qnrCF	GGGTTGTACATTTATTGAATCG	307
	qnrCR	CACCTACCCATTTATTTTCA	
qnrS	qnrSmF	GCAAGTTCATTGAACAGGGT	428
	qnrSmR	TCTAAACCGTCGAGTTCGGCG	
aac(6′)-Ib	aacIbF	TTGCGATGCTCTATGAGTGGCTA	482
	aacIbR	CTCGAATGCCTGGCGTGTTT	
qepA	qepAF	AACTGCTTGAGCCCGTAGAT	596
	qepAR	GTCTACGCCATGGACCTCAC	
gyrA	gyrAWF	AAATCTGCCCGTGTCGTTGGT	344
	gyrAWR	GCCATACCTACGGCGATACC	
parC	parCWF	CTGAATGCCAGCGCCAAATT	168
	parCWR	GCGAACGATTTCGGATCGTC	

2.7. Virulence Genotyping

All isolates belonging to phylogenetic groups B2 and D were investigated for the following eight ExPEC related virulence genes by two quadroplex PCRs (table 3) : *kpsM II* (group 2 capsular polysaccharide), *papA* and *papC* (P fimbriae), *iutA* (iron acquisition system), *sfaS* (S fimbriae), *focG* (F1C fimbriae), *afa* (afimbrial adhesion), and *hlyD* (cytolytic protein toxin) (Johnson *et al.*, 2005b). Four strains were used as positive controls in the PCR screening: *E. coli* RS218 *(kpsMT II, papA, papC, sfaS, hlyD)*, *E. coli* V27 (*kpsMT II, papA, papC, iutA, focG), E. coli* 2H16 (*papC, iutA, afa, hlyD*), and *E. coli* J96 (*papA, focG). E. coli* MG1655 was used as a negative control in the reactions. All the reference *E. coli* strains were kindly provided by James R. Johnson (VA Medical Center, Minneapolis, MN, US). *E. coli* isolates were defined as ExPEC if they tested positive for at least two of *papA* and/or *papC*, *sfa/foc*, *afa/dra*, *kpsMT II* and *iutA* genes (Johnson *et al.*, 2005b).

Table 3. Primers, amplicon size, and annealing temperature for the Multiplex PCR reactions to detect VFs in this study

Target class	Target gene	Primer designation	Primer sequence (5′–3′)	Amplicon size (bp)	Annealing temp. (°C)	Reference
First quadroplex PCR						
Group 2 polysaccharide capsule	*kpsM* II	kpsMII F kpsMII R	GCG CAT TTG CTG ATA CTG TTG CAT CCA GAC GAT AAG CAT GAG CA	272	61	Johnson and O'Bryan (2004)
P fimbriae	*papA*	papA F papA R	ATG GCA GTG GTG TCT TTT GGT G CGT CCC ACC ATA CGT GCT CTT C	717		Johnson and Stell (2000)
S fimbriae	*sfaS*	sfaS F sfaS R	GTC TCT CAC CGG ATG CCA GAA TAT GCA TTA CTT CCA TCC CTG TCC TG	138		Johnson and Stell (2000)
F1C fimbriae	*focG*	focG F focG R	CGT ACC TGT ACC ATT GGT AAT GGA GG TGA ATT AAT ACT TCC CGC ACC AGC	366		Johnson and Stell (2000)
Second quadroplex PCR						
Iron acquisition system	*iutA*	iutA F iutA R	ATC GGC TGG ACA TCA TGG GAA C CGC ATT TAC CGT CGG GAA CGG	314	61	Jakobsen et al. (2008)
P fimbriae	*papC*	papC F papC R	GTG GCA GTA TGA GTA ATG ACC GTT A ATA TCC TTT CTG CAG GGA TGC AAT A	203		Johnson and Stell (2000)
Cytolytic protein toxin	*hlyD*	hlyD F hlyD R	CTC CGG TAC GTG AAA AGG AC GCC CTG ATT ACT GAA GCC TG	904		Rodriguez-Siek et al. (2005)
Afimbrial adhesion	*afa*	afa F afa R	GGC AGA GGG CCG GCA ACA GGC CCC GTA ACG CGC CAG CAT CTC	594		Johnson and Stell (2000)

2.8. Detection of ST131

For the detection of ST131, all group B2 isolates were screened by PCR for (i) the ST131-associated O25b rfb variant (Clermont) and (ii) ST131-associated SNPs in mdh (i.e., C288T and C525T) and gyrB (i.e., C621T, of gyrB SNPs were gyrB47_forward (5ʹ-CGC GAT AAG CGC GAC-3ʹ) and gyrB47_reverse (5ʹ-ACC GTC TTT TTC GGT GGA A-3ʹ) and the primers used for the detection of mdh SNPs were mdh36_forward (5ʹ-GTT TAA CGT TAA CGC CGG T-3ʹ) and mdh36_reverse (5ʹ-GGT AAC ACC AGA GTG ACC A-3ʹ). Amplification was done with 4.0 mM MgCl2 and a cycling protocol of 95°C for 10 min; then 32 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 2 min; and then a hold at 4°C. *E. coli* MVAST131, jj2663 and BUTI 1-2-1 (H17) were used as positive control strains (90).

2.9. H30 subclone detection

All ST131 isolates were tested by allele-specific primers for allele 30 of *fimH* (encoding a variant of the type 1 fimbrial adhesin) corresponding with the main FQ resistanceʹassociated subset within ST131, the *H*30 subclone(114). Primers fimH30F-21 (5ʹ-CCG CCA ATG GTA CCG CTA TT-3ʹ) and fimH30R-20 (5ʹ-CAG CTT TAA TCG CCA CCC CA-3ʹ) (354 bp product) underwent PCR as follows: 8 at 95°; 30 cycles of (20 seconds at 94° and 45 seconds at 68°); 5 at 72°; hold at 4° . *E. coli* strains MVAST131 and jj1886 were used as positive control strains.

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2.10. ERIC-PCR analysis

Enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) was performed on all isolates. The PCR amplifications were performed in 25- l volumes containing 2.5 mM MgCl$_2$, 0.5U of *Taq* polymerase, 0.25mMeach deoxynucleoside triphosphate, 2.5 ul of 10X amplification buffer, 10 ng of crude template DNA, and 25 pmol each of primers ERIC1R (5ø-ATG TAA GCT CCT GGG GAT TCA-3ø) and ERIC2 (5ø-AAG TAA GTG ACT GGG GTG AGC G-3ø. The samples were amplified as follows: 95°C for 5 min; four-low stringency cycles of 94°C for 1 min, 26°C for 1 min, and 72°C for 2 min; 40 cycles of 94°C for 30 s, 40°C for 30 s, and 72°C for 1 min; and finally, 72°C for 10 min. BioNumerics software (Applied Maths, Belgium) was used for analysis of ERIC-PCR fingerprints. Cluster analysis using Dice algorithm was performed on ERIC-PCR proŁles. The similarities in amplicon profiles were compared using a Dice coefficient at 1% tolerance and 0.5% optimization, and a dendrogram was constructed with the UPGMA clustering method with a cut-off of 80% similarity (115).

2.11. Raman spectroscopy

Raman spectroscopy was performed at Toplab of University of Applied Science Leiden, Leiden, Netherlands and according to the manufacturerøs guidelines (River Diagnostics, Netherlands) and as described by Willemse-Erix et al(110). Briefly, all the phylogroup B2 and D isolates were grown on trypticase soy agar (TSA). Dilutions of colony suspensions were prepared and plated on a TSA plate that was incubated for 20 h at 35 °C. Biomass from TSA plates was collected with a 1- l inoculation loop and suspended in 5 l of demineralised water. After brief centrifugation to remove air bubbles, the wet pellet was transferred to a fused silica slide and allowed to dry. The slide was then placed in the measurement stage. All isolates were measured in triplicate.

2.11.1. Data analysis.

Spectrum pretreatment and cluster analysis were performed using the SpectraCell RA software (River Diagnostics). Histogram plots and correlation matrices were created using MATLAB version 7.1 (The MathWorks, USA).

2.11.2. Similarity between spectra.

In the BioNumerics software, version 6.1, (Applied Maths, Sint-Martens-Laten, Belgium), the similarity between two measured samples is expressed as the squared Pearson correlation coefficient (R2 value).

2.11.3. Reproducibility of RA and discrimination between isolates.

To be able to use RA for bacterial typing, the similarity between spectra of unrelated isolates should be lower than the similarity between spectra obtained from replicate cultures. The distribution of similarities can be visualized in a graph. The overlap between both curves indicates the discriminatory power of RA. The smaller the overlap, the better RA is able to discriminate isolates.

2.11.4. Determination of the similarity threshold and cutoff.
Two different similarity values were used to indicate relatedness between isolates. The similarity threshold (breakpoint at a lower R2 value) is chosen such that 99% of all replicate spectra have an R2 value above this threshold (i.e., 99% of the red curve is positioned above this value). Two isolates with an R2 value below the similarity threshold are considered different by RA and are assigned different RA types. This implies that for 1% of the replicates a misidentification as unrelated is allowed. The cutoff (breakpoint at a higher R2 value) is set such that 97% of all genetically unrelated isolates show R2 values below this threshold (i.e., 97% of the blue curve is positioned below the cutoff). Two isolates with an R2 value above the cutoff are considered indistinguishable by RA and are assigned the same RA type. This implies that for 3% of the

unrelated isolates a misidentification as indistinguishable is allowed. If an R2 value between two isolates is found in the area between the similarity threshold and the cutoff, these isolates are considered to be potentially related.

