Molecular Epidemiology and Mechanisms of Cephalosporin Resistance in *Escherichia coli* of Different Origins; Broilers, Flies and White Storks

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Molecular Epidemiology and Mechanisms of Cephalosporin Resistance in *Escherichia coli* of Different Origins; Broilers, Flies and White Storks

Epidemiología Molecular y Mecanismos de Resistencia a Cefalosporinas en *Escherichia coli* de Diferentes Orígenes; Pollos de Engorde, Moscas y Cigüeñas Blancas

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PhD in Animal and Human Health

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2017-2018

UAB

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Memoria presentada por Marc Solà Ginés para aspirar al Grado de Doctor en Medicina y Sanidad Animal por la Universidad Autónoma de Barcelona (UAB). La tesis doctoral titulada "Epidemiología molecular y mecanismos de resistencia a cefalosporinas en *Escherichia coli* de diferentes orígenes; pollos de engorde, moscas y cigüeñas blancas" ha sido realizada en el Centro de Investigación en Sanidad Animal (CReSA-IRTA), dentro del Subprograma Infecciones bacterianas y endoparasitarias, y resistencia a antimicrobianos (BACPAR). La Dra. Lourdes Migura García, investigadora del centro y como directora de la tesis, y la Dra. Natàlia Majó, profesora asociada de la UAB, investigadora del centro y como tutora de la tesis, así lo certifican.

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A la meva estrella polar, gràcies papa per ajudar-me a seguir el meu camí.

“Nadie, ni yo ni nadie, puede andar este camino por ti. Habrás de recorrerlo tú solo. No está lejos, lo tienes a tu alcance. Tal vez estás en él desde que naciste. Tal vez está en todas partes: en el mar y en la tierra.”

Canto de mí mismo 46, Walt Whitman

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Abbreviations

AEMPS, Agencia Española de Medicamentos y Productos Sanitarios

afa/dra, Dr binding proteins

AFEC, avian faecal *E. coli*

Am, ampicillin

AMPs, antimicrobial peptides/péptidos antimicrobianos

APEC, avian pathogenic *E. coli*

BLEE, betalactamasas de espectro extendido

BHI, brain heart infusion

C. ciconia, *Ciconia ciconia*

CamCon, Campylobacter control

Caz, ceftazidime

CDC, Centers for Disease Control and Prevention

Ci, ciprofloxacin

CIs, confidence intervals

Cm, chloramphenicol

ColV, colicin V from serum resistance-associated plasmids

Cplx, clonal complex

CR, cephalosporin resistant

Cs, colistin

Ctx, cefotaxime

DNA, deoxyribonucleic acid

E. coli, *Escherichia coli*

EAEC, enteroaggregative *Escherichia coli*

EAF, EPEC adherence factor

EARS-Net, European Antimicrobial Resistance Surveillance Network

EAST1, enteroggregative *Escherichia coli* heatstable enterotoxin I

EHEC, entherohemorragic *Escherichia coli*

EIEC, enteroinvasive *Escherichia coli*

ESBL, extended-spectrum beta-lactamase

EPEC, entheropathogenic *Escherichia coli*

ETEC, enterotoxigenic *Escherichia coli*

EU/UE, European Union/Unión Europea

EXPEC, extraintestinal pathogenic *Escherichia coli*

Ff, florfenicol

FQ, flouroquinolone resistance gene

fyuA, yersiniabactin system receptor

GLMM, Generalized mixed linear model

Gm, gentamicin

GzLM, Generalized linear model

INIA, Instituto Nacional de Investigación y Tecnología Agraría y Alimentaría

Inc, incompatibility group

irp-2, iron repressible associated with yersiniabactin synthesis

ISCR, Insertion sequence common regions

iss, increasead serum survival

iutA, aerobactin receptor

Kb, kilobases

Km, kanamycin

kpsM II, group 2 capsule synthesis

kpsM II-K1, K1 capsular antigen

LREC, Laboratorio de Referencia de *Escherichia coli*

M. domestica, *Musca domestica*

mcr-1, plasmid-mediated colistin resistance gene

MIC, minimum inhibitory concentration

MLST, multilocus sequence typing

Nal, nalidixic acid

OMS, Organización Mundial de la Salud

OLS, ordinary least squares

OR, odds ratio

pap, P fimbriae

PCR, polymerase chain reaction

PQMR, plasmid-mediated quinolone resistance

PFGE, pulsed-field gel electrophoresis

RNA, ribonucleic acid

sfa/foc, S/F1C fimbriae

Sm, streptomycin

SMA, standardised major axis

ST, sequence type

STEC, shiga toxin-producing *Escherichia coli*

stx1, shiga toxin 1

stx2, shiga toxin 2

Su, sulfamethoxazole

Tc, tetracycline

Tm, trimethoprim

tsh, temperature-sensitive hemagglutinin

UTI, urinary tract infections

UV, ultraviolet

VF, virulence-associated factor

VTEC, verocytotoxin-producing *Escherichia coli*

WGS, whole genome-sequencing

WHO, World Health Organization

WT, wild type

Summary

Bacteria resistant to antimicrobials, and specifically to extended-spectrum beta-lactamases (ESBLs), have become of increasing concern. Its prevalence has increased fast worldwide due to a pandemic dissemination of plasmids and the introduction of ESBL resistance genes into successful clones (Coque, Baquero et al. 2008, Brolund 2014). According to the World Health Organization (WHO), the most critical antimicrobials in human and animal health are third-generation cephalosporins, fluoroquinolones, macrolides and aminoglycosides (Collignon, Powers et al. 2009, Collignon, Conly et al. 2016). Therefore, this thesis has focused in resistance to cephalosporins and particularly to ESBLs and AmpC type of resistance.

Due to the overuse of antimicrobials in humans and veterinary medicine, the emergence of multi-drug resistance has increased in the last decades. Most of the research has focused on the epidemiology of antibiotic resistance in human and veterinary medicine; however, in the last years it has been an increasing attention on how multiresistant bacteria circulates in natural environments and how these niches can act as reservoirs of resistant traits (Allen, Donato et al. 2010). Once the resistant bacteria are present in the environment, it can easily get in contact with humans.

Through the studies compiled in this thesis, we have investigated the occurrence and mechanisms of dissemination of cephalosporin resistance (CR) *Escherichia coli* from different ecological niches; broilers as an example of food-producing animals, houseflies (*Musca domestica*) as mechanical vectors and reservoir of resistance genes contributing to the spread of resistance in the farm environment; and

finally white storks (*Ciconia ciconia*) as an example of long-distance vectors and sentinels of human pressure. With this approach, we intend to understand the transmissibility of these resistances among different niches; and identify clones, resistance genes and plasmids involved.

Resumen

Las bacterias resistentes a antibióticos, y en concreto a betalactamasas de espectro extendido (BLEEs), se han convertido en un problema grave. Su prevalencia ha incrementado rápidamente a nivel mundial debido a una diseminación pandémica de plásmidos y a la introducción de genes de resistencia a BLEEs en clones exitosos (Coque, Baquero et al. 2008, Brolund 2014). Según la Organización Mundial de la Salud (OMS), los antimicrobianos más críticos en sanidad humana y animal son las cefalosporinas de tercera generación, las fluoroquinolonas, los macrólidos y los aminoglicósidos (Collignon, Powers et al. 2009, Collignon, Conly et al. 2016). Por lo tanto, esta tesis se ha centrado en las resistencias a cefalosporinas, y en particular resistencias a BLEEs y betalactamasas tipo AmpC.

La mayoría de las investigaciones se han centrado en la epidemiología de las resistencias a antibióticos en medicina humana y veterinaria; sin embargo, en los últimos años ha habido un aumento de los estudios dirigidos a bacterias multiresistentes que circulan en ambientes naturales actuando como reservorios (Allen, Donato et al. 2010). Una vez las bacterias están presentes en el ambiente, pueden transmitirse a los humanos.

A través de los estudios incluidos en la actual tesis, hemos investigado la aparición y los mecanismos de diseminación de *Escherichia coli* resistentes a cefalosporinas de diferentes nichos ecológicos; pollos de engorde como ejemplo de animales de producción, moscas (*Musca domestica*) como vectores mecánicos y reservorios de genes de resistencia que contribuyen a la diseminación de resistencias en el ambiente de granja; y finalmente, cigüeñas

blancas (*Ciconia ciconia*) como ejemplo de vectores de larga distancia y centinelas de la presión humana. Con este enfoque, intentamos entender la transmisibilidad de las resistencias entre nichos diferentes; e identificar los clones, los genes de resistencia y los plásmidos involucrados.

Introduction

Escherichia coli

General characteristics of *E. coli*

Enterobacteriaceae family encloses a high number of bacteria, including *E. coli* that is one of the most prevalent. The family *Enterobacteriaceae* contains 47 genera and a few non-classified enterobacteria with a high homology and physiology (https://www.ncbi.nlm.nih.gov/taxonomy).

E. coli usually belongs to the commensal microflora of the intestinal tract of humans and warm-blooded animals, although several variants can colonize other ecological niches such as water, soil or vegetative cells (Madigan, Martiniko et al. 2003). *E. coli* is a Gram-negative bacteria of 4-5 µm long, non-spore forming rod and they may or not be mobile, depending if they are flagellated or not (Figure 1). The microorganism is facultative anaerobe and ferments simple sugars like glucose to form acetic, lactic and formic acids. It can grow with a range from 6ºC to 50ºC, being the optimal conditions a temperature of 37ºC.

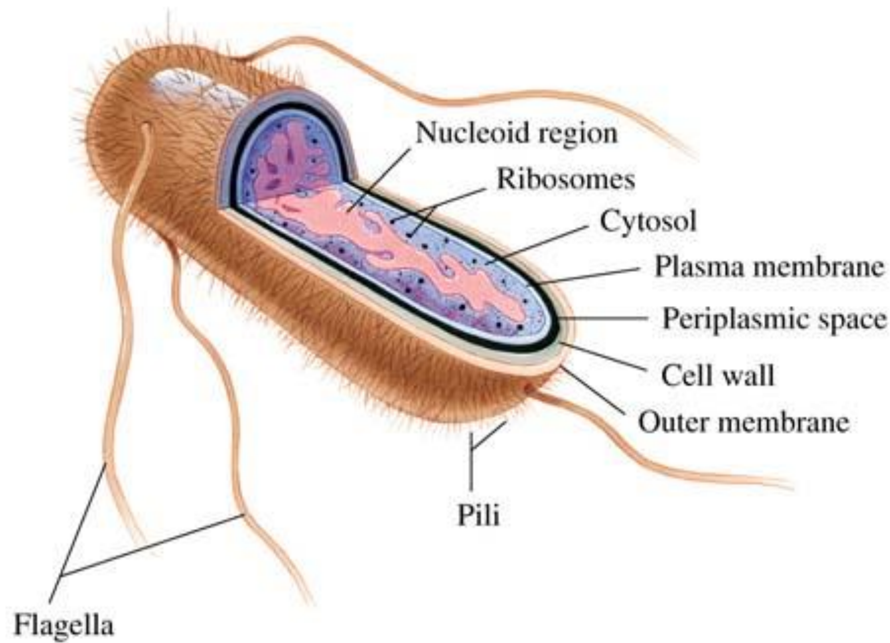


Figure 1. Structure and different elements of *E. coli* bacteria.

E. coli is used as an indicator of faecal contamination when evaluating the safty of water and food. This is due to its high prevalence in faeces and gastrointestinal tract (FAO 2011). Generally, *E. coli* is non-pathogenic, however, they can cause infection of the gastrointestinal system or induce disease in bodily sites outside of the gastrointestinal tract (extraintestinal pathogenic *E. coli*) (Kaper, Nataro et al. 2004).

Classification of *E. coli* as a pathogen

Most of *E. coli* strains in mammals and birds belong to the microbiota. They contribute with their metabolism suppressing the growth of harmful bacteria and synthetizing vitamins (Russo and Johnson 2003). Nevertheless, there are some strains that can cause intestinal illness in different hosts. These pathogenic strains have the ability to cause disease with mechanisms, such as adhesion and invasion of host cells or toxin production; and depending on the

symptomatology produced in humans can be divided in different groups.

There are many different pathogenic strains grouped and named according to their ability to produce diferent type of disease in the host. Most important are enterohemorragic *E. coli* (EHEC), which is a subset of shiga toxin-producing *E. coli* (STEC) responsible for producing the citotoxin named shiga that causes a blooded diarrhea; enterotoxigenic *E. coli* (ETEC) is the responsible for the also called "turist diarrhea" in countries with poor sanitation and hygiene; enteropathogenic *E. coli* (EPEC) is associated to infant diarrhea in developing countries. Enteroinvasive *E. coli* (EIEC) are characterized for causing diarrhea and dysentery, due to a complex pathogenic mechanism producing a strong inflammation reaction and ulcers. Enteroaggregative *E. coli* (EAEC) are able to aggregate and adhere to epithelial cells and are responsible for causing diarrhea in adults and children in several countries. Both ETEC and EHEC pathotypes can be found in different animal species, becoming their reservoirs (Nataro and Kaper 1998).

There are some *E. coli* pathotypes that can cause important extraintestinal infections resulting in high morbidity, which are not related with epidemics or alimentary toxoinfections. These strains are able to successfully colonize the intestine (Russo and Johnson 2000). Same autors make a new designation for the mentioned strains as ExPEC, extraintestinal pathogenic *E. coli*. Johnson et al (2003) described the virulence-associated factors recognized as extraintestinal such as *papC* o *papAH*, *sfa/foc*, *afa/dra*, *iutA* and *kpsM II*. Accordingly, the strains are considered ExPEC if they contain more than two of these markers (Johnson, Murray et al. 2003).

Some ExPEC strains can affect broiler farms and avian industry causing a lot of economical losses (Schouler, Schaeffer et al. 2012). This pathotype is called APEC (avian pathogenic *E. coli*) and causes extraintestinal infections in poultry, such as colibacilosis (Morley and Thomson 1984).

Virulence-associated factors of *E. coli*

Virulence is the ability of bacteria to cause disease in a healthy host (Peterson 1996). In the case of *E. coli* there are different virulence genes that are implicated in several functions, such as motility, colonization, dissemination and nutrient acquisition. The number of virulence-associated factors (VF) could be an approximation of the capability of a strain to cause disease by the effect of the different VF needed, being a pathogenic or non-pathogenic commensal strain (Peterson 1996). In this work, we described the most studied VF for the interest of the studies, in particular the VF associated to ExPEC and APEC infections.

VF comprise mechanisms that allow pathogenic bacteria to cause infections. Althought the presence of a single virulence factor will not make a strain virulent, a combination and levels of expression of those factors would determine if a bacteria could cause infection (Dobrindt 2005).

VF can be classified in functional categories; such as adhesins, toxins, anti-host defense factors or protectins, iron acquisition systems and other with additional functions. Adhesins allow pathogenic *E. coli* to colonize different sites where they are not normally found, being necessary for bacteria-cell interaction during the colonization. In the case of APEC, the most common adhesins are type 1 fimbriae and P

fimbrial (Mellata, Dho-Moulin et al. 2003). Iron acquisition systems are essential VF in the infectivity process of *E. coli* pathogenic strains. They are required to capture iron molecules either by having receptors of iron transporters like haemoglobin or transferrin, or secreting siderophores able to quelate Fe^{+3} that are associated to transporting proteins (Mokady, Gophna et al. 2005).

In case of ExPEC strains, they exhibit an extensive diversity of VF, which are showed in Table 1, generally encoded in mobile genetic elements, although they can also be integrated in the chromosome. The combination of VF contributes to fitness cost of these isolates, increasing their adaptability, competitiveness and the ability to colonize the host (Mokady, Gophna et al. 2005)

There are some VF described and expressed by APEC strains, such as; temperature-sensitive hemagglutinin (*tsh*), aeorbactin siderophore and yersiniabactin system (*fyuA* and *irp-2*), colicin V from serum resistance-associated plasmids (*colV*), K1 capsular antigen (*kpsM II-K1*), increasead serum survival (*iss*), shiga toxins 1 and 2 (*stx1* and *stx2*) (Blanco, Blanco et al. 1997, Schubert, Rakin et al. 1998, Pfaff-McDonough, Horne et al. 2000).

	Virulence-associated factor
Adhesins	
papA	Major structural subunit of P fimbrial shaft
papC	Pilus assembly; central region of P fimbrae operon
papEF	Pilus associated with pyelonephritis (P fimbriae)
papG	Gal(α1-4) Gal-specific pilus tip adhesin molecule (P fimbrae)
sfa/focDE	Central region of S and F1C fimbrae operons
fimH	D-mannose-specific adhesin of type 1 fimbriae
afa/draBC	Dr antigen-specific adhesin operons (AFA, Dr, F1845)
Toxin	
hlyD	α-Haemolysin
hlyF	Haemolysin F
sat	Secreted autotransporter toxin

astA	Enteroaggregative E. coli heat stable toxin (EAST 1)
cnf1	Cytotoxic necrotizing factor 1
cdtB	Cytolethal distending toxin
tsh	Temperature-sensitive haemagglutinin-serine protease
stx1	Shiga toxin 1
stx2	Shiga toxin 2
Siderophores	
iroN	Novel catecholate siderophore receptor
fyuA	Yersinia siderophore receptor (ferric yersiniabactin uptake)
ireA	Iron-regulated element (novel siderophore receptor)
iutA	Ferric aerobactin receptor (iron uptake: transport)
Protectins	
kpsM II	Group II capsule
kpsM II-K2	K2 subgroup II capsule
kpsM II-K5	K5 subgroup II capsule
kpsM II-K1	K1 subgroup II capsule
kpsM III	Group III capsule
cvaC	ColV; colicin V from serum resistance-associated plasmids
Iss	Increased serum survival (outer membrane protein)
traT	Surface exclusion, serum survival (outer membrane protein)
Miscellaneous	
usp	Uropathogenic-specific protein
ompT	Outer membrane protein (protease) T
fliC H7	Flagellin variant H7
malX	PAI; pathogenicity-associated island marker
ibeA	Invasion of brain endothelium
clbN	Codes for non-ribosomal synthetase (colibactin)
clbB	Codes for hybrid peptide-polyketide synthase (colibactin)

Table 1. Virulence associated factors characteristics of ExPEC, including those related to APEC strains. Adapted from Pitout et al (Pitout 2012).

Zoonotic potential of *E. coli*

E. coli is known to be a carrier of different antibiotic resistance genes, such as cephalosporins, fluoroquinolones, aminogylcosides and colistin (Bennett 2008, Hawser, Bouchillon et al. 2009, Brolund, Edquist et al. 2014, Liu, Wang et al. 2016). The selection pressure applied by antimicrobials used in agricultural or clinical frame has contributed to the evolution and spreading of resistance genes (Figure 2). Even physichal forces, like those created by wind and watershed,

can be important drivers of spreading antibiotic resistant genes (Allen, Donato et al. 2010).

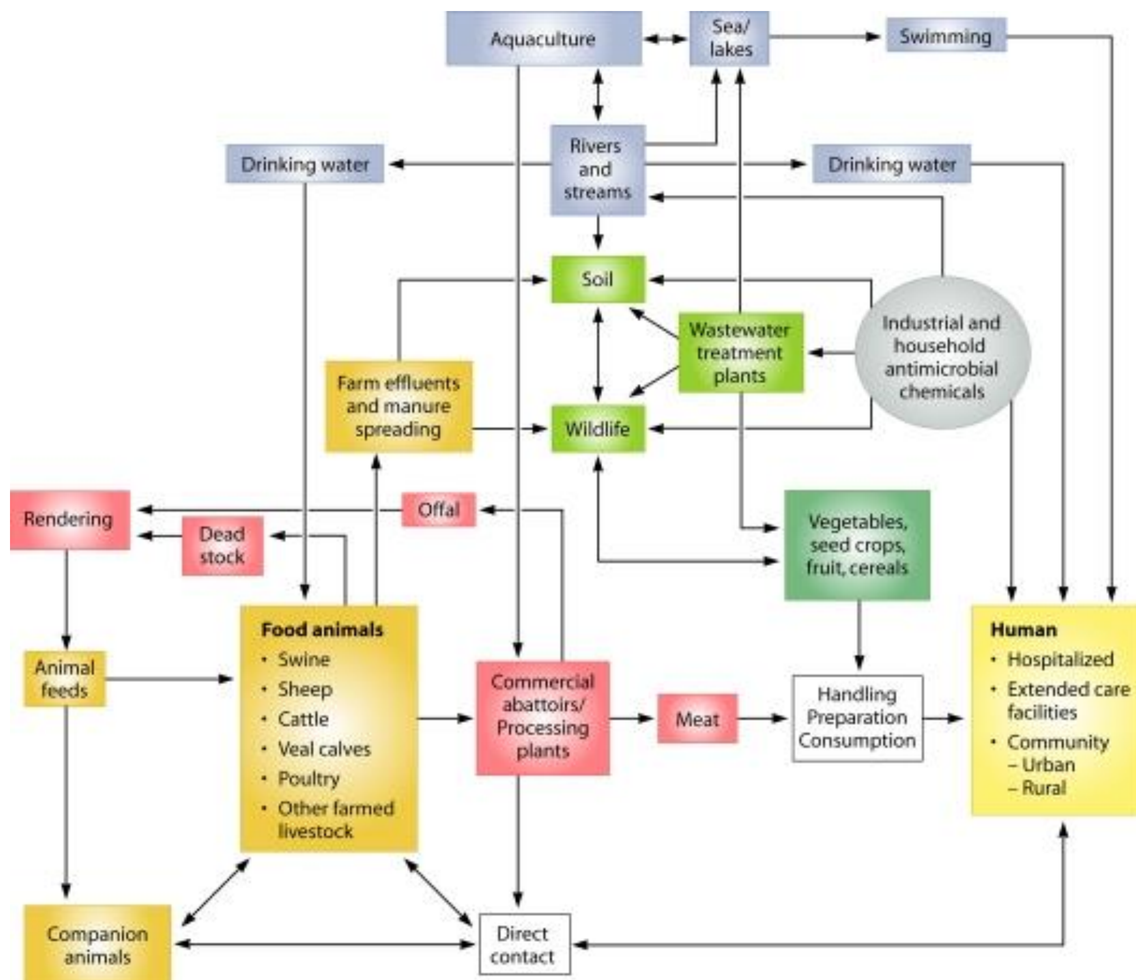


Figure 2. Dissemination of antibiotics and its resistance within all the environments; such as hospitals, agriculture, human community, wastewater treatment, and all the related niches (Davies and Davies 2010).

ExPEC infections are increasing worldwide causing an enormous burden in public health (Barnes, Vaillancourt et al. 2003). In addition, some pathotypes are also responsible for huge economical losses in food-producing animals, particulary in the poultry industry (Schouler, Schaeffer et al. 2012). Many studies have suggested that avian products could serve as an important source of ExPEC to humans, causing sepsis infections because of their virulence. Poultry meat has

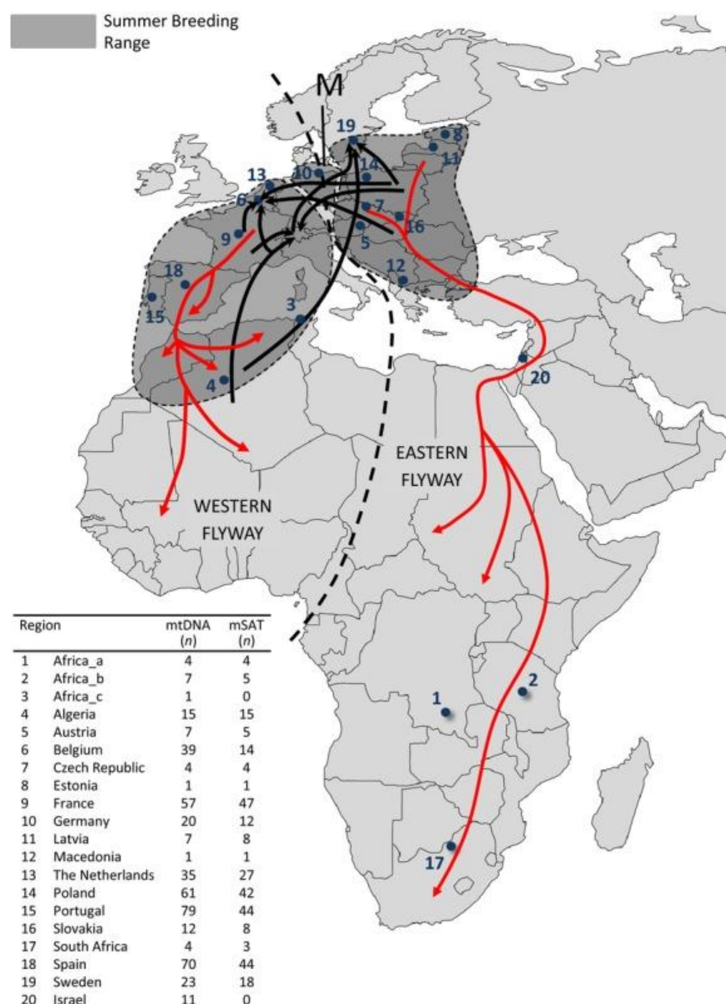
the highest levels of multi-drug resistant *E. coli* compared to other type of meat (FDA 2010). The increased of poultry meat production have contributed to the emergence of foodborne ExPEC infections and antibiotic resistance in humans, being an important zoonotic risk (Manges and Johnson 2012). One of the sources of these bacteria, which are causing important health problems in humans, may be the excessive use of these antimicrobials in agriculture and livestock (Johnson, Sannes et al. 2007).

APEC is the main cause of colibacillosis in poultry farms; which is a syndrome associated to different pathological symptoms; such as respiratory infections (airsacculitis), perihepatitis, pericarditis, and sometimes ending with a fatal septicaemia. Moreover, APEC strains can cause local infections, such as cellulitis and salpingitis (Barnes, Vaillancourt et al. 2003). *E. coli* infections are responsible for the mortality of 1–10% of the animals in farms, which increases in case of broilers (Omer, Abusalab et al. 2010). Avian colibacillosis treatment depends mainly on the use of antimicrobials. In the poultry industry, the most commonly used antimicrobials to treat *E. coli* infections are tetracyclines, flouorquinolones and sulphonamides (Zhao, Maurer et al. 2005). ESBL genes have been detected in APEC and fecal *E. coli* from healhy poultry, and the emergence for instance in Canada has been associated to the use of ceftiofur, a third-generation cephalosporin that is injected in chicken eggs to control *E. coli* omphalitis (Dutil, Irwin et al. 2010). However, ESBL-producing *E. coli* has been detected also in poultry not treated with cephalosporins, so this assumption would not be the only explanation. In the case of Europe, there are studies associating this emergence to vertical transmission from breeders to broilers (Bortolaia, Guardabassi et al. 2010).

Multi-drug resistance bacteria have been detected in the microbiota of many wild animals, even in long-distance migrants. The non-well treated human wastes and the use of antimicrobial in livestock have contributed to the spread of resistance bacteria in wildlife (Arnold, Williams et al. 2016). Wildlife animals, and specifically wild birds, are well known reservoirs and spreaders of pathogenic and multi-drug resistance bacteria that can cause disease in human population (Skurnik, Ruimy et al. 2006). Moreover, the level of antimicrobial resistant bacteria in wild animals seems to follow a correlation with the degree of human activity of the habitat (Skurnik, Ruimy et al. 2006).

Many bird species are considered migratory, covering long distances, crossing continents and increasing the potential of disease along the migration routes (Figure 3). These birds can move antimicrobial resistant bacteria from hotspots to vulnerable populations, having a huge potential of dissemination of resistance (Arnold, Williams et al. 2016). Therefore, wild birds are good sentinels of multidrug-resistant bacteria from the environment, since they are not treated with antibiotics. Many antimicrobials are excreted in an active form and persist in the environment. White stork, as an exemple of wild migratory bird, can acquire bacteria from faecal contamination of surface waters or contaminated pastures. They also cohabit with humans using man-made constructions. So, they can be a potential source of ESBL-producing *E. coli* for livestock and humans. Additionally, bacteria of wildlife origin may infect human or transfer resistance genes with human pathogens (Vittecoq, Godreuil et al. 2016). There are many studies showing a direct correlation beween antibiotic resistant bacteria isolated from different wildlife animals

and human population (Stoddard, Atwill et al. 2008, Williams, Sherlock et al. 2011); in addition, there are also studies showing that in the most remote and unhabited places, antimicrobial resistance bacteria can be detected from wildlife animlals (Cristobal-Azkarate, Dunn et al. 2014). A recent study has, reported cephalosporin resistant *E. coli* in white storks for the first time (Alcalá, Alonso et al. 2016, Camacho, Hernandez et al. 2016).



Region		mtDNA (*n*)	mSAT (*n*)
1	Africa_a	4	4
2	Africa_b	7	5
3	Africa_c	1	0
4	Algeria	15	15
5	Austria	7	5
6	Belgium	39	14
7	Czech Republic	4	4
8	Estonia	1	1
9	France	57	47
10	Germany	20	12
11	Latvia	7	8
12	Macedonia	1	1
13	The Netherlands	35	27
14	Poland	61	42
15	Portugal	79	44
16	Slovakia	12	8
17	South Africa	4	3
18	Spain	70	44
19	Sweden	23	18
20	Israel	11	0

Figure 3. Distribution of white storks sampled regions within eastern and western flyway, divided by a black dotted line. Summer breading locations are indicated with dark grey. Red arrows show the direction of overwintering migration, whereas black arrows indicate the translocation routes of individuals between flyways as part of reintroducing activities (Shephard, Ogden et al. 2013).

Typing methods for *E. coli*

In order to assess a health risk for survilance purposes, *E. coli* isolates are normaly typed following different methods, such as, serotyping, phenotyping, phatotyping, plasmids characterization and DNA-based fingerprints including pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Nowadays, whole-genome sequencing (WGS) of bacteria is being used for identifying pathogenic species, detecting resistance, virulence genes and providing a useful tool for phylogenetic analyses. WGS will be soon routinely used in microbiology laboratories, since it is becoming cheaper in price and the bioinformatic packages are more user friendly, providing faster and more accurate results than classical molecular techniques (Didelot, Bowden et al. 2012).

A serologic classification of *E. coli* was firstly described by Kauffman in 1947. This technique is useful to determinate those serotypes (O:H:K) more prone to cause infections or those belonging to commensal strains (Kauffman 1947). In 1981, Guinée *et al* modified the method; the different serotypes are classified according to their cell surface antigens, allowing an epidemiologic classification at sub-species level. During the technique procedure, all antisera are obtained and absorbed with the corresponding cross-reacting antigens to remove nonspecific agglutinins (Guinée 1981). Serologic studies cannot be used to discriminate virulence degree. For this reason, the combination of molecular techniques, such as phylogeny, MLST, PFGE, plasmid DNA analysis and the virulence profile of the strains may help to identify pathogenic clones.

In the case of the phylotyping, *E. coli* is divided in phylogenetical groups according to difference genes (*chuA*, *yjaA*, *arpA*) and a specific

DNA fragment (TSPE4.C2) resulting in a different combination for each group (A, B1, B2, C, D, E and F) (Clermont, Bonacorsi et al. 2000, Clermont, Christenson et al. 2013). A multiplex PCR followed by an electrophoresis agarose gel is performed to obtain the results, as shown in Figure 4.

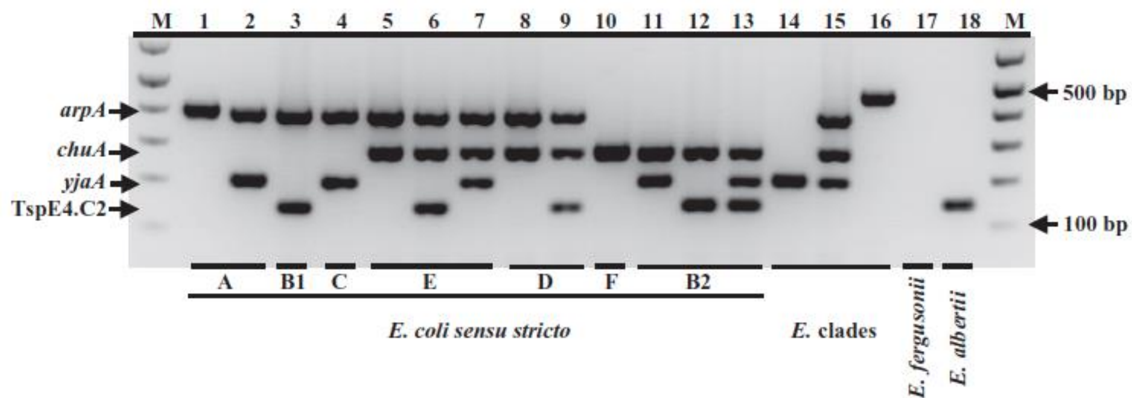


Figure 4. Different multiplex PCR profiles of the phylo-typing method for E. coli (Clermont, Christenson et al. 2013).

PFGE was developed to detect multilocality foodborne outbreaks. The application of relevant molecular subtyping techniques and the accessibility to sophisticated computers for analysing all data have increased in laboratories. Not all laboratories use the same method, so comparisions between laboratories were difficult (Barrett, Lior et al. 1994). PFGE has been shown to be a highly discriminating method used for subtyping pathogen bacteria. Currently, it is the “gold standard” for subtyping foodborne pathogens, despite of intra-and interlaboratory reproductible problems. However, it will be soon replaced for next generation sequencing methods. This was the purpose of PulseNet created by CDC in 1996, to have a comparable molecular subtyping-based surveillance method (Swaminathan, Barrett et al. 2001). So nowadays, standardized PulseNet protocols can

be performed in 24-28h. During the protocol, chromosomal DNA contained in the PFGE plugs is digested by *XbaI* restriction enzyme, and the resultant bands from electrophoresis (Figure 4) are analysed by a software called Fingerprinting II Informatix (Applied Maths, Sint-Martens-Latem, Belgium). In our laboratory strains with one band difference in their profile have been considered different (Ribot, Fair et al. 2006).