2.11.5. Correlation matrix.

To analyze spectral relationships between different isolates, a correlation matrix was created. This matrix displays the similarity of each pair of spectra using a color index. The diagonal indicates R2 values of 1, since this represents the similarity of each isolate with itself. The values above the diagonal are the reverse graphic image of the values below this diagonal. In each matrix, red clusters indicate isolates that are indistinguishable based on the previously set cutoff. The gray areas indicate samples that are classified as unrelated based on the previously set similarity threshold. The samples that are potentially related are indicated by yellow to orange. Spectra were sorted based on similarity. Each horizontal line in the matrix represents all R2 values of an isolate with all other isolates in the matrix. Correlation coefficients were calculated between each group of R2 values. By sorting these correlation coefficients based on height, the isolates with high similarity are grouped together.

2.12. Data analysis

BioNumerics software, version 6.1, (Applied Maths, Sint-Martens-Laten, Belgium) was used for analyzing the ERIC-PCR fingerprints. The similarities in amplicon profiles were compared using a Dice coefficient at 1% tolerance and 0.5% optimization, and a dendrogram was constructed with the UPGMA clustering method with a cut-off of 80% similarity (115).

The similarity between pairs of spectra was calculated using the Ranked squared Pearson correlation coefficient (R^2) and expressed as percentage. Then, cluster analysis of sets of spectra was performed to yield a dendrogram.

3.Results

3.1. E.coli *Isolates and antibiogram results*

E. coli were isolated from all of the chicken samples. However, multi-drug resistant (MDR) *E. coli* were isolated from 152 (84.4%) of the 180 chicken samples. MDR was defined when an isolate was resistant to at least three classes of antibiotics (Table 3.1). From 109 chicken meat samples more than one *E. coli* were isolated, these isolates were pylogeneticaly different, or they were different in antibiogram resistance pattern. No MDR *E. coli* isolates were found in other types of food samples. Resistance to ceftazidime and ciprofloxacin was predominant, being found in 232 (97.8%) and 218 (91.9%). All of the isolates showed sensitivity to meropenem. All of the isolates were positive for ESBL production by double disk synergy test. Table 3.1 describes frequency of resistance to the antibacterial drugs tested according with phylogroups.

Phylogenetic group	Antibacterial drug*								
	AMC nr. (%)	CAZ nr. (%)	CTX nr. (%)	FEP nr. (%)	FOX nr. (%)	GEN nr. (%)	CIP nr. (%)	SXT nr. (%)	TET nr. (%)
A	21 (70.0)	26 (87.0)	26 (87.0)	3 (10.0)	0 (0.0)	4 (13.3)	23 (76.7)	21 (70.0)	27 (90.0)
B1	87 (76.5)	110 (96.8)	110(96.8)	30 (26.5)	5 (4.7)	14 (12.5)	103 (90.6)	91 (79.7)	107(93.7)
B2	7 (53.8)	13(100)	13 (100)	7 (53.8)	2 (16.5)	0 (0.0)	13 (100)	5 (38.4)	13 (100)
D	45 (55.9)	68 (85.3)	68 (85.3)	19 (23.5)	19 (23.5)	10 (11.8)	75 (94.1)	75 (94.1)	71 (88.8)

* tested by the disk diffusion method

AMC, amoxicillin-clavulanic acid; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; GEN, gentamicin; CIP. ciprofloxacin; SXT, sulfamethoxazole-trimethoprim; TET, tetraycline

3.2. Phylogenetic diversity of Isolates

Triplex PCR to detect different phylogenetic groups showed that while among *E. coli* isolates from chicken meat sampless phylogenetic group B1 was the most prevalent (114 isolates, 48.1%) followed by groups D (80 isolates, 33.7%), A (30 isolates, 12.7%) and B2 (13 isolates, 5.5%); phylogenetic group B2 was the most prevalent phylogroup among clinical isolates (136 isolates, 68.5%) followed by groups D (33 isolates, 16.8%), A (20 isolates, 10.6%) and B2 (9 isolates, 4.6%), respectively (Figure 3.1). Figure 3.2 shows the Triplex PCR products on 2% agarose gel after one hour immigration.
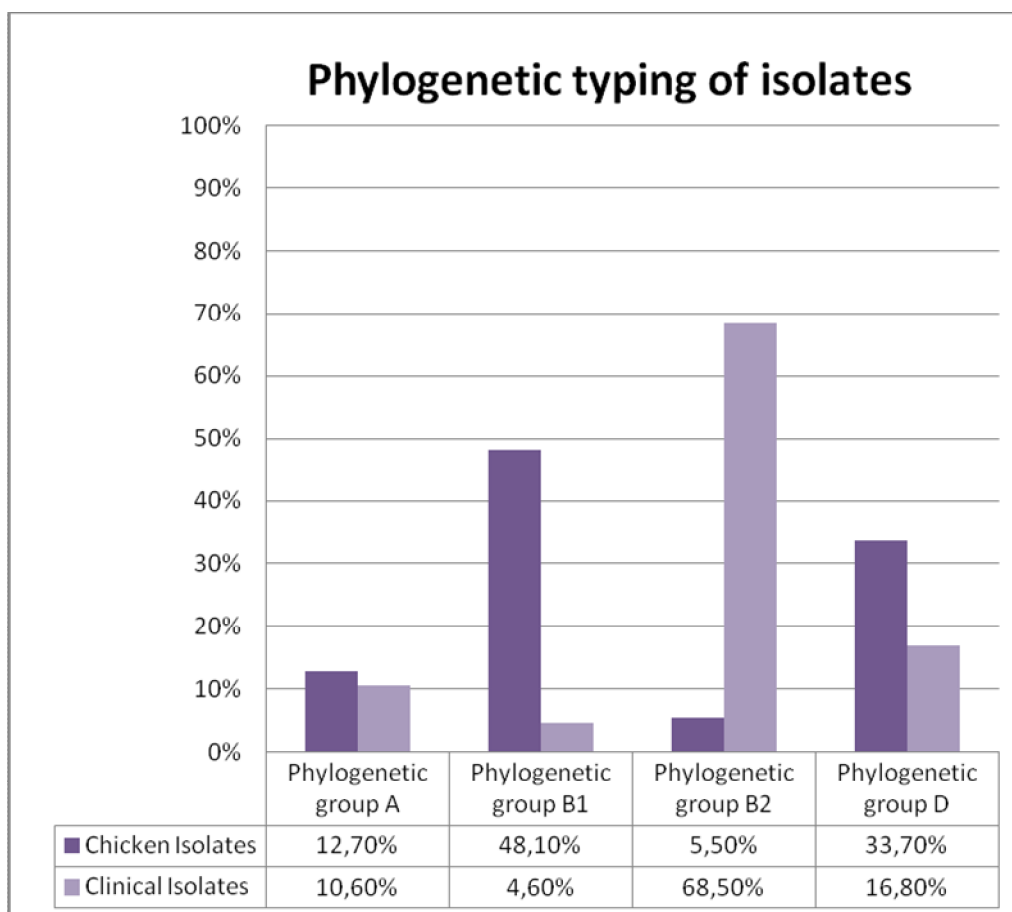

Figure 3.1. Distribution of different phylogenetic groups among isolates
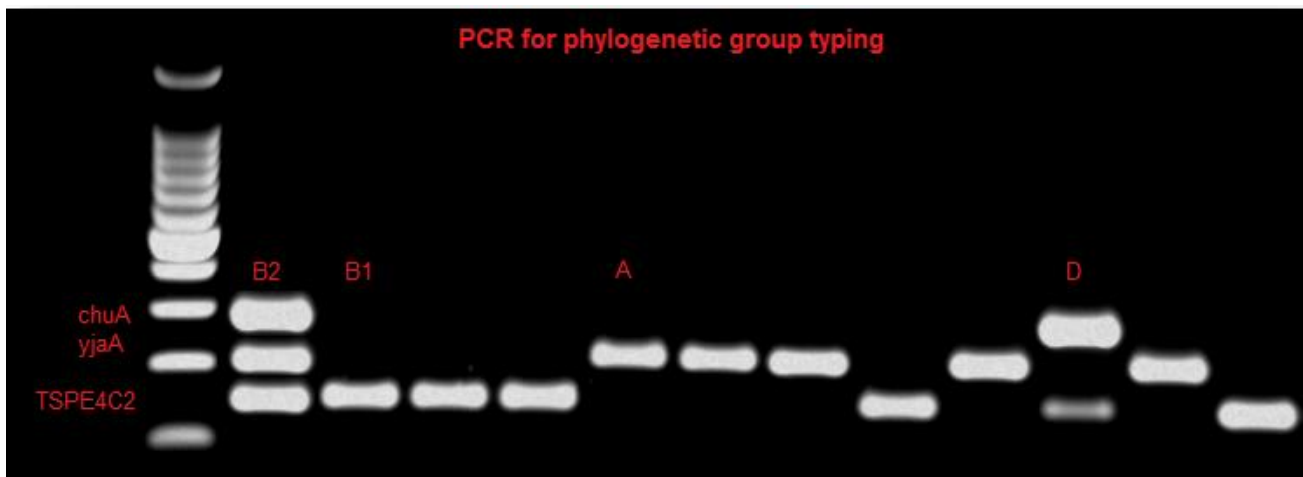
Figure 3.2. Different phylogenetic groups of *E. coli* on agarose gel