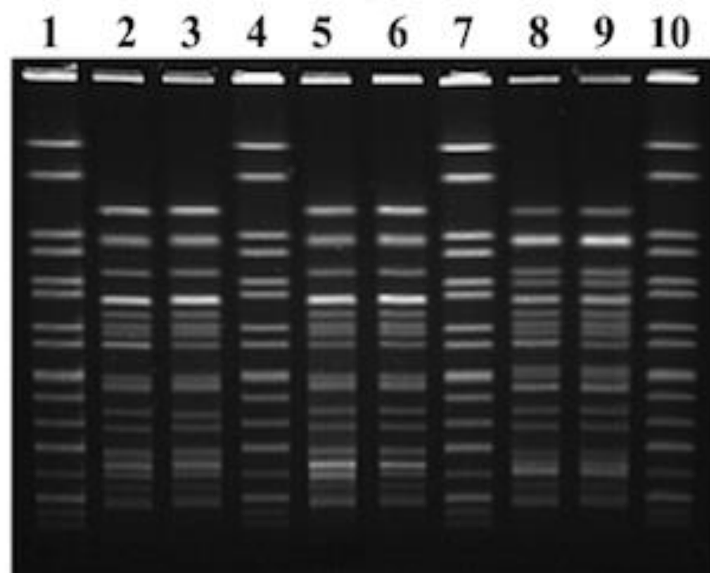


Figure 4. PFGE image of *E. coli* O157:H7 using 1 day protocol. Universal standard strains (*Salmonella Branderup H9812*) are in lanes 1, 4, 7 and 10. The electrophoresis run time was 18h. Adapted from Ribot *et al* (Ribot, Fair et al. 2006).

MLST technique was developed in order to determinate the potential evolutionary relatedness among the different bacteria isolates. The protocol consists in a gene amplification by PCR of seven housekeeping gene fragments (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) and their following sequencing and analysis of the sequences (Wirth, Falush et al. 2006). The resultant allelic profile and the sequence types (STs) obtained via an MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli). Results can appear in an MSTREE

showing the strains relationship, and allowing to see the evolution of virulent phenotypes (Figure 5).

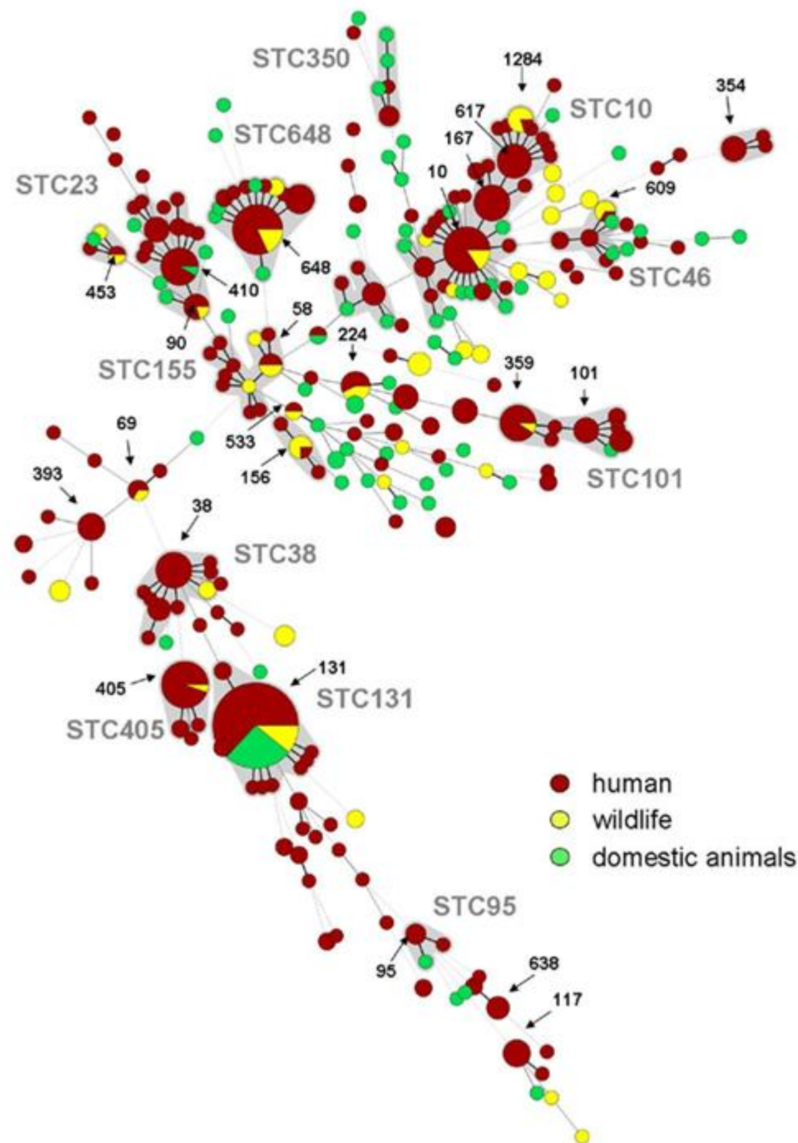


Figure 5. An example of MS_{TREE} of different niches sequence types known for ESBL production based on data from MLST database (Guenther, Ewers et al. 2011).

Antimicrobials

Antimicrobials are considered compounds produced in nature by different type of microorganisms. The term also includes semisynthetic derivates of themselves or totally synthetics. They can act as bacteriostatic when they inhibit bacterial grow, or as bactericidal killing them. Despite of the bactericidal or bacteriostatic effect of antimicrobials in the use of disease treatment, antimicrobials also act exerting a selective pressure on microbiota bacteria from animals and humans, which favours the selection of resistant bacteria.

Alexander Fleming discovered accidentally penicillin in 1928. This discovery is considered one of the most important health events of modern time, and a crucial moment for human history (Ligon 2004). This was the first natural compound with antimicrobial activity described. Since then, pharmaceutic industry started to obtain new antimicrobial molecules from different microorganisms, discovering the most important families such as beta-lactams, aminoglycosides or macrolides. This time was called the golden era, when also new syntetic antimicrobials were developed; and the effect of these agents together with an improved sanitation and vaccination, contributed to the reduction of infectious diseases and mortality. During the next decades, new discoveries of antimicrobial families were achived, but in the last decades this rhythm stopped and only a few new antimicrobial molecules were developed. This situation, together with the alarming increase of antimicrobial resistance, makes difficult the treatment of many bacterial infecctions.

Beta-lactam antimicrobials

Beta-lactam antibiotics have in common the structure of the betalactam ring (Figure 6), which is responsible of its action inhibiting the last step of the bacteria cell wall synthesis. They have been the primary choice for treatment diseases worldwide since 1940s due to their wide spectrum of activitiy (Marin and Gudiol 2003). Nevertheless, the increasing of resistance and the spread of beta-lactamases in Gram-negative bacteria represents difficulties at the time of a successful treatment. This group of antimicrobials is the most numerous family. Among the most used beta-lactams are penicillins and cephalosporins (Johnson and Woodford 2013).

Beta-lactam inhibitors were introduced to treat clinical cases to overcome beta-lactam antimicrobials resistance. These inhibitors, such as clavulanate acid or sulbactam, can make an efficient use of penicilins in clinical important infections. They usally are administrated together, reducing the action of the bacteria for inhibiting beta-lactam antimicrobials. The most commonly used inhibitor in clinical cases is clavulanate acid together with ampicillin or amoxicillin. However, the selective pressure for the excesive use of antimicrobials promoted the emergence of resistance to these inhibitors (Drawz and Bonomo 2010).

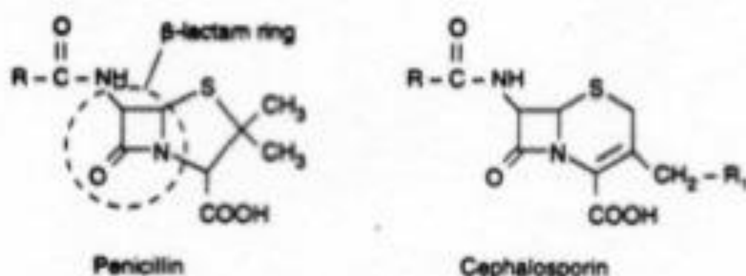


Figure 6. Chemical structure of the most used beta-lactam antimicrobials (penicillins and cephalosporins) with the common beta-lactam ring.

Cephalosporins

Cephalosporins are a group of beta-lactam antimicrobials derived from a fungus called *Cephalosporium*. The first cephalosporin developed was cephalosporin C and it was discovered in 1955. This was capable of absorbing UV light, to keep the stability in acid media, with a low toxicity and resistant to enzymes able to degradate penicillin (Abraham, Newton et al. 1955). The mechanism of action is common to all beta-lactam antimicrobials, interfering in the cell wall formation of the bacteria by joining the active enzymes during the peptideglican synthesis (Marshall and Blair 1999).

After 1960s, and with the knowledge of the biosynthesis route of cephalosporin C, many different groups of semisynthetic cephalosporins were obtained. They were divided in generations depending on their historic development, its structural and antimicrobial characteristics (Marshall and Blair 1999):

- First generation cephalosporins presented an activity against Gram-postive coccus (*Staphylococcus* and *Streptococcus*) but with a very limited activity against Gram-negative bacteria. Cefradin, cefapirin or cefalexin are examples.
- Second-generation cephalosporins have a very similar activity against Gram-positive but with an increment against Gram-negative; such as, *Haempohilus*, *Neisseria* or *Moraxella*. They were resistant to penicillins and first

generation cephalosporins. Cefoxitin and cefaclor are included in this group.

- Third generation cephalosporins are semisynthetic compounds with an enormous activity against enterobacteria, except for ESBL-producing isolates. Important antimicrobials of this group are ceftriaxone, ceftiofur and cefotaxim.
- Fourth-generation cephalosporins have a broad-spectrum activity against Gram-negative bacteria with a large stability and more resistant to beta-lactamases. Cefepime and cefquinome are the most important.

Use of beta-lactam antimicrobials in human and veterinary medicine

Antimicrobials are needed for the treatment, control and prevention of infectious diseases in human and veterinary medicine. Moreover, they are used also in agriculture and aquaculture but in less quantity.

Since 1950s antimicrobials have been used as growth promoters, especially in pigs and broilers, but its use was banned in the European Union (EU) in 2006 due to the corelation with a huge increment of antimicrobial resistance (Sarmah, Meyer et al. 2006). Despite of it, in other continents is still permitted.

Spain is one of countries from the EU that more antimicrobials uses to produce one kg of meat (418 mg/PCU population correction unit) (EMA 2016). The regulation of antimicrobial cosumption for livestock depends on the country. For instance, prophylaxis is forbidden in Scandinavian countries, whereas this practice is still common in other EU countries. In EU, approximately more than 50% of

antimicrobials were used for veterinary medicine (Grave, Torren-Edo et al. 2010). Additionally, more than 80% of the antimicrobials used in animals in the EU are prescribed in livestock, whereas the rest are used in companion animals (Ungemach, Muller-Bahrdt et al. 2006).

Beta-lactams are one of the antimicrobials that provide more benefits for the treatment of bacterial infections. Several beta-lactams are currently lisenced to be used in veterinary medicine. This situation presents an opportunity for selection of resistant bacteria, in particular the presence of resistance to cephalosporins has been observed in bacteria from animal origin. Ceftiofur and cefquinome, third and fourth-generation cephalosporins respectively, have been developed exclusively for their use in veterinary medicine, mainly to treat pigs and bovine infections (Hornish and Kotarski 2002).

Antibiotic resistant bacteria

Origin and development of resistance

Unfortunately, the use of antimicrobials came with a rapid appearance of resistance isolates in parallel to the emergence of these drugs (Figure 7). The world is saturated with antimicrobials since their introduction in 1937. During 1950s, genetically transferable genes for antibiotic resistance were identified in Japan, where the concept of conjugation was introduced, explaining how antibiotic resistance genes could be disseminated over the whole bacterial population (Davies 1995). During those years a "battle" took place between pharmaceutic industries creating and modyfiying new antimicrobials, and bacteria that was developing new mechanisms of resistance to those antimicrobials (Figure 7), being an extraordinary model of evolution (Torres 2012). Currently, old antimicrobials such as colistin are the unique effective treatment to kill pathogenic bacteria, (Pulcini, Mohrs et al. 2017).

The overuse of antibiotics together with the genetic capacity of bacteria acquiring genes, has increased antibiotic resistance in humans, animals and the environment becoming an important socioeconomical problem (Davies and Davies 2010). Since antibiotics are essential for the treatment of bacterial infections, there is a priority to preserve their efficiency. During the last decades, scientists have called for the prudent use of antimicrobials, making an effort to decrease the epidemic dissemination of resistance (Davies 2007). Antimicrobials from urban and agricultural sources persist in soil and aquatic environments, and the selective pressure applied by them may reach the consumer affecting the treatment of human diseases. This

selection pressure exerted by antimicrobials has caused the evolution and spreading of resistance genes despite its origin (Allen, Donato et al. 2010). So, there is a necessity to ensure a much better and immediate control of antimicrobials release and its disposal in the environment from all population.

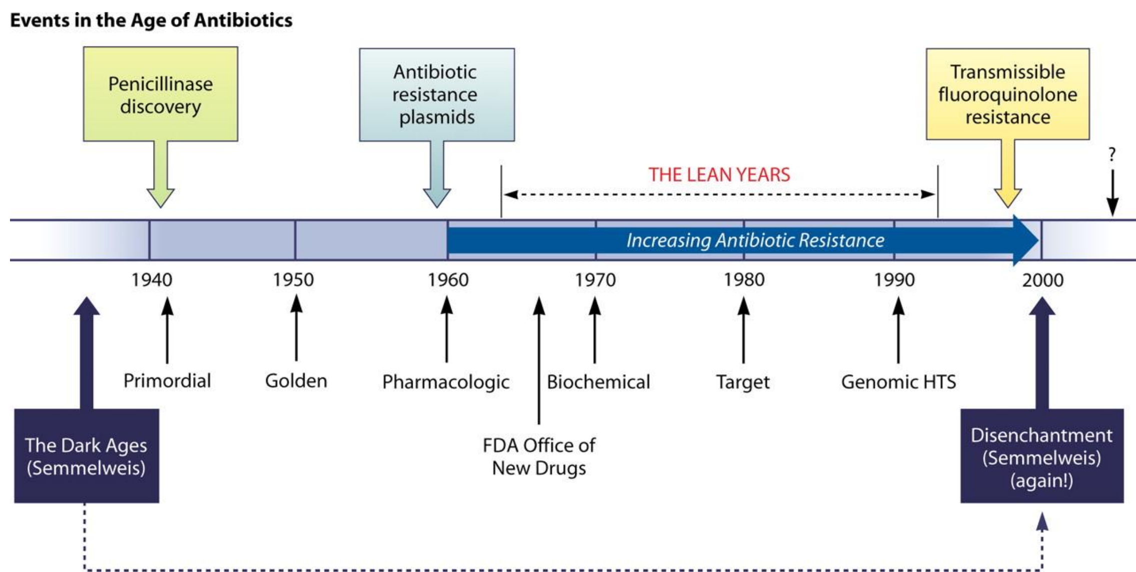


Figure 7. Summary of history of antibiotic discovery and most relevant events on development of antibiotic resistant (Davies and Davies 2010).

Intrinsic and acquired resistance

The type of antimicrobial resistance depends on the nature of it, being natural or "intrinsic", which refers to the lack of target for a certain antibiotic, or to the existence of genes in its genome common in all group of bacteria that can generate different resistance phenotype. In contrast, "acquired" resistance referred as a result of exposure to antibiotics that push resistance by a selection of bacteria in a population with genetic characteristics conferring resistance, being very variable (Daza Pérez 1998). Thereby, antimicrobial resistance selection in pathogenic and commensal bacteria both, in humans and

livestock, may be a final consequence of the incressing selective pressure by clinical antimicrobial use (Arnold, Williams et al. 2016).

The term multirresistance is defined as the non-susceptibility to at least three or more different families of antibiotics (Schwarz, Silley et al. 2010).

Mechanisms of resistance

Antimicrobials need to find and achive a target of action in the bacteria to cause the death or inhibit its growth. So, bacteria have different resistance mechanisms against antimicrobials (Figure 8):

- Enzymatic modification of the antibiotic making changes in its structure and consequently losing its functionality. In the case of beta-lactamases, which are the most prevalent, they are inactivating the antimicrobial by hydrolysiation of the betalactam ring (Tafur, Torres et al. 2008).
- Modifying the target of action of antimicrobial interrupting the vital function of the bacteria. Mainly used by Gram-positive bacteria, which change the sites of action of betalactamic antimicrobials (Cavaco, Abatih et al. 2008).
- Efflux pumps removing antimicrobials and environmental stressors like heavy metals avoiding that the antibiotics reach the target. Mainly used by Gram-negative bacteria. There are efflux pumps for multidrugs in the bacteria wall that permit the expulsion of several antimicrobials (Vila, Marti et al. 2007).
- Changes in the permeability of bacteria wall avoiding that the antimicrobial penetrates into the bacteria by the action of porines, which modify the external cell wall or make the membrane as an impermeable barrier (Vila, Marti et al. 2007).

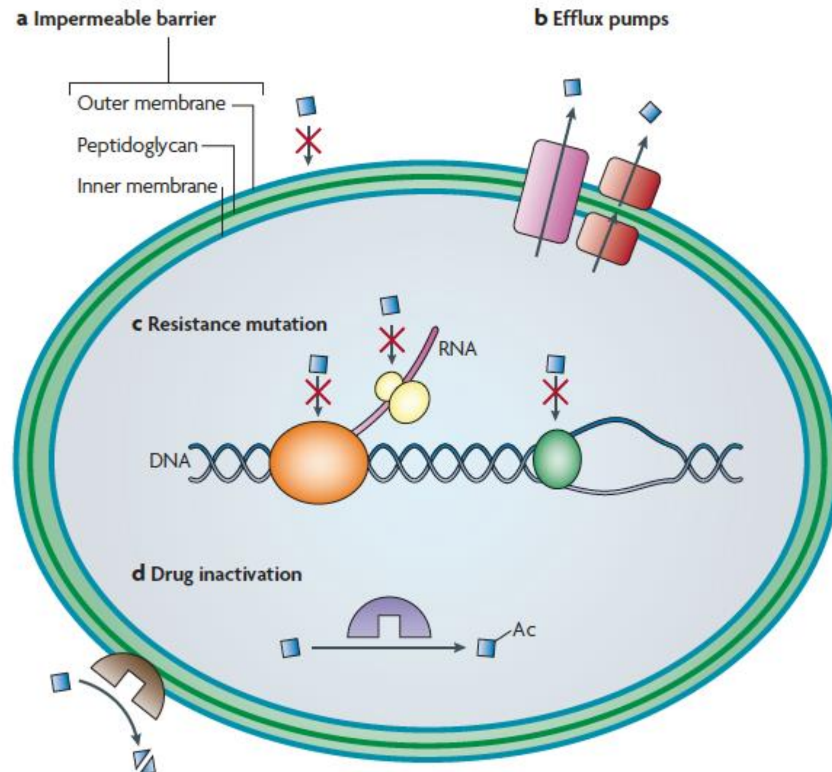


Figure 8. Different mechanisms of antibiotic resistance in Gram-negative bacteria. Such as impermeable barriers, multi-drug resitant efflux pumps, resistant mutations in different points acting in replication or traduction of DNA or inactivation of the antibiotic by different enzymes (Allen, Donato et al. 2010).

Actual antibiotics are acting at different levels of the cell (Table 2):

- Beta-lactam antibiotics are interfering with the synthesis of the bacterial cell wall.
- Quinolones have different mechanisms; inhibiting bacterial gyrase enzymes that are involved in the replication of DNA, efflux pumps export the antibiotic outside the cell, and *qnr* genes acting DNA-binding protein regulators.
- Rifampicin inhibits the transcription binding to RNA-polymerase.

- Aminoglycosides and tetracyclines inhibit 70S-ribosomal function inhibiting protein synthesis.
- Macrolides act binding different sites in the peptide exit tunnel of the 50S-ribosome unit by modification of RNA or protein components.

The targets of these antibiotics are genes that are essential for the bacteria. Knowing how resistance develops and spreads in a population, there is a necessity of introducing methods to prevent or at least delay the spread (Normark and Normark 2002, Davies and Davies 2010).

Antibiotic class	Example(s)	Target	Mode(s) of resistance
Beta-lactams	Penicillins (ampicillin), cephalosprins (ceftriaxona), penems (meropenem), monobactams (aztreonam)	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracyclines	Minocycline, tigecycline	Translation	Monooxygenation, efflux, altered target
Quinolones	Ciprofloxacin, nalidixic acid	DNA replication	Acetylation, efflux, altered target
Macrolides	Erythromycin, azithromicin	Translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicols	Chloramphenicol	Translation	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C_1 metabolism	Efflux, altered target
Sulfonamides	Sulfamethoxazole	C_1 metabolism	Efflux, altered target
Rifamycins	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target
Cationic peptides	Colistin	Cell membrane	Altered target, efflux

Table 2. Resistance mechanisms and modes of action of the most common antimicrobials. Adapted from Davies and Davies (Davies and Davies 2010).

Resistance genes

Antimicrobial-producing bacteria has resistant mechanisms to defend themselves against the compound that they synthesized. Also, structural similarities have been observed between antimicrobials and other molecules that participate in bacteria metabolism. It is possible that some resistance genes have a double action; inactivating antimicrobials and acting in cellular processes, such as detoxification or intracellular signalling (Wright 2007). This topic is more complex than originally thought. Hence recently the antimicrobial resistome concept has emerged, which include all genes that contribute to antimicrobial resistance; such as those from environmental bacteria, genes from pathogenic bacteria, intrinsic genes from bacterial chromosomes and genes encoding metabolic proteins that might be precursors of antimicrobial resistance genes (Wright 2010).

Transference of resistance

Bacteria have different strategies to have or acquire new mechanisms of resistance. They can get resistance to a particular antimicrobial by mutations of genes that are coding for the synthesis of proteins responsible for the antimicrobial action. This is an amazing evolutive adaptation strategy of bacteria to avoid its inhibition or death (Baquero, Blázquez et al. 2002).

Another important mechanism of the bacteria is to aquire resistance by exogen gene mobilization of genetic platforms. There are different complex systems either to acomulate resistance genes (integrons) or for its dissemination afterwards to other bacteria (plasmids and transposons).

Plasmids are extracromosomic genetic elements with the ability of self-replication that contain genes not vital for bacteria, but with ventatges to survive in adverse environments. Tranposons are DNA sequences with an enormous movement capacity with the ability to jump to diferent parts of cell genome. So, having resistance genes in this easyly disseminated plasmids or conjugative transposons is an important threat for the global resistance (Torres 2012).

On the other hand, integrons are amazing effective mechanisms to catch and acomulate different resistance genes. This system has an enzyme known to integreate a cassette of resistance genes; so most of integrons have many resistance genes together (Vinue, Jove et al. 2011). Integrons can be included in transposons, and these transposons in plasmids, which will be multirresistant (Baquero 2004). All these genetic elements can be horizontally transferred among different bacteria by conjugation, transformation and transduction.

- Conjugation can take place in environments that have many bacteria and very close one to each other through a sexual pilus (Figure 9), such as in the microbiota of the large intestine of animals and humans (Sommer, Dantas et al. 2009).
- During transformation, resistance genes are transferred between bacteria as “nude” DNA. When the bacteria die its DNA is released and captured by other bacteria that can incorporate this genetic material (Figure 9).
- Transduction is the transfer of genetic material between bacteria through a virus called bacteriophage that can infect bacteria (Figure 9); this virus can be integrated in

bacteria DNA, adding the resistance genes transported (von Wintersdorff, Penders et al. 2016).

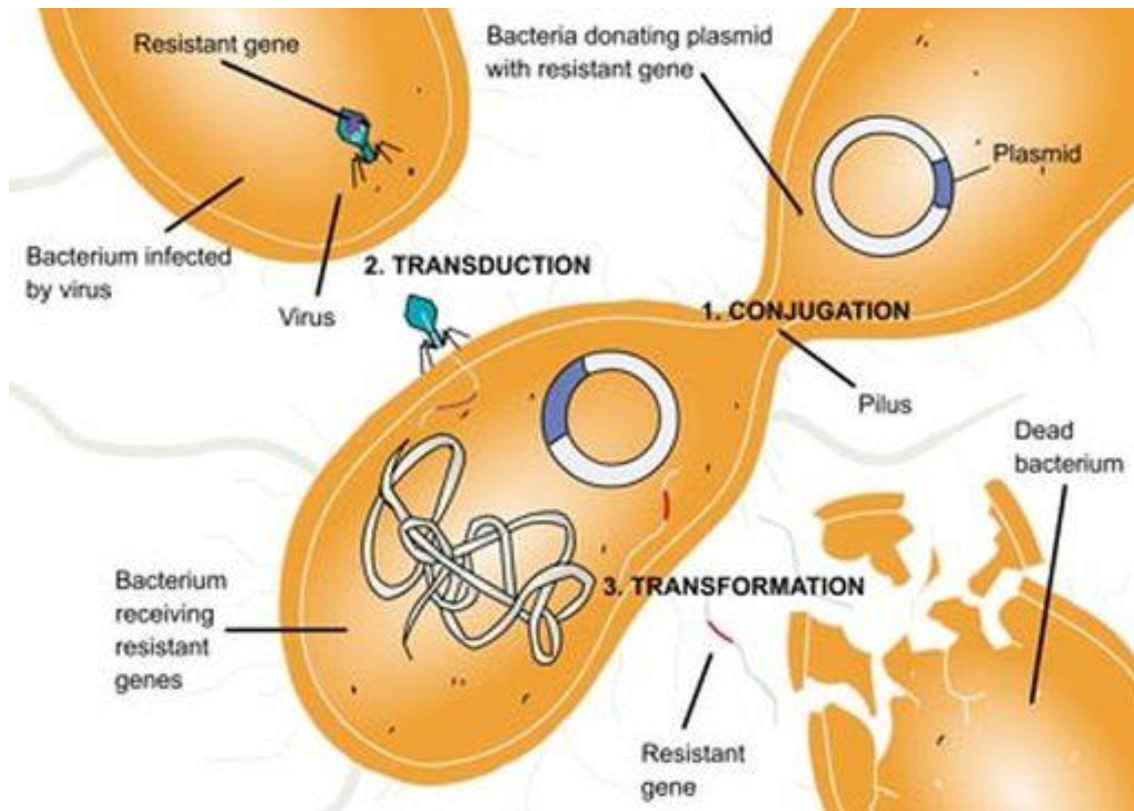


Figure 9. Different mechanisms of horizonzal transference of antimicrobial resistance genes (http://eu.wiley.com/WileyCDA/).

Plasmid classification

Plasmids are double-stranded extracromosomic DNA elements, mainly circular, and present in prokaryotic and a few eukaryotes organisms (Couturier, Bex et al. 1988). Their sizes are variable ranging between one to hundreds of kilobases (Kb), and the number of them in the cells can also be variable. The capacity of self-replication and maintenance into the host bacteria is due to genetic elements involve in plasmidic replication, called replicons, which contains the proteins responsible of plasmid replication (Couturier, Bex et al. 1988). Plasmid genes are often disseminated with mobile genetic elements that can move them within the same plasmid or between chromosome and

plasmid. Insertion sequence common regions (ISCR) are also mobile genetic elements that can be found on plasmids (Bennett 2008).

Plasmids were classified in different groups according to the incompatibility of being two equal plasmids in the same cell, since one inhibits the stability of the other (Couturier, Bex et al. 1988). Nowadays, there are more than 30 incompatibility groups (Inc) in *Enterobacteriaceae*. Plasmids may cause an extra cost for the bacteria, so they have developed a complex control mechanism of number of copies and maintenance to avoid their elimination (Bennett 2008). Replicons are a highly conserved part of plasmids, where the genes encoding for the origin of replication are situated, control and copy number, etc. (Figure 10). The type of Inc determines the host number of plasmids. The most common Inc associated to CTX-M genes is IncF, which are plasmids with a large host range and low copy number. Some of the endemic plasmids in *Enterobacteriaceae* are IncF, IncA/C, IncL/M, IncN and IncI (Carattoli 2011). Moreover, in *E. coli* plasmids harbouring $bla_{\text{CTX-M}}$ genes belong to the families IncF, IncA/C, IncL/M, IncN, IncHI2, IncI, IncK, IncX4, IncU and RCR (Carattoli 2013).

Liu and collegues (2016) recently described a plasmid encoding the genes conferring resistance to colistin, which is considered a frontline antibiotic against infections caused by multiresistance bacteria (Liu, Wang et al. 2016).

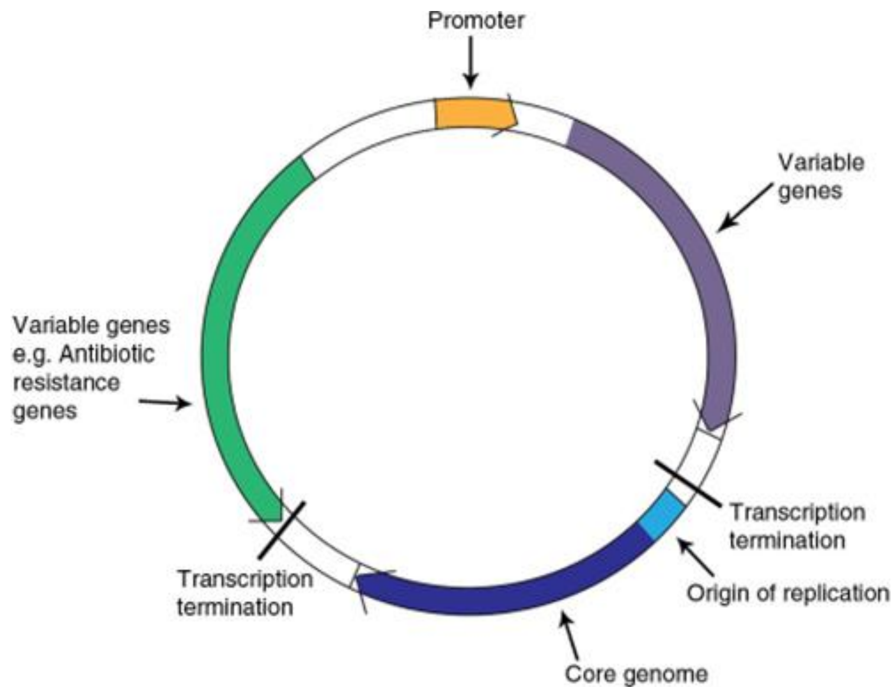


Figure 10. Illustration of a plasmid and its parts (Brolund 2014).

Plasmid typing methods

Couturier and colleagues (Couturier, Bex et al. 1988) developed a method based in hibridation of plasmid DNA with specific probes for the most common replicons in *Enterobacteriaceae*.

Another method for plasmids typing was created by Gotz et al (Gotz, Pukall et al. 1996) using PCR-based technique with specific primers for the detection of plasmid replication origin, accordingly with the most common replicons for the species studied (Carattoli, Bertini et al. 2005, Carattoli 2009). This charaterization has been tested for those strains exhibiting cephalosporin resistance to localize the responsible resistance gene. Plasmid detection and its sizing can be performed by PFGE of the total DNA of the strains digested with S1 nucelase restriction enzyme (Barton, Harding et al. 1995). Afterwards, the restriction fragments from S1-PFGE gel are transferred onto positively charged nylon membranes and hybridised with specific

probes of the cephalosporin resistance genes and the replicons previously identified. Same method has been used to identify a chromosomal location of the resistant gene, digesting with nuclease I-*CeuI*, and the hybrydisation by specific probes for the 16S rRNA gene and for the different cephalosporin resistance genes (Liu, Hessel et al. 1993).

Extended-spectrum beta-lactamases

Extended spectrum beta-lactamases (ESBL) are enzymes produced by the bacteria that allow them hydrolyse beta-lactams antibiotics, such as third-generation cephalosporins (Paterson and Bonomo 2005). This is the most important resistance mechanism against beta-lactams. Many Gram-negative bacteria possess a naturally occurring resistance, chromosmally mediated beta-lactamase (Philippon, Arlet et al. 2002). But these enzymes have evolved from penicillin-binding protein due to the selective pressure in the environment of beta-lactam-producing soil bacteria (Rawat and Nair 2010).

Enterobacteriaceae is the most numerous family associated to ESBL production; in particular the most important are *E. coli* and *Klebsiella pneumonia* (Brolund 2014). More than 150 ESBLs have been reported and one thousand resistance-related beta-lactamases have been described (Bradford 2001). Often, ESBL-producing *Enterobacteriaceae* displays multi-drug resistant phenotypes limiting antibiotic range options in treatment. Co-resistance with aminoglycosides, flouroquinolones or trimethoprim is common (Brolund, Edquist et al. 2014).