3.3. Anibiotic resistance determinants among isolates

ESBLs and AmpC -lactamases were detected by PCR in 233 (98.3%) and 29 (12.2%) of chicken meat isolates and 194 (97.5%) and 34 (17.2%) of clinical isolates, respectively. PMQR determinants were detected in 215 (90.7%) of chicken isolates and 183 (91.9%) of clinical isolates, respectively. Moreover, all isolates testing positive for the PMQR genes had also ESBLs. In particular, among chicken meat isolates, 143 isolates (60.3%) tested positive for SHV, 59 (29.9%) for TEM and 104 (43.9%) for both TEM and SHV and in clinical isolates 32 isolates (16.2%) tested positive for SHV, 164 (69.1%) for TEM and 26 (13.1%) for both TEM and SHV, respectively (Figure 3.3) . While none of the chicken meat isolates was positive for the OXA-1-like gene, in clinical isolates OXA-1-like positive strains were detected in 121 (60.9%) of isolates. Moreover, among chicken isolates, 56 isolates (23.6%) were positive for CTX-M-1 group, 41 isolates (17.3%) for CTX-M-2 group and 25 isolates (10.6%) for CTX-M-9 group and among clinical isolates 154 isolates (77.7%) were positive for CTX-M-1 group, 21 isolates (10.6%) for CTX-M-2 group and 23 isolates (21.3%) for CTX-M-9 group , respectively. Twenty nine chicken meat isolates (11.2%) and 34 clinical isolates (17.2%) carried AmpC genes which all proved to be CIT-like. Two isolates of D phylogroup were positive for SHV, TEM, CTX-M and plasmidic AmpC -lactamases. Among the

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PMQR determinants, only *qnr*A was detected in all fluoroquinolone resistant isolates. The differential distribution of the ESBL, AmpC and PMQR genes under study according with the phylogroup is showed in Figure 3.4.
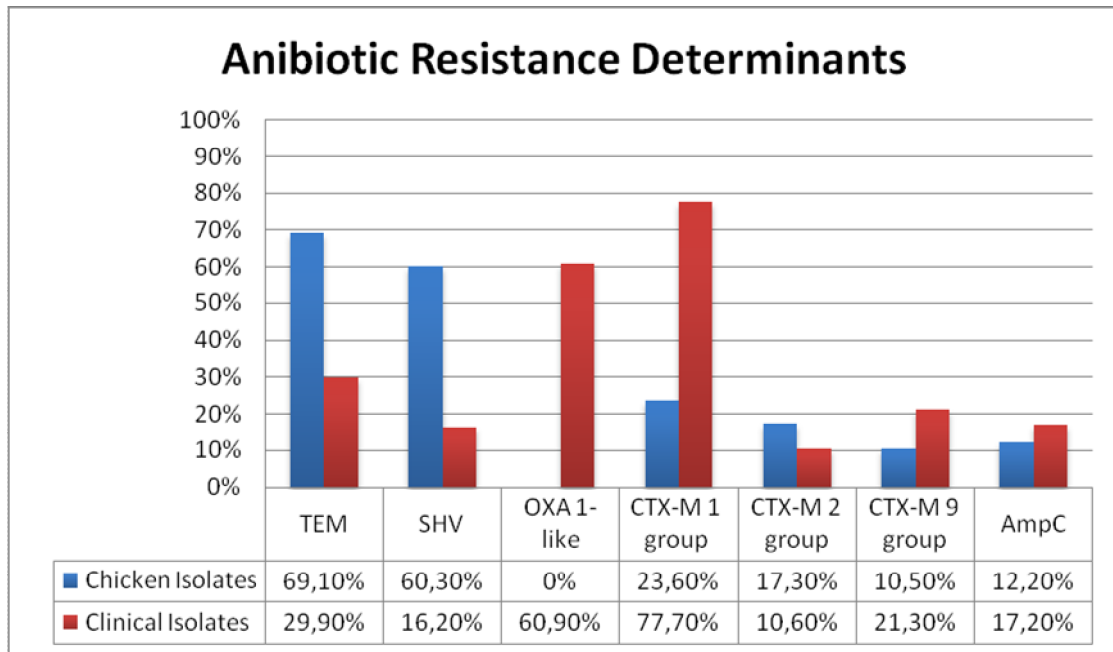

Anibiotic Resistance Determinants

	TEM	SHV	OXA 1-like	CTX-M 1 group	CTX-M 2 group	CTX-M 9 group	AmpC
■ Chicken Isolates	69,10%	60,30%	0%	23,60%	17,30%	10,50%	12,20%
■ Clinical Isolates	29,90%	16,20%	60,90%	77,70%	10,60%	21,30%	17,20%

Figure 3.3. Distribution of antibiotic resistance determinants among isolates

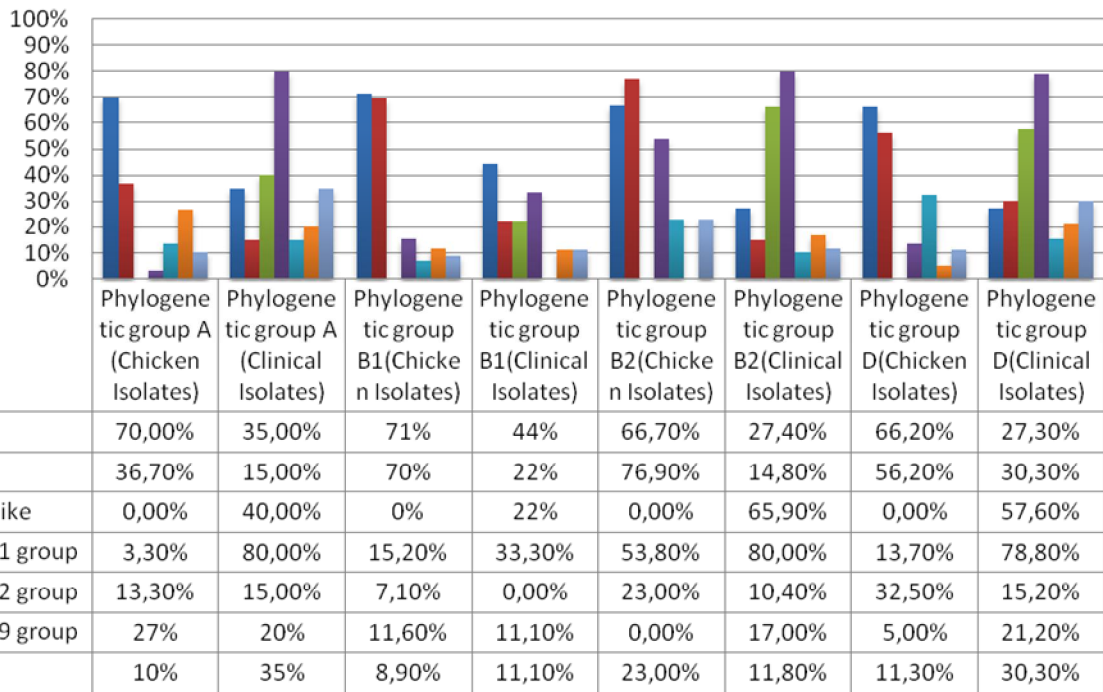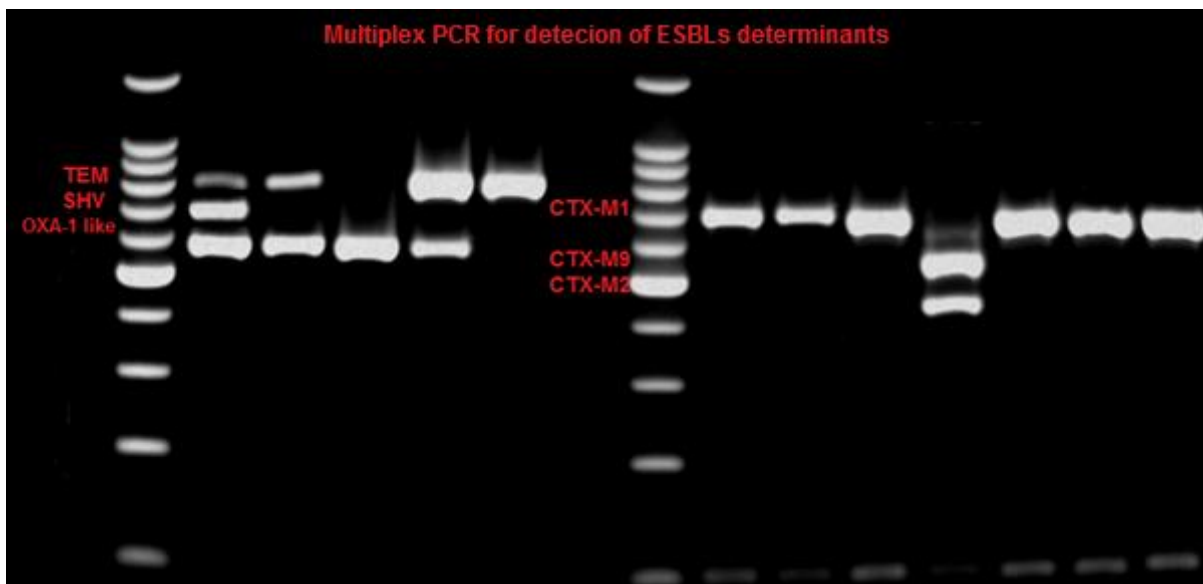**Figure 3.4.** Distribution of antibiotic resistance determinants among isolates according with the phylogroup

The chart title: **Distribution of Antibiotic resistance determinants among Isolates**

	Phylogenetic group A (Chicken Isolates)	Phylogenetic group A (Clinical Isolates)	Phylogenetic group B1(Chicken Isolates)	Phylogenetic group B1(Clinical Isolates)	Phylogenetic group B2(Chicken Isolates)	Phylogenetic group B2(Clinical Isolates)	Phylogenetic group D(Chicken Isolates)	Phylogenetic group D(Clinical Isolates)
■ TEM	70,00%	35,00%	71%	44%	66,70%	27,40%	66,20%	27,30%
■ SHV	36,70%	15,00%	70%	22%	76,90%	14,80%	56,20%	30,30%
■ OXA 1-like	0,00%	40,00%	0%	22%	0,00%	65,90%	0,00%	57,60%
■ CTX-M 1 group	3,30%	80,00%	15,20%	33,30%	53,80%	80,00%	13,70%	78,80%
■ CTX-M 2 group	13,30%	15,00%	7,10%	0,00%	23,00%	10,40%	32,50%	15,20%
■ CTX-M 9 group	27%	20%	11,60%	11,10%	0,00%	17,00%	5,00%	21,20%
■ CIT	10%	35%	8,90%	11,10%	23,00%	11,80%	11,30%	30,30%

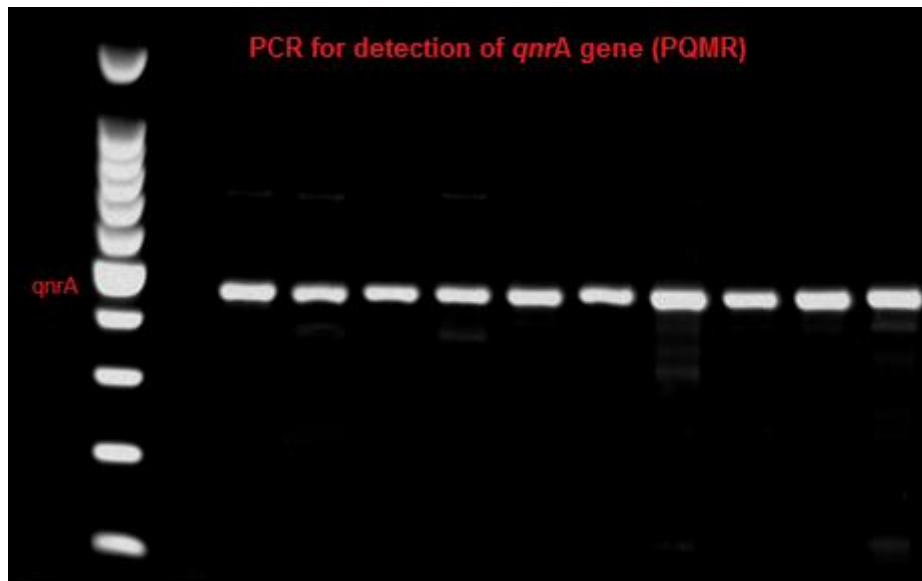**Figure 3.5**. Different ESBLs determinats among *E. coli* isolates on agarose gel

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Figure 3.6. PQMR of *qnr*A type among *E. coli* isolates on agarose gel

3.4. ExPEC among E. coli *isolates*

Based on the molecular definition of ExPEC, in chicken isolates 58 isolates out of 92 belonging to phylogroups B2 and D were attributed with the status of ExPEC (all the B2 isolates and 45 out of 80 [56.2%] D isolates). Among clinical isolates 156 isolates out of 166 belonging to phylogroups B2 and D were attributed with the status of ExPEC (130 out of 136 B2 isolates [95.6%] and 27 out of 33 [81.8%] D isolates). The most prevalent virulence factors among both clinical and chicken meat samples were *kps*MT II and *iut*A (figure 3.7).

Figure 3.7. Distribution of virulence factors among isolates
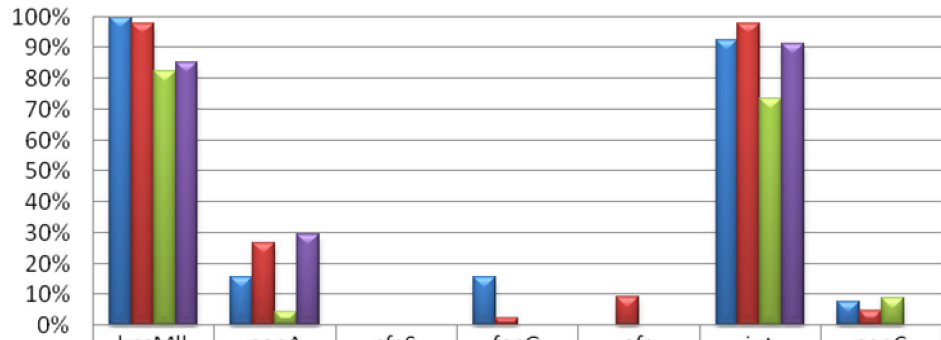
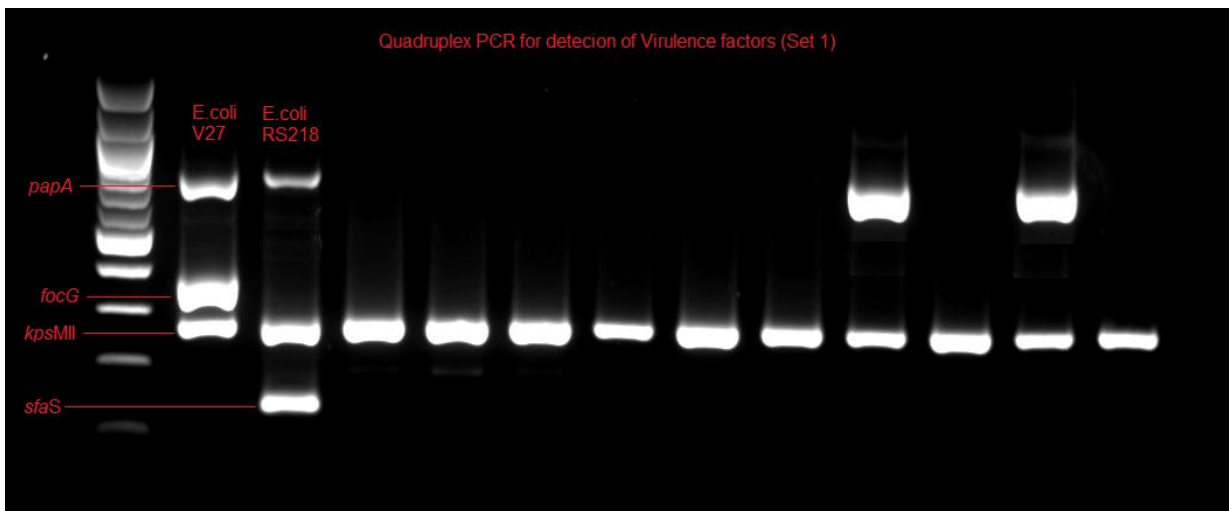|	kpsMII	papA	sfaS	focG	afa	iut	papC
■ Phylogenetic group B2(Chicken Isolates)	100%	15,40%	0%	15,40%	0%	92,30%	7,70%
■ Phylogenetic group B2(Clinical Isolates)	97,70%	26,90%	0%	2,30%	9,20%	97,70%	4,60%
■ Phylogenetic group D(Chicken Isolates)	82,20%	4,40%	0%	0%	0%	73,30%	8,90%
■ Phylogenetic group D(Clinical Isolates)	85,10%	29,60%	0%	0%	0%	90,90%	0%