ESBL enzymes, according to Giske et al (Giske, Sundsfjord et al. 2009) definition, are divided in three main groups; $ESBL_A$, $ESBL_M$ and $ESBL_{CARBA}$. In $ESBL_A$ group are included the most frequently found enzymes CTX-M, SHV and TEM. In contrast, $ESBL_M$ is a miscellaneous group where AmpC is a most representing type, and $ESBL_{CARBA}$ is a group of enzymes with carbapenemase activity.

During last years, a huge increment in the prevalence of ESBL-producing *E. coli* has been isolated in hospitals, being especially important the emergence of CTX-M enzymes, mostly in patients with urinary tract infections (UTI). These strains usually present a multirresistant phenotype including antimicrobials with high relevance, such as flouroquinolones. A few years ago, a new family of plasmid mediated ESBLs has appeared, called CTX-M. This gene that hydrolyse cefotaxime is common in different genera of *Enterobacteriacae*, mainly in *E. coli* and *Salmonella* Typhmurium. Several studies reported that CTX-M enzyme is the most frequent ESBL isolated in clinical cases (Sabaté, Tarrago et al. 2000).

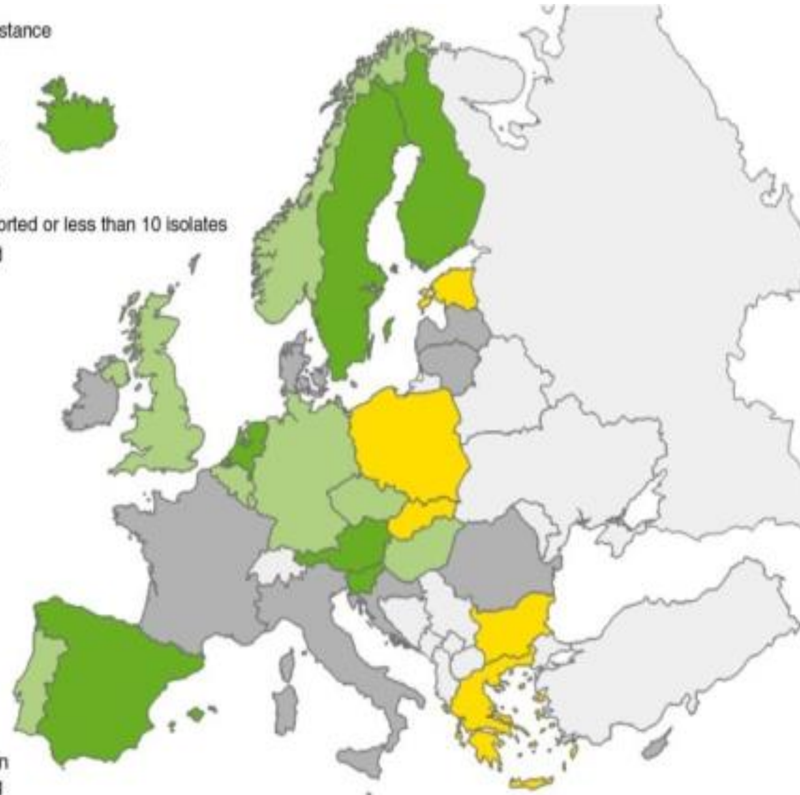
Different studies showed that the emergence of ESBL-producing *E. coli* CTX-M type in hospitals has been in parallel to the emergence of those strains in microbiota from heathy humans and animals and in food from animal origin. Same CTX-M types and same plasmids were described in different environments (EFSA 2011, Leverstein-van Hall, Dierikx et al. 2011). Thus, microbiota from food, healthy humans and healthy animals are an important reservoir of CTX-M resistance gene. The presence of ESBL-producing *E. coli* CTX-M type in feaces from wild animals has also been reported (Costa, Poeta et al. 2006, Alcalá, Alonso et al. 2016), which are contributing to the dissemination of this resistance gene in natural environments.

CTX-Ms were discovered in the 1980s (Brolund 2014). By the 1990s they have spread to all continents and nowadays the numbers have increased hugely (Figure 5). The *bla*$_{CTX-M}$ genes are grouped in five phylogroups; CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. CTX-M-1 phylogroup includes *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-15}$ among the most relevant genes, whereas CTX-M-9 includes *bla*$_{CTX-M-9}$ and *bla*$_{CTX-M-14}$ as the most importants of the group (D'Andrea, Arena et al. 2013).

The ESBL genes types TEM and SHV, which are the most numerous, maintain the capacity to hydrolyse penicillins (Bush and Singer 1989). TEM-1 is the most common ESBL gene in Gram-negative bacteria, responsible of 90% of ampicillin resistance and able to hydrolyse penicillins and early cephalosporins (Livermore 1995). In case of SHV genes, it has several variants in a few positions in the structural gene; the most common substitution is Gly238Ser, which is critical for the hydrolysis of ceftazidime (Huletsky, Knox et al. 1993).

OXA-type enzyme in an ESBL family that is the less found in *E. coli*, mainly described in *Pseudomonas aeruginosa*, and confering resistance to ampicillin and cefalotin.

In Europe, the prevalence of infections in the community due to ESBL-producing bacteria changes a lot between countries, being less frequent in the north and more common in the southern and eastern parts (Figure 11). Every year, there is a report from European network of national survillence systems of antimicrobial resistance (EARS-Net) summarizing the levels of invasive infections that have been caused by resistant bacteria (htttp://ecdc.europa.eu/).


Percentage resistance
< 1%
1 to < 5%
5 to < 10%
10 to < 25%
25 to < 50%
≥ 50%
No data reported or less than 10 isolates
Not included
Liechtenstein
Luxembourg
Malta

< 1%
1% to <5%
5% to <10%
10% to <25%
25% to <50%
≥ 50 %
Less than 10 isolates reported
No data

Luxembourg

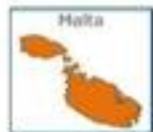

Malta

Figure 11. Maps illustrating the evolution of invasive *E. coli* isolates resistant to third-generation cephalosporins from reporting countries. a) 2001 and b) 2015 (ECDC/EARS-Net 2016).

Dissemination of ESBLs

Transmission of the genes encoding ESBL enzymes can happen by the spread of a particular clon or by horizontal dissemination of these genes as mentioned above (Figure 9).

Clonal expansion occurs when a bacteria cell line is multiplying and disseminating into a particular community or even causing an outbreak (Brolund 2014). A good exemple is the emergent clone *E. coli* ST131, found over the world and highly associated to $bla_{\text{CTX-M-15}}$ (Blanco, Alonso et al. 2009). Even so, many studies revealed that not all of *E. coli* ST131 isolates are genetically related, which indicates that horizontal gene dissemination by plasmids is more common for the spreading of ESBLs (Brolund, Edquist et al. 2014).

Hypothesis and objectives

The overall aim of this project was to study the occurrence of cephalosporin resistant *E. coli* from different origins; livestock, flies and wildlife and characterize the mechanisms involved in the transmission of resistance. The molecular epidemiology, resistance genes and mechanisms involved in the transfer of plasmids have also been investigated.

The specific objectives are:

I. To characterize phenotypic and genotypicaly *E. coli* strains causing colibacilosis in broilers and compare them with commensal isolates.

II. To evaluate the flies as potential vectors of introduction of cephalosporin resistant genes and virulence genes into broiler farms.

III. To assess the role of white storks as sentinels of anthropogenic pressure in the transmission of cephalsoporin resistant *E. coli*.

IV. To study the plasmids carrying cephalosporin resistance genes in *E. coli* isolated from different niches and assess their capacity to transfer the resistant genes.

Results

Study I: Diversity of multi-drug resistant avian pathogenic *Escherichia coli* (APEC) causing outbreaks of colibacillosis in broilers during 2012 in Spain

Abstract

Avian pathogenic *Escherichia coli* (APEC) are the major cause of colibacillosis in poultry production. In this study, a total of 22 *E. coli* isolated from colibacillosis field cases and 10 avian faecal *E. coli* (AFEC) were analysed. All strains were characterised phenotypically by susceptibility testing and molecular typing methods such as pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). The presence of 29 virulence genes associated to APEC and human extraintestinal pathogenic *E. coli* (ExPEC) was also evaluated. For cephalosporin resistant isolates, cephalosporin resistance genes, plasmid location and replicon typing was assessed. Avian isolates belonged to 26 O:H serotypes and 24 sequence types. Out of 22 APEC isolates, 91% contained the virulence genes predictors of APEC; *iutA*, *hlyF*, *iss*, *iroN* and *ompT*. Of all strains, 34% were considered ExPEC. PFGE analysis demonstrated a high degree of genetic polymorphism. All strains were multi-resistant, including those isolated from healthy animals. Eleven strains were resistant to cephalosporins; six contained $bla_{CTX\text{-}M\text{-}14}$, two $bla_{SHV\text{-}12}$, two $bla_{CMY\text{-}2}$ and one $bla_{SHV\text{-}2}$. Two strains harboured *qnrA*, and two *qnrA* together with *aac*(6')-Ib-cr. Additionally, the emergent clone O25b:H4-B2-ST131 was isolated from a healthy animal which harboured $bla_{CMY\text{-}2}$ and *qnrS* genes. Cephalosporin resistant genes were mainly associated to the presence of IncK replicons. This study demonstrates a very diverse population of multi-drug resistant *E. coli* containing a high number of virulent genes. The *E. coli* population among broilers is a reservoir of resistance and virulence-associated genes that could be transmitted into the community through the food chain. More epidemiological studies are

necessary to identify clonal groups and resistance mechanisms with potential relevance to public health.

Introduction

Escherichia coli is a bacterium widespread in the intestine of animals and humans, and a pathogen that can induce enteric and extraintestinal infections. In particular, avian pathogenic *E. coli* (APEC) is the main cause of colibacillosis in poultry farms; a syndrome associated to airsacculitis, perihepatitis, pericarditis, and sometimes fatal septicemia. APEC strains are responsible for the mortality of 3-4% of the animals in a farm, and for the reduction of 2-3% of egg production (Morley and Thomson 1984), resulting in an economic burden to the poultry industry (Schouler, Schaeffer et al. 2012). In many cases, the fundamental cause of the disease remains unclear, since the infection with *E. coli* is associated to the presence of *Mycoplasma gallisepticum* or respiratory viruses, such as Newcastle virus or Infectious Bronchitis virus (Gross 1991).

Several virulence genes are implicated in avian colibacillosis such as adhesins, toxins, anti-host defence factors, iron acquisition systems, autotransporters and the IbeA protein (Nakazato, de Campos et al. 2009). Subtractive hybridization studies have demonstrated sequence homology between specific DNA regions of APEC and human extraintestinal pathogenic *E. coli* (ExPEC) (de Campos, Lago et al. 2008). Additionally, the presence of similar virulence genes found in both, APEC and ExPEC strains, suggested that APEC strains may act as zoonotic pathogens and reservoir of virulence causing human infections (Ewers, Janssen et al. 2004, Ewers, Li et al. 2007, Mora, Viso et al. 2013). According to Johnson *et al.* (2003), a strain could be considered ExPEC if exhibits two or more of the following virulence genes; *pap* (P fimbriae), *sfa/foc* (S/F1C fimbriae), *afa/dra* (Dr binding adhesins), *iutA* (aerobactin receptor), and *kpsM* II (group 2 capsule

synthesis) (Johnson, Murray et al. 2003). ExPEC strains are more often derived from virulence-associated B2 and D phylogroups (Russo and Johnson 2000).

The successful treatment of avian colibacillosis caused by APEC strains mainly depends on the use of antimicrobials. However, increasing resistance to critically important antimicrobials, such as third-generation cephalosporins and fluoroquinolones, is nowadays common in *E. coli* from poultry origin (Dierikx, van der Goot et al. 2013). These resistances can be transmitted to humans via the food supply (Johnson, Sannes et al. 2007, Mora, Herrera et al. 2010). In particular, *E. coli* producing extended-spectrum beta-lactamases (ESBLs) and plasmid mediated AmpC beta-lactamases have increased considerably in the last years (Cantón, Novais et al. 2008). Normally, these genes are located on plasmids, and can be transferred by conjugation to other bacterial species (Dierikx, van der Goot et al. 2013). Some of the virulence factors for APEC and ExPEC can also be harboured on plasmids. Particularly, ColV plasmids yield some virulence genes such as *hlyF*, *ompT*, *iss* and *cvaC* surrounding the replicon RepFIB (Johnson and Nolan 2009).

Several studies have described APEC strains in the literature (Maluta, Logue et al. 2014). However, not many studies have combined extensive characterization at the serotype level, virulence-associated genes, molecular typing techniques, molecular determination of resistance mechanisms and mobile genetic elements involved in transfer of resistance. For this reason, the objective of this study was to discriminate and to perform such characterization of highly pathogenic *E. coli* causing outbreaks of colibacillosis in 13 different broiler farms throughout Spain, and compare them to avian faecal *E. coli* (AFEC)

obtained from healthy animals. Additionally, the identification of clones more prone to cause disease has been assessed.

Materials and Methods

Isolation

A total of 22 tissue swabs of culled-animals affected with colibacillosis arrived to the laboratory between January and March 2012. The samples were obtained as part of routine care. Samples were taken from chickens already sacrificed for diagnostic purposes following the procedures according to the requirements of the Ethics Committee of Animal and Human Experimentation of the Universitat Autònoma de Barcelona (Permit Number DMAH-4239 that specifically permits euthanasia of chickens). Animals were euthanized using intravenous sodium pentobarbital (100 mg/kg, Dolethal®, Vétoquinol, Cedex, France) in the wing vein. The method to sacrifice the animals follows the welfare rules stated in the European Directive 86/609/CEE. None of the authors of this manuscript were involved in manipulating or sacrificing the chickens. All the samples were collected from clinical cases submitted to the Diagnostic Service of the Veterinary School of the Universitat Autònoma de Barcelona. Swabs were taken from 13 broiler farms located in nine different regions of Spain (Fig 1). Ten *E. coli* strains isolated from faeces of healthy animals collected in nine farms were also included in the study. The samples were plated onto MacConkey agar and incubated overnight at 37ºC. Three lactose-positive colonies for each plate were selected and confirmed to be *E. coli* by PCR (Heininger, Binder et al. 1999). Subsequently, one representative was selected for further studies.

Serotyping

Determination of O and H antigens was carried out using the method previously described by Guinée *et al*. with all available O (O1 to O181) and H (H1 to H56) antisera (Guinée 1981). Non-typeable isolates were denoted as ONT or HNT and non-motile isolates were denoted as HNM. All antisera were obtained and absorbed with the corresponding cross-reacting antigens to remove the nonspecific agglutinins. The O and H antisera were produced in the Laboratorio de Referencia de *E. coli* (LREC, Lugo, Spain). O25a and O25b subtypes were determined by PCR (Clermont, Lavollay et al. 2008).

Phylogeny, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

Isolates were separated in phylogroups (A, B1, B2, C, D, E or F) according to a method previously described (Clermont, Bonacorsi et al. 2000, Clermont, Christenson et al. 2013).

PFGE was performed as described elsewhere (Ribot, Fair et al. 2006). The results were analysed by Fingerprinting II Informatix software (Applied Maths, Sint-Martens-Latem, Belgium). PFGE-types were separated based on differences of at least one band in the restriction profiles. The analysis of the bands generated was carried out using the Dice coefficient and unweighted pair group method with arithmetic averages (optimization of 1.25% and position tolerance 1.25%).

MLST was carried out as previously described according to the protocol and primers specified on the *E. coli* MLST web site (http://mlst.ucc.ie/mlst/dbs/Ecoli) (Wirth, Falush et al. 2006).

Sequences were analysed with Vector NTI advance 11 software (InforMax, Inc., Bethesda, MD).

Detection of virulence-associated genes

All strains were tested by PCR for 29 ExPEC and APEC virulence-associated genes (Tables 1 and 2) (Johnson, Wannemuehler et al. 2008, Mora, Herrera et al. 2010). The genes described previously by Johnson *et al.* (2008) as the minimal predictors of APEC virulence; *iroN*, *ompT*, *hlyF*, *iutA* and *iss* were detected by a multiplex PCR (Johnson, Wannemuehler et al. 2008). Virulence scores were calculated for each isolate as the sum of all virulence-associated genes detected; *pap, sfa-foc* and *kpsM* II were counted only once.

Statistical analysis

Differences in the prevalence between different groups were determined by Fisher's exact test as described before (Solà-Ginés, Gonzalez-Lopez et al. 2015). Virulence scores were compared by the use of Mann-Whitney U test. Statistical analyses were performed using GraphPad Prsim, version 3.1 software (GraphPad Software, Inc., San Diego, CA).

Antimicrobial susceptibility testing

Disc diffusion was performed according to CLSI guidelines using the following discs (Oxoid, Basingstoke, UK): cefoxitin, 30 mg; cefepime, 30 mg; ceftazidime, 30 mg; cefotaxime, 30 mg; cefotaxime+clavulanic acid, 30+10 mg; and ceftazidime+clavulanic acid, 30+10 mg. The disc combinations of cefotaxime and cefotaxime/clavulanic acid, ceftazidime and ceftazidime/clavulanic

acid were used for the identification of ESBLs; cefoxitin was used for the detection of AmpC-type beta-lactamase (CLSI 2008). All isolates were susceptibility tested using a minimum inhibitory concentration (MIC)-based broth microdilution (VetMIC GN-mo, National Veterinary Institute, Uppsala, Sweden) as described before (Solà-Ginés, Gonzalez-Lopez et al. 2015). Isolates were considered to be wild type (WT) or non-WT based on epidemiological cut-off values according to EUCAST (http://www.eucast.org/).

Resistance genes

All strains exhibiting resistance to third-generation cephalosporins (cefotaxime and ceftazidime) were tested by PCR methods for the presence of the *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CMY-1}$ and *bla*$_{CMY-2}$ genes as described by Hasman *et al.* (Hasman, Mevius et al. 2005). Detection of plasmid-mediated AmpC beta-lactamase genes was assessed by multiplex PCR (Pérez-Pérez and Hanson 2002). Sequencing of both strands of amplicons was performed. The presence of the fluoroquinolone resistance genes *aac(6')-Ib-cr*, *qnrA*, *qnrB*, *qnrS*, *qepA* and *oqxAB* was also assessed (Coelho, Mirelis et al. 2009, Chen, Zhang et al. 2012).

Plasmid DNA analysis

Isolates exhibiting resistance to cephalosporins were selected for plasmid characterization. Plasmid replicons tested were elected according to the presence of determinant resistance genes (HI1, HI2, I1, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIA and K) (Carattoli 2009) and were identified using the PCR-based replicon typing method previously described (Carattoli, Bertini et al. 2005, Garcia-Fernandez,

Fortini et al. 2009). Plasmids detection and sizing was performed on all the isolates by S1-nuclease PFGE of total DNA (Barton, Harding et al. 1995). Restriction fragments from S1-PFGE gels were transferred onto a positively charged nylon membrane and hybridised with specific probes for $bla_{CTX\text{-}M\text{-}14}$, bla_{TEM}, bla_{SHV}, bla_{CMY} and for each replicon that was previously identified.

Results

A total of 22 *E. coli* were recovered from 13 different farms distributed throughout Spain during 2012. Additionally 10 isolates from healthy animals collected in nine different farms were also included in the study to make a total of 32 *E. coli* isolates.

Serotypes

A total of 16 different O serogroups, 15 flagellar H antigens and 26 different O:H serotypes were identified (Table 1). The most prevalent serotypes were: O3:H26, O5:H10, O5:H51 and O78:H9. Additionally, the emergent clone O25b:H4 was detected in a commensal isolate.

Table 1. Distribution of virulence-associated gene profiles, phylogeny, serotyping and MLST results among all 32 strains.

	Isolate	**Phylo.**	**Serotype**	**ST**	**Cplx**	**Virulence gene profiles[a]**
AFEC						
	GN-2215	D	ONT:H4	ST117		***iss***, *fimH, fyuA, irp-2, cdtB, traT, malX, tsh*
	GN-2216	A	O53:H18	ST10	ST10 Cplx	***iss***, *fimH, traT*
	GN-2221	B2	O25b:H4	ST131		***iroN***, ***ompT***, ***iutA***, ***iss***, *fimH, cvaC, irp-2, kpsM II-K1, traT, ibeA, malX, usp, tsh*
	GN-2222	F	O83:HNT	ST648		***iroN***, ***ompT***, ***iutA***, ***iss***, *fimH, cvaC, astA, kspM II-K5, traT, malX, tsh*
	GN-2223	A	ONT:HNT	ST10	ST10 Cplx	*fimH, fyuA, irp-2, traT*
	GN-2233	A	O3:H26	ST165	ST165 Cplx	*fimH, fimAvMT78, traT*
	GN-2234	A	O2:H40	ST10	ST10 Cplx	***iroN***, ***ompT***, ***iss***, *fimH, fyuA, traT*
	GN-2235	A	O3:HNM	ST165	ST165 Cplx	*fimH, fimAvMT78, traT*
	GN-2236	B1	O127:H37	ST297		***ompT***, ***iutA***, ***iss***, *fimH, cvaC, traT, malX, tsh*
	GN-2237	A	O3:H26	ST189	ST165 Cplx	*fimH, fimAvMT78, traT*
APEC						
	N107	B1	ONT:H16	ST295		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, cvaC, traT, tsh*
	N108	C	O78:H9	ST23	ST23 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, fyuA, astA, traT, tsh*
	N109	B1	ONT:H28	ST156	ST156 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, astA, irp-2, traT*
	N116	D	O25a:[H4]	ST624		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, cvaC, kpsM III, traT, malX*
	N113	A	O5:H10	ST93	ST168 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *astA, kpsM II-K2*
	N114	A	O6:H16	ST48	ST10 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, fimAvMT78, kpsM III, traT*

	N125	A	O88:HNM	ST1137		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, cvaC, astA, fimAvMT78, traT*
	N126	B1	ONT:H51	ST889		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, cvaC, traT, tsh*
	N127b	D	ONT:H51	ST156	ST156 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, traT, tsh*
	N128	E	ONT:H27	ST350	ST350 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, cvaC, astA, irp-2, fimAvMT78, papEF, papG, traT, tsh*
	N129	B1	O15:H10	ST101	ST101 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *cvaC, astA, sfa/focDE, traT, tsh*
	N130	A	O5:H51	ST93	ST168 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *astA, kspM II-K2*
	N131	A	O5:H51	ST93	ST168 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *astA, kspM II-K2*
	N132	E	O102:H25	ST57	ST350 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, traT, tsh*
	N133	A	O5:H10	ST93	ST168 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *kpsM II-K2*
	N134	B1	O159:H28	ST539		***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, irp-2, traT*
	N135	B2	O2:H1	ST429		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, fyuA, cvaC, irp-2, kpsM II-K1, traT, ibeA, malX, usp, tsh*
	N136	D	ONT:H4	ST830		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, fyuA, traT, malX*
	N137	D	O11:H15	ST3161		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, cvaC, traT*
	N138	C	O78:H9	ST650	ST23 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH, traT*
	N139	B1	O45:H8	ST533		***iroN***, ***ompT***, ***hlyF***, *fimH, astA, traT, malX*
	N140	E	O119:H27	ST350	ST350 Cplx	***iutA***, *fimH, astA, fimAvMT78*

Phylo, phylogroup; ST, sequence type; Cplx, clonal complex.

Adhesins *fimH* (D-mannose-specific adhesin of type I fimbriae), *fimAvMT78* (FimA variant MT78 of type 1 fimbriae), *papEF* and *papG* (P fimbria subunits), and *sfa/focDE* (S fimbrial adhesin/putative F1C fimbrial adhesin); toxins *cdtB* (cytolethal distending toxin), *hlyF* (hemolysin F), and *astA* (EAST1, enteroaggregative E. coli heat-stable toxin); siderophores fyuA (yersiniabactin), *iutA* (aerobactin), *iroN* (novel catecholate siderophore receptor), and *irp-2* (iron repressible associated with yersiniabactin synthesis); protectins *kpsM* (groups

II and III, specifically targeting the K1, K2 and K5 genes of group II capsules), *cvaC* (ColV, colicin V from serum resistance-associated plasmids), *iss* (surface exclusion serum survival protein), and *traT* (serum resistance); miscellaneous virulence genes *ompT* (protease), *ibeA* (invasion of brain endothelium), *malX* (PAI, pathogenicity island marker), and *usp* (uropathogenic-specific protein, bacteriocin).

[a] Virulence-associated genes shown in boldface are the five genes characteristics of APEC strains.

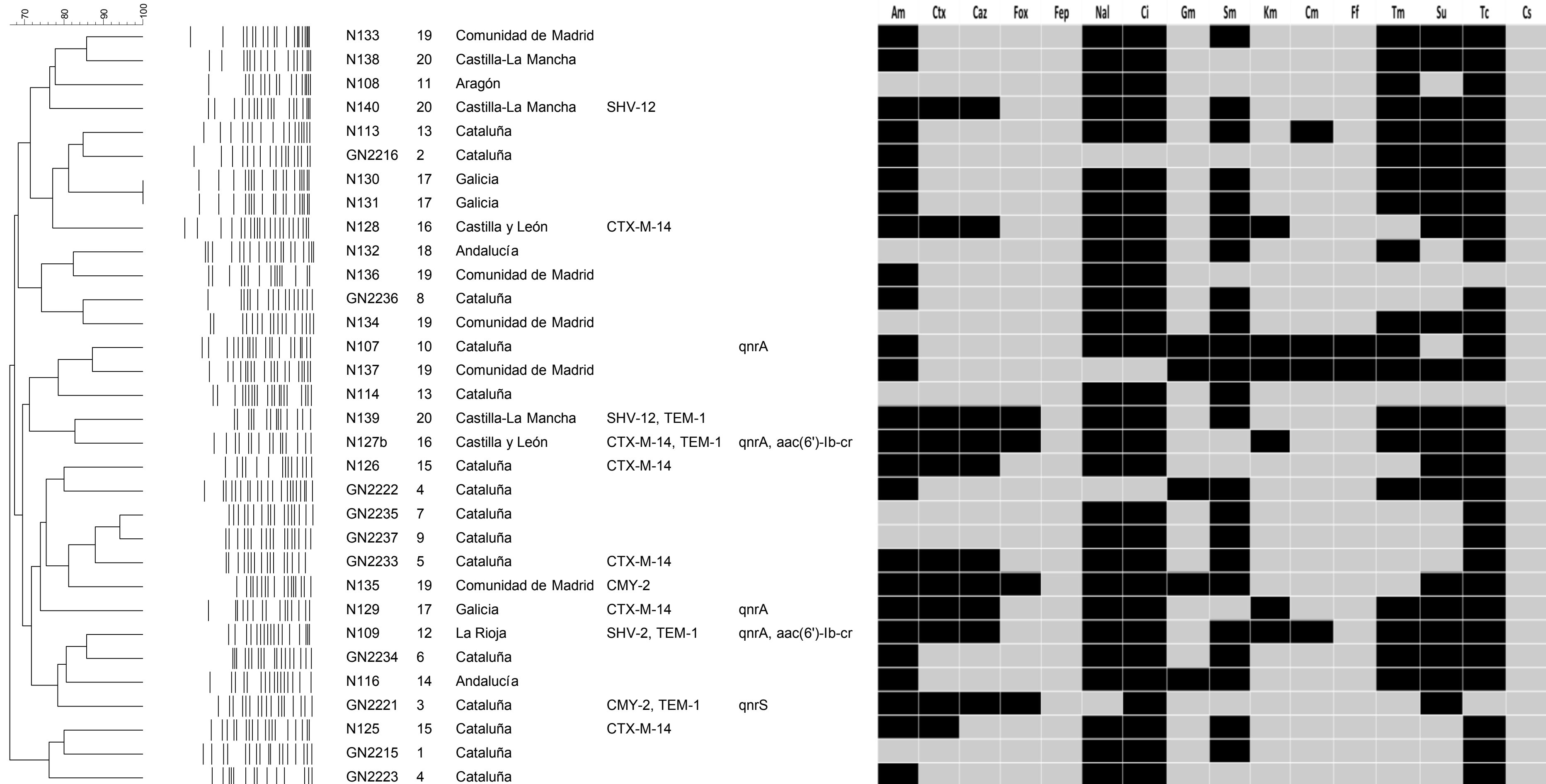
Phylogeny, PFGE and MLST

Within the APEC strains (n=22) the phylotyping identified six strains belonging to group A (27%), six to group B1 (27%), four to group D (18%), three to group E (14%), two to group C (9%) and one to group B2 (5%). For the commensal strains (n=10), 60% belonged to phylogroup A, and the remaining 40% to B1, B2, D and F phylogroups (10% each) (Table 1).

XbaI-PFGE analysis showed a high degree of genetic polymorphism. A total of 31 different PFGE restriction profiles were identified among the 32 *E. coli* isolates (Fig 1). Only two isolates were epidemiologically related and belonged to the same farm.

Among the APEC subgroup (n=22), MLST analyses identified 18 STs, while six STs were found among AFEC isolates (n=10) (Table 1). Within the APEC strains, four belonged to the ST168 clonal complex (Cplx), three to the ST350 Cplx, two to ST156 Cplx, and two to the highly pathogenic ST23 Cplx (Table 1). Within the AFEC isolates, the most common Cplx was ST10 (n=3), followed by ST165 Cplx (n=3) (Table 1). The emergent pandemic multirresistant clone O25b:H4-B2-ST131 was found among the AFEC isolates.

Fig 1. PFGE dendogram illustrating the phenotypic and genotypic relationship of the strains and the cephalosporin resistance genes.



PFGE, pulsed-field gel electrophoresis; CR., cephalosporin resistance genes; FQ., flouoroquinolone resistance genes; Am: Ampicillin (WT≤8mg/L); Ctx: Cefotaxime (WT≤0.25mg/L); Caz: Ceftazidime (WT≤0.5mg/L); Fox: Cefoxitin (WT≤8mg/L); Fep: Cefepime (WT≤0.125mg/L); Nal: Nalidixic acid (WT≤16mg/L); Ci: Ciprofloxacin (WT≤0.064mg/L); Gm: Gentamicin (WT≤2mg/L); Sm: Streptomycin (WT≤16mg/L); Km: Kanamycin (WT≤8mg/L);. Cm: Chloramphenicol (WT≤16mg/L); Ff: Florfenicol (WT≤16mg/L); Tm: Trimethoprim (WT≤2mg/L); Su: Sulphamethoxazole (WT≤64mg/L); Tc: Tetracycline (WT≤8mg/L); Cs: Colistin (WT≤2mg/L).

[a] Isolates are divided in APEC (N) and AFEC (GN) strains.

[b] Location of the strains is named in order to the different regions of Spain where farms were localized.

Detection of virulence-associated genes

The prevalence of 29 virulence-associated genes is shown in Tables 1 and 2. Regarding the five virulence genes associated to APEC (Fig 1); 81%, 78%, 75%, 72% and 72% of the 32 *E. coli* strains yielded amplicons for *iss*, *ompT*, *iutA*, *iroN* and *hlyF*, respectively (Table 1). The prevalence of these genes was higher in APEC isolates (91% of the APEC strains harboured all of the mentioned genes), when compared to the AFEC isolates. In general, the presence of virulence-associated genes in AFEC strains was low, with 80% of the strains having from zero to three of the previously mentioned virulence genes (Table 1). According to the number of virulence-associated genes, 34% of the isolates were considered ExPEC.

Statistical analysis

Significant differences (P = 0.035) were observed in the number of virulence-associated genes (*hlyF*, *astA*, *iroN*, *iutA* and *ompT*) found between APEC (mean, 9.2; range 5 to 16) and AFEC (mean, 6.7; range 3 to 13) isolates (Table 2). Statistical difference was found also comparing the virulence-associated genes of the isolates of phylogroup A for APEC and AFEC (P = 0.013; virulence score 7.2 vs 4.2). The strains belonging to phylogroup B2 exhibited the highest virulence score (mean, 14.5; range, 13 to 16) whereas the phylogroup A exhibited the lowest (mean, 5.8; range, 3 to 11). Additionally, significant differences were observed comparing B2 and D phylogroups (mean, 11.1; range 8 to 16) with A and B1 phylogroups (mean 7.4; range 3 to 13) (P = 0.008).

Table 2. Distribution and characterization of virulence-associated genes and phylogroups of the 32 isolates.

Virulence gene (s)[a]	Total isolates (%) (n=32)	APEC isolates (%) (n=22)	AFEC isolates (%) (n=10)	A (%) (n=12)	B1 (%) (n=7)	B2 (%) (n=2)	D (%) (n=5)	C, E and F (%) (n=6)	B2/D (%) (n=7)	A/B1 (%) (n=19)	A/APEC (%) (n=6)	A/AFEC (%) (n=6)	*P* value[b] APEC vs AFEC	*P* value[b] A/APEC vs A/AFEC	*P* value[b] B2/D vs A/B1
Adhesins															
fimH	28 (88%)	18 (82%)	10 (100%)	8 (67%)	7 (100%)	2 (100%)	5 (100%)	5 (83%)	7 (100%)	15 (79%)	2 (33%)	6 (100%)			
fimAvMT78	7 (22%)	4 (18%)	3 (30%)	5 (42%)	0	2 (100%)	0	2 (33%)	2 (29%)	5 (26%)	2 (33%)	3 (50%)			
papEF	1 (3%)	1 (5%)	0	0	0	0	0	1 (17%)	0	0	0	0			
papG	1 (3%)	1 (5%)	0	0	0	0	0	1 (17%)	0	0	0	0			
sfa/focDE	1 (3%)	1 (5%)	0	0	1 (14%)	0	0	0	0	1 (5%)	0	0			
afa/draBC	0	0	0	0	0	0	0	0	0	0	0	0			
Toxins															
cnf1	0	0	0	0	0	0	0	0	0	0	0	0			
cdtB	1 (3%)	0	1 (10%)	0	0	0	1 (20%)	0	1 (15%)	0	0	0			
sat	0	0	0	0	0	0	0	0	0	0	0	0			
hlyA	0	0	0	0	0	0	0	0	0	0	0	0			
*hlyF**	22 (72%)	22 (100%)	0	6 (50%)	6 (86%)	1 (50%)	4 (80%)	4 (67%)	5 (71%)	12 (63%)	6 (100%)	0	<0.0001	0.0022	
astA	12 (38%)	11 (50%)	1 (10%)	5 (42%)	3 (43%)	0	1 (20%)	3 (50%)	0	8 (42%)	5 (83%)	0	0.0496	0.0152	
tsh	12 (38%)	8 (36%)	4 (40%)	0	4 (57%)	2 (100%)	2 (40%)	4 (67%)	4 (57%)	4 (21%)	0	0			
Siderophores															
fyuA	9 (28%)	5 (23%)	4 (40%)	2 (17%)	1 (14%)	2 (100%)	3 (60%)	1 (17%)	5 (71%)	3 (16%)	0	2 (33%)			0.0138
*iutA**	24 (75%)	21 (95%)	2 (20%)	6 (50%)	6 (86%)	2	4 (80%)	6 (100%)	6 (86%)	12	6	0	<0.0001	0.0022	

						(100%)				(63%)	(100%)				
*iroN**	24 (72%)	21 (95%)	3 (30%)	7 (58%)	6 (86%)	2 (100%)	4 (80%)	5 (83%)	6 (86%)	13 (68%)	6 (100%)	1 (17%)	0.0003	0.0152	
irp-2	8 (25%)	5 (23%)	3 (30%)	1 (8%)	3 (43%)	2 (100%)	1 (20%)	1 (17%)	3 (43%)	4 (21%)	0	1 (17%)			
Protectins															
kpsM II	7 (22%)	5 (23%)	2 (20%)	4 (33%)	0	2 (100%)	0	1 (17%)	2 (29%)	4 (21%)	4 (67%)	0			
kpsM II-K1	2 (6%)	1 (5%)	1 (10%)	0	0	2 (100%)	0	0	2 (29%)	0	0	0			
kpsM II-K2	4 (13%)	4 (18%)	0	4 (33%)	0	0	0	0	0	4 (2%)	4 (67%)	0			
kpsM II-K5	1 (3%)	0	1 (10%)	0	0	0	0	1 (17%)	0	0	0	0			
kpsM III	2 (6%)	2 (9%)	0	1 (8%)	0	0	1 (20%)	0	1 (15%)	1 (5%)	1 (17%)	0			
cvaC	11 (34%)	9 (41%)	3 (30%)	1 (8%)	4 (57%)	2 (100%)	2 (40%)	2 (33%)	4 (57%)	5 (26%)	1 (17%)	0			
*iss**	26 (81%)	20 (91%)	6 (60%)	8 (67%)	7 (100%)	2 (100%)	5 (100%)	5 (83%)	7 (100%)	15 (79%)	6 (100%)	2 (33%)			
traT	27 (84%)	17 (77%)	10 (100%)	8 (67%)	7 (100%)	2 (100%)	5 (100%)	5 (83%)	7 (100%)	15 (79%)	2 (33%)	6 (100%)			
Miscellaneous															
*ompT**	26 (78%)	22 (100%)	4 (40%)	7 (58%)	7 (100%)	2 (100%)	4 (80%)	3 (50%)	6 (86%)	14 (74%)	6 (100%)	1 (17%)	0.0002	0.0152	
ibeA	2 (6%)	1 (5%)	1 (10%)	0	0	2 (100%)	0	0	2 (29%)	0	0	0			
malX	8 (25%)	4 (18%)	4 (40%)	0	1 (14%)	2 (100%)	3 (60%)	1 (17%)	5 (71%)	1 (5%)	0	0			0.0018
usp	2 (6%)	1 (5%)	1 (10%)	0	0	2 (100%)	3 (60%)	0	5 (71%)	0	0	0			0.0003
Mean (range)	8.2 (3-16)	9.2 (5-16)	6.7 (3-13)	5.8 (3-	10 (8-	14.5 (13-	9.8 (8-	9.2 (5-14)	11.1 (8-	7.4 (3-	7.2 (5-	4.2 (3-6)	0.035	0.013	0.008

virulence score[c]				11)	13)	16)	11)		16)	13)	11)				

Adhesins *fimH* (D-mannose-specific adhesin of type I fimbriae), *fimAvMT78* (FimA variant MT78 of type 1 fimbriae), *papEF* and *papG* (P fimbria subunits), *sfa/focDE* (S fimbrial adhesin/putative F1C fimbrial adhesin), and *afa/draBC* (Dr antigen specific adhesin); toxins *cnf1* (cytotoxic necrotizing factor 1), *cdtB* (cytolethal distending toxin), *sat* (secreted autotransporter toxin), *hlyA* (α-hemolysin), *hlyF* (hemolysin F), and *astA* (EAST1, enteroaggregative E. coli heat-stable toxin); siderophores fyuA (yersiniabactin), *iutA* (aerobactin), *iroN* (novel catecholate siderophore receptor) , and *irp-2* (iron repressible associated with yersiniabactin synthesis); protectins *kpsM* (groups II and III, specifically targeting the K1, K2 and K5 genes of group II capsules), *cvaC* (ColV, colicin V from serum resistance-associated plasmids), *iss* (surface exclusion serum survival protein), and *traT* (serum resistance); miscellaneous virulence genes *ompT* (protease), *ibeA* (invasion of brain endothelium), *malX* (PAI, pathogenicity island marker), and *usp* (uropathogenic-specific protein, bacteriocin).

[a] Virulence-associated genes shown with asterisk are the five genes characteristics of APEC strains.

[b] *P* values (by Fisher's exact test) are shown where $P<0.05$.

[c] The virulence score was the number of virulence genes detected, adjusted for multiple detection of the *pap*, *sfa* and *foc*, and *kpsM* II operons. Virulence scores were compared by use of the Mann-Whitney U test.

Antimicrobial susceptibility testing and resistance genes

All the analyzed strains were multi-resistant (resistant to more than 3 antimicrobial families), including those isolated from healthy animals. Furthermore, 50% were resistant to more than eight antimicrobials. Susceptibility testing detected 11 strains resistant to cephalosporins (34%); six *bla*$_{CTX-M-14}$, two *bla*$_{SHV-12}$, two *bla*$_{CMY-2}$ and one *bla*$_{SHV-2}$. Two of these strains belonged to AFEC isolates. Two isolates were resistant to cefoxitin, and the resistance mechanism involved could not be determined.

In addition, 88% of the isolates were resistant to nalidixic acid and 91% to ciprofloxacin. Additionally, 91% of the strains were resistant to tetracycline, 78% to ampicillin, 69% to streptomycin, 63% to sulfamethoxazole, 59% to trimethoprim 34% to cefotaxime, 31% to ceftazidime, 19% to kanamycin, 16% to gentamicin, 13% to cefoxitin, 13% to chloramphenicol and 6% to florfenicol. No resistance to cefepime and colistin was observed among the isolates (Fig 1).

The presence of the *qnrS* gene was only confirmed in the isolate belonging to O25b:H4-B2-ST131. Finally, two of the APEC strains exhibited *qnrA* and two *qnrA* together with *aac(6')-Ib-cr*. The genes *qnrB*, *qepA* and *oqxAB* were not found in this strain collection.

Plasmid localisation of cephalosporin resistance genes

PCR-based replicon typing among the 11 cephalosporin resistant *E. coli* isolates showed that IncFIB replicon was present in all analysed isolates (Table 3). The replicons IncI1, IncN, IncK, IncY, IncP, IncFIA, IncHI1 and IncHI2 were also detected (Table 3).

Plasmid-sizes varied between 90 and 120-kb, with the exception of two plasmids; pST131-2 and pST350-1 with 250 and 180-kb,

respectively (Table 3). The most common incompatibility group detected by Southern blot was IncK (n=5). Specific probe hybridization of S1-digested DNA demonstrated that isolate GN2221 harboured *bla*$_{CMY-2}$ gene in a plasmid containing replicon IncK (pST131-3). The isolate N135 harbouring also *bla*$_{CMY-2}$ gene in a 90-kb plasmid contained IncK and IncP replicons. Isolate GN2233 yielded *bla*$_{CTX-M-14}$ in a 90-kb plasmid with two replicons (IncK and IncY). The presence of *bla*$_{SHV-12}$ was confirmed in two isolates having a 120 and 90-kb plasmids of the IncI1 and unknown replicon family, respectively. N109 harboured *bla*$_{SHV-2}$ in an IncK plasmid (pST156-2). Finally, no plasmid location could be confirmed for the CTX-M-14 gene of isolate N127b.

Table 3. Identification and characterisation of the location of *bla*$_{CTX-M-14}$, *bla*$_{SHV-2}$, *bla*$_{SHV-12}$, *bla*$_{CMY-2}$ and *bla*$_{TEM}$ among 11 cephalosporin resistant *E. coli* isolates.

Isolate[a]	**ST**	**Replicons**[b]	***bla* type**	**Plasmid**[c]	**Inc**[d]	**Plasmid size (kb)**
N129	101	FIB	CTX-M-14	pST101	-	90
GN2221	131	N, FIB, K	TEM-1	pST131-1	N	60
			TEM-1	pST131-2	N	250
			CMY-2	pST131-3	K	120
N109	156	HI1, FIB, K	TEM-1	pST156-1	HI1	250
			SHV-2	pST156-2	K	120
N127b	156	HI2, FIB	TEM-1	-	-	-
			CTX-M-14	-	-	-
GN2233	165	FIB, Y, K	CTX-M-14	pST165	K, Y	90
N128	350	FIB	TEM-1	pST350-1	-	180
			CTX-M-14	pST350-2	-	90
N140	350	I1, FIB, K	SHV-12	pST350-3	-	90
N135	429	FIB, P, K	CMY-2	pST429	P, K	90
N139	533	I1, FIB	TEM-1	pST533	I1	120
			SHV-12	pST533	I1	120
N126	889	FIA, FIB, K	CTX-M-14	pST889	K	90
N125	1137	I1, FIB	CTX-M-14	pST1137	I1	95

p(ST number), plasmid location; Inc, identified replicon.

[a] Isolates are divided in APEC (N) and AFEC (GN) strains.

[b] Replicon identifications are based on positive amplifications from the PCR-based replicon typing method.

[c] Plasmids were named based on the source strains sequence type and plasmid size.

[d] In all *E. coli* isolates, replicons from plasmids containing the different *bla* genes were identified by PCR-positive amplification and by Southern hybridisation of the S1-digested fragments.

Discussion

This study has demonstrated the presence of different clones of APEC causing outbreaks of colibacillosis during the same period of time in different broiler farms from different regions of Spain. All these isolates were multirresistant, therefore therapeutic success may have been compromised, causing a serious economic burden to the broiler industry. Moreover, the AFEC strains were also resistant to critically important antimicrobials such as cephalosporins and fluoroquinolones. Several studies have demonstrated that the *E. coli* population of broilers are a reservoir of antimicrobial resistance genes that may be transferred by mobile genetic elements to the community via the food chain (Johnson, Sannes et al. 2007). Although efforts have been implemented to reduce the use of antimicrobials in the poultry industry, these are sometimes overused in food producing animals, and particularly in broiler farms (Collignon, Aarestrup et al. 2013). The restriction of fluoroquinolones and cephalosporins usage in livestock for human consumption, and the implementation of measures to limit the dissemination are needed (Liebana, Carattoli et al. 2013).

This study have also demonstrated the presence of virulence genes associated to APEC strains in commensal *E. coli*, indicating a possible reservoir of virulence-associated genes in this population. However, the presence of the minimal predictors of APEC virulence was much higher in APEC isolates than AFEC isolates. Furthermore, the similarity of avian pathogenic strains with human pathogenic *E. coli* (ExPEC) based on virulence-associated genes was confirmed in this study, since a total of 34% of the isolates could be considered ExPEC. As also suggested by other studies, certain APEC subgroups,

specifically a large proportion of phylogroup A isolates, may be considered potential zoonotic agents (Ewers, Li et al. 2007).

The information provided by different typing methods is usually unrelated. MLST results, phylogenetic groups and serotyping showed similarities between strains that with PFGE were not significant. For instance, the two highly pathogenic strains O78:H9-C-ST23 shared 80% of identity by PFGE, as well as the two strains O5:H10-A-ST93, which shared 70% similarity. Additionally, two AFEC strains O3:H26-A-ST165 exhibited 90% similarity by PFGE. In these cases, the virulence gene content and the results of the susceptibility testing were equal or almost equal. However, the combination of all these techniques may be useful to discriminate between strains causing outbreaks and those with potential risk to cause disease in animals or humans. For instance, combining serotyping, phylotyping and MLST the strain GN-2221 (O25b:H4-B2-ST131) could be identified as a potential zoonotic agent causing infections in humans. Still, further studies based in animal models are necessary to properly confirm the pathogenicity of the strain.

All the serotypes found within this strain collection have been well described in previous studies (Blanco, Blanco et al. 1998, Rodriguez-Siek, Giddings et al. 2005, Wang, Tang et al. 2010, Mora, Viso et al. 2013, Maluta, Logue et al. 2014). In contrast, different results have been observed from the ST data, where several ST types (ST10, ST23, ST48, ST57, ST93, ST117, ST131, ST156, ST350, ST429 and ST648) have been previously associated to APEC strains (Ozawa, Baba et al. 2010, Manges and Johnson 2012, Mora, Viso et al. 2013, Pires-dos-Santos, Bisgaard et al. 2013, Maluta, Logue et al. 2014), whereas other STs (ST101, ST165, ST189, ST297, ST533, ST539, ST624, ST650,

ST889 and ST3161) have not been related to infections cause by APEC before.

The phylogroup B2 is known to harbour many more virulence-encoding genes than the rest of the *E. coli* phylogroups (Russo and Johnson 2000). In our study, the two B2 strains found were O25b:H4-B2-ST131, which previously has been associated to human infections (Lopez-Cerero, Navarro et al. 2014), and O2:H1-B2-ST429 which is frequently associated with poultry disease (Dziva, Hauser et al. 2013). They exhibited 14 and 16 of the 29 virulence genes tested, respectively. Additionally, both strains were resistant to cephalosporins by production of CMY-2. The typical highly pathogenic APEC clonal group O78-H9-C-ST23 was found twice in this strain collection. Studies on sequencing and phylogenetic analysis of this clonal line revealed that O78 is more closely related to human strains (i. e. ST23 enterotoxigenic *E. coli* (ETEC)) than other APEC strains (Dziva, Hauser et al. 2013). These results suggest that the *E. coli* population of broilers may be a potential reservoir of virulence-associated genes that could be transferred to humans through the food chain.

Our results are in line with the most commonly described ESBLs and AmpC producing *E. coli* in poultry production, which are CTX-M-14, CTX-M-1, CMY-2 and SHV-12 (Ewers, Bethe et al. 2012). Also, these data provided evidence to the known genetic heterogeneity among ESBL-harboring *E. coli* isolates in broilers. In the last years, an increase in the presence of *E. coli* O25b:H4-B2-ST131 producing CTX-M-15 with a high virulence potential has been reported in human infections (Coque, Baquero et al. 2008, Blanco, Alonso et al. 2009, Coelho, Mora et al. 2011). The high prevalence of this clonal group among multi-drug

resistant isolates has important clinical and public health implications, due to the risk of treatment failure. The first time that the human ExPEC clonal group O25b:H4-B2-ST131 was detected in poultry was in Spain in 2010 (Cortes, Blanc et al. 2010, Mora, Herrera et al. 2010). In both cases, the genes encoding resistance to cephalosporins were CTX-M-9. Additionally, other studies have described ST131 isolates of human origin carrying CTX-M-15 and *qnrS* in IncN plasmids (Novais, Pires et al. 2012). It is noteworthy that our study describes for the first time a poultry *E. coli* isolate clonal group O25b:H4-B2-ST131 producing CMY-2 with co-resistance to flouroquinolones (*qnrS*). Interestingly, this isolate presented an exceptional phenotype, since it was resistant to fluoroquinolones (MIC = 0.25) but was susceptible to nalidixic acid (MIC = 4) according to epidemiological cut-off values. This isolate was isolated from a healthy animal, corroborating the true zoonotic potential.

Up to date, the most common plasmids carrying cephalosporin resistance genes in *E. coli* isolated in poultry farms belong to the IncI1, IncFIB and IncN families (Bortolaia, Guardabassi et al. 2010). However, in this study, cephalosporin resistance genes are mostly associated to IncK plasmids. Moreover, in some occasions two replicas of the same ESBL gene were harboured in two different plasmids. Interestingly, isolate O25b:H4-B2-ST131 harboured two plasmids from the same incompatibility group (IncN), in the same host cell. Therefore, it is probable that another unknown replicon is present and expressed in at least one of these plasmids.

In conclusion, this study demonstrated a very diverse population of multi-drug resistant *E. coli* in broiler farms containing a high number of virulence-associated genes. This is probably the

combination of virulence and resistance genes transferring from one strain to another via mobile genetic elements creating a multi-clonal scenario, together with *E. coli* strains acquiring the genes and becoming clonally successful. More epidemiological studies are necessary to identify clonal groups and resistance mechanisms with potential relevance to public health. Additionally, prudent use of antimicrobials in animal production should be implemented to reduce the burden of resistance organisms entering the food chain.

Study II: Housefly (*Musca domestica*) as a vector for ESBL-producing *Escherichia coli* in Spanish broiler farms

Abstract

Flies may act as potential vectors for the spread of resistant bacteria into different environments. This study intends to evaluate the presence of *E. coli* resistant to cephalosporins in flies captured in the surroundings of five broiler farms. Phenotypic and molecular characterization of the resistant population was performed by different methods: minimum inhibitory concentration, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and phylotyping. The presence of extended spectrum beta-lactamase (ESBL) genes, its plasmid location and the characterization of the mobile genetic elements involved in its mobilization were studied. Additionally, presence of 35 genes associated to virulence was evaluated. Out of 682 flies captured, 42 yielded ESBL-producing *E. coli*. Of those, 23 contained $bla_{\text{CTX-M-1}}$, 18 $bla_{\text{CTX-M-14}}$ and one $bla_{\text{CTX-M-9}}$. ESBL genes were mainly associated to the presence of IncI1 and IncFIB replicons. Additionally, all the strains were multi-resistant and five of them also harboured *qnrS*. Identical PFGE profiles were found in *E. coli* obtained from flies at different sampling times indicating persistence of the same clones in the farm environment over the months. According to virulence genes, 81% of the isolates were considered avian pathogenic *E. coli* (APEC) and 29% extraintestinal pathogenic *E. coli* (ExPEC). The entrance of flies to broiler houses constitutes a considerable risk for colonization of broilers with multi-drug resistant *E. coli.* ESBLs in the flies reflect the contamination status of the farm environment. Additionally, this study demonstrates the potential contribution of flies to the dissemination of virulence and resistance genes into different ecological niches.

Introduction

Escherichia coli is a commensal bacterium commonly found in nature and in the lower intestine of warm-blooded organisms. However, some serotypes can cause enteric and extraintestinal infections in humans and animals (Yang, Chen et al. 2004). For instance, avian pathogenic *E. coli* (APEC) is the major cause of colibacillosis in poultry production. It is a syndrome that causes respiratory infections associated to airsacculitis, pericarditis, and septicemia, resulting in large economical burden for the poultry industry due to loss of production and mortality (Schouler, Schaeffer et al. 2012). Hybridization studies detected APEC specific DNA sequences presenting high homology with DNA sequences of human extraintestinal pathogenic *E. coli* (ExPEC) strains (de Campos, Lago et al. 2008). Both have virulence determinants in common, suggesting that APEC could serve as a reservoir and a source of virulence for ExPEC (Mokady, Gophna et al. 2005). Moreover, it has been suggested that APEC strains are potential zoonotic pathogens (Ewers, Janssen et al. 2004).

Antimicrobials are the common treatment for the avian colibacillosis caused by APEC. During the last years, increased resistance to frontline antimicrobials, such as fluroquinolones and third generation cephalosporins, have been reported in *E. coli* isolated from poultry (Yang, Chen et al. 2004). Additionally, the emergence of bacteria resistant to critically important antimicrobials is a major concern in human and veterinary medicine. Presence of isolates producing extended-spectrum beta-lactamases (ESBLs) and plasmid mediated AmpC beta-lactamases among *E. coli* from broilers has increased substantially in the last decade (Cantón, Novais et al. 2008).

Generally, the genes encoding for beta-lactamases are located on plasmids, which can be transferred to other bacteria (Dierikx, van der Goot et al. 2013).

Persistence of *E. coli* in the environment has been described in the literature (Brennan, Abram et al. 2010). However, the relevance of wildlife vectors in the persistence and spread of ESBL-producing *E. coli* in the farm environment has not been studied thoroughly (Usui, Iwasa et al. 2013, Blaak, Hamidjaja et al. 2014). In particular, flies are one of the most important vectors of human diseases worldwide (Greenberg 1973). They have the capacity to horizontally transfer pathogens from different environments (Wales, Carrique-Mas et al. 2010), posing a high risk for human health (Olsen 1998). Due to their movements, the capacity to fly long distances, and the attraction to decaying organic materials and food, houseflies amplify the risk of human exposure to foodborne pathogens (Murvosh and Thaggard 1966, Nazni, Luke et al. 2005, Barreiro, Albano et al. 2013). Moreover, they may also spread antibiotic resistance genes within microbial communities (Boulesteix, Le Dantec et al. 2005). The digestive tract of flies provides a suitable environment for the horizontal transfer of genes among bacteria, which contributes to the spread of resistance and virulence genes (Petridis, Bagdasarian et al. 2006). Several studies have demonstrated that flies are carrying multi-drug resistant bacteria in hospital environments, and also their role in transmission of human pathogens within hospitals (Boulesteix, Le Dantec et al. 2005).

The objective of this study was to assess the potential contribution of flies to the spread of ESBL/AmpC-producing *E. coli* into the farm environment. For this purpose the isolates, the resistant genes and mobile genetic elements involved in the transmission of

resistant genes were fully characterized. The genomic relationship among strains and the virulence content associated to APEC and ExPEC strains were also evaluated.

Materials and Methods

Study design

The study was conducted in five broiler farms (Farms 1 to 5) each one with one or two houses, located in Catalonia region (Spain). Broiler houses capacity ranged from 15,000 to 46,000 birds per house. Only Farm 2 presented cats at the premises. Minimum and maximum distance between farms was 15 Km and 200 Km. From May to November 2012, each broiler farm was visited twice per rearing cycle to capture the flies (6 to 8 visits per farm in total). Overall, 23 broiler flocks were reared in the 5 study houses during the study period. Flies sampling was performed when chickens were about 14 and 28 days-old. At each visit, up to 50 flies were collected always outside the same broiler houses (within 10 meters periphery). Overall, 682 flies were captured individually, placed in disposable sterile plastic bags and transported refrigerated alive to the laboratory. Once at the laboratory, flies were anesthetized with CO_2, identified to genus or species level, and subsequently processed for *Campylobacter* (unpublished data), and thereafter for cephalosporin resistant *E. coli* isolation. Each individual fly was aseptically macerated in plastic bags with 2.5 ml Bolton broth (CM0983 with SR0183 and SR0048, Oxoid, Basingstoke, UK), and incubated at 42ºC for 24 h in 2 ml screw cap tubes. A 10 µl loop of broth was plated onto MacConkey agar (Oxoid, Basingstoke, UK) supplemented with ceftriaxone (1mg/L). Three lactose-positive colonies for each plate were selected and confirmed as *E. coli* by PCR

(Heininger, Binder et al. 1999). Subsequently, one representative was selected for further studies.

Phylogeny, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

Isolates were discriminated in phylogenetic groups by PCR (A, B1, B2, C, D, E or F) as previously described by Clermont *et al.* (Clermont, Bonacorsi et al. 2000, Clermont, Christenson et al. 2013).

PFGE was performed to analyze the genomic relatedness among *E. coli* isolates. PFGE of chromosomal DNA digested with the restriction enzyme *XbaI* was carried out according to the PulseNet protocol (Ribot, Fair et al. 2006). *Salmonella* serovar Branderup H9812 was used as size marker. The results were analyzed by Fingerprinting II Informatix software (Applied Maths, Sint-Martens-Latem, Belgium). Isolates were considered to have a different pattern when at least one band difference was detected. The analysis of the bands generated was performed using the Dice coefficient and unweighted pair group method with arithmetic averages (optimization of 1.25% and position tolerance 1.25%).

MLST was performed to determine potential evolutionary relatedness between isolates. MLST was carried out as previously described by gene amplification and sequencing of the seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA* and *recA*), according to the protocol and primers specified on the *E. coli* MLST web site (http://mlst.ucc.ie/mlst/dbs/Ecoli) (Wirth, Falush et al. 2006). Sequences were analysed with Vector NTI advance 11 (InforMax, Inc., Bethesda, MD). The allelic profile of the gene sequences

and the sequence types (STs) were obtained via electronic database at *E. coli* MLST web site.

Antimicrobial susceptibility testing

Disc diffusion was performed according to CLSI guidelines using the following discs (Oxoid, Basingstoke, UK): cefoxitin, 30 mg; cefepime, 30 mg; ceftazidime, 30 mg; cefotaxime, 30 mg; cefotaxime+clavulanic acid, 30+10 mg; and ceftazidime+clavulanic acid, 30+10 mg. The synergy between cefotaxime and cefotaxime/clavulanic acid, ceftazidime and ceftazidime/clavulanic acid were used as suggestive evidence of ESBL production; cefoxitin was used for the detection of AmpC-type beta-lactamase (CLSI 2008). Additionally, all isolates were tested for antimicrobial susceptibility using a minimum inhibitory concentration (MIC)-based broth microdilution (VetMIC GN-mo, National Veterinary Institute, Uppsala, Sweden) for the following antimicrobial agents: ampicillin (1 - 128 mg/L), cefotaxime (0.016 - 2 mg/L), ceftazidime (0.25 - 16 mg/L), nalidixic acid (1 -128 mg/L), ciprofloxacin (0.008 - 1 mg/L), gentamicin (0.12 – 16 mg/L), streptomycin (2 - 256 mg/L), kanamycin (8 - 16 mg/L), chloramphenicol (2 - 64 mg/L), florfenicol (4 - 32 mg/L), trimethoprim (1 - 128 mg/L), sulfamethoxazole (8 - 1024 mg/L), tetracycline (1 - 128 mg/L) and colistin (0.5 - 4 mg/L). *E. coli* ATCC 25922 was used as a control strain. Isolates were considered to be wild type (WT) or non-WT based on epidemiological cut-off values according to EUCAST (http://www.eucast.org/) (Schwarz, Silley et al. 2010).

Resistance genes

All strains were tested by PCR for the presence of the *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CMY-2}$ genes as described by Hasman *et al.* (Hasman, Mevius et al. 2005). Sequencing of both strands of amplicons was performed. The presence of the genes *aac(6')-Ib-cr*, *qnrA*, *qnrB*, *qnrS*, *qepA* and *oqxAB* conferring resistance to fluoroquinolones was also assessed (Coelho, Mirelis et al. 2009).

Plasmid DNA analysis

One isolate from each PFGE clonal cluster was selected for plasmid characterization. The presence of the plasmid replicons HI1, HI2, I1, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIAs, and K was assessed by the PCR-based replicon typing method described previously (Carattoli, Bertini et al. 2005, Carattoli 2009), including screening for the IncR replicon (Garcia-Fernandez, Fortini et al. 2009). The detection of plasmids and sizing was carried out in all the isolates by PFGE of total DNA digested with S1-nuclease (Barton, Harding et al. 1995). Restriction fragments from S1-PFGE gels were transferred onto positively charged nylon membranes and hybridised with specific probes for *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$ and with specific probes for each previously identified replicon.

Detection of virulence-associated genes

All 42 strains were tested for a pool of 35 virulence-associated genes (Table 2), including seven adhesins, four siderophores, nine toxins, eight capsule synthesis-associated genes or protectins and seven miscellaneous genes, by PCR using primers described previously (Johnson, O'Bryan et al. 2000, Johnson, Johnston et al. 2008). The five virulence factors for ExPEC detection (*pap*, *sfa/foc*, *afa/dra*, *iutA* and

kpsM II) (Johnson, Murray et al. 2003) together with the five potential APEC virulence genes (*iutA*, *hlyF*, *iss*, *iroN* and *ompT*) (Johnson, Wannemuehler et al. 2008) were included in the PCR analysis. Virulence scores were calculated for each isolate as the sum of all virulence genes detected; *pap, sfa-foc, clbB-clbN* and *kpsM* II were counted only once regardless of the number of elements or subunits identified (maximum possible score 27).

Statistical analysis

Differences in the prevalence of phylogroups and STs between the distinct groups were determined by Fisher's exact test. The associations between groups were assessed by calculation of the odds ratio (OR) with 95% confidence intervals (CIs). The null hypothesis was rejected for data with P values of <0.05. Statistical analyses were performed using GraphPad Prsim, version 3.1, software (GraphPad Software, Inc., San Diego, CA). Virulence scores were compared by the Mann-Whitney U test.

Results

During the course of the study, a total of 682 flies were captured from the surrounding environment of five different broiler farms. The 42 ESBL-producers were collected from Farm 1 (9%, n=193), Farm 2 (3%, n=138), Farm 3 (15%, n=109) and Farm 4 (4%, n=134). Finally, all flies collected from Farm 5 (n=108) were negative for ESBL-producing *E. coli*. Most of the fly species were classified as *M. domestica* (n=615) followed by *Ophyra* spp. (n=33), *Stomoxys calcitrans* (n=15), *Muscina stabulans* (n=7), *Fannia canicularis* (n=6) and others (n=6). A

total of 42 ESBL-producing *E. coli* were isolated mainly from *M. domestica* (n=41), and one from *Muscina stabulans*.

PFGE, phylogeny and MLST

XbaI-PFGE analysis revealed a total of 29 different PFGE restriction profiles among the 42 *E. coli* isolates (Figure 1). The number of fragments generated ranged from 14 to 21, and their sizes varied from 20 to 1135 kb. In almost all cases, isolates epidemiologically related belonged to the same farm, except for three isolates (F1F8M17, F3F6M21 and F3F6M7) from Farms 1 and 3 that presented identical fingerprints (Figure 1).

Four different phylogroups were represented among the 42 isolates. Of these, 15 were of group A (36%), 18 of group B1 (43%), seven of group C (17%), one of group E (2%) and one unknown (U) (2%) (Figure 1).

MLST analyses identified 21 STs belonging to 11 different clonal complexes (Cplx) (Figure 1). The most common Cplx was ST10 (n=10) containing four different ST types (ST10, ST48, ST195 and ST617), followed by Cplx ST446 (n=7; all ST602) and Cplx ST23 (n=7) comprising four different ST types (ST88, ST90, ST410 and ST650). Only two different Cplx were represented by more than one phylogenetic group (ST10-A and E; ST23-C and A).

Figure 1. PFGE dendogram illustrating the phenotypic and genotypic relationship of the strains, phylogeny and cephalosporin resistance genes.

Isolate[a]	Phylo.	ESBL	FQ	ST	Cplx
F1F8M11	A	CTX-M-1	qnrS	ST650	ST23 Cplx
F2F8M3	A	CTX-M-1	qnrS	ST226	ST226 Cplx
F4F6M16	A	CTX-M-14		ST398	ST398 Cplx
F1F8M8	A	CTX-M-1		ST10	ST10 Cplx
F1F8M4	A	CTX-M-1		ST10	ST10 Cplx
F1F8M10	A	CTX-M-1	qnrS	ST48	ST10 Cplx
F1F8M29	A	CTX-M-1		ST48	ST10 Cplx
F1F8M27	A	CTX-M-1		ST48	ST10 Cplx
F1F8M33	A	CTX-M-1		ST48	ST10 Cplx
F4F7M38	A	CTX-M-14		ST48	ST10 Cplx
F1F8M17	C	CTX-M-1		ST88	ST23 Cplx
F3F6M21	C	CTX-M-1		ST88	ST23 Cplx
F3F6M7	C	CTX-M-1		ST88	ST23 Cplx
F2F7M9	B1	CTX-M-14		ST101	ST101 Cplx
F1F8M15	C	CTX-M-1	qnrS	ST90	ST23 Cplx
F1F8M25	U	CTX-M-1	qnrS	ST155	ST155 Cplx
F4F5M17	B1	CTX-M-14		ST602	ST446 Cplx
F4F6M10	B1	CTX-M-14		ST602	ST446 Cplx
F4F5M2	B1	CTX-M-14		ST602	ST446 Cplx
F4F5M5	B1	CTX-M-14		ST602	ST446 Cplx
F4F5M7	B1	CTX-M-14		ST602	ST446 Cplx
F4F7M41	B1	CTX-M-14		ST602	ST446 Cplx
F4F7M24	C	CTX-M-14		ST410	ST23 Cplx
F4F5M11	B1	CTX-M-14		ST876	
F4F7M28	B1	CTX-M-14		ST876	
F2F7M18	B1	CTX-M-14		ST58	ST155 Cplx
F3F7M17	A	CTX-M-14		ST354	ST354 Cplx
F4F5M8	B1	CTX-M-14		ST602	ST446 Cplx
F1F8M19	E	CTX-M-1		ST195	ST10 Cplx
F1F8M3	A	CTX-M-1		ST165	ST165 Cplx
F1F8M39	A	CTX-M-1		ST165	ST165 Cplx
F4F7M23	B1	CTX-M-1		ST155	ST155 Cplx
F1F8M14	C	CTX-M-9		ST10	ST10 Cplx
F3F6M10A	A	CTX-M-1		ST165	ST165 Cplx
F3F6M10B	C	CTX-M-1		ST88	ST23 Cplx
F4F7M48	A	CTX-M-1		ST617	ST10 Cplx
F1F8M2	B1	CTX-M-1		ST212	
F1F8M34	B1	CTX-M-1		ST162	ST469 Cplx
F1F8M37	B1	CTX-M-1		ST162	ST469 Cplx
F2F8M39	B1	CTX-M-14		ST641	ST86 Cplx
F4F5M4	B1	CTX-M-14		ST117	
F4F5M1	B1	CTX-M-14		ST117	

PFGE, pulsed field gel electrophoresis; Phylo, phylogroups; ESBL, extended spectrum beta-lactamase (ESBL) genes; FQ, flouoroquinolone resistance genes; ST, sequence types; Cplx, clonal complexes; U, unknown; Am: Ampicillin (WT≤8mg/L); Ctx: Cefotaxime (WT≤0.25mg/L); Caz: Ceftazidime (WT≤0.5mg/L); Nal: Nalidixic acid (WT≤16mg/L); Ci: Ciprofloxacin (WT≤0.064mg/L); Gm: Gentamicin (WT≤2mg/L); Sm: Streptomycin (WT≤16mg/L); Km: Kanamycin (WT≤8mg/L); Cm: Chloramphenicol (WT≤16mg/L); Ff: Florfenicol (WT≤16mg/L); Tm: Trimethoprim (WT≤2mg/L); Su: Sulfamethoxazole (WT≤64mg/L); Tc: Tetracycline (WT≤8mg/L); Cs: Colistin (WT≤2mg/L).