Figure 3.8. Quadroplex PCR results for detection of *pap*A, *foc*G, *kps*MII, *sfa*S on agarose gel
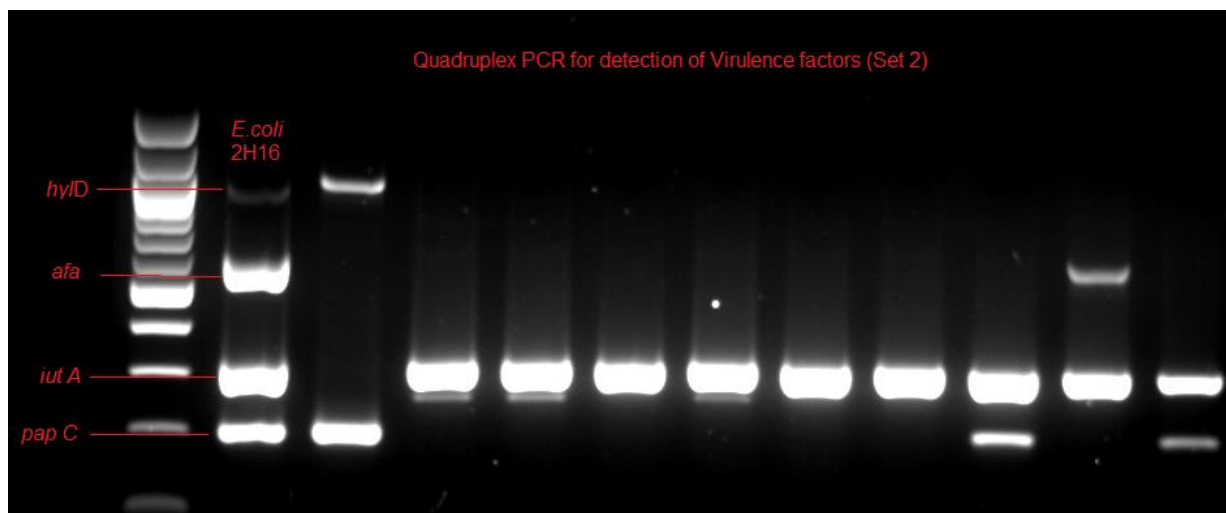
Figure 3.9. Quadroplex PCR results for detection of *pap*C, *afa, iut*A, *hyl*D on agarose gel

3.5. Prevalence of ST131 *E. coli*

A total 149 *E. coli* isolates belonging to phylogroup B2 (136 clinical and 13 chicken meat isolates) were tested by PCR of specific single nucleotide polymorphism for *mdh* and *gyrB*. Figure 3.10 shows the agarose gel electrophoresis of PCR-amplified products of *mdh* and *gyrB* respectively. Overall, 134 *E. coli* isolates (9 chicken meat isolates and 125 clinical) gave positive results in PCR of specific single nucleotide polymorphism for *mdh* and *gyrB* and they were identified as ST131. Hence, total of 91.9% (125/136) and 69.2% (9/13) of the clinical and chicken meat isolates of *E. coli* population were identified as ST131, respectively.
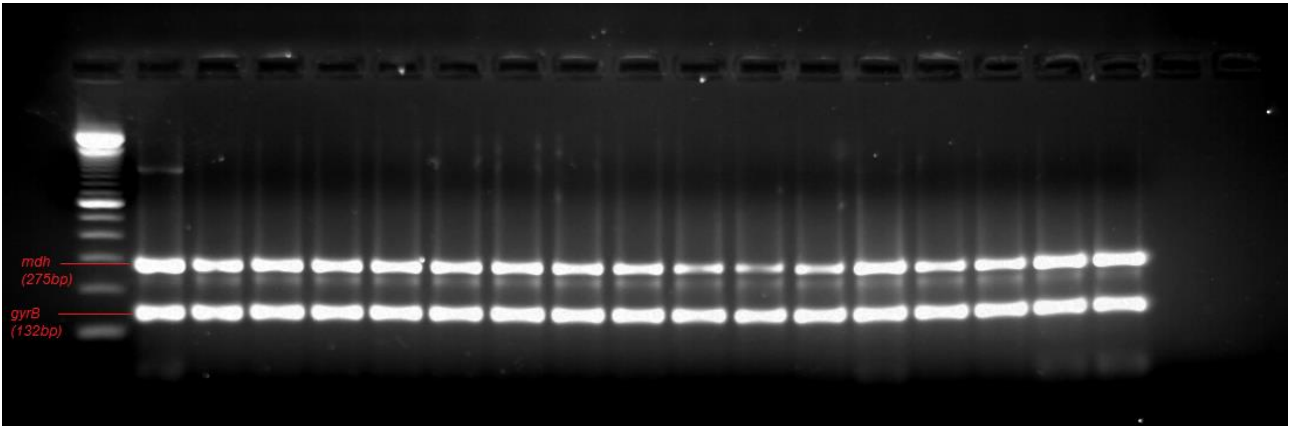
Figure 3.10. The agarose gel electrophoresis of PCR-amplified products of *mdh* and *gyrB*. Lane 1 , 2 E. coli MVST131

and JJ1886 as positive controls, lane 19 E. coli Puti 1 as negative control

3.6. Prevalence of subclone H30 among ST131 *E. coli*

A total 134 *E. coli* isolates belonging to phylogroup B2 (125 clinical and 9 chicken meat ST131 *E. coli* isolates) were tested by PCR of specific single nucleotide polymorphism for H30 subclone. Figure 3.11 shows the agarose gel electrophoresis of PCR-amplified products of *fim*H30.

SNP-PCR for H30 subclone showed that an overall of 118 ST131 *E. coli* isolates (113 and 5 clinical and chicken meat ST131 *E. coli* isolates) were positive for H30.

Hence, total of 90.4% (113/125) and 55.6% (5/9) of the clinical and chicken meat isolates of   *E. coli* population were identified as H30 subclone of *E. coli* ST131 clone, respectively.
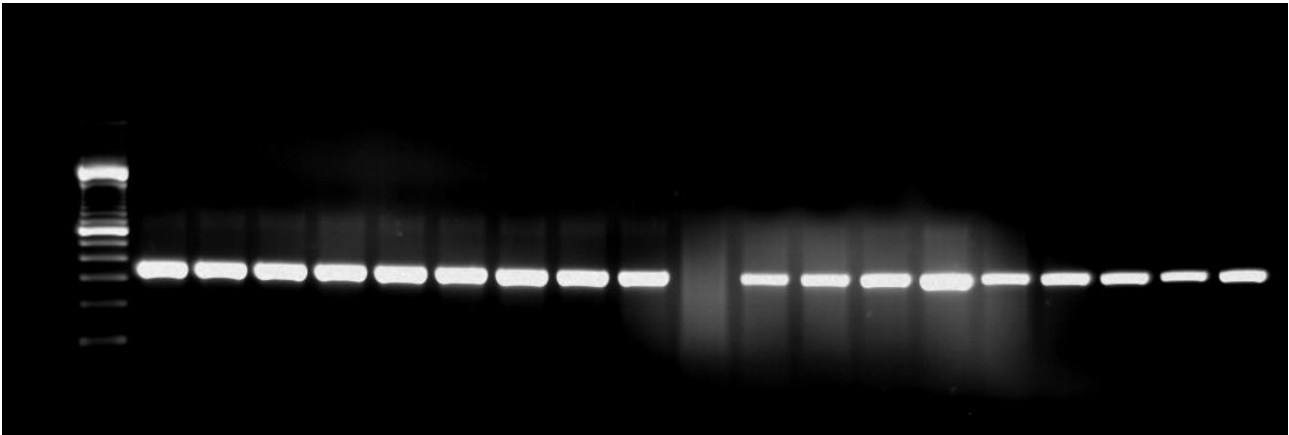
Figure 3.11. The agarose gel electrophoresis of PCR-amplified products of

3.7. Molecular typing of E. coli *isolates*

3.7.1. ERIC-PCR typing of isolates

BioNumerics software, version 6.1, (Applied Maths, Sint-Martens-Laten, Belgium) was used for analyzing the ERIC-PCR fingerprints. The similarities in amplicon profiles were compared using a Dice coefficient at 1% tolerance and 0.5% optimization, and a dendrogram was constructed with the UPGMA clustering method with a cut-off of 80% similarity.

Three sets of analysis were performed :