[a] Isolates were named based on the number of farms (F), flocks (F) and flies (M).

Antimicrobial susceptibility testing and resistance genes

Disc diffusion demonstrated that all isolates presented the ESBL phenotype. All the strains were multi-resistant (resistant to more than 3 antimicrobial families). Furthermore, 79% of the isolates were non-WT to more than eight antimicrobials. MIC determination confirmed that all strains were non-WT to cephalosporins (100% to cefotaxime and 83% to ceftazidime) with 23 isolates yielding amplicons for *bla*$_{CTX-M-1}$, 18 *bla*$_{CTX-M-14}$ and one *bla*$_{CTX-M-9}$. Out of the 42 isolates, 33 isolates harboured the *bla*$_{TEM}$ gene. None of the isolates were positive for *bla*$_{SHV}$ or *bla*$_{CMY-2}$. In addition, 93% of the isolates were non-WT to nalidixic acid and 98% to ciprofloxacin. The presence of *qnrS* genes was detected in five isolates obtained from Farms 1 and 2. The genes *aac(6')-Ib-cr*, *qnrA*, *qnrB*, *qepA* and *oqxAB* were not found in this collection. Additionally, 100% of the strains were non-WT to ampicillin, trimethoprim and tetracycline, 98% to sulfamethoxazole, 86% to streptomycin, 45% to chloramphenicol, 36% to gentamicin, 17% to kanamycin and 2% to colistin. All isolates were WT to florfenicol.

Localisation of *bla*$_{CTX-M}$

Replicons IncFIB and IncI1 were detected in the majority of the isolates (90% and 83% respectively), being or not associated with any of the CTX-M genes. However, IncP, IncK, IncY, Inc FIA, IncHI1, IncH12 and IncN were also detected (Table 1).

All *bla*$_{CTX-M-1}$ isolates hybridised with a plasmid of aprox. 110-kb containing an IncI1 replicon, except for the following exceptions; two isolates (F1F8M15 and F3F7M23) containing both, IncI1 and IncFIB in a 120-kb plasmid and one extra isolate (F1F8M25) that contained

IncI1 together with IncFIB in two plasmids of 120 and 190-kb (Table 1). Additionally, isolate F4F7M48 carried a second copy of the gene on a large plasmid of 300-kb (Table 1). The IncI1 and IncFIB replicons were identified on *bla*$_{CTX-M-14}$-carrying plasmids of different sizes. The isolate carrying *bla*$_{CTX-M-9}$ exhibited three different replicons (IncI1, IncFIB and IncP) on the same plasmid (Table 1).

Table 1. Identification and characterisation of the plasmid location of *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-9}$ among 29 CTX-M-producing *E. coli* isolates, one representative for each PFGE cluster.

Isolate[a]	ST	Cplx	Replicons[b]	*bla*$_{CTX-M-1}$		
				Plasmid[c]	Inc[d]	Plasmid size (kb)
F1F8M8	10	10	I1, FIB	pST10-1	I1	110
F1F8M10	48	10	I1, FIB	pST48-1	I1	110
F1F8M17	88	23	I1, FIB, P	pST88	I1	110
F1F8M15	90	23	I1, FIB	pST90	I1, FIB	120
F1F8M25	155	155	I1, FIB, P	pST155-1	I1, FIB	120
				pST155-2	I1, FIB	190
F1F8M34	162	469	I1, FIB	pST162	I1	110
F1F8M3	165	165	I1, FIB, P	pST165-1	I1	110
F1F8M19	195	10	I1, FIB, P, K	pST195	I1	110
F1F8M2	212		I1, FIB, Y, P	pST212	I1	110
F1F8M11	650	23	I1, FIB, P	pST650	I1	110
F3F6M10B	88	23	I1, FIB, P	pST88	I1	110
F3F6M10A	165	165	I1	pST165-2	I1	120
F4F7M23	155	155	I1, FIB	pST155-3	I1, FIB	110
F4F7M48	617	10	I1, FIA, FIB	pST617-1	-	50
				pST617-2	-	300
F2F8M3	226	226		pST226	-	50
				bla$_{CTX-M-14}$		
				Plasmid[c]	Inc[d]	Plasmid size (kb)
F4F7M38	48	10	I1, FIB	pST48-2	I1	90
F4F5M4	117		FIB	pST117	FIB	145
F4F6M16	398	398	FIB	pST398	-	75
F4F7M24	410	23	HI1, HI2, I1, FIB, P	pST410	I1	110
F4F5M8	602	446	HI1, HI2, I1, FIB, P	pST602-1	I1	110
F4F5M2	602	446	HI1, HI2, I1, FIB, P	pST602-1	I1	110
F4F5M17	602	446	HI1, HI2, I1, FIB, P	pST602-1	I1	110
F4F7M41	602	446	HI1, HI2, I1, FIB, P	pST602-2	I1	90
F4F5M11	876		HI1, HI2, I1, FIB, P	pST876	-	90
F2F7M18	58	155	I1, FIB	pST58	-	90
F2F7M9	101	101	FIB	pST101	-	90

F2F8M39	641	86	I1, N	pST641	I1	100
F3F7M17	354	354	FIA, FIB	pST602-2	FIB	90
				bla$_{CTX-M-9}$		
				Plasmid[c]	Inc[d]	Plasmid size (kb)
F1F8M14	10	10	I1, FIB, P	pST10-2	I1, FIB, P	120

ST, sequence types; Cplx, clonal complexes, p(ST number), plasmid location; cr, chromosomal location; Inc, identified replicon.

[a] Isolates were named based on the number of farms (F), flocks (F) and flies (M).

[b] Replicon identifications are based on positive amplifications from the PCR-based replicon typing method.

[c] Plasmids were named based on the source strains sequence type and plasmid size.

[d] In all *E. coli* isolates, replicons from plasmids containing the different *bla* genes were identified by PCR-positive amplification and by Southern hybridisation of the S1-digested fragments.

Detection of virulence genes

The prevalence of 35 virulence-associated genes, including the genes associated to APEC and ExPEC, is illustrated in Table 2. The virulence genes detected with the highest prevalence were *fimH* (100%), *traT* (88%), *clbB* (76%), *cdts* (67%) and *cvaC* (48%). The presence of *astA* (29%), *tsh* (29%), *papEF* (26%) and *kpsM* III (24%) was confirmed in an intermediate percentage of the isolates. On the contrary, the presence of *fyuA* (14%), *ireA* (14%), *papC* (10%), *papA* (7%), *papG* (7%), *kpsM* II (5%), *sfa/focDE* (2%), *kpsM* II-K2 (2%), *kpsM* II-K5 (2%), *ibeA* (2%), *malX* (2%), *usp* (2%) and $fliC_{H7}$ (2%) was confirmed in a lower number of strains. None of the isolates were positive for *afa/draBC*, *cnf1*, *cdtB*, *sat*, *hlyD*, *stx1*, *stx2*, *kpsM* II-K1 and *clbN*. A total of 12 (29%) isolates from this study were identified as ExPEC according to the ExPEC definition. Additionally, 79%, 88%, 88%, 76% and 86% of the strains yielded amplicons for *iroN*, *ompT*, *hlyF*, *iutA* and *iss*, respectively; these genes are described as the minimal predictors of APEC virulence. A total of 34 (81%) isolates were considered APEC, since they harboured between 4 and 5 of these genes. Moreover, 11 (26%) of the isolates were considered ExPEC and APEC at the same time.

Table 2. Distribution of virulence genes among the 42 isolates, the largest phylogenetic groups and relevant ST Cplx.

Virulence gene (s)	Description	Total isolates (%) (n=42)	A (%) (n=15)	B1 (%) (n=18)	C (%) (n=7)	A/ST10 Cplx (%) (n=8)	A/non-ST10 Cplx (%) (n=7)	*P* value[a] A/ST10 Cplx vs A/non-ST10 Cplx
Adhesins								
fimH	D-mannose-specific adhesin of type 1 fimbriae	42 (100%)	15 (100%)	18 (100%)	7 (100%)	8 (100%)	7 (100%)	
papEF	pilus associated with pyelonephritis (P fimbriae)	11 (26%)	5 (33%)	4 (22%)	0	5 (63%)	0	0.0256
papG	Gal(α1-4) Gal-specific pilus tip adhesin molecule	3 (7%)	0	2 (11%)	0	0	0	
papA	major structural subunit of P fimbrial shaft	3 (7%)	0	3 (17%)	0	0	0	
papC	pilus assembly; central region of pap operon	4 (10%)	0	3 (17%)	0	0	0	
sfa/focDE	central region of sfa and foc operons	1 (2%)	0	1 (6%)	0	0	0	
afa/draBC	Dr antigen-specific adhesin operons (AFA, Dr, F1845)	0	0	0	0	0	0	
Toxins								
cnf1	cytotoxic necrotizing factor 1	0	0	0	0	0	0	
cdtB	cytolethal distending toxin	0	0	0	0	0	0	
sat	secreted autotransporter	0	0	0	0	0	0	
hlyD	α-haemolysin	0	0	0	0	0	0	
hlyF	haemolysin F	37 (88%)	12 (80%)	16 (89%)	7 (100%)	8 (100%)	4 (57%)	

astA	(EAST 1) enteroaggregative E. coli heat stable toxin	12 (29%)	7 (47%)	4 (22%)	0	7 (86%)	0	0.0014
tsh	temperature-sensitive haemagglutinin-serine protease	12 (29%)	2 (13%)	4 (22%)	6 (86%)	2 (25%)	0	
stx1	shiga toxin 1	0	0	0	0	0	0	
stx2	shiga toxin 2	0	0	0	0	0	0	
Siderophores								
fyuA	Yersinia siderophore receptor (ferric yersiniabactin uptake)	6 (14%)	0	3 (17%)	3 (43%)	0	0	
iutA	ferric aerobactin receptor (iron uptake: transport)	32 (76%)	9 (60%)	15 (83%)	6 (86%)	8 (100%)	1 (14%)	0.0014
iroN	novel catecholate siderophore receptor	33 (79%)	9 (60%)	15 (83%)	7 (100%)	8 (100%)	1 (14%)	0.0014
ireA	iron-regulated element (novel siderophore receptor)	6 (14%)	0	5 (28%)	0	0	0	
Protectins								
kpsM II	group II capsule	2 (5%)	1 (7%)	0	0	0	1 (14%)	
kpsM II-K2	K2 subgroup II capsule	1 (2%)	1 (7%)	0	0	0	1 (14%)	
kpsM II-K5	K5 subgroup II capsule	1 (2%)	0	1 (6%)	0	0	0	
kpsM II-K1	K1 subgroup II capsule	0	0	0	0	0	0	
kpsM III	group III capsule	10 (24%)	7 (47%)	2 (11%)	0	7 (86%)	0	0.0014
cvaC	ColV; colicin V from serum resistance-associated plasmids	20 (48%)	1 (7%)	12 (67%)	6 (86%)	1 (13%)	0	
iss	increased serum survival (outer membrane	36 (86%)	10	17	7	8 (100%)	2 (29%)	0.0070

	protein)		(67%)	(94%)	(100%)		
traT	surface exclusion, serum survival (outer membrane protein)	37 (88%)	12 (80%)	17 (94%)	6 (86%)	8 (100%)	4 (57%)
Miscellaneous							
ompT	outer membrane protein (protease) T	37 (88%)	12 (80%)	16 (89%)	7 (100%)	8 (100%)	4 (57%)
ibeA	invasion of brain endothelium	1 (2%)	1 (7%)	0	0	0	1 (14%)
malX	PAI; pathogenicity-associated island marker	1 (2%)	1 (7%)	0	0	1 (13%)	0
usp	uropathogenic-specific protein (bacteriocin)	1 (2%)	1 (7%)	0	0	0	1 (14%)
clbB	codes for hybrid peptide-polyketide synthase (colibactin)	32 (76%)	12 (80%)	13 (72%)	6 (86%)	6 (75%)	6 (86%)
clbN	codes for non ribosomal synthetase (colibactin)	0	0	0	0	0	0
$fliC_{H7}$	H7 flagellin variant	1 (2%)	0	1 (6%)	0	0	0
APEC		34 (81%)	9 (60%)	17 (94%)	7 (100%)	8 (100%)	1 (14%)	0.0014
ExPEC		12 (29%)	6 (40%)	4 (22%)	0	5 (63%)	1 (14%)
Mean (range) virulence score[b]		8.8 (1-12)	7.8 (1-12)	9.1 (4-12)	9.7 (6-11)	10.6 (10-12)	4.6 (1-7)	<0.0001

[a] *P* values (by Fisher's exact test) are shown where $P<0.05$.

[b] The virulence score was the number of virulence genes detected, adjusted for multiple detection of the *pap*, *sfa* and *foc*, *clbB* and *clbN*, and *kpsM* II operons. Virulence scores were compared by use of the Mann-Whitney U test.

Statistical analysis

No significant differences were observed in the number of virulence genes found between phylogroups. Phylogroups A, B1 and C exhibited a virulence score between 7.8 and 9.7 (Table 2). C/ST23 Cplx (mean, 9.8; range, 6 to 11), A/ST10 Cplx (mean, 10.6; range, 10 to 12) and B1/ST446 Cplx (mean, 9.6; range, 9 to 12) exhibited similar virulence scores, but different gene content (data not shown). Significant differences were observed in virulence score between A/ST10 Cplx isolates and A/non-ST10 Cplx ($P < 0.0001$). The virulence factors that were significantly different were characteristic of APEC (*iss, iutA, iroN* and *astA*) and ExPEC (*papEF* and *kpsM* III) (Table 2).

Discussion

M. domestica is an arthropod distributed worldwide and the most abundant fly species in animal production and food at homes and restaurants. Flies are suspected reservoirs and vectors for human and animal pathogens due to their contact with animal manure, food and humans. They can pick up bacteria present in those sites and transport them to the kitchen (Forster, Sievert et al. 2009). Some studies have suggested that flies can also play an important role in the dissemination of antimicrobial resistance genes within the bacterial community (Johnson and Stell 2000, Liu, Yang et al. 2013). In our study, the presence of multi-drug resistant *E. coli* isolated from flies, and in particular ESBL-producing *E. coli*, demonstrates the capacity of houseflies to disseminate and transport resistance genes located in mobile genetic elements. Additionally, five of the isolates also harboured plasmid mediated quinolone resistance genes. *qnrS* genes have been previously associated to the same plasmids harbouring

ESBL genes (Robicsek, Jacoby et al. 2006). The continuous increase in antimicrobial resistant bacteria has been associated to the use of these drugs to treat human and animal infections, and the presence of ESBLs- producing *E. coli* in flies suggest that animals and the farm environment are colonized and inhabited by these microorganisms. Flies are a reservoir of resistant bacteria and can contribute to the spread of resistance genes between different ecological niches.

Some studies have suggested the relationship between different *E. coli* phylogenetic groups and the virulence capabilities of the strains (Picard, Garcia et al. 1999, Johnson and Stell 2000). Commensal isolates belong mainly to phylogenetic groups A, B1 and C (Moissenet, Salauze et al. 2010, Clermont, Olier et al. 2011). In contrast, the most virulent phylogroups described in the literature are B2 followed by D, which are mainly responsible for extra-intestinal infections (Picard, Garcia et al. 1999, Johnson and Stell 2000). None of the ESBL-producing isolates from this study belonged to B2 and D phylogroups, most of them belonged to A, B1 and C and possessed quite high virulence scores. Furthermore, the ESBL-producing *E. coli* A/ST10 Cplx isolates from this study have a significantly higher virulence score than other ST types from the same phylogroup. Similar results were obtained in other studies, where strains A/ST10 of APEC and ExPEC origin were described as emerging pathogens; suggesting that this ST complex may have a relevant zoonotic potential (Dissanayake, Octavia et al. 2014, Maluta, Logue et al. 2014).

PFGE results demonstrated the same clonal groups in the same farms, suggesting dissemination of epidemiologically related clones within farm environments. An exception was the three strains from Farm 1 and 3 belonging to the same PFGE. These farms were about 25

Km apart. This observation would reinforce what has been previously reported that flies can travel long distances in a short time, spreading the resistant bacteria (Murvosh and Thaggard 1966, Nazni, Luke et al. 2005, Barreiro, Albano et al. 2013). Additionally, identical fingerprints have been recovered from different flies belonging to the same farm at different time points, including different broiler cycles, demonstrating the capacity of these bacteria to survive and persist in the environment for long periods of time.

This study has also demonstrated the presence of *E. coli* isolates with virulence-associated genes characteristic of both, APEC (81%) and ExPEC (29%), and the capacity of flies to transport them. Some of these virulence genes are also associated to mobile genetic elements, highlighting the relevance of flies in the transmission of virulence determinants in broiler farms and hospital settings (Boulesteix, Le Dantec et al. 2005).

In the present study we have found that $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-14}}$ are the most prevalent ESBL genes detected in *E. coli* isolates obtained from flies captured at the surroundings of broiler farms (55% and 43%, respectively). This result is in agreement with previous studies, which demonstrate that $bla_{\text{CTX-M-1}}$ is one of the most prevalent ESBL genes detected in *Enterobacteriacea* from broiler origin (Accogli, Fortini et al. 2013, Belmar Campos, Fenner et al. 2014, Ben Sallem, Ben Slama et al. 2014, Zurfluh, Wang et al. 2014). In line also with studies performed in broiler farms, the most common replicons encountered in this study were IncI1 and IncFIB (Bortolaia, Guardabassi et al. 2010, Wang, Stephan et al. 2013). Moreover, we have found five isolates with the same ESBL gene harboured in two different plasmids. Having two or more copies of the resistance mechanism in different locations

would ensure the maintenance and persistence of these genes even if the selected pressure enforce the lost of one of these copies.

In conclusion, this study has demonstrated a very diverse population of multi-drug resistant *E. coli* recovered from flies at different broiler farms. ESBL-producing *E. coli* in the flies reflect the colonization status of the farm environment. Flies are probably not the source, but the result of the colonization of the animals. These isolates contained a high number of virulence-associated genes and ESBLs genes, which could be easily introduced and disseminated into the farms through the flies, and subsequently could potentially colonise the animals. Additional biosecurity measures, aimed at blocking or reducing the entrance of flies into broiler houses, should be overtaken. Otherwise, zoonoses control and antimicrobial resistance reduction may be frustrated. Flies are also contributing to pathogens evolution since transfer of resistance and virulence-associated genes between different strains could be facilitated through the flies.

1

Study III: Does exploitation of human residues make the white stork (*Ciconia ciconia*) a reservoir of extended-spectrum beta-lactamases-producing *Escherichia coli*?

Summary

Ciconia ciconia (white stork) may act as a reservoir and vehicle of cephalosporin resistant (CR) *Escherichia coli*. Between 2011 and 2014 white storks from colonies exposed to different degrees of anthropic pressure were sampled. *E. coli* isolates were phenotypically characterized, presence of CR genes was confirmed and plasmids were classified. Risk factors for acquiring these genes were assessed. Overall, 8.8% (41 out of 467) storks carried CR *E. coli* in their cloaca and five were identified from recently deposited droppings; therefore, 46 isolates were further characterized. Of them 21 contained $bla_{CTX\text{-}M\text{-}1}$, 10 $bla_{CMY\text{-}2}$, 6 $bla_{CTX\text{-}M\text{-}14}$, 3 $bla_{SHV\text{-}12}$, 3 $bla_{CTX\text{-}M\text{-}15}$, 2 $bla_{CTX\text{-}M\text{-}1}$ together with $bla_{CMY\text{-}2}$, and 1 $bla_{CTX\text{-}M\text{-}1}$ together with $bla_{SHV\text{-}12}$. All were multi-resistant, and three harboured the plasmid-mediated colistin resistance *mcr-1* gene. CR genes were associated with the presence of IncI1, IncFIB and IncN replicon families. Pulsed-field gel electrophoresis demonstrated a high degree of polymorphism, but showed also identical profiles from isolates obtained from different locations. Carriage of CR *E. coli* was strongly associated to use of landfills as food source. This study demonstrates that the proximity of white storks to human activities, where the antimicrobial pressure is high, contributes to the acquisition and dissemination of CR *E. coli*.

Introduction

Over the past years, the presence of multidrug resistant pathogens in wildlife, particularly cephalosporin resistant (CR) *Escherichia coli* has become a problem of increasing concern in public health (Guenther, Ewers et al. 2011). The genes encoding these enzymes are frequently located in plasmids and can be horizontally transferred to other bacteria (Carattoli 2013, Dierikx, van der Goot et al. 2013). The overuse of antimicrobials in both, human and veterinary medicine has been considered one of the main factors contributing to the dissemination of antimicrobial resistant bacteria, increasing their detection in humans, food-producing animals and food (Jouini, Vinue et al. 2007, Smet, Martel et al. 2009). Furthermore, antibiotics from urban and livestock sources persist over time in soil and aquatic environments. As a consequence, the structure of bacterial communities could be affected, increasing the occurrence of resistant bacteria in the environment (Martinez 2009).

Some studies have demonstrated the presence of biologically active antibiotic residues in animal and human waste, such as sewage and manure (Kummerer 2009). In the case of wild birds, most of the studies have associated the influence of human activities such as farming, presence of dumpsites, or even tourism with the detection of antibiotic resistant bacteria (Allen, Donato et al. 2010). Also, several reports have suggested the importance of wildlife in the dissemination of CR *E. coli* (Pinto, Radhouani et al. 2010, Veldman, van Tulden et al. 2013, Baez, Hernandez-Garcia et al. 2015). Since wild birds in their natural environment are not treated with antibiotics (Santos, Silva et al. 2013), they are potential sentinels of multidrug resistant bacteria discharged into the environment.

The role of wild birds as disseminators of antibiotic-resistant bacteria between distant ecosystems is difficult to estimate (Baez, Hernandez-Garcia et al. 2015). White storks (*Ciconia ciconia*), like other free-living birds, can become long-distance vectors of CR *E. coli*. They feed nearby pastures and ploughed fields, marshy wetlands, rice fields and more recently on landfills, and due to their mobility, they can effectively acquire and spread disease and resistant traits (Szczepanska, Kaminski et al. 2015) through faecal contamination of pastures and surface waters, becoming a potential source of CR *E. coli* for humans and farm animals (Tryjanowski, Sparks et al. 2006, Keller, Shriver et al. 2011). White storks may breed in open farmland with access to marshy wetlands but also very often they cohabit with humans, making use of man-made facilities, such as roofs of buildings, telephone or electric power line poles or other constructions for their nests. If such individuals acquire CR *E. coli* during feeding, they can act as potential reservoirs of resistant bacteria.

There have been several studies reporting detection of CR *E. coli* in wild birds (Veldman, van Tulden et al. 2013, Zurfluh, Nuesch-Inderbinen et al. 2013, Baez, Hernandez-Garcia et al. 2015) and Alcalá and colleagues (Alcalá, Alonso et al. 2016) described for the first time the presence of CR *E. coli* in a white stork in Spain. However, to our knowledge this is the first work that studies the carriage of CR *E. coli* in white storks in detail in a number of colonies subjected to different degrees of anthropic pressure with the aim of determining the epidemiology of CR *E. coli* in this species and their role as potential spreaders of CR *E. coli.* For this purpose, the isolates have been extensively characterized by antimicrobial susceptibility testing, identification of $bla_{ESBL/AmpC}$ genes and plasmids involved in the

transmission, determination of phylogenetic groups, genomic relationship among the isolates and detection of virulence genes associated to avian pathogenic *E. coli* (APEC).

Experimental procedures

Ethical statement

Sampling of white storks was in all cases associated to ringing or radio-tagging activities. None of the storks was specifically captured or handled for the purpose of this study. All ringing and radio-tagging activities were carried out under the pertinent permits from the local authorities (regional governments of Castilla – La Mancha, Madrid, Extremadura, Castilla y Leon and Andalucía) and by registered specialized personnel. Handling and sampling of the storks was carried out following all applicable international, national, and/or institutional guidelines for the care and ethical use of animals.

Study design

Our study was based on the analysis of cloacal swabs (n=467) as well as recently deposited droppings collected at the base of nesting platforms (n=70). The study was designed to take specific aspects of the feeding and spatial ecology of white storks into account. During the reproductive season (approximately February-July) white storks are spatially bound to their colony/nest. Foraging occurs in a spatially defined area and can be linked fairly clearly to a specific type of habitat/food source, allowing for the comparison of the effect of feeding on natural and human origin (waste) resources (Alonso, Alonso et al. 1991, Tortosa, Caballero et al. 2002). In contrast, during the migration and wintering periods the spatial distribution of foraging

increases, as well as the intensity of use of landfills as predictable food source, making association of storks to a specific habitat impossible (Ciach and Kruszyk 2010). The largest part of the swab samples, one per bird (n=441) was collected from chicks at ringing in the nests (nestlings). This allows for the collection of a considerable number of samples without the need of stressful, costly and time-consuming capture. In addition, swab samples were obtained from 26 breeding adults captured for satellite/gps data logger transmitter fitting, in the nest (the presence of small chicks at their nest was certified), meaning that the samples were obtained in a period were foraging was also limited to a defined type of habitat. For comparison and with view to cost-effective, non-invasive sampling recently deposited droppings of adult birds (n=70) were collected below the nesting platforms in three colonies during the reproductive period.

Study area and period

The study was designed to cover most of the area of natural distribution of white storks in Spain and thus includes swab samples obtained from individuals belonging to twelve different colonies from six different regions (n=467). Sampling occurred more intensively and in consecutive years in colonies situated in south-central Spain. Hence, nestlings from six colonies in south-central Spain were sampled in 2013 and 2014, samples from nestlings of four colonies were obtained in 2013 and samples from nestlings from five different colonies were collected in 2014 (Figure and Table 1).

In March 2011 (just prior to egg-laying) fresh faecal samples were collected below nesting platforms in three colonies in central Spain (n=70), thus presumably belonging to the adults breeding on this

particular platform. Swab samples from adult and nestling white storks were collected during the breeding period (May to July) in 2013 (n=226) and 2014 (n=241).

The adult storks that were sampled during this study were fitted with satellite transmitter or gps/gsm data logger tags in the context of studies on the spatial ecology and migratory behaviour of white storks. Distinctive analysis of the spatial and migratory data is the objective of other publications. However the collected data showed very different migratory patterns, and for most individuals an increase in the exploitation of landfills as food source during the migratory and wintering period, regardless of their behaviour during the breeding period (De la Puente, Höfle et al. 2014).

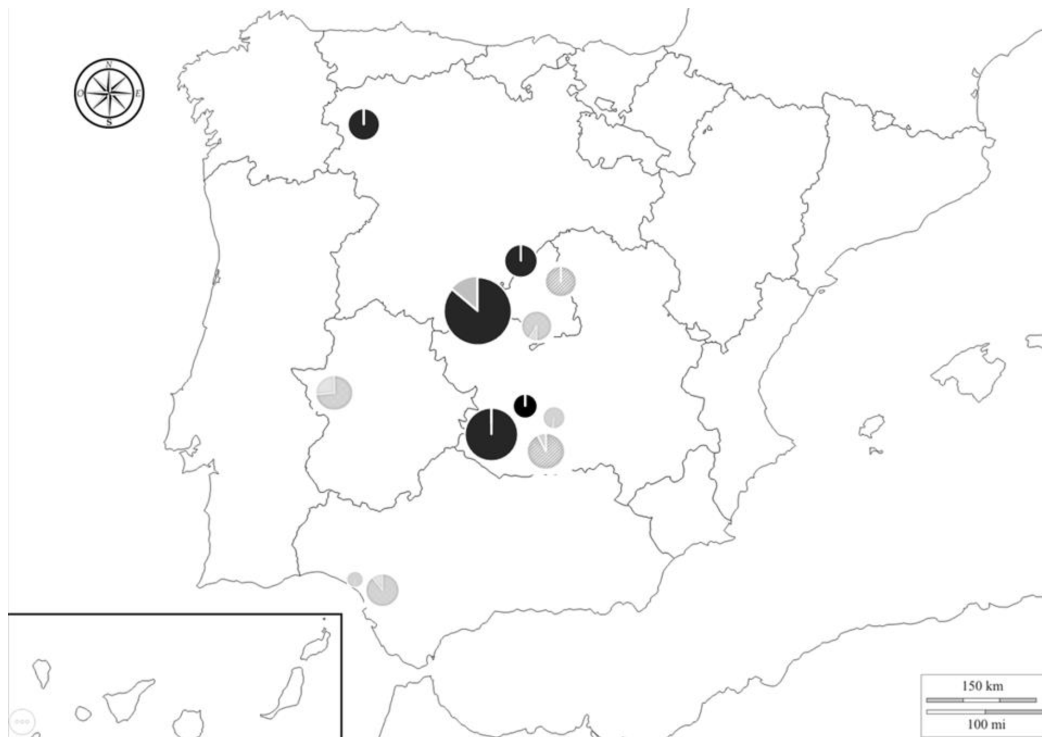


Figure 1. Distribution of studied colonies in Spain. Dark grey circles represent colonies from natural habitat colonies and light grey circles colonies in which adults forage on landfills. The size of the circles reflects sample size and the lighter shaded areas CR *E. coli* prevalence.

Table 1. Location, characteristics, number of samples and presence of CR *E. coli* obtained from the different white stork colonies sampled.

Location	Province	Landfill use	Year	Age	Number of samples	CR *E. coli*
Abenójar	Ciudad Real	No	2013	Nestling	28	0 (0%)
				Adult	2	0 (0%)
			2014	Nestling	29	0 (0%)
Almodóvar	Ciudad Real	Yes	2013	Nestling	30	2 (6.7%)
				Adult	2	0 (0%)
			2014	Nestling	30	3 (10%)
Alcázar de San Juan	Ciudad Real	Yes	2013	Nestling	8	0 (0%)
				Adult	3	0 (0%)
			2014	Nestling	13	1 (7.7%)
Cabañeros	Ciudad Real	No	2013	Nestling	15	0 (0%)
				Adult	2	0 (0%)
			2014	Nestling	19	0 (0%)
Alcalá de Henares	Madrid	Yes	2014	Nestling	14	0 (0%)
Pinilla del Valle	Madrid	No	2013	Nestling	31	0 (0%)
				Adult	2	0 (0%)
			2014	Nestling	29	0 (0%)
Prado Herrero	Madrid	No	2013	Nestling	30	6 (20%)
			2014	Nestling	57	8 (14%)
Rivas	Madrid	Yes	2014	Nestling	20	2 (10%)
Malpartida	Cáceres	Yes	2014	Nestling	43	16 (37.2%)
				Adult	1	0 (0%)
Doñana, Dehesa de Abajo	Sevilla	Yes	2013	Nestling	18	1 (5.6%)
				Adult	7	2 (28.6%)
Doñana, Matasgordas	Huelva	Yes	2014	Adult	6	0 (0%)
León	León	No	2013	Nestling	28	0 (0%)
				Adult	1	0 (0%)

Sampling

Fresh faecal samples (n=70) were collected below nesting platforms using sterile cotton tip swabs and small zip-lock bags. The samples were kept at 4 °C until arrival at the laboratory and divided into a subsample for storage at -80 °C and a sample for immediate processing.

In total 467 cloacal swabs were collected at ringing (nestlings) or after capture (adults) in the colony (n=467). Tarsus length and body weight were recorded in all white storks. Cloacal samples were obtained using sterile cotton swabs in AMIES transport medium (Deltalab, Barcelona, Spain). They were kept at 4 °C until arrival to the laboratory, where they were processed in less than 12 hours after sampling. Samples were plated onto plain MacConkey agar (Oxoid, Basingstoke, United Kingdom) or MacConkey agar supplemented with 4 µg/ml cefotaxime (Sigma-Aldrich Chemical, Madrid, Spain), and incubated overnight at 37 °C. Lactose positive colonies morphologically compatible with *E. coli* were considered indicative of growth above breakpoint concentrations of cefotaxime. From these plates, three lactose-positive colonies were stored at -80 °C in 30% BHI glycerol. Subsequently, one representative sample was selected for further studies and confirmed as *E. coli* by PCR (Heininger, Binder et al. 1999).

Phylogeny and pulsed-field gel electrophoresis (PFGE)

The strains were classified in phylogenetic groups (A, B1, B2, C, D, E or F) by PCR, according to a method previously described by Clermont et al (Clermont, Bonacorsi et al. 2000, Clermont, Christenson et al. 2013).

PFGE was performed to determine the genomic relatedness among the isolates. The experiments were carried out as described in the PulseNet protocol (Ribot, Fair et al. 2006). *Salmonella enterica* serovar Branderup H9812 was used as a size marker. The results were analysed by Fingerprinting II Informatix software (Applied Maths, Sint-Martens-Latem, Belgium). The strains were considered to have a different PFGE-type when changes in at least one band were detected after digestion with XbaI. The analysis of the bands generated by the program was carried out using the Dice coefficient and unweighted-pair group method with arithmetic averages (optimization of 1.25% and position tolerance of 1.25%).

Detection of virulence-associated genes

All 46 isolates were tested by multiplex PCR for the genes described previously by Johnson *et al* (Johnson, Wannemuehler et al. 2008) as the minimal predictors of APEC virulence; *iroN*, *ompT*, *hlyF*, *iutA* and *iss*.

Antimicrobial susceptibility testing

Disc diffusion tests were performed according to CLSI guidelines, using the following discs (Oxoid, Basingstoke, UK): cefoxitin, 30 mg; cefepime, 30 mg; ceftazidime, 30 mg; cefotaxime, 30 mg; cefotaxime+clavulanic acid, 30+10 mg; and ceftazidime+clavulanic acid, 30+10 mg. The synergies between cefotaxime and cefotaxime/clavulanic acid, ceftazidime and ceftazidime/clavulanic acid were used for the identification of ESBL production; cefoxitin was used for the detection of AmpC-type beta-lactamase (CLSI, 2008). Moreover, all strains were susceptibility tested using a minimum

inhibitory concentration (MIC) based broth microdilution (VetMIC GN-mo, National Veterinary Institute, Uppsala, Sweden) as described before (Solà-Ginés, Gonzalez-Lopez et al. 2015). Isolates were considered to be wild type (WT) or non-WT based on epidemiological cut-off values defined by EUCAST (http://www.eucast.org/).

Resistance genes

All isolates were tested by PCR methods for the presence of the *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{CMY-1}$ and *bla*$_{CMY-2}$ genes as previously described by Hasman *et al.* (Hasman, Mevius et al. 2005). The isolates showing resistance to colistin were also tested for the presence of *mcr-1* gene (Liu, Wang et al. 2016). Sequencing of both strands of amplicons was performed.

Mating experiments and plasmid characterization

Filter mating experiments were carried out to assess the capacity of the plasmids to conjugate. For the analysis, 42 strains were selected. They comprised representative isolates from three PFGE clusters and 39 PFGE types. Mating assays were performed as previously described (Bielak, Bergenholtz et al. 2011), using the isolates as donors and rifampicin-resistant *E. coli* HB101 as a recipient. Transconjugants were selected on LB agar plates supplemented with rifampicin (150 mg/L) and ceftriaxone (1 mg/L). The identity of the transconjugants was confirmed by PFGE.

Plasmid DNA was purified from the subset of 42 isolates using a Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Plasmids were introduced to electrocompetent plasmid free *E. coli* BL21 cells by electroporation.

Transformants were selected in LB agar plates containing ceftriaxone (1 mg/L), and PCR was performed for the confirmation of ESBL resistance genes. The presence of a unique plasmid in the transformants and their corresponding sizes were analysed using S1 nuclease digestion followed by PFGE (S1-PFGE) (Barton, Harding et al. 1995). Additionally, plasmids were classified by PCR-based replicon typing (Carattoli, Bertini et al. 2005).

Statistical analysis

For statistical purposes we considered nestlings with any degree of exposure to food foraged at landfills as "landfill diet" and nestlings fed on diets foraged in natural habitat "natural diet". We calculated the body condition of white stork nestlings and adults sampled in the field according to the scaled mass index proposed by Peig and Green (Peig and Green 2009). This index can be computed as: $M = M_i\ (L_0/L_i)^{bsma}$, where M_i and L_i are the body mass and the structural size measurement (tarsus length) of each individual respectively; bsma is the scaling exponent estimated by the standardised major axis (SMA) regression of M on L,$L_{0''}$ is the arithmetic mean value for the study population; M is the predicted body mass for individual i when the structural size body measure is standardized to L_0. The scaling exponent "bsma" has been calculated indirectly by dividing the slope from an ordinary least squares (OLS) regression (bols) by the Pearson's correlation coefficient r. Normal distribution of the continuous variable body condition index M was confirmed using the Kolmogórov-Smirnov and Shapiro-Wilks test prior to further analysis. Haematocrit and total plasma solids did not follow normal distribution even after logarithmic transformation. We compared body condition

between CR. *E. coli* infected and uninfected white stork nestlings, APEC infected and uninfected nestlings and nestlings fed natural or landfill derived diets from field populations using student's t-test. We employed Generalized linear mixed models (GLMM) with a binary response and a logit regression and the nest of origin within the colony as random factors, to determine the effect of sampling year, sex, number of siblings in the nest and the association to natural or landfill habitat on the prevalence of commensal CR *E. coli* and APEC respectively in white stork nestlings. All analysis were carried out using SPSS statistical software, version 22.0 (IBM®, SPSS Inc., Chicago, USA).

Results

Prevalence of CR *E. coli* in white stork samples

Overall CR *E. coli* prevalence in swab samples was 8.8% (41 out of 467). Only nine of the 34 phenotypically cefotaxime resistant *E. coli* strains from fresh faecal material (n=70) could be studied further and five of these were identified as CR *E. coli*. For this reason, statistical analysis concerning CR *E. coli* was only carried out on results from swab samples.

Phylogeny and PFGE

The phylotyping analysis identified six different phylogenetic groups among the 46 isolates (Figure 2). Of them, 14 belonged to phylogroup B1 (30%), 12 to group A (26%), eight to group E (17%), four to group B2 (9%), four to group F (9%) and one to group C (2%). No phylogroup could be determined for three of the isolates (7%).

XbaI-PFGE analysis revealed a high degree of genetic polymorphism (Figure 2). Among the 46 CR *E. coli* isolates, 42 different macro-restriction profiles were identified. During 2013, three epidemiologically related isolates were obtained from two different colonies located within a radius of about 250 km, in Madrid and Cáceres.

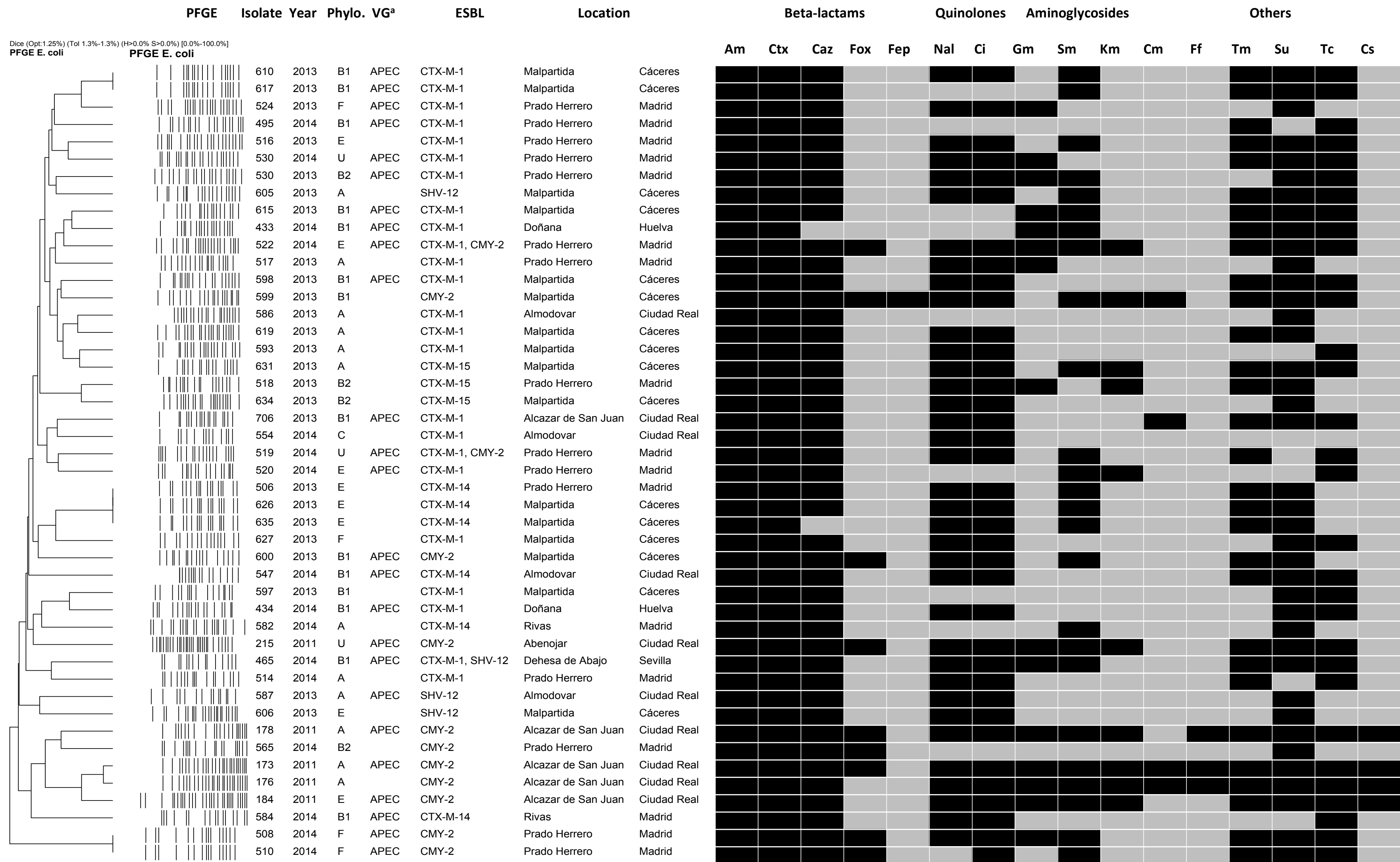

PFGE Isolate Year Phylo. VGa ESBL Location
Beta-lactams Quinolones Aminoglycosides Others
Dice (Opt:1.25%) (Tol 1.3%-1.3%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE E. coli
PFGE E. coli
Am Ctx Caz Fox Fep Nal Ci Gm Sm Km Cm Ff Tm Su Tc Cs
610 2013 B1 APEC CTX-M-1 Malpartida Cáceres
617 2013 B1 APEC CTX-M-1 Malpartida Cáceres
524 2013 F APEC CTX-M-1 Prado Herrero Madrid
495 2014 B1 APEC CTX-M-1 Prado Herrero Madrid
516 2013 E CTX-M-1 Prado Herrero Madrid
530 2014 U APEC CTX-M-1 Prado Herrero Madrid
530 2013 B2 APEC CTX-M-1 Prado Herrero Madrid
605 2013 A SHV-12 Malpartida Cáceres
615 2013 B1 APEC CTX-M-1 Malpartida Cáceres
433 2014 B1 APEC CTX-M-1 Doñana Huelva
522 2014 E APEC CTX-M-1, CMY-2 Prado Herrero Madrid
517 2013 A CTX-M-1 Prado Herrero Madrid
598 2013 B1 APEC CTX-M-1 Malpartida Cáceres
599 2013 B1 CMY-2 Malpartida Cáceres
586 2013 A CTX-M-1 Almodovar Ciudad Real
619 2013 A CTX-M-1 Malpartida Cáceres
593 2013 A CTX-M-1 Malpartida Cáceres
631 2013 A CTX-M-15 Malpartida Cáceres
518 2013 B2 CTX-M-15 Prado Herrero Madrid
634 2013 B2 CTX-M-15 Malpartida Cáceres
706 2013 B1 APEC CTX-M-1 Alcazar de San Juan Ciudad Real
554 2014 C CTX-M-1 Almodovar Ciudad Real
519 2014 U APEC CTX-M-1, CMY-2 Prado Herrero Madrid
520 2014 E APEC CTX-M-1 Prado Herrero Madrid
506 2013 E CTX-M-14 Prado Herrero Madrid
626 2013 E CTX-M-14 Malpartida Cáceres
635 2013 E CTX-M-14 Malpartida Cáceres
627 2013 F CTX-M-1 Malpartida Cáceres
600 2013 B1 APEC CMY-2 Malpartida Cáceres
547 2014 B1 APEC CTX-M-14 Almodovar Ciudad Real
597 2013 B1 CTX-M-1 Malpartida Cáceres
434 2014 B1 APEC CTX-M-1 Doñana Huelva
582 2014 A CTX-M-14 Rivas Madrid
215 2011 U APEC CMY-2 Abenojar Ciudad Real
465 2014 B1 APEC CTX-M-1, SHV-12 Dehesa de Abajo Sevilla
514 2014 A CTX-M-1 Prado Herrero Madrid
587 2013 A APEC SHV-12 Almodovar Ciudad Real
606 2013 E SHV-12 Malpartida Cáceres
178 2011 A APEC CMY-2 Alcazar de San Juan Ciudad Real
565 2014 B2 CMY-2 Prado Herrero Madrid
173 2011 A APEC CMY-2 Alcazar de San Juan Ciudad Real
176 2011 A CMY-2 Alcazar de San Juan Ciudad Real
184 2011 E APEC CMY-2 Alcazar de San Juan Ciudad Real
584 2014 B1 APEC CTX-M-14 Rivas Madrid
508 2014 F APEC CMY-2 Prado Herrero Madrid
510 2014 F APEC CMY-2 Prado Herrero Madrid

Figure 2. PFGE dendogram showing the phenotypic and genotypic relationships of the strains, phylogenies, virulence-associated genes and cephalosporin and colistin resistance genes. Phylo., phylogroup; VG, virulence-associated genes; Am: Ampicillin (WT≤8mg/L); Ctx: Cefotaxime (WT≤0.25mg/L); Caz: Ceftazidime (WT≤0.5mg/L); Fox: Cefoxitin (WT≤8mg/L); Fep: Cefepime (WT≤0.125mg/L); Nal: Nalidixic acid (WT≤16mg/L); Ci: Ciprofloxacin (WT≤0.064mg/L); Gm: Gentamicin (WT≤2mg/L); Sm: Streptomycin (WT≤16mg/L); Km: Kanamycin (WT≤8mg/L);. Cm: Chloramphenicol (WT≤16mg/L); Ff: Florfenicol (WT≤16mg/L); Tm: Trimethoprim (WT≤2mg/L); Su: Sulphamethoxazole (WT≤64mg/L); Tc: Tetracycline (WT≤8mg/L); Cs: Colistin (WT≤2mg/L).

Detection of virulence genes associated to APEC

Out of 46 isolates, 70%, 70%, 70%, 57% and 52% yielded amplicons for *ompT*, *hlyF*, *iutA*, *iss*, and *iroN*, respectively. Accordingly, 25 isolates (54%) were considered APEC, since they had from four to five of the genes mentioned above (Figure 2).

Antimicrobial susceptibility testing and resistance genes

Disc diffusion experiments confirmed that all isolates presented extended spectrum betalactamase (ESBL) or AmpC phenotypes. All strains were multi-resistant (resistant to more than three antimicrobial families). Furthermore, 54% were resistant to more than seven antimicrobials. MIC values determined that all isolates exhibited a non-wild type (WT) phenotype for cephalosporins (100% resistance to cefotaxime and 96% to ceftazidime), with 21 strains yielding amplicons for $bla_{\text{CTX-M-1}}$, ten $bla_{\text{CMY-2}}$, six $bla_{\text{CTX-M-14}}$, three $bla_{\text{SHV-12}}$, three $bla_{\text{CTX-M-15}}$, two $bla_{\text{CTX-M-1}}$ together with $bla_{\text{CMY-2}}$, and one $bla_{\text{CTX-M-1}}$ together with $bla_{\text{SHV-12}}$.

In addition, four isolates from Alcazar de San Juan (9%) presented a non-WT phenotype to colistin. The presence of *mcr-1* gene was detected in three of these isolates (id. 173, 176 and 178). Additionally, 100% of the isolates presented a non-WT phenotype to ampicillin, 85% to sulfamethoxazole, 80% to ciprofloxacin, 78% to nalidixic acid, 67% to tetracycline, 65% to trimethoprim, 54% to streptomycin, 33% to gentamicin, 22% to kanamycin, 9% to chloramphenicol and 7% to florfenicol (Figure 2).

Conjugation and transformation experiments

From the subset of 42 isolates, 15 were able to transfer the CR genes by conjugation. Additionally, 33 transferred the CR genes to the BL21 electrocompetent strain. S1-nuclease PFGE confirmed the presence of a unique plasmid in all selected isolates except in two of them (518 and 598), which contained more than one plasmid. Sizes of plasmids varied between 40 kb and 150 kb approximately (Table 2). PCR-based replicon typing showed that IncI1 was the most common replicon associated to the CR genes, present in 25 of the isolates, followed by IncFIB and IncN. The replicons IncFIA, IncFIC, IncA/C, IncK and IncL/M were also detected (Table 2). Four isolates presented between two and five replicons on the same plasmid (517, 631, 176 and 184), and none of the tested replicons were detected in seven of the transformants or conjugants (434, 514, 554, 508, 173, 626 and 634).

Table 2. Identification and characterization of the plasmid locations of *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{SHV-12}}$, and *bla*$_{\text{CTX-M-15}}$ among 42 CR *E. coli* isolates. Transconjugants (TC) and transformants (TF) used for PCR-based replicon typing method are in boldface.

Gene and isolate	Conjugation result	Transformation result	Inc type(s)								Plasmid size (kb)
			I1	FIB	N	K	FIA	FIC	A/C	L/M	
bla$_{\text{CTX-M-1}}$											
516	**TC1b**		+								100
517		**TF2a**		+				+	+		130
524	**TC3a**		+								90
530,13		**TF4a**	+								60
586	**TC5a**	TF5a	+								100
593		**TF6a**	+								100
597	TC7a	**TF7a**	+								100
606		**TF8b**	+								100
610		**TF9a**	+								100
615		**TF10b**	+								90
619		**TF11a**	+								100
627		**TF12a**	+								100
631		**TF13a**		+			+				150
706		**TF14a**	+								100
433		**TF15a**	+								110
434	**TC16b**	TF16a									110
495	TC17a	**TF17a**		+							50
514		**TF18a**									60
520		**TF19a**	+								100
530,14		**TF20a**			+						40
554	TC21a	**TF21a**									50
bla$_{\text{CMY-2}}$											
599		**TF22a**							+		120
508		**TF23a**									100
565	**TC24a**	TF24a	+								80
173		**TF25a**									90
176		**TF26a**	+	+	+			+		+	90
178		**TF27a**	+								90
184	**TC28b**		+		+	+					40
215		**TF29b**			+						40
bla$_{\text{CTX-M-14}}$											
626		**TF30a**									110
547		**TF31a**	+								110
582	TC32a	**TF32a**				+					80
584	TC33a	**TF33a**	+								90
bla$_{\text{SHV-12}}$											
587	**TC34a**		+								90
600	**TC35c**		+								90
605		**TF36a**	+								100
bla$_{\text{CTX-M-1}}$ + bla$_{\text{CMY-2}}$											
519		**TF37a**	+								100
522		**TF38a**	+								110
bla$_{\text{CTX-M-15}}$											
634	**TC39b**										60
bla$_{\text{CTX-M-1}}$ + bla$_{\text{SHV-12}}$											
465	**TC40a**			+							110

Risk factors for the acquisition of CR *E.coli* by white storks

CR *E.coli* prevalence did not vary significantly between nestling (8.8%, 39 out of 441) and adult storks (7.7%, 2 out of 26). Sex, sampling year and number of siblings in the nest did not affect CR *E. coli* carriage in white stork nestlings, but white stork nestlings that could be associated to the use of human waste from landfills as food source were significantly more likely to carry CR *E. coli* (14.3%, 25 out of 175, GLMM F=9.235, d.f.=1, p=0.003), than white stork nestlings with no known contact to food from landfills (5.3%, 14 out of 266) during the sampling period (Figure and Table 1). Carriage of CR *E. coli* or APEC had no significant effect on body condition, haematocrit or plasma proteins of white stork nestlings. However, white stork nestlings from colonies associated to foraging at landfills were in significantly better body condition than nestlings from colonies with foraging in natural habitats (t (359)= -2.22, p = 0.027).

Discussion

White storks are free-living colonial birds frequently associated to humanized habitats. In the recent past, they have adapted to the exploitation of human origin residues in landfills and introduced American crayfish (*Procambarus clarkii*) in rice fields and small water bodies as predictable food source during winter. This has led to a significant modification of migratory behaviour of a large part of the adult stork population of Western Europe as well as, in the case of landfills, to the exposure to numerous contaminants and pathogens (Flack, Fiedler et al. 2016, Gilbert, Correia et al. 2016).

As storks cohabit in the proximities of human and food producing animals, they are able to acquire bacteria from these and

transport them to other locations in their annual or regular movements (Tryjanowski, Sparks et al. 2006). The present study takes advantage of the fact that even though during winter most storks forage on human waste, during breeding the nesting site and colony determine the foraging ecology and allow for the characterization of risk factors for the exposure of white storks to CR *E. coli* (Alonso, Alonso et al. 1991, Tortosa, Caballero et al. 2002). We found that stork nestlings fed landfill residues by their parents were significantly more likely to carry CR *E. coli* than nestlings fed natural diets. Also, CR *E. coli* carriage did not affect body condition in infected nestlings. Hence, using data from white stork nestlings the present study has demonstrated the presence of CR *E. coli* in white storks feeding on human residues and their capacity to disseminate and transport the resistant bacteria. In particular, the same clones with the same resistant genes have been detected in nestlings from colonies from Madrid and Caceres, located within 250 km distance. These results suggest a common source of contamination, together with the capacity of the birds to spread the same clones during their movements. According to our results, extensive livestock farming in natural habitats (present in the proximity of at least five of six natural habitat colonies) appears to be less of a risk factor for the acquisition of CR *E. coli* by white storks, except for one farm that raises cattle and where storks and cattle share the pastures (Prado Herrero, Table 1). In fact the latter was the only natural feeding habitat in which CR *E. coli* was detected. Storks frequently collect cattle dung and place it in the nest just prior to, and a few weeks after hatching, presumably to help chicks to maintain body temperature (Tortosa and Villafuerte 1999). This behaviour as well as foraging of the adults on contaminated pastures

(especially on dung beetles) is a likely route of exposure of the nestlings to CR *E. coli* excreted by cattle. Moreover, CTX-M-1 is the most common ESBL gene found in this stork colony and it is also frequently described in cattle in Europe (Schmid, Hormansdorfer et al. 2013).

Some studies suggest that wildlife play also an important role in the global dissemination of antimicrobial resistance genes, transferring mobile genetic elements between bacterial communities (Allen, Donato et al. 2010). In our study, the presence of multi-resistant *E. coli* demonstrated the potential capacity of white stork to disseminate and transport ESBL genes located in mobilizable plasmids. These isolates were all resistant to third generation cephalosporins and most of them were also resistant to fluoroquinolones. These two families of antimicrobials are nominated of critical importance in human health since they are used in hospitals for the treatment of enteric infections (Liebana, Carattoli et al. 2013). In addition, three of the isolates, obtained in 2011 from recently deposited droppings of three different animals of the same colony harboured CMY-2 together with *mcr-1*, conferring to these isolates resistance to almost all last-line antimicrobials available, including cephalosporins and colistin. This also demonstrates that these plasmids have been circulating in wildlife for many years. The fact that a large proportion of the European white stork population is migratory may facilitate the spread of the *mcr*-1 gene between different continents and emphasises the importance of studies monitoring wildlife as potential reservoirs of resistant genes with an impact in public health along their migration routes. Also, as especially juvenile storks with different geographical origins may share the same areas during wintering in Africa this could potentially

lead to cross contamination between storks from different regions (Flack, Fiedler et al. 2016).

Several studies have suggested a relationship between the different *E. coli* phylogroups and the virulence of the isolates (Picard, Garcia et al. 1999, Clermont, Olier et al. 2011). Accordingly, non-virulent isolates mainly belong to A, B1 and C phylogenetic groups, whereas the most virulent phylogroups are associated to B2 followed by D (Russo and Johnson 2000). In this study, most of the isolates belonged to phylogroups A and B1, and only four isolates were B2. On the contrary, analysing phylogroups and virulence genes in this strain collection, we have observed that almost all B1 strains had the APEC virulence-associated genes, whereas only one B2 strain had the APEC genes. This may be due to the presence of virulent factors associated to mobile genetic elements such is the case of the previously described ColV plasmid (Johnson and Nolan 2009), which contained several virulence genes within the same plasmid (*hlyF*, *ompT*, *iss* and *cvaC*). So, an acquisition of such type of plasmid may confer B1 isolates with more virulence factors than expected. Presence of APEC virulence-associated genes carrying *E. coli* strains in the cloaca of white stork nestlings was apparently not related to landfill origin of food or any of the other factors, neither did it affect body condition.

The present study showed a high variability of CR genes detected among the isolates, being the most prevalent $bla_{\text{CTX-M-1}}$ (45%), followed by $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-14}}$ (22% and 13% respectively). These $bla_{\text{CTX-M}}$ types are the most frequent variants in ESBL producers in animals and food of animal origin, while $bla_{\text{CTX-M-15}}$ has been mostly described in human medicine (Valverde, Turrientes et al. 2015) and occasionally on farms (Cameron-Veas, Sola-Gines et al. 2015). This study has

demonstrated the presence of CTX-M-15 gene in three isolates of white stork harboured in plasmids of different sizes and containing IncFIB replicon, the most common incompatibility group for CTX-M-15 isolated from humans in Spain (Carattoli 2009). They were obtained from two different colonies, one of which is closely associated to a village with part of the nests on urban structures, and confirmed consumption of human residues. The other colony is in a natural environment with no confirmed consumption of human residues during the breeding season, nevertheless this colony is situated 50 km flight distance from Madrid, and thus exposure to human residues cannot be excluded completely. In fact satellite transmitter data from an adult stork from this colony shows extensive use of landfills outside the breeding period (De la Puente, Höfle et al. 2014).

Significant differences were found between location of the colonies and presence of CR genes. For instance, in the colony located in Cáceres, the most prevalent gene was CTX-M-1 associated to 90 and 100 kb plasmids, whereas in the colony located in Alcázar de San Juan (Ciudad Real) the most prevalent was CMY-2 harboured in two types of plasmids (40 and 90 kb). The former was also the colony in which nestlings carried the greatest variety of CR genes (5) as compared to the other colonies.

The most common replicons found in the study were IncI1 (60%), followed by IncFIB (12%) and IncN (10%). To date, these three replicons are the most commonly found in plasmids carrying CR genes in *E. coli* isolated from poultry (Wang, Stephan et al. 2013, Solà-Ginés, Cameron-Veas et al. 2015). Almost all of the CR genes containing IncI1 replicons were located in plasmids of the same sizes, between 90 and 110 kb; specifically 100 kb in the case of $bla_{\text{CTX-M-1}}$. However, in the case

of two isolates, isolation of the plasmid carrying the CR gene could not be achieved. During transformation and conjugation experiments co-transfer of plasmids of different molecular weights was observed. The entry of two or more plasmids into a single transformation procedure is not uncommon; Weston *et al* (1979) demonstrated that 20% of the transformants could be double transformants (Weston, Humphreys et al. 1979). This type of transformation can be frequent if it is performed with high amounts of plasmid DNA (Goldsmith, Kiss et al. 2007).

In conclusion, results from this study confirm that by exposure of white storks to human residues they can acquire CR genes. As all individuals included in this study are free-living birds, they have not been treated with antimicrobials. Since they are not subjected to any direct antibiotic pressure and they cohabit in the proximities of humans, the presence of these resistant traits is a reflection of the anthropic pressure of human activities and particularly exposure to human residues. In fact outside the breeding season adult and juvenile storks have been observed to use landfill indistinctive of the habitat of the colony of origin (De la Puente, Höfle et al. 2014). The distinctive pattern of prevalence of CR genes in white stork nestlings in association to foraging behaviour of their parents during the breeding season, in addition to recent findings that show that a large proportion of storks are exposed to human residues during wintering, suggests that persistence of CR genes in bacteria of the digestive tract of white storks is relatively short. This makes white storks excellent sentinels for local antibiotic pressure in their foraging habitat. Nevertheless, due to their great mobility and migratory habits, the white stork can also act as carriers of CR *E. coli*, contributing to the global dissemination of antimicrobial resistance genes and plasmids.

Discussion

Es indiscutible la enorme importancia y el valor de los antimicrobianos, ya que el éxito de tratamientos para diferentes infecciones y durante procesos quirúrgicos depende totalmente de ellos. Actualmente, los mecanismos de resistencia a estos antimicrobianos son pandémicos y esto es un punto muy importante para el cuidado de la salud en el mundo entero; donde acciones estrictas ya se están poniendo en marcha para solventar este grave problema. Afortunadamente, no todos los patógenos son resistentes, y por lo tanto, en estos casos los antibióticos funcionan con éxito. Sin embargo, es absolutamente necesaria la búsqueda de nuevos agentes con propiedades antimicrobianas (Boucher, Talbot et al. 2009).

E. coli es un candidato ideal para la realización de estudios de vigilancia sobre la resistencia a los antimicrobianos, ya que es una bacteria comensal pero que también puede causar infecciones, y se encuentra en todos los ecosistemas posibles. Se considera una bacteria centinela de resistencias; por lo tanto, para conocer la diseminación global y sus mecanismos de resistencia, son necesarios estudios de tal manera que se pueda predecir su evolución y diseñar estratégias para su control.

En el ambiente de granjas avícolas hay una necesidad de evitar cualquier medicación profiláctica, a pesar de que actualmente sea prácticamente nula. La administración de antimicrobianos se puede cambiar por una buena higiene de las incubadoras y una buena gestión de la producción de las granjas de cría. Las cefalosporinas de tercera y cuarta generación no están registradas para su uso en avicultura; sin embargo, en aquellos sectores donde están registradas, deben usarse

prudentemente y únicamente en casos basados en pruebas de susceptibilidad, debido al gran riesgo que puede suponer su transmisión a los humanos (EU 2015). El estudio I corrobora que bacterias productoras de betalactamas de espectro extendido (BLEEs) se encuentran con cierta prevalencia en granjas de aves de corral debido a una transmisión vertical en los diferentes niveles de producción o a la coselección por el uso de otros antimicrobianos (Blaak, Hamidjaja et al. 2014). Dichas bacterias estan causando infecciones muy importantes en humanos y avicultura (APEC). El uso de antimicrobianos en aves de corral, conjuntamente con el incremento del consumo de su carne, han llevado a la diseminación y la persistencia de estas resistencias, lo cual puede llegar a ser un grave problema para la salud humana y animal. Debido a la gran variabilidad de estas cepas se deben realizar controles de vigilancia (Mellata 2013).

En España, desde el 2010 se han detectado en granjas de pollos de engorde cepas pandémicas y emergentes de *E. coli* O25b-B2-ST131 portadoras de CTX-M-9 (Cortes, Blanc et al. 2010, Mora, Herrera et al. 2010). Este mismo tipo de cepa patogénica, pero portadora de genes CTX-M-15, causa infecciones en humanos (Coque, Baquero et al. 2008). Sin embargo, en el estudio I y por primera vez para nuestro conocimiento, se detectó el grupo clonal O25b-B2-ST131 en pollos de engorde albergando el gen CMY-2 y con corresistencia a fluoroquinolonas, y además, esta cepa contenía 13 de los 29 genes asociados a virulencia testados. Este aislado pertenecía a un animal sano, lo que implica un posible potencial zoonótico en animales de producción que finalmente pueden causar infecciones en la población humana. Adicionalmente, varios trabajos realizados recientemente describen que este linaje de cepas ya se encuentra bien establecido en

la comunidad y que en su evolución va adquiriendo diferentes genes de resistencia y virulencia (Cagnacci, Gualco et al. 2008).

Los factores de virulencia en gran medida están correlacionados, un solo gen de virulencia no puede convertir una cepa commensal en patógena (Bonnet, Diarrassouba et al. 2009). Pero también es cierto que en nuestros estudios se identificaron un conjunto común de factores asociados a virulencia en las cepas APEC, el cual ya había sido descrito anteriormente (Johnson, Wannemuehler et al. 2008). Según Johnoson y colaboradores (2009) la mayoría de estos genes se localizan en el mismo plásmido llamado ColV (Johnson and Nolan 2009). Por otra parte, en los estudios I y II no se identificaron muchas cepas virulentas y según el análisis filogenético no pertenecen a los filogrupos mas patógenos como B2 y D (Russo and Johnson 2000). Pero hemos encontrado resultados similares en otros estudios, donde los filogrupos A, B1 y D contenian diversos VF en comparación con B2, lo que hace que estas cepas se puedan considerar potencialmente virulentas. Estos resultados sugieren que el número de factores de virulencia y el filogrupo son independientes (Johnson and Kuskowski 2000).