1. ERIC PCR analysis of all ExPEC isolates from chicken meat samples showed the isolates are very heterogeneous. Among all ExPEC isolates belong to B2 and D phylogroups , only 12 isolates belong to phylogroup D grouped in six clusters of two isolates in each cluster with the similarity more than 80% (figure 3.13).
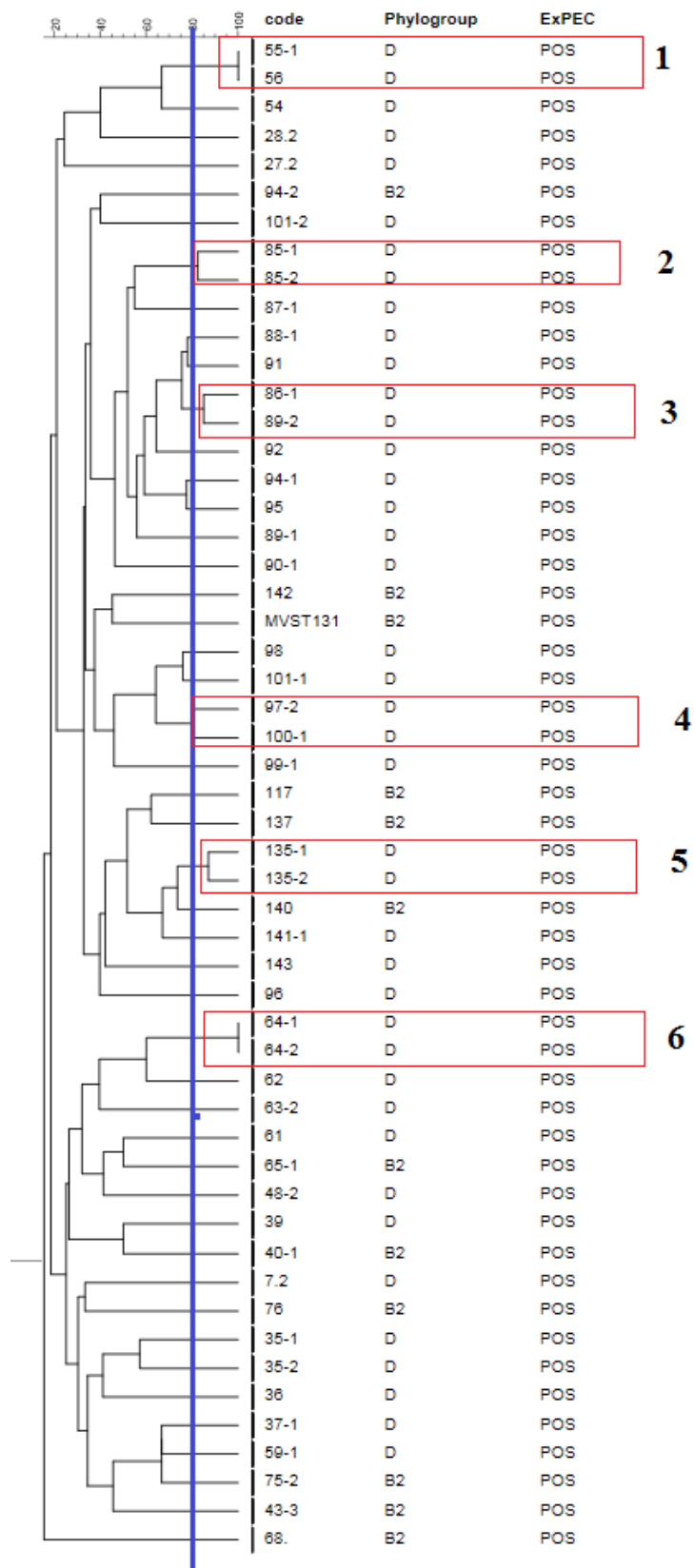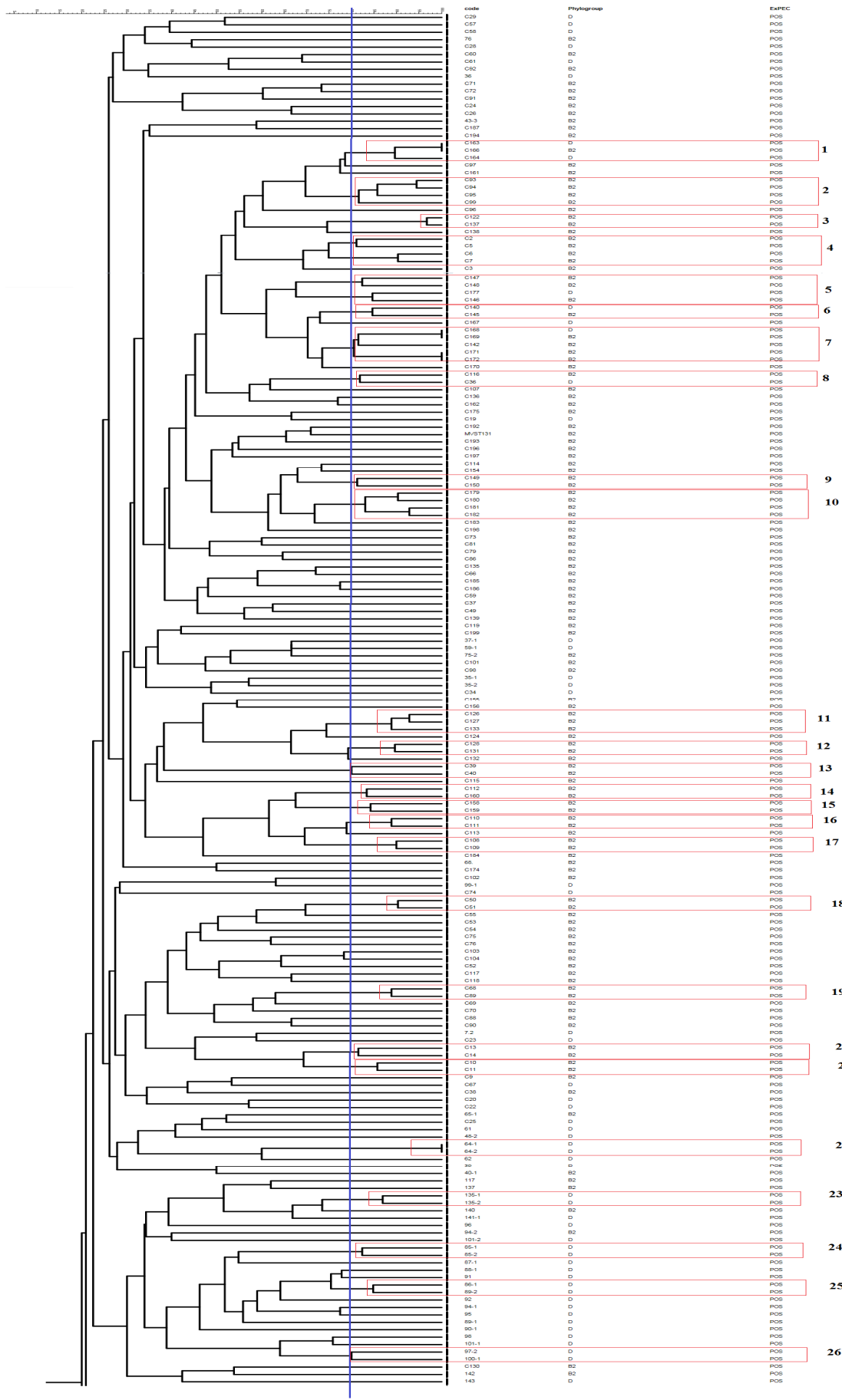
Figure 3.13. ERIC PCR analysis of ExPEC isolates from chicken meat samples

2. ERIC PCR analysis of all ExPEC isolates from chicken meat and clinical samples showed that only 71 out of 210 isolates grouped in 29 clusters and the remaining 139 isolates create unique ERIC patterns. The largest cluster is cluster number 7 which consist of five isolates (four isolates belong to group B2 and one D isolate). Each of the clusters number 2, 4 and 10 consist of 4 isolates and each of the clusters 1 and 11 consist of 3 isolates. The remaining 23clusters consist of 2 isolates each (figure 3.14).
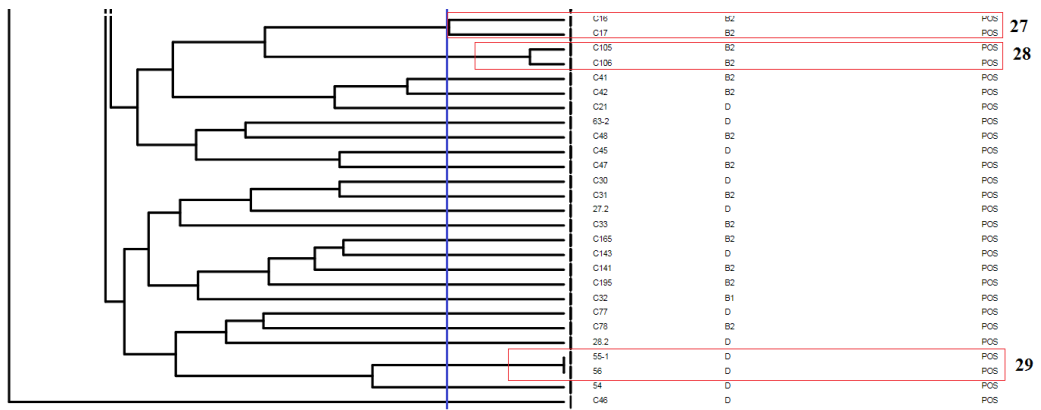
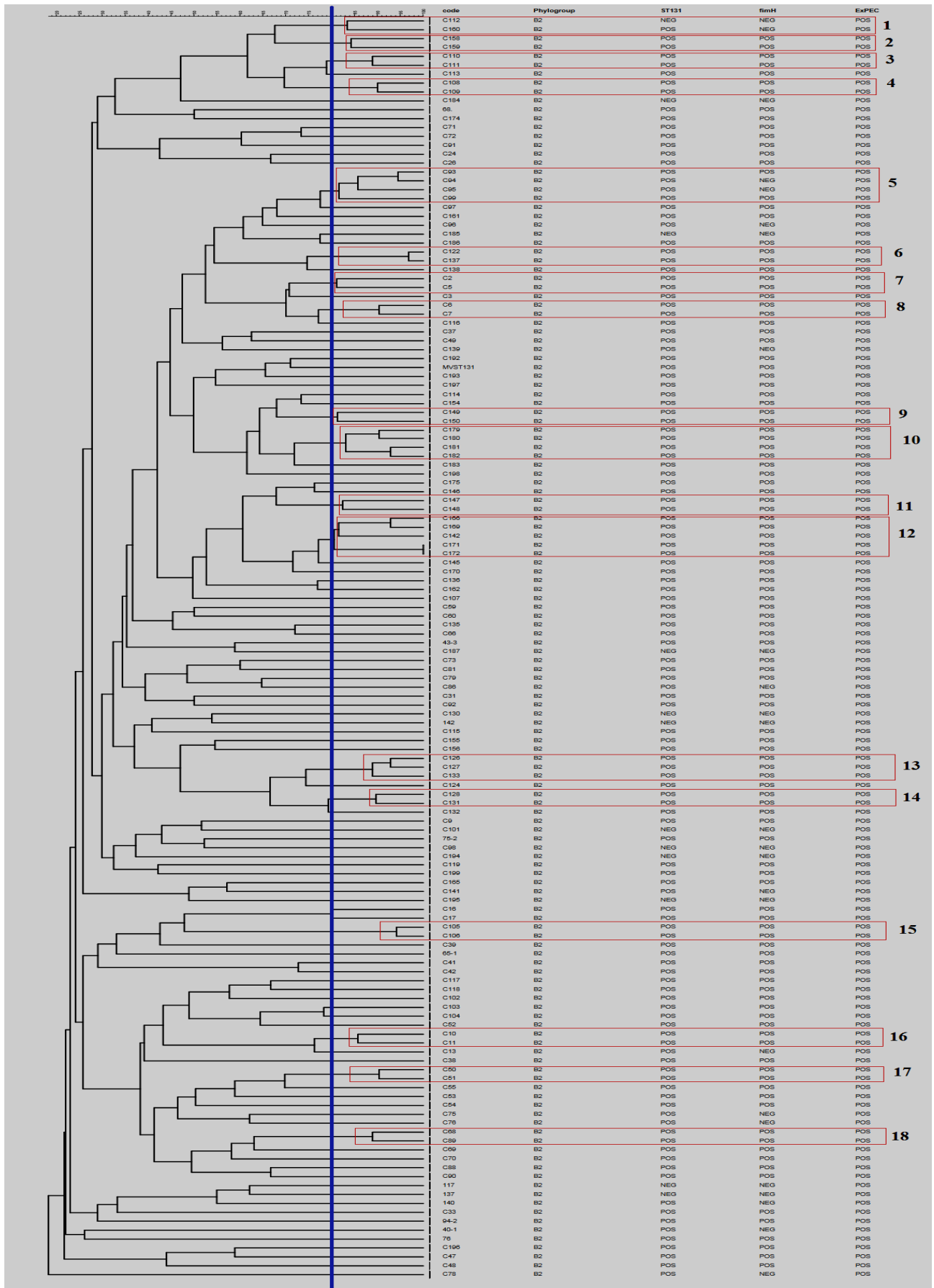**Figure 3.13.** ERIC PCR analysis of all ExPEC isolates from chicken meat and clinical samples