Las moscas, como representantes de los vectores mecánicos más importantes en el mundo, han demostrado ser un reflejo de la persistencia y del contenido de resistencias a antibióticos en el medio ambiente (Johnson and Stell 2000). En el caso del estudio II, las moscas antes de entrar a las naves de pollos contenían un elevado número de genes BLEEs y de VF, los cuales se pueden introducir en la granja y colonizar a los pollos. Las moscas no son solo buenas diseminadoras de bacterias resistentes, sino que también son unos enormes reservorios

en los que se dan las condiciones óptimas para la transferencia horizontal de genes (Petridis, Bagdasarian et al. 2006).

Además, las cigüeñas blancas del estudio III corroboran que los animales que se encuentran en un habitat salvaje son también diseminadores y reservorios de genes de resistencia a los antibióticos, así como un buen reflejo de la presión humana ejercida en sus hábitats. Como hemos demostrado, la prevaléncia de *E. coli* productoras de BLEEs en colonias de cigüeñas blancas cercanas a residuos humanos es mucho mayor que en las colonias residentes en parques naturales. Poder llegar a controlar la transmisión de resistencias en fauna silvestre es un punto muy complicado y desafiante, ya que mejorar la higiene o la restricción de movimientos es algo imposible de implementar en animales de vida libre (Arnold, Williams et al. 2016). La resistencia a los antimicrobianos en fauna silvestre ha de ser estudiada debido al potencial peligro que puede suponer para los humanos y la seguridad alimentaria, ya que se ha descrito que el 40% de las enfermedades emergentes en humanos se han originado en fauna silvestre (Jones, Patel et al. 2008). Por lo tanto, ya no es un problema clínico, es también un problema ecológico. En el tercer estudio, cepas clonales que contenían el mismo gen de resistencia a cefalosporinas fueron encontradas en dos colonias distintas de cigüeña blanca localizadas en los alrededores del centro de España, y separadas por una distáncia de 250 km. Estas colonias se encuentran situadas con diferentes grados de presión antrópica, anidando desde parques naturales hasta vertederos. Esta situación sugiere una capacidad asombrosa de diseminación de las cigüeñas en sus movimientos, independientemente de la presión selectiva ejercida por los humanos.

En el mundo globalizado en el que vivimos, humanos y animales estan viajando continuamente largas distancias, existiendo un flujo e intercambio importante de bacterias resistentes y sus genes entre diferentes ambientes (Canton 2009). Las BLEEs son un ejemplo espectacular de la evolución bacteriana, ya que un gen de resistencia que se encuentra en una bacteria en la naturaleza puede transmitirse a otras y esparcirse entre los animales, humanos y el entorno natural. En ambos estudios II y III encontramos que los genes BLEEs más comunes fueron los mismos; $bla_{CTX-M-1}$ seguido de $bla_{CTX-M-14}$ y bla_{CMY-2}; así como los replicones plasmídicos; IncI1 seguido de IncFIB y IncN. Se ha descrito que $bla_{CTX-M-1}$ es el gen BLEE con mayor prevalencia en la carne para el consumo humano, mientras que en humanos el gen más prevalente es $bla_{CTX-M-15}$ (Randall, Clouting et al. 2011). Los replicones plasmídicos más comunes en granjas de pollos de engorde son IncI1 y IncFIB (Wang, Stephan et al. 2013). En el estudio I, $bla_{CTX-M-14}$ fue el gen de BLEE más común, y IncFIB fue el replicón plasmídico más prevalente, presente en todos los aislados de cepas de *E. coli* productoras de BLEEs. Todos los aislados contenían los genes más comunmente descritos en granjas de pollos de engorde, como CTX-M-14, CTX-M-1, CMY-2 y SHV-12 (Ewers, Bethe et al. 2012). Los plásmidos de los grupos de incompatibilidad IncI y IncF se encuentran normalmente en *E. coli* de origen humano y animal, y están asociados a los genes BLEEs (Carattoli 2009). En particular, la família IncI está asociada con la resistencia a BLEE de los tipos CTX-M y CMY, mientrás que los replicones IncF (IncFIA, IncFIB y IncFIC) juegan un papel importante en la diseminación de las resistencias en *Enterobacteriacae*, y han sido relacionados con genes que confieren

resistencias a betalactamasas, quinolonas y aminoglicósidos (Hopkins, Liebana et al. 2006, Marcade, Deschamps et al. 2009).

En los tres estudios incluidos en esta tesis, todas las cepas que eran resistentes a cefalosporinas fueron multiresistentes. Los resultados del estudio I indicaron que incluso los pollos de engorde para el consumo humano son un reservorio potencial de bacteria multiresistente, siendo un riesgo importante si estas cepas entran en la cadena alimentaria. Estos aislados que contienen genes BLEEs son más propensos a presentar coresistencia, debido a que se ha demostrado que los genes que confieren resistencia para diferentes antimicrobianos se pueden transmitir a otras bacterias conjuntamente a través de elementos genéticos móviles, como es el caso de fluoroquinolonas y colistina, encontrados en los estudios realizados (Robicsek, Jacoby et al. 2006). En los estudios I y II detectamos genes *qnr* que se asocian a fluoroquinolonas, y en el estudio III se encontró *mcr-1*, el cual se asocia a resistencia a colistina. En ambos casos estos genes se asocian a plásmidos, resistencia a quinolonas mediada por plásmido (PMQR) y resistencia a colistina mediada por plásmido respectivamente (Stokes and Gillings 2011, Liu, Wang et al. 2016). Está también descrito que los genes asociados a virulencia se encuentran en los mismos plásmidos que los genes de resistencia a antimicrobianos (Johnson and Nolan 2009).

En el estudio I describimos que dos réplicas del mismo gen de BLEE pueden estar localizadas en dos plásmidos diferentes. Además, tanto en el estudio I como en el II, hemos encontrado que en algunos casos los genes BLEEs pueden estar situados en un plásmido y en el cromosoma al mismo tiempo (los resultados no se muestran en la tesis). La presencia de un gen de resistencia en el cromosoma puede

ser debida a una transferencia horizontal entre el cromosoma bacteriano y un plásmido residente, el cual implica una mayor estabilidad del gen en la población bacteriana. Estas cepas pueden ser epidemiológicamente relevantes y se deben tener en consideración. Los plásmidos fueron divididos en los que tienen un rango ancho o un rango estrecho de huéspedes según la facilidad de replicación entre huéspedes, y esta interacción huésped-plasmido puede ser una de las claves de la evolución y estabilidad de los plásmidos (De Gelder, Ponciano et al. 2007).

La resistencia a los antimicrobianos se ha convertido en un problema de salud muy importante, y la predicción es que esta cifra de muertes causada por las multiresistencia vaya en aumento hasta 10 millones en el 2050, causando incluso más muertes que las actuales asociadas con cáncer. Por esta razón y la trágica disminución de nuevos antibióticos aprobados, hay una necesidad inmediata de desarrollar nuevos agentes, como por ejemplo drogas alternativas basadas en peptidos antimicrobianos (AMPs). Los AMPs son un componente importante de la immunidad innata, y son candidatos idóneos para el diseño de nuevos agentes antimicrobianos por su actividad de amplio espectro y la baja susceptbilidad al desarrollo de resistencia bacteriana (Wang, Zeng et al. 2016). Se han descrito aplicaciones potenciales con AMPs en la producción de pollos de engorde (Choi, Ingale et al. 2013).

Las bacterias han desarrollado muchas estratégias a través de la evolución para defenderse de los antimicrobianos. Se han descrito muchas dianas interesantes para crear nuevos inhibidores; un buen ejemplo es la combinación de antibióticos betalactámicos conjuntamente con inhibidores de las betalactamasas (Normark and

Normark 2002). Sin embargo, la bacteria ha desarrollado también mecanismos de resistencia a estos inhibidores (Drawz and Bonomo 2010).

Debido a la situación actual, se están implementando medidas más estrictas en el uso de los antibióticos a nivel de animales de producción para poder mantener un uso exitoso de los antibióticos de primera línia en salud humana. Para controlar las bacterias multiresistentes y su potencial zoonótico, se deben de potenciar medidas de reducción de uso de antimicrobianos en todos los niveles involucrados; autoridades sanitarias, investigadores, industrias farmacéuticas, ganadería, hospitales y atención primaria siguiendo la perspectiva "Una Sola Salud"; ya que es un problema multifactorial. Por otro lado, esta legislación debe ser a escala internacional y común en todos los países, los cuales han de ser responsables de entrenar a sus profesionales y transmitir el compromiso en el uso prudente de los antimicrobianos. En España, la Agencia Española de Medicamentos y Productos Sanitarios (AEMPS) publicó en 2014 un "Plan estratégico y de acción para reducir el riesgo de selección y diseminación de la resistencia a los antibióticos", tal y como la UE sugirió en 2012. Cada estado ha de desarrollar e implementar su propio plan nacional sobre la contención de resistencia a antimicrobianos (AEMPS 2014). Por otra parte, son necesarios estudios más profundos en investigación sobre resistencias a antimicrobianos para descubrir factores de riesgo asociados a la emergencia de cepas resistentes, así como alternativas a los antimicrobianos (planes de vacunación, desarrollo de prebióticos/probióticos, péptidos con actividad antimicrobiana, etc.). Un buen objeto de estudio son también los diferentes nichos involucrados para poder entender mejor todo lo relacionado con la

resistencia a antimicrobianos; tales como su diseminación, su persisténcia, su emergencia y sus riesgos. Todo en beneficio de un control exitoso del riesgo de aparición de resistencias (Ungemach, Muller-Bahrdt et al. 2006). Si no mejora, una regresión a una nueva era preantibiótica les espera a nuestros descendentes (MacDougall and Polk 2005).

Conclusions

I. The outbreaks of colibacilosis detected in different Spanish broiler farms during the same period of time were caused by unrelated multi-drug resistant APEC strains.

II. Commensal *E. coli* from broilers contained both, cephalosporin resistant genes and virulence genes endowing them with a zoonotic potential.

III. ESBL-producing *E. coli* were isolated from flies outside the farm becoming a potential vector of resistance genes into the animals.

IV. The presence of CR *E. coli* in white storks demonstrated their capacity to disseminate and transport resistance genes and evidences that wild animals are good centinels of anthropogenic pressure.

V. CTX-M genes were the most prevalent in all niches studied. CTX-M-1 and CTX-M-14 associated to plasmid replicons IncI1 and IncFIB were detected in flies and white storks.

VI. All cephalosporin resistant isolates from the three studies were multirresistant, including in some cases, resistance to fluoroquinolone mediated by *qnr* genes associated to plasmids and *mcr*-1 (colistin resistant) also associated to mobile genetic elements.

V. Cephalosporin resistant genes from all ecological niches studied were harboured in mobilizable plasmids that can be transferred horizontally among bacteria.

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Curriculum Vitae

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Publications

- Solà-Ginés, M., Camacho, M. C., De la Puente, J., Pineda-Pampliega, J., Aguirre, J. I., Torres-Medina, F., Ramis, A., Majó, N., Höfle-Hansen, U. and Migura-Garcia, L. (2018). Does exploitation of human residues make the white stork (*Ciconia ciconia*) a reservoir of extended-spectrum beta-lactamases-producing *Escherichia coli*? Submitted.
- Solà-Ginés, M., Cameron-Veas, K., Badiola, I., Dolz, R., Majó, N., Dahbi, G., Viso, S., Mora, A., Blanco, J., Piedra-Carrasco, N., González-López, J. J. and Migura-García, L. (2015). Diversity of multi-drug resistant avian pathogenic *Escherichia coli* (APEC) causing outbreaks of colibacillosis in broilers during 2012 in Spain. *PLoS One.* **10** (11): e0143191.
- Solà-Ginés, M., González-López, J. J., Cameron-Veas, K., Piedra-Carrasco, N., Cerdà-Cuellar, M. and Migura-García, L. (2015). Houseflies (*Musca domestica*) as vectors for extended-spectrum β-lactamase-producing *Escherichia coli* on Spanish broiler farms. *Appl Environ Microbiol.* **81** (11): 3604-11.
- Cameron-Veas, K., Solà-Ginés, M., Moreno, M. A., Fraile, L. and Migura-García, L. (2015). Impact of the use of beta-lactam antimicrobials on the emergence of *Escherichia coli* isolates resistant to cephalosporins under standard pig-rearing conditions. *Appl Environ Microbiol*. **81** (5): 1782-7.

Conferences and courses

- 7th Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE). Braunschweig, Germany (June 2017). Poster presentation: “Does exploitation of human residues make the white stork (*Ciconia ciconia*) a reservoir of extended-spectrum beta-lactamases-producing *Escherichia coli*?”
- Curso de formación de Personal Investigador Usuario de Animales para Experimentación y otras Finalidades Científicas (FELASA). UAB, Bellaterra, Spain (October 2016).
- 27th Congress of the European Society of Veterinary Dermatology and the European College of Veterinary Dermatology. Salzburg, Austria (September 2014). Poster presentation: “In vitro determination of the minimum bactericidal concentration of a honey-based ointment against *Staphylococcus pseudintermedius* isolated from canine bacterial pyoderma.”
- 24th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID). Barcelona, Spain (May 2014). Poster presentation: “Presence of cephalosporin resistant *Salmonella* from pig farms using different medication regimes.”
- Curso básico de Citometría de Flujo. CReSA, Bellaterra, Spain (March 2014).
- 10th International Meeting on Microbial Epidemiological Markers (IMMEM-10). Paris, France (October 2013). Poster presentation: “Diversity of multi-drug resistant avian pathogenic *Escherichia coli* (APEC) isolated from different outbreaks in Spain during 2012.”

- 50 Congreso Científico de Avicultura, Simposio WPSA-AECA. Lleida, Spain (October 2013). Oral presentation: “Diversidad de cepas de *Escherichia coli* multirresistentes y con genes de virulencia causantes de brotes de colibacilosis en aves en España durante el año 2012.”
- 5th Congress of European Microbiologists, FEMS. Leipzig, Germany (July 2013). Poster presentation: “Impact of the use of celtiofur on the emergence of *Escherichia coli* resistant to cephalosporins in a pig farm.”
- 5th Symposium on Antimicrobial Resistance in Animals and the Environment. Ghent, Belgium (July 2013). Poster presentation: “Amoxicilin as a risk factor associated to the emergence *Escherichia coli* resistant to cephalosporins in a pig farm.”

Acknowledgments

Agradecer al mundo la oportunidad que me puso delante, los años en el CReSA han sido un aprendizaje profesional y de vida que nunca olvidaré. Por esas puertas entró un niño inocente e inseguro en el gran y apasionante mundo de la investigación. Y tras varias etapas de todos los colores, se ha forjado un investigador confiado e ilusionado en seguir adelante.

Gracias de corazón Lourdes por ser la culpable de que hoy pueda escribir estas líneas. Pero sobretodo, gracias por tu paciencia y tu confianza, por contagiarme la pasión por las resistencias a los antibióticos y por compartir conmigo tus conocimientos. Me quedo con la ayuda de los primeros meses en el laboratorio, durante la escritura de mi primer artículo o al presentar mi primer póster en París. Mil y una gracias.

A mis padres, mis grandes educadores, gracias por enseñarme que con constancia y esfuerzo puedo llegar donde quiera. A mi padre, por compartir tanto hasta el final y enseñarme a no rendirme, este libro va por ti. A mi madre, por su alegría, bondad y fortaleza. Gracias por ser la mejor beca que existe. Gracias por apoyarme ciegamente en todo. Eternamente agradecido y orgulloso, os debo mi esencia.

A Saray, mi mujer y mi gran apoyo. Vivir esta tesis de principio a fin no ha sido fácil; sin tu paciencia y comprensión esto no hubiera sido posible, parte de esto es tuyo. Gracias por entenderme y empatizar durante este el ultimo año. Gracias por abrir mi mente científica y enseñarme tantas cosas de la vida, gracias por cada uno de los momentos que hemos vivido, gracias por ser mi mejor espejo y evolucionar juntos. Gracias por todo, te amo incondicionalmente.

Gracias a la gran família del CReSA; investigadores, técnicos, becarios, ha sido un honor formar parte. Gracias sobretodo a la gente de "Bacter", vuestra experiencia y ayuda contínua me ha hecho crecer. Gracias al despacho de becarios por hacer esto más ameno, la compañia ha sido muy grata. Guille, Eli y Karla gracias por compartir tantos momentos en el laboratorio y ayudarme a acabar protocolos interminables que no me daba tiempo por el trabajo. Guille, que felicidad conocerte y compartir viajes, paellas en casa de mis padres y mucho más. Al otro lado del charco tienes un hermano y una casa para cuando quieras, eres una grandísima persona.

Gracias a todos los colaboradores que han formado parte de los trabajos recogidos en esta tesis; al Laboratorio de Referencia de *E. coli* de la Universidad de Santiago de Compostela, al Instituto de Investigación de Recursos Cinéticos (IREC) de la Universidad de Castilla-La Mancha, y muy especialmente al Servei de Microbiologia del Hospital de la Vall d'Hebron en Barcelona, donde estuve unos meses como en casa. Gracias Juanjo, Núria, Toni y Thaís, guardo un gran recuerdo.

Gracias a la Escola Garbí, a mis compañeros de trabajo y a sus maravillosos niños y niñas, que han hecho que estos años sean muy agradecidos y divertidos. Trabajar media jornada aquí ha hecho posible la continuidad del doctorado. ¡Gracias a todos!

Gracias también a mis grandes hobbies; el deporte, los viajes y la cocina, y a los tantos amigos con los que tengo la suerte de compartirlos. Gracias a ellos he disfrutado como un niño del poco tiempo libre que me ha quedado. Me siento agradecido y afortunado nuevamente de las maravillas que te brinda la vida.

Y un especial agradecimiento a mi actual família y equipo, los "I+Dienses" de APC Europe. No creo en las casualidades, pero si en el destino y la ley de la atracción. Gracias Javier y Carmen por la oportunidad y la confianza depositada en mi. Gracias Carmen, Neus y Jesús por compartir el día a día y enseñarme tanto, me siento muy afortunado de poder trabajar al lado de tan grandes personas y profesionales. Gracias Ciro, ¡es viernes y el pollo lo sabe! Deseo seguir aprendiendo, creciendo y disfrutando como investigador a vuestro lado. Gracias de corazón por tanto…

Annexes

RESEARCH ARTICLE

Diversity of Multi-Drug Resistant Avian Pathogenic *Escherichia coli* (APEC) Causing Outbreaks of Colibacillosis in Broilers during 2012 in Spain

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OPEN ACCESS

Citation: Solà-Ginés M, Cameron-Veas K, Badiola I, Dolz R, Majó N, Dahbi G, et al. (2015) Diversity of Multi-Drug Resistant Avian Pathogenic *Escherichia coli* (APEC) Causing Outbreaks of Colibacillosis in Broilers during 2012 in Spain. PLoS ONE 10(11): e0143191. doi:10.1371/journal.pone.0143191

Editor: W.C. Yam, The University of Hong Kong, CHINA

Received: June 3, 2015

Accepted: November 1, 2015

Published: November 23, 2015

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Data Availability Statement: All relevant data are within the paper.

Funding: This work was partially supported by the grants AGL2011- 28836 and AGL2013-47852-R from the Ministerio de Economía y Competitividad (España) and grants CN2012/303 and EM2014/001 (Consellería de Cultura, Educación e Ordenación Universitaria, Xunta de Galicia and the European Regional Development Fund, ERDF). Work from LMG is supported by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) and the European Social Fund. The funders

Abstract

Avian pathogenic *Escherichia coli* (APEC) are the major cause of colibacillosis in poultry production. In this study, a total of 22 *E. coli* isolated from colibacillosis field cases and 10 avian faecal *E. coli* (AFEC) were analysed. All strains were characterised phenotypically by susceptibility testing and molecular typing methods such as pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). The presence of 29 virulence genes associated to APEC and human extraintestinal pathogenic *E. coli* (ExPEC) was also evaluated. For cephalosporin resistant isolates, cephalosporin resistance genes, plasmid location and replicon typing was assessed. Avian isolates belonged to 26 O:H serotypes and 24 sequence types. Out of 22 APEC isolates, 91% contained the virulence genes predictors of APEC; *iutA*, *hlyF*, *iss*, *iroN* and *ompT*. Of all strains, 34% were considered ExPEC. PFGE analysis demonstrated a high degree of genetic polymorphism. All strains were multi-resistant, including those isolated from healthy animals. Eleven strains were resistant to cephalosporins; six contained $bla_{CTX\text{-}M\text{-}14}$, two $bla_{SHV\text{-}12}$, two $bla_{CMY\text{-}2}$ and one $bla_{SHV\text{-}2}$. Two strains harboured *qnrA*, and two *qnrA* together with *aac*(6')-Ib-cr. Additionally, the emergent clone O25b:H4-B2-ST131 was isolated from a healthy animal which harboured $bla_{CMY\text{-}2}$ and *qnrS* genes. Cephalosporin resistant genes were mainly associated to the presence of IncK replicons. This study demonstrates a very diverse population of multi-drug resistant *E. coli* containing a high number of virulent genes. The *E. coli* population among broilers is a reservoir of resistance and virulence-associated genes that could be transmitted into the community through the food chain. More epidemiological studies are necessary to identify clonal groups and resistance mechanisms with potential relevance to public health.

had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Escherichia coli is a bacterium widespread in the intestine of animals and humans, and a pathogen that can induce enteric and extraintestinal infections. In particular, avian pathogenic *E. coli* (APEC) is the main cause of colibacillosis in poultry farms; a syndrome associated to airsacculitis, perihepatitis, pericarditis, and sometimes fatal septicemia. APEC strains are responsible for the mortality of 3–4% of the animals in a farm, and for the reduction of 2–3% of egg production [1], resulting in an economic burden to the poultry industry [2]. In many cases, the fundamental cause of the disease remains unclear, since the infection with *E. coli* is associated to the presence of *Mycoplasma gallisepticum* or respiratory viruses, such as Newcastle virus or Infectious Bronchitis virus [3].

Several virulence genes are implicated in avian colibacillosis such as adhesins, toxins, anti-host defence factors, iron acquisition systems, autotransporters and the IbeA protein [4]. Subtractive hybridization studies have demonstrated sequence homology between specific DNA regions of APEC and human extraintestinal pathogenic *E. coli* (ExPEC) [5]. Additionally, the presence of similar virulence genes found in both, APEC and ExPEC strains, suggested that APEC strains may act as zoonotic pathogens and reservoir of virulence causing human infections [6–8]. According to Johnson *et al.* (2003), a strain could be considered ExPEC if exhibits two or more of the following virulence genes; *pap* (P fimbriae), *sfa/foc* (S/F1C fimbriae), *afa/dra* (Dr binding adhesins), *iutA* (aerobactin receptor), and *kpsM* II (group 2 capsule synthesis) [9]. ExPEC strains are more often derived from virulence-associated B2 and D phylogroups [10].

The successful treatment of avian colibacillosis caused by APEC strains mainly depends on the use of antimicrobials. However, increasing resistance to critically important antimicrobials, such as third-generation cephalosporins and fluoroquinolones, is nowadays common in *E. coli* from poultry origin [11]. These resistances can be transmitted to humans via the food supply [12, 13]. In particular, *E. coli* producing extended-spectrum beta-lactamases (ESBLs) and plasmid mediated AmpC beta-lactamases have increased considerably in the last years [14]. Normally, these genes are located on plasmids, and can be transferred by conjugation to other bacterial species [11]. Some of the virulence factors for APEC and ExPEC can also be harboured on plasmids. Particularly, ColV plasmids yield some virulence genes such as *hlyF*, *ompT*, *iss* and *cvaC* surrounding the replicon RepFIB [15].

Several studies have described APEC strains in the literature [16]. However, not many studies have combined extensive characterization at the serotype level, virulence-associated genes, molecular typing techniques, molecular determination of resistance mechanisms and mobile genetic elements involved in transfer of resistance. For this reason, the objective of this study was to discriminate and to perform such characterization of highly pathogenic *E. coli* causing outbreaks of colibacillosis in 13 different broiler farms throughout Spain, and compare them to avian faecal *E. coli* (AFEC) obtained from healthy animals. Additionally, the identification of clones more prone to cause disease has been assessed.

Materials and Methods

Isolation

A total of 22 tissue swabs of culled-animals affected with colibacillosis arrived to the laboratory between January and March 2012. The samples were obtained as part of routine care. Samples were taken from chickens already sacrificed for diagnostic purposes following the procedures according to the requirements of the Ethics Committee of Animal and Human Experimentation of the Universitat Autònoma de Barcelona (Permit Number DMAH-4239 that specifically

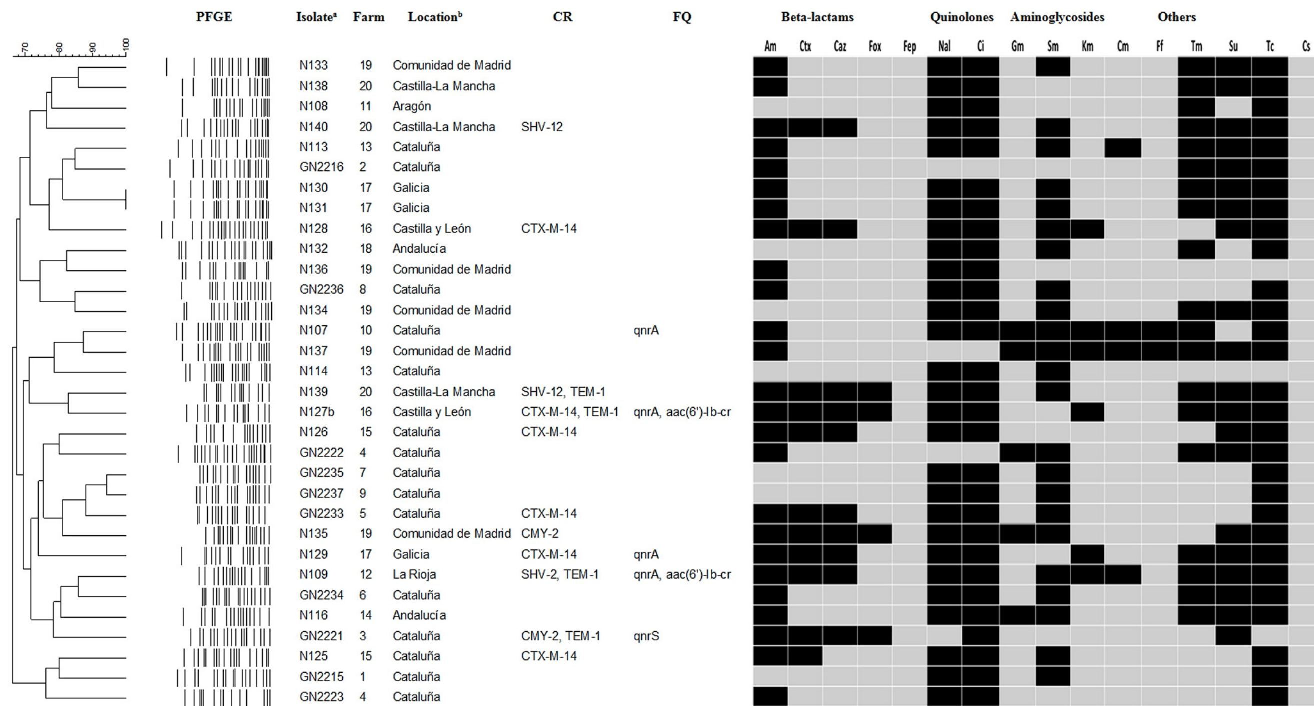

Fig 1. PFGE dendogram illustrating the phenotypic and genotypic relationship of the strains and the cephalosporin resistance genes. PFGE, pulsed-field gel electrophoresis; CR., cephalosporin resistance genes; FQ., flouoroquinolone resistance genes; Am: Ampicillin (WT≤8mg/L); Ctx: Cefotaxime (WT≤0.25mg/L); Caz: Ceftazidime (WT≤0.5mg/L); Fox: Cefoxitin (WT≤8mg/L); Fep: Cefepime (WT≤0.125mg/L); Nal: Nalidixic acid (WT≤16mg/L); Ci: Ciprofloxacin (WT≤0.064mg/L); Gm: Gentamicin (WT≤2mg/L); Sm: Streptomycin (WT≤16mg/L); Km: Kanamycin (WT≤8mg/L);. Cm: Chloramphenicol (WT≤16mg/L); Ff: Florfenicol (WT≤16mg/L); Tm: Trimethoprim (WT≤2mg/L); Su: Sulphamethoxazole (WT≤64mg/L); Tc: Tetracycline (WT≤8mg/L); Cs: Colistin (WT≤2mg/L). [a] Isolates are divided in APEC (N) and AFEC (GN) strains. [b] Location of the strains is named in order to the different regions of Spain where farms were localized.

doi:10.1371/journal.pone.0143191.g001

permits euthanasia of chickens). Animals were euthanized using intravenous sodium pentobarbital (100 mg/kg, Dolethal, Vétoquinol, Cedex, France) in the wing vein. The method to sacrifice the animals follows the welfare rules stated in the European Directive 86/609/CEE. None of the authors of this manuscript were involved in manipulating or sacrificing the chickens. All the samples were collected from clinical cases submitted to the Diagnostic Service of the Veterinary School of the Universitat Autònoma de Barcelona. Swabs were taken from 13 broiler farms located in nine different regions of Spain (Fig 1). Ten *E. coli* strains isolated from faeces of healthy animals collected in nine farms were also included in the study. The samples were plated onto MacConkey agar and incubated overnight at 37°C. Three lactose-positive colonies for each plate were selected and confirmed to be *E. coli* by PCR [17]. Subsequently, one representative was selected for further studies.

Serotyping

Determination of O and H antigens was carried out using the method previously described by Guinée *et al.* with all available O (O1 to O181) and H (H1 to H56) antisera [18]. Non-typeable isolates were denoted as ONT or HNT and non-motile isolates were denoted as HNM. All antisera were obtained and absorbed with the corresponding cross-reacting antigens to remove the nonspecific agglutinins. The O and H antisera were produced in the Laboratorio de Referencia de *E. coli* (LREC, Lugo, Spain). O25a and O25b subtypes were determined by PCR [19].

Phylogeny, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

Isolates were separated in phylogroups (A, B1, B2, C, D, E or F) according to a method previously described [20, 21].

PFGE was performed as described elsewhere [22]. The results were analysed by Fingerprinting II Informatix software (Applied Maths, Sint-Martens-Latem, Belgium). PFGE-types were separated based on differences of at least one band in the restriction profiles. The analysis of the bands generated was carried out using the Dice coefficient and unweighted pair group method with arithmetic averages (optimization of 1.25% and position tolerance 1.25%).

MLST was carried out as previously described according to the protocol and primers specified on the *E. coli* MLST web site (http://mlst.ucc.ie/mlst/dbs/Ecoli) [23]. Sequences were analysed with Vector NTI advance 11 software (InforMax, Inc., Bethesda, MD).

Detection of virulence-associated genes

All strains were tested by PCR for 29 ExPEC and APEC virulence-associated genes (Tables 1 and 2) [13, 24]. The genes described previously by Johnson *et al.* (2008) as the minimal predictors of APEC virulence; *iroN*, *ompT*, *hlyF*, *iutA* and *iss* were detected by a multiplex PCR [24]. Virulence scores were calculated for each isolate as the sum of all virulence-associated genes detected; *pap*, *sfa-foc* and *kpsM* II were counted only once.

Statistical analysis

Differences in the prevalence between different groups were determined by Fisher's exact test as described before [25]. Virulence scores were compared by the use of Mann-Whitney U test. Statistical analyses were performed using GraphPad Prsim, version 3.1 software (GraphPad Software, Inc., San Diego, CA).

Antimicrobial susceptibility testing

Disc diffusion was performed according to CLSI guidelines using the following discs (Oxoid, Basingstoke, UK): cefoxitin, 30 mg; cefepime, 30 mg; ceftazidime, 30 mg; cefotaxime, 30 mg; cefotaxime+clavulanic acid, 30+10 mg; and ceftazidime+clavulanic acid, 30+10 mg. The disc combinations of cefotaxime and cefotaxime/clavulanic acid, ceftazidime and ceftazidime/clavulanic acid were used for the identification of ESBLs; cefoxitin was used for the detection of AmpC-type beta-lactamase [26]. All isolates were susceptibility tested using a minimum inhibitory concentration (MIC)-based broth microdilution (VetMIC GN-mo, National Veterinary Institute, Uppsala, Sweden) as described before [25]. Isolates were considered to be wild type (WT) or non-WT based on epidemiological cut-off values according to EUCAST (http://www.eucast.org/).

Resistance genes

All strains exhibiting resistance to third-generation cephalosporins (cefotaxime and ceftazidime) were tested by PCR methods for the presence of the bla_{CTX-M}, bla_{SHV}, bla_{TEM}, bla_{CMY-1} and bla_{CMY-2} genes as described by Hasman *et al.* [27]. Detection of plasmid-mediated AmpC beta-lactamase genes was assessed by multiplex PCR [28]. Sequencing of both strands of amplicons was performed. The presence of the fluoroquinolone resistance genes *aac(6')-Ib-cr*, *qnrA*, *qnrB*, *qnrS*, *qepA* and *oqxAB* was also assessed [29, 30].

Plasmid DNA analysis

Isolates exhibiting resistance to cephalosporins were selected for plasmid characterization. Plasmid replicons tested were elected according to the presence of determinant resistance genes (HI1, HI2, I1, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIA and K) [31] and were identified using the PCR-based replicon typing method previously described [32, 33]. Plasmids detection and sizing was performed on all the isolates by S1-nuclease PFGE of total DNA [34]. Restriction fragments from S1-PFGE gels were transferred onto a positively charged nylon membrane and hybridised with specific probes for $bla_{\text{CTX-M-14}}$, bla_{TEM}, bla_{SHV}, bla_{CMY} and for each replicon that was previously identified.