Figure 3.14. ERIC PCR analysis of all B2 ExPEC isolates from chicken meats and clinical samples

3.7.2. Raman typing of isolates

All ExPEC isolates from chicken meats and clinical samples were typed with SpectraCell Raman Spectroscopy (RA). Using the Ranked squared Pearson correlation coefficient (R^2) with cut-off 95%, Raman typing of 50 ExPEC from chicken meat confirmed a high rate of heterogeneity among isolates with 37 unique profiles and only 7 clusters. Expect cluster 5 which includes two isolates from B2 phylogroup , the other 6 clusters include only isolates from D phylogroup (Figure 3.15). Moreover Raman typing of 214 ExPEC from chicken meat and clinical samples also confirmed a high rate of heterogeneity among isolates with 130 unique profiles and only 28 clusters consist of 84 isolates tottaly.
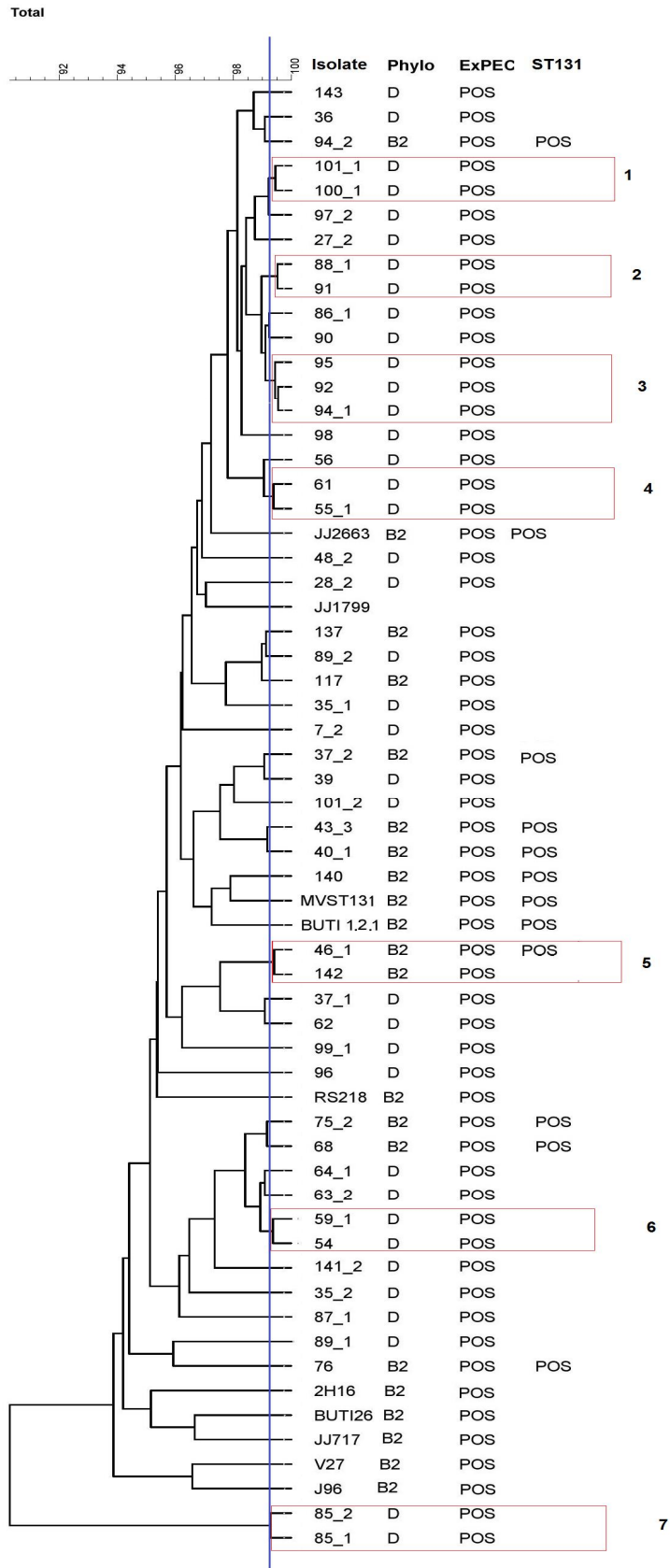
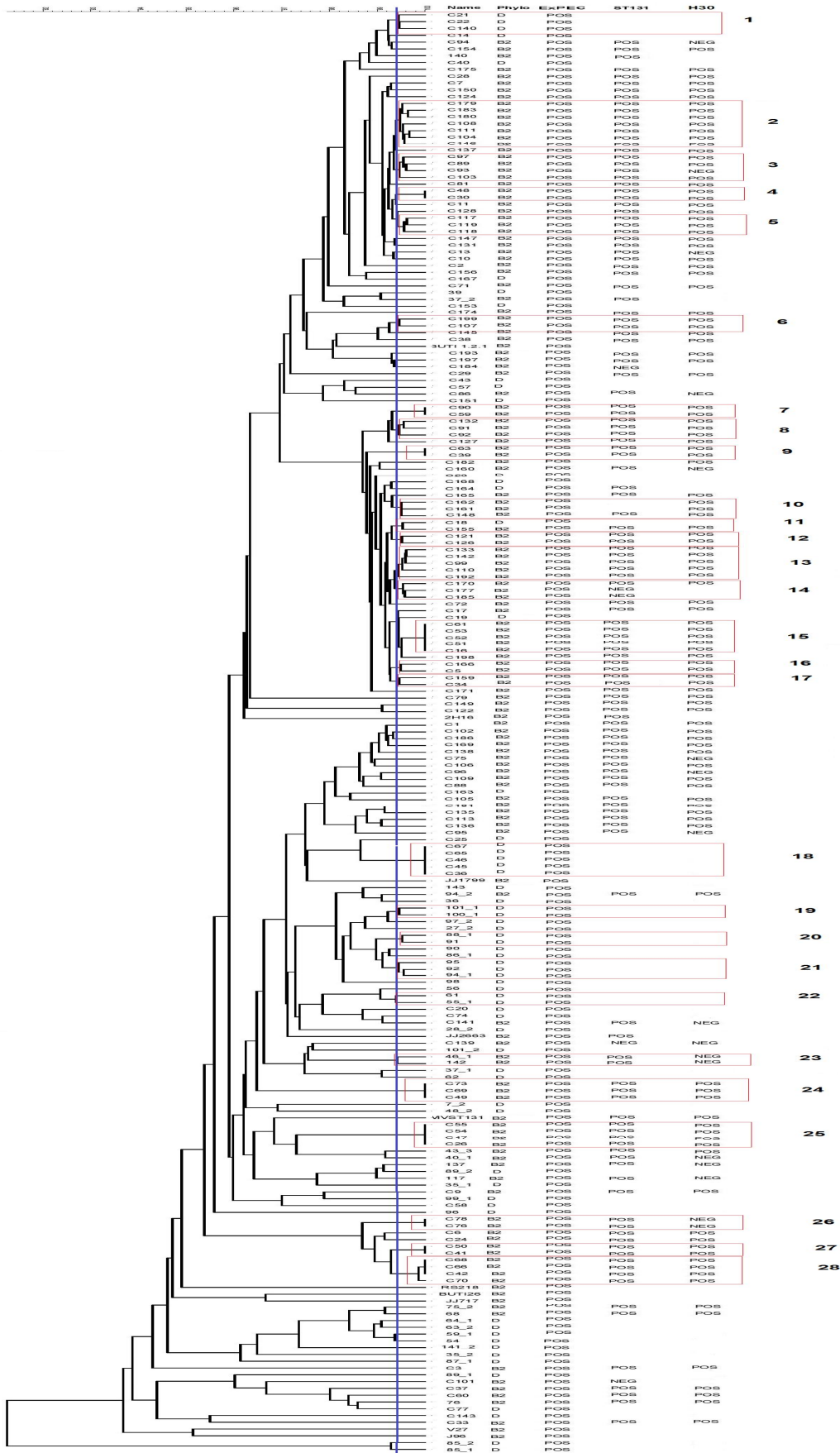**Figure 3.15.** SpectraCell Raman typing analysis of ExPEC isolates from chicken meat samples

Figure 3.16. SpectraCell Raman typing analysis all ExPEC isolates from chicken meat and clinical samples

4.Discussion

Antimicrobial resistance is an issue of great concern because of the serious limitation and even the risk for loss of effective antimicrobial treatment of infections. There are undeniable evidences that foods from many different animal sources and in all stages of processing contain resistant bacteria and their resistance genes (116). It has been as well documented that they can be transferred to humans (55, 83, 116). Food-producing animals, indeed, play a critical role in this transmission chain. According with literature, antimicrobial-resistant *E. coli* strains carried by healthy animals seem to link directly with human infection cases (117). In particular, colonization of the intestinal tract with resistant *E. coli* from chickens has been shown in human volunteers and resistance to the same drugs has been also described in programs undertaken in different countries to monitor bacterial resistance (118). Moreover, studies about bloodstream infections caused by *E. coli* in Europe suggest that poultry might be an important source of antibiotic-resistant isolates (119). Johnson *et al.* (25, 36), after comparing genetic profiles of fluoroquinolone-resistant *E. coli* strains from human blood and fecal samples and from slaughtered chickens, determined that human isolates were virtually identical to resistant isolates from geographically linked chickens. Drug-susceptible human *E. coli* strains, however, were genetically distinct from poultry strains, suggesting that the fluoroquinolone-resistant *E. coli* strains in humans were more likely imported from poultry than derived from susceptible human *E. coli* (3, 29).

In this study, MDR *E. coli* were recovered from about 84.4% of poultry meat samples on sale in Palermo, Italy. These results are in line with other findings from European and non-European countries in accordance to EFSA Panel on Biological Hazards (BIOHAZ) (120). In three similar studies in Portugal, Spain and Netherlands, MDR *E. coli* strains were isolated in 60%, 90% and 94% of chicken carcasses at the retail level, respectively(43, 87, 121). However, results from different studies are generally difficult to compare because of different settings and isolation and testing methods.

In our study, ESBLs and/or AmpC -lactamases were carried by about 95% of isolates which is a higher figure compared with data from other European countries. Indeed, in three similar studies in Germany, Spain and Netherlands, ESBLs were found in 88.6%, 79.7% and 79.8%, respectively, (13, 88, 102). Finally, Mevius *et al.* (103) reported a prevalence of ESBL positive *E.coli* isolates from poultry as high as 86% .

The most predominant ESBL gene families found in our *E. coli* isolates were bla_{SHV}, bla_{TEM}, and $bla_{CTX-M-1}$ group, which is again comparable with previous reports from European countries. TEM, SHV and CTX-M-1 group are reported as the most prevalent -lactam resistance determinants in the food animal reservoir (13, 84, 93, 98, 102). AmpC -lactamases are less frequently reported among *E. coli* isolates and the family CMY-2 is the most geographically spread. In our study plasmid mediated AmpC producing *E. coli* were detected in about 10% of isolates which is comparable with the findings of other studies in Europe (86). However, among our *E. coli* isolates plasmidic AmpC was belonging to the CIT-like type. The absence of AmpC genes in some phenotype-positive isolates might indicate a different mechanism of resistance, such as overexpression of chromosomal AmpC, usually resulting from mutations in the promoter/attenuator region (122).

The prevalence of plasmid-mediated quinolone resistance was very high among our isolates which highlights a serious clinical and public health issue of concern. *qnrA* was the only PMQR determinant detected in all ciprofloxacin resistant isolates accounting for a prevalence of about 90%, substantially higher than any other previous report. For instance, in two studies from Czech Republic and Portugal, PMQR genes were detected only in 4% and 5.5% of strains, respectively (32, 83, 123). This finding might be related to the reportedly extensive use of fluoroquinolones in the poultry industry in the southern European countries, including Italy (124). Transmission of *qnr*-positive *E. coli* isolates to humans through the food chain has been previously hypothesized (125).

According to increased demand for poultry meat and poultry products and the growing poultry industry around the world, the importance of poultry meat safety is a critical public health issue.

The ExPEC isolated from retail poultry meats have been associated with potential infections in humans in Europe, North America and Australia(4, 55). The prevalence and epidemiology of FQ resistant and/or ESBL producing *E. coli* ST131 are changing rapidly. However, only a few studies have investigated the presence of *E. coli* ST131 in animal foods and the current study is one of the first studies to investigate the presence of ExPEC ST131 subclone H30 in retail chickens meat. The prevalence of FQ resistant and/or ESBL-producing ExPEC ST131 from chicken meat in this study was higher than any other studies. Vincent et al. reported a single non-ESBL-producing ST131 isolate from 417 retail chicken samples analyzed(59). None of the 141 ESBL/AmpC-producing *E. coli* isolates from raw chickens imported into the United Kingdom from South America were identified as ST131(53). Similarly, Egea et al. did not detect the ST131 clone among ESBL-producing *E. coli* isolates from 33 raw retail meat samples(89). In contrast, *E. coli* ST131 was isolated from seven retail chicken meat samples from the 100 analyzed in Spain (prevalence, 7%). Three of these isolates both were resistant to FQ and produced CTX-M-9; one produced CTX-M-9, and one was resistant to FQ(10). In current study nine (3.8%) isolates of *E. coli* ST131 were found among 237 *E. coli* isolates which all of them were FQ resistant and ESBL producers. However, until now there is no report of finding ExPEC ST131, subclone H30 from retail chicken meat. This finding may be attributed to several factors, such as the initial colonization status of broilers before processing and the degree of fecal contamination of carcasses during the slaughter operation at the processing facility. This contamination is possible particularly if the persistence/growth of these *E. coli* isolates is favored by specific food processing steps, such as scalding, evisceration, washing and deboning(44, 88). It has been suggested that clinical *E. coli* isolates from sick chickens carry similar sets of virulence genes as those identified in ExPEC isolates from humans and animals(17, 56). Moreover, (7, 12, 38, 48), so it is possible that entry of diseased birds into the poultry slaughter plant might contribute to the contamination of retail meats.

Although next generation sequencing (NGS) can probably provide the necessary resolution for the typing of E. coli, and it is often referred to as a fast and cost-effective method, in a routine setting Raman spectroscopy is cheaper, faster, and easier to perform (126). It does not require the complicated sample preparation steps that are needed for NGS nor does it require special DNA/bioinformatics skills and facilities. The data analysis of Raman spectroscopy can be performed in minutes for Raman vs hours for NGS, and data analysis of Raman spectra is performed in a semiautomated almost real-time fashion while NGS usually requires manual input from someone with a bioinformatics background and may take days to complete. The running costs of Raman are significantly less as they are limited to a simple sample carrier vs NGS that requires costly DNA isolation and sequencing kits.

5.Conclusion

To conclude, this study demonstrated that ST131 is widespread among *E. coli* clinical isolates in our area and the dissemination of ST131-H30 isolates has been observed in our community. Moreover, it was found that ST131, especially the H30 subclone are present in poultry meat and they are closely related to antimicrobial resistant strains. Importantly, the antimicrobial resistance rates of several antibiotics commonly used for UTI have been increased dramatically and the choice of empirical therapy has been limited in our locality. The findings highlight the importance of clonal expansion in dissemination of antimicrobial resistance among urinary pathogens. In summary, our results show that in Sicily a large proportion of retail chicken meat samples carry cephalosporin and ciprofloxacin resistant *E. coli* and especially Extraintestinal pathogenic *E. coli*. A high prevalence of ESBL and PMQR determinants was also found in these isolates, which raises a serious public health concern. A prudent use of antimicrobial agents is urgent in veterinary as well as in human medicine to minimize the selection and spread of antibiotic resistant strains and their resistance genetic determinants.

While NGS is a promising technique, currently it is still a relatively slow and costly method, especially when single isolates that are suspected to belong to an outbreak have to be typed, whereas Raman spectroscopy provides cheap real-time typing of isolates. It can be concluded that Raman spectroscopy is able to cluster isolates that are suspected to be related based on epidemiological data and their antibiotic profiles. Without typing data and only based on available epidemiological data and antibiograms the isolates would have been grouped into four clusters, but typing information proved to be very useful for the analysis of these potential outbreaks as several patients could be excluded from the outbreaks. However, these data need further confirmation before the method can be adapted for routine typing.

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