Results

A total of 22 *E. coli* were recovered from 13 different farms distributed throughout Spain during 2012. Additionally 10 isolates from healthy animals collected in nine different farms were also included in the study to make a total of 32 *E. coli* isolates.

Serotypes

A total of 16 different O serogroups, 15 flagellar H antigens and 26 different O:H serotypes were identified (Table 1). The most prevalent serotypes were: O3:H26, O5:H10, O5:H51 and O78:H9. Additionally, the emergent clone O25b:H4 was detected in a commensal isolate.

Phylogeny, PFGE and MLST

Within the APEC strains (n = 22) the phylotyping identified six strains belonging to group A (27%), six to group B1 (27%), four to group D (18%), three to group E (14%), two to group C (9%) and one to group B2 (5%). For the commensal strains (n = 10), 60% belonged to phylogroup A, and the remaining 40% to B1, B2, D and F phylogroups (10% each) (Table 1).

XbaI-PFGE analysis showed a high degree of genetic polymorphism. A total of 31 different PFGE restriction profiles were identified among the 32 *E. coli* isolates (Fig 1). Only two isolates were epidemiologically related and belonged to the same farm.

Among the APEC subgroup (n = 22), MLST analyses identified 18 STs, while six STs were found among AFEC isolates (n = 10) (Table 1). Within the APEC strains, four belonged to the ST168 clonal complex (Cplx), three to the ST350 Cplx, two to ST156 Cplx, and two to the highly pathogenic ST23 Cplx (Table 1). Within the AFEC isolates, the most common Cplx was ST10 (n = 3), followed by ST165 Cplx (n = 3) (Table 1). The emergent pandemic multirresistant clone O25b:H4-B2-ST131 was found among the AFEC isolates.

Detection of virulence-associated genes

The prevalence of 29 virulence-associated genes is shown in Tables 1 and 2. Regarding the five virulence genes associated to APEC (Fig 1); 81%, 78%, 75%, 72% and 72% of the 32 *E. coli* strains yielded amplicons for *iss*, *ompT*, *iutA*, *iroN* and *hlyF*, respectively (Table 1). The prevalence of these genes was higher in APEC isolates (91% of the APEC strains harboured all of the mentioned genes), when compared to the AFEC isolates. In general, the presence of virulence-associated genes in AFEC strains was low, with 80% of the strains having from zero to three of the previously mentioned virulence genes (Table 1). According to the number of virulence-associated genes, 34% of the isolates were considered ExPEC.

Table 1. Distribution of virulence-associated gene profiles, phylogeny, serotyping and MLST results among all 32 strains.

	Isolate	Phylo.	Serotype	ST	Cplx	Virulence gene profiles[a]
AFEC						
	GN-2215	D	ONT:H4	ST117		***iss***, *fimH*, *fyuA*, *irp-2*, *cdtB*, *traT*, *malX*, *tsh*
	GN-2216	A	O53:H18	ST10	ST10 Cplx	***iss***, *fimH*, *traT*
	GN-2221	B2	O25b:H4	ST131		***iroN***, ***ompT***, ***iutA***, ***iss***, *fimH*, *cvaC*, *irp-2*, *kpsM II-K1*, *traT*, *ibeA*, *malX*, *usp*, *tsh*
	GN-2222	F	O83:HNT	ST648		***iroN***, ***ompT***, ***iutA***, ***iss***, *fimH*, *cvaC*, *astA*, *kspM II-K5*, *traT*, *malX*, *tsh*
	GN-2223	A	ONT:HNT	ST10	ST10 Cplx	*fimH*, *fyuA*, *irp-2*, *traT*
	GN-2233	A	O3:H26	ST165	ST165 Cplx	*fimH*, *fimAvMT78*, *traT*
	GN-2234	A	O2:H40	ST10	ST10 Cplx	***iroN***, ***ompT***, ***iss***, *fimH*, *fyuA*, *traT*
	GN-2235	A	O3:HNM	ST165	ST165 Cplx	*fimH*, *fimAvMT78*, *traT*
	GN-2236	B1	O127:H37	ST297		***ompT***, ***iutA***, ***iss***, *fimH*, *cvaC*, *traT*, *malX*, *tsh*
	GN-2237	A	O3:H26	ST189	ST165 Cplx	*fimH*, *fimAvMT78*, *traT*
APEC						
	N107	B1	ONT:H16	ST295		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *cvaC*, *traT*, *tsh*
	N108	C	O78:H9	ST23	ST23 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *fyuA*, *astA*, *traT*, *tsh*
	N109	B1	ONT:H28	ST156	ST156 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *astA*, *irp-2*, *traT*
	N116	D	O25a:[H4]	ST624		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *cvaC*, *kpsM III*, *traT*, *malX*
	N113	A	O5:H10	ST93	ST168 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *astA*, *kpsM II-K2*
	N114	A	O6:H16	ST48	ST10 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *fimAvMT78*, *kpsM III*, *traT*
	N125	A	O88:HNM	ST1137		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *cvaC*, *astA*, *fimAvMT78*, *traT*
	N126	B1	ONT:H51	ST889		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *cvaC*, *traT*, *tsh*
	N127b	D	ONT:H51	ST156	ST156 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *traT*, *tsh*
	N128	E	ONT:H27	ST350	ST350 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *cvaC*, *astA*, *irp-2*, *fimAvMT78*, *papEF*, *papG*, *traT*, *tsh*
	N129	B1	O15:H10	ST101	ST101 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *cvaC*, *astA*, *sfa/focDE*, *traT*, *tsh*
	N130	A	O5:H51	ST93	ST168 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *astA*, *kspM II-K2*
	N131	A	O5:H51	ST93	ST168 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *astA*, *kspM II-K2*
	N132	E	O102:H25	ST57	ST350 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *traT*, *tsh*
	N133	A	O5:H10	ST93	ST168 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *kpsM II-K2*
	N134	B1	O159:H28	ST539		***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *irp-2*, *traT*
	N135	B2	O2:H1	ST429		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *fyuA*, *cvaC*, *irp-2*, *kpsM II-K1*, *traT*, *ibeA*, *malX*, *usp*, *tsh*
	N136	D	ONT:H4	ST830		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *fyuA*, *traT*, *malX*

(*Continued*)

Table 1. (*Continued*)

Isolate	Phylo.	Serotype	ST	Cplx	Virulence gene profiles[a]
N137	D	O11:H15	ST3161		***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *cvaC*, *traT*
N138	C	O78:H9	ST650	ST23 Cplx	***iroN***, ***ompT***, ***hlyF***, ***iutA***, ***iss***, *fimH*, *traT*
N139	B1	O45:H8	ST533		***iroN***, ***ompT***, ***hlyF***, *fimH*, *astA*, *traT*, *malX*
N140	E	O119:H27	ST350	ST350 Cplx	***iutA***, *fimH*, *astA*, *fimAvMT78*

Phylo, phylogroup; ST, sequence type; Cplx, clonal complex. Adhesins *fimH* (D-mannose-specific adhesin of type I fimbriae), *fimAvMT78* (FimA variant MT78 of type 1 fimbriae), *papEF* and *papG* (P fimbria subunits), and *sfa/focDE* (S fimbrial adhesin/putative F1C fimbrial adhesin); toxins *cdtB* (cytolethal distending toxin), *hlyF* (hemolysin F), and *astA* (EAST1, enteroaggregative E. coli heat-stable toxin); siderophores fyuA (yersiniabactin), *iutA* (aerobactin), *iroN* (novel catecholate siderophore receptor), and *irp-2* (iron repressible associated with yersiniabactin synthesis); protectins *kpsM* (groups II and III, specifically targeting the K1, K2 and K5 genes of group II capsules), *cvaC* (ColV, colicin V from serum resistance-associated plasmids), *iss* (surface exclusion serum survival protein), and *traT* (serum resistance); miscellaneous virulence genes *ompT* (protease), *ibeA* (invasion of brain endothelium), *malX* (PAI, pathogenicity island marker), and *usp* (uropathogenic-specific protein, bacteriocin).

[a] Virulence-associated genes shown in boldface are the five genes characteristics of APEC strains.

doi:10.1371/journal.pone.0143191.t001

Statistical analysis

Significant differences ($P = 0.035$) were observed in the number of virulence-associated genes (*hlyF*, *astA*, *iroN*, *iutA* and *ompT*) found between APEC (mean, 9.2; range 5 to 16) and AFEC (mean, 6.7; range 3 to 13) isolates (Table 2). Statistical difference was found also comparing the virulence-associated genes of the isolates of phylogroup A for APEC and AFEC ($P = 0.013$; virulence score 7.2 vs 4.2). The strains belonging to phylogroup B2 exhibited the highest virulence score (mean, 14.5; range, 13 to 16) whereas the phylogroup A exhibited the lowest (mean, 5.8; range, 3 to 11). Additionally, significant differences were observed comparing B2 and D phylogroups (mean, 11.1; range 8 to 16) with A and B1 phylogroups (mean 7.4; range 3 to 13) ($P = 0.008$).

Antimicrobial susceptibility testing and resistance genes

All the analyzed strains were multi-resistant (resistant to more than 3 antimicrobial families), including those isolated from healthy animals. Furthermore, 50% were resistant to more than eight antimicrobials. Susceptibility testing detected 11 strains resistant to cephalosporins (34%); six $bla_{CTX-M-14}$, two bla_{SHV-12}, two bla_{CMY-2} and one bla_{SHV-2}. Two of these strains belonged to AFEC isolates. Two isolates were resistant to cefoxitin, and the resistance mechanism involved could not be determined.

In addition, 88% of the isolates were resistant to nalidixic acid and 91% to ciprofloxacin. Additionally, 91% of the strains were resistant to tetracycline, 78% to ampicillin, 69% to streptomycin, 63% to sulfamethoxazole, 59% to trimethoprim 34% to cefotaxime, 31% to ceftazidime, 19% to kanamycin, 16% to gentamicin, 13% to cefoxitin, 13% to chloramphenicol and 6% to florfenicol. No resistance to cefepime and colistin was observed among the isolates (Fig 1).

The presence of the *qnrS* gene was only confirmed in the isolate belonging to O25b:H4-B2-ST131. Finally, two of the APEC strains exhibited *qnrA* and two *qnrA* together with *aac (6')-Ib-cr*. The genes *qnrB*, *qepA* and *oqxAB* were not found in this strain collection.

Plasmid localisation of cephalosporin resistance genes

PCR-based replicon typing among the 11 cephalosporin resistant *E. coli* isolates showed that IncFIB replicon was present in all analysed isolates (Table 3). The replicons IncI1, IncN, IncK, IncY, IncP, IncFIA, IncHI1 and IncHI2 were also detected (Table 3).

Table 2. Distribution and characterization of virulence-associated genes and phylogroups of the 32 isolates.

Virulence gene (s)[a]	Total isolates (%) (n = 32)	APEC isolates (%) (n = 22)	AFEC isolates (%) (n = 10)	A (%) (n = 12)	B1 (%) (n = 7)	B2 (%) (n = 2)	D (%) (n = 5)	C, E and F (%) (n = 6)	B2/D (%) (n = 7)	A/B1 (%) (n = 19)	A/ APEC (%) (n = 6)	A/ AFEC (%) (n = 6)	*P* value[b] APEC vs AFEC	*P* value[b] A/ APEC vs A/ AFEC	*P* value[b] B2/D vs A/ B1
Adhesins															
fimH	28 (88%)	18 (82%)	10 (100%)	8 (67%)	7 (100%)	2 (100%)	5 (100%)	5 (83%)	7 (100%)	15 (79%)	2 (33%)	6 (100%)			
fimAvMT78	7 (22%)	4 (18%)	3 (30%)	5 (42%)	0	2 (100%)	0	2 (33%)	2 (29%)	5 (26%)	2 (33%)	3 (50%)			
papEF	1 (3%)	1 (5%)	0	0	0	0	0	1 (17%)	0	0	0	0			
papG	1 (3%)	1 (5%)	0	0	0	0	0	1 (17%)	0	0	0	0			
sfa/focDE	1 (3%)	1 (5%)	0	0	1 (14%)	0	0	0	0	1 (5%)	0	0			
afa/draBC	0	0	0	0	0	0	0	0	0	0	0	0			
Toxins															
cnf1	0	0	0	0	0	0	0	0	0	0	0	0			
cdtB	1 (3%)	0	1 (10%)	0	0	0	1 (20%)	0	1 (15%)	0	0	0			
sat	0	0	0	0	0	0	0	0	0	0	0	0			
hlyA	0	0	0	0	0	0	0	0	0	0	0	0			
*hlyF**	22 (72%)	22 (100%)	0	6 (50%)	6 (86%)	1 (50%)	4 (80%)	4 (67%)	5 (71%)	12 (63%)	6 (100%)	0	<0.0001	0.0022	
astA	12 (38%)	11 (50%)	1 (10%)	5 (42%)	3 (43%)	0	1 (20%)	3 (50%)	0	8 (42%)	5 (83%)	0	0.0496	0.0152	
tsh	12 (38%)	8 (36%)	4 (40%)	0	4 (57%)	2 (100%)	2 (40%)	4 (67%)	4 (57%)	4 (21%)	0	0			
Siderophores															
fyuA	9 (28%)	5 (23%)	4 (40%)	2 (17%)	1 (14%)	2 (100%)	3 (60%)	1 (17%)	5 (71%)	3 (16%)	0	2 (33%)			0.0138
*iutA**	24 (75%)	21 (95%)	2 (20%)	6 (50%)	6 (86%)	2 (100%)	4 (80%)	6 (100%)	6 (86%)	12 (63%)	6 (100%)	0	<0.0001	0.0022	
*iroN**	24 (72%)	21 (95%)	3 (30%)	7 (58%)	6 (86%)	2 (100%)	4 (80%)	5 (83%)	6 (86%)	13 (68%)	6 (100%)	1 (17%)	0.0003	0.0152	
irp-2	8 (25%)	5 (23%)	3 (30%)	1 (8%)	3 (43%)	2 (100%)	1 (20%)	1 (17%)	3 (43%)	4 (21%)	0	1 (17%)			
Protectins															
kpsM II	7 (22%)	5 (23%)	2 (20%)	4 (33%)	0	2 (100%)	0	1 (17%)	2 (29%)	4 (21%)	4 (67%)	0			
kpsM II-K1	2 (6%)	1 (5%)	1 (10%)	0	0	2 (100%)	0	0	2 (29%)	0	0	0			
kpsM II-K2	4 (13%)	4 (18%)	0	4 (33%)	0	0	0	0	0	4 (2%)	4 (67%)	0			
kpsM II-K5	1 (3%)	0	1 (10%)	0	0	0	0	1 (17%)	0	0	0	0			
kpsM III	2 (6%)	2 (9%)	0	1 (8%)	0	0	1 (20%)	0	1 (15%)	1 (5%)	1 (17%)	0			
cvaC	11 (34%)	9 (41%)	3 (30%)	1 (8%)	4 (57%)	2 (100%)	2 (40%)	2 (33%)	4 (57%)	5 (26%)	1 (17%)	0			
*iss**	26 (81%)	20 (91%)	6 (60%)	8 (67%)	7 (100%)	2 (100%)	5 (100%)	5 (83%)	7 (100%)	15 (79%)	6 (100%)	2 (33%)			

(*Continued*)

Table 2. (Continued)

Virulence gene (s)[a]	Total isolates (%) (n = 32)	APEC isolates (%) (n = 22)	AFEC isolates (%) (n = 10)	A (%) (n = 12)	B1 (%) (n = 7)	B2 (%) (n = 2)	D (%) (n = 5)	C, E and F (%) (n = 6)	B2/D (%) (n = 7)	A/B1 (%) (n = 19)	A/ APEC (%) (n = 6)	A/ AFEC (%) (n = 6)	P value[b] APEC vs AFEC	P value[b] A/ APEC vs A/ AFEC	P value[b] B2/D vs A/ B1
traT	27 (84%)	17 (77%)	10 (100%)	8 (67%)	7 (100%)	2 (100%)	5 (100%)	5 (83%)	7 (100%)	15 (79%)	2 (33%)	6 (100%)			
Miscellaneous															
*ompT**	26 (78%)	22 (100%)	4 (40%)	7 (58%)	7 (100%)	2 (100%)	4 (80%)	3 (50%)	6 (86%)	14 (74%)	6 (100%)	1 (17%)	0.0002	0.0152	
ibeA	2 (6%)	1 (5%)	1 (10%)	0	0	2 (100%)	0	0	2 (29%)	0	0	0			
malX	8 (25%)	4 (18%)	4 (40%)	0	1 (14%)	2 (100%)	3 (60%)	1 (17%)	5 (71%)	1 (5%)	0	0			0.0018
usp	2 (6%)	1 (5%)	1 (10%)	0	0	2 (100%)	3 (60%)	0	5 (71%)	0	0	0			0.0003
Mean (range) virulence score[c]	8.2 (3–16)	9.2 (5–16)	6.7 (3–13)	5.8 (3–11)	10 (8–13)	14.5 (13–16)	9.8 (8–11)	9.2 (5–14)	11.1 (8–16)	7.4 (3–13)	7.2 (5–11)	4.2 (3–6)	0.035	0.013	0.008

Adhesins *fimH* (D-mannose-specific adhesin of type I fimbriae), *fimAvMT78* (FimA variant MT78 of type 1 fimbriae), *papEF* and *papG* (P fimbria subunits), *sfa/focDE* (S fimbrial adhesin/putative F1C fimbrial adhesin), and *afa/draBC* (Dr antigen specific adhesin); toxins *cnf1* (cytotoxic necrotizing factor 1), *cdtB* (cytolethal distending toxin), *sat* (secreted autotransporter toxin), *hlyA* (α-hemolysin), *hlyF* (hemolysin F), and *astA* (EAST1, enteroaggregative E. coli heat-stable toxin); siderophores fyuA (yersiniabactin), *iutA* (aerobactin), *iroN* (novel catecholate siderophore receptor), and *irp-2* (iron repressible associated with yersiniabactin synthesis); protectins *kpsM* (groups II and III, specifically targeting the K1, K2 and K5 genes of group II capsules), *cvaC* (ColV, colicin V from serum resistance-associated plasmids), *iss* (surface exclusion serum survival protein), and *traT* (serum resistance); miscellaneous virulence genes *ompT* (protease), *ibeA* (invasion of brain endothelium), *malX* (PAI, pathogenicity island marker), and *usp* (uropathogenic-specific protein, bacteriocin).

[a] Virulence-associated genes shown with asterisk are the five genes characteristics of APEC strains.

[b] *P* values (by Fisher's exact test) are shown where $P<0.05$.

[c] The virulence score was the number of virulence genes detected, adjusted for multiple detection of the *pap*, *sfa* and *foc*, and *kpsM* II operons. Virulence scores were compared by use of the Mann-Whitney U test.

doi:10.1371/journal.pone.0143191.t002

Plasmid-sizes varied between 90 and 120-kb, with the exception of two plasmids; pST131-2 and pST350-1 with 250 and 180-kb, respectively (Table 3). The most common incompatibility group detected by Southern blot was IncK (n = 5). Specific probe hybridization of S1-digested DNA demonstrated that isolate GN2221 harboured $bla_{\text{CMY-2}}$ gene in a plasmid containing replicon IncK (pST131-3). The isolate N135 harbouring also $bla_{\text{CMY-2}}$ gene in a 90-kb plasmid contained IncK and IncP replicons. Isolate GN2233 yielded $bla_{\text{CTX-M-14}}$ in a 90-kb plasmid with two replicons (IncK and IncY). The presence of $bla_{\text{SHV-12}}$ was confirmed in two isolates having a 120 and 90-kb plasmids of the IncI1 and unknown replicon family, respectively. N109 harboured $bla_{\text{SHV-2}}$ in an IncK plasmid (pST156-2). Finally, no plasmid location could be confirmed for the CTX-M-14 gene of isolate N127b.

Discussion

This study has demonstrated the presence of different clones of APEC causing outbreaks of colibacillosis during the same period of time in different broiler farms from different regions of Spain. All these isolates were multirresistant, therefore therapeutic success may have been compromised, causing a serious economic burden to the broiler industry. Moreover, the AFEC strains were also resistant to critically important antimicrobials such as cephalosporins and

Table 3. Identification and characterisation of the location of $bla_{CTX-M-14}$, bla_{SHV-2}, bla_{SHV-12}, bla_{CMY-2} and bla_{TEM} among 11 cephalosporin resistant *E. coli* isolates.

Isolate[a]	ST	Replicons[b]	*bla* type	Plasmid[c]	Inc[d]	Plasmid size (kb)
N129	101	FIB	CTX-M-14	pST101	-	90
GN2221	131	N, FIB, K	TEM-1	pST131-1	N	60
			TEM-1	pST131-2	N	250
			CMY-2	pST131-3	K	120
N109	156	HI1, FIB, K	TEM-1	pST156-1	HI1	250
			SHV-2	pST156-2	K	120
N127b	156	HI2, FIB	TEM-1	-	-	-
			CTX-M-14	-	-	-
GN2233	165	FIB, Y, K	CTX-M-14	pST165	K, Y	90
N128	350	FIB	TEM-1	pST350-1	-	180
			CTX-M-14	pST350-2	-	90
N140	350	I1, FIB, K	SHV-12	pST350-3	-	90
N135	429	FIB, P, K	CMY-2	pST429	P, K	90
N139	533	I1, FIB	TEM-1	pST533	I1	120
			SHV-12	pST533	I1	120
N126	889	FIA, FIB, K	CTX-M-14	pST889	K	90
N125	1137	I1, FIB	CTX-M-14	pST1137	I1	95

p(ST number), plasmid location; Inc, identified replicon.

[a] Isolates are divided in APEC (N) and AFEC (GN) strains.

[b] Replicon identifications are based on positive amplifications from the PCR-based replicon typing method.

[c] Plasmids were named based on the source strains sequence type and plasmid size.

[d] In all *E. coli* isolates, replicons from plasmids containing the different *bla* genes were identified by PCR-positive amplification and by Southern hybridisation of the S1-digested fragments.

doi:10.1371/journal.pone.0143191.t003

fluoroquinolones. Several studies have demonstrated that the *E. coli* population of broilers are a reservoir of antimicrobial resistance genes that may be transferred by mobile genetic elements to the community via the food chain [12]. Although efforts have been implemented to reduce the use of antimicrobials in the poultry industry, these are sometimes overused in food producing animals, and particularly in broiler farms [35]. The restriction of fluoroquinolones and cephalosporins usage in livestock for human consumption, and the implementation of measures to limit the dissemination are needed [36].

This study have also demonstrated the presence of virulence genes associated to APEC strains in commensal *E. coli*, indicating a possible reservoir of virulence-associated genes in this population. However, the presence of the minimal predictors of APEC virulence was much higher in APEC isolates than AFEC isolates. Furthermore, the similarity of avian pathogenic strains with human pathogenic *E. coli* (ExPEC) based on virulence-associated genes was confirmed in this study, since a total of 34% of the isolates could be considered ExPEC. As also suggested by other studies, certain APEC subgroups, specifically a large proportion of phylogroup A isolates, may be considered potential zoonotic agents [6].

The information provided by different typing methods is usually unrelated. MLST results, phylogenetic groups and serotyping showed similarities between strains that with PFGE were not significant. For instance, the two highly pathogenic strains O78:H9-C-ST23 shared 80% of identity by PFGE, as well as the two strains O5:H10-A-ST93, which shared 70% similarity. Additionally, two AFEC strains O3:H26-A-ST165 exhibited 90% similarity by PFGE. In these

cases, the virulence gene content and the results of the susceptibility testing were equal or almost equal. However, the combination of all these techniques may be useful to discriminate between strains causing outbreaks and those with potential risk to cause disease in animals or humans. For instance, combining serotyping, phylotyping and MLST the strain GN-2221 (O25b:H4-B2-ST131) could be identified as a potential zoonotic agent causing infections in humans. Still, further studies based in animal models are necessary to properly confirm the pathogenicity of the strain.

All the serotypes found within this strain collection have been well described in previous studies [8, 16, 37–39]. In contrast, different results have been observed from the ST data, where several ST types (ST10, ST23, ST48, ST57, ST93, ST117, ST131, ST156, ST350, ST429 and ST648) have been previously associated to APEC strains [8, 16, 40–42], whereas other STs (ST101, ST165, ST189, ST297, ST533, ST539, ST624, ST650, ST889 and ST3161) have not been related to infections cause by APEC before.

The phylogroup B2 is known to harbour many more virulence-encoding genes than the rest of the *E. coli* phylogroups [10]. In our study, the two B2 strains found were O25b:H4-B2-ST131, which previously has been associated to human infections [43], and O2:H1-B2-ST429 which is frequently associated with poultry disease [44]. They exhibited 14 and 16 of the 29 virulence genes tested, respectively. Additionally, both strains were resistant to cephalosporins by production of CMY-2. The typical highly pathogenic APEC clonal group O78-H9-C-ST23 was found twice in this strain collection. Studies on sequencing and phylogenetic analysis of this clonal line revealed that O78 is more closely related to human strains (i. e. ST23 enterotoxigenic *E. coli* (ETEC)) than other APEC strains [44]. These results suggest that the *E. coli* population of broilers may be a potential reservoir of virulence-associated genes that could be transferred to humans through the food chain.

Our results are in line with the most commonly described ESBLs and AmpC producing *E. coli* in poultry production, which are CTX-M-14, CTX-M-1, CMY-2 and SHV-12 [45]. Also, these data provided evidence to the known genetic heterogeneity among ESBL-harboring *E. coli* isolates in broilers. In the last years, an increase in the presence of *E. coli* O25b:H4-B2-ST131 producing CTX-M-15 with a high virulence potential has been reported in human infections [46–48]. The high prevalence of this clonal group among multi-drug resistant isolates has important clinical and public health implications, due to the risk of treatment failure. The first time that the human ExPEC clonal group O25b:H4-B2-ST131 was detected in poultry was in Spain in 2010 [13, 49]. In both cases, the genes encoding resistance to cephalosporins were CTX-M-9. Additionally, other studies have described ST131 isolates of human origin carrying CTX-M-15 and *qnrS* in IncN plasmids [50]. It is noteworthy that our study describes for the first time a poultry *E. coli* isolate clonal group O25b:H4-B2-ST131 producing CMY-2 with co-resistance to flouroquinolones (*qnrS*). Interestingly, this isolate presented an exceptional phenotype, since it was resistant to fluoroquinolones (MIC = 0.25) but was susceptible to nalidixic acid (MIC = 4) according to epidemiological cut-off values. This isolate was isolated from a healthy animal, corroborating the true zoonotic potential.

Up to date, the most common plasmids carrying cephalosporin resistance genes in *E. coli* isolated in poultry farms belong to the IncI1, IncFIB and IncN families [51]. However, in this study, cephalosporin resistance genes are mostly associated to IncK plasmids. Moreover, in some occasions two replicas of the same ESBL gene were harboured in two different plasmids. Interestingly, isolate O25b:H4-B2-ST131 harboured two plasmids from the same incompatibility group (IncN), in the same host cell. Therefore, it is probable that another unknown replicon is present and expressed in at least one of these plasmids.

In conclusion, this study demonstrated a very diverse population of multi-drug resistant *E. coli* in broiler farms containing a high number of virulence-associated genes. This is probably

the combination of virulence and resistance genes transferring from one strain to another via mobile genetic elements creating a multi-clonal scenario, together with *E. coli* strains acquiring the genes and becoming clonally successful. More epidemiological studies are necessary to identify clonal groups and resistance mechanisms with potential relevance to public health. Additionally, prudent use of antimicrobials in animal production should be implemented to reduce the burden of resistance organisms entering the food chain.

Acknowledgments

This work was partially supported by the grants AGL2011- 28836 and AGL2013-47852-R from the Ministerio de Economía y Competitividad (Gobierno de España) and grants CN2012/303 and EM2014/001 (Consellería de Cultura, Educación e Ordenación Universitaria, Xunta de Galicia and the European Regional Development Fund, ERDF).

MSG is a Ph.D. student registered with the Universidad Autónoma de Barcelona. Work from LMG is supported by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) and the European Social Fund.

Author Contributions

Conceived and designed the experiments: MSG LMG. Performed the experiments: MSG KCV GD SV AM NPC. Analyzed the data: MSG LMG JJGL JB. Contributed reagents/materials/analysis tools: RD NM IB JB. Wrote the paper: MSG LMG. Corrections and suggestions to the manuscript: JJGL JB.

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Houseflies (*Musca domestica*) as Vectors for Extended-Spectrum β-Lactamase-Producing *Escherichia coli* on Spanish Broiler Farms

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Flies may act as potential vectors for the spread of resistant bacteria to different environments. This study was intended to evaluate the presence of *Escherichia coli* strains resistant to cephalosporins in flies captured in the areas surrounding five broiler farms. Phenotypic and molecular characterization of the resistant population was performed by different methods: MIC determination, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and phylotyping. The presence of extended-spectrum beta-lactamase (ESBL) genes, their plasmid location, and the mobile genetic elements involved in their mobilization were studied. Additionally, the presence of 35 genes associated with virulence was evaluated. Out of 682 flies captured, 42 yielded ESBL-producing *E. coli*. Of these isolates, 23 contained $bla_{CTX-M-1}$, 18 contained $bla_{CTX-M-14}$, and 1 contained $bla_{CTX-M-9}$. ESBL genes were associated mainly with the presence of the IncI1 and IncFIB replicons. Additionally, all the strains were multiresistant, and five of them also harbored *qnrS*. Identical PFGE profiles were found for *E. coli* isolates obtained from flies at different sampling times, indicating a persistence of the same clones in the farm environment over months. According to their virulence genes, 81% of the isolates were considered avian-pathogenic *E. coli* (APEC) and 29% were considered extraintestinal pathogenic *E. coli* (ExPEC). The entrance of flies into broiler houses constitutes a considerable risk for colonization of broilers with multidrug-resistant *E. coli*. ESBLs in flies reflect the contamination status of the farm environment. Additionally, this study demonstrates the potential contribution of flies to the dissemination of virulence and resistance genes into different ecological niches.

Escherichia coli is a commensal bacterium commonly found in nature and in the lower intestine of warm-blooded organisms. However, some serotypes can cause enteric and extraintestinal infections in humans and animals (1). For instance, avian-pathogenic *E. coli* (APEC) is the major cause of colibacillosis in poultry production. It is a syndrome that causes respiratory infections associated with airsacculitis, pericarditis, and septicemia, resulting in a large economical burden for the poultry industry due to the loss of production and mortality (2). Hybridization studies detected APEC-specific DNA sequences presenting a high level of homology with DNA sequences of human extraintestinal pathogenic *E. coli* (ExPEC) strains (3). Both types have virulence determinants in common, suggesting that APEC could serve as a reservoir and a source of virulence for ExPEC (4). Moreover, it has been suggested that APEC strains are potential zoonotic pathogens (5).

Antimicrobials are the common treatment for avian colibacillosis caused by APEC. During the last years, increased resistance to frontline antimicrobials, such as fluoroquinolones and third-generation cephalosporins, has been reported for *E. coli* isolates from poultry (1). Additionally, the emergence of bacteria resistant to critically important antimicrobials is a major concern in human and veterinary medicine. The presence of isolates producing extended-spectrum beta-lactamases (ESBLs) and plasmid-mediated AmpC beta-lactamases among *E. coli* isolates from broilers has increased substantially in the last decade (6). Generally, the genes encoding beta-lactamases are located on plasmids, which can be transferred to other bacteria (7).

The persistence of *E. coli* in the environment has been described in the literature (8). However, the relevance of wildlife vectors in the persistence and spread of ESBL-producing *E. coli* in the farm environment has not been studied thoroughly (9, 10). In particular, flies are one of the most important vectors of human diseases worldwide (11). They have the capacity to horizontally transfer pathogens from different environments (12), posing a high risk to human health (13). Due to their movements, their capacity to fly long distances, and their attraction to decaying organic materials and food, houseflies amplify the risk of human exposure to foodborne pathogens (14–16). Moreover, they may also spread antibiotic resistance genes within microbial communities (17). The digestive tract of flies provides a suitable environment for the horizontal transfer of genes among bacteria, which contributes to the spread of resistance and virulence genes (18). Several studies have demonstrated that flies carry multidrug-resistant bacteria in hospital environments and have also demonstrated their role in the transmission of human pathogens within hospitals (17).

The objective of this study was to assess the potential contribution of flies to the spread of ESBL/AmpC-producing *E. coli* in the

Received 7 January 2015 **Accepted** 9 March 2015

Accepted manuscript posted online 20 March 2015

Citation Solà-Ginés M, González-López JJ, Cameron-Veas K, Piedra-Carrasco N, Cerdà-Cuéllar M, Migura-Garcia L. 2015. Houseflies (*Musca domestica*) as vectors for extended-spectrum β-lactamase-producing *Escherichia coli* on Spanish broiler farms. Appl Environ Microbiol 81:3604–3611. doi:10.1128/AEM.04252-14.

Editor: H. L. Drake

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doi:10.1128/AEM.04252-14

farm environment. For this purpose, isolates, resistant genes, and mobile genetic elements involved in the transmission of resistant genes were fully characterized. The genomic relationship among strains and the virulence content associated with APEC and ExPEC strains were also evaluated.

MATERIALS AND METHODS

Study design. The study was conducted in five broiler farms (farms 1 to 5) each one with one or two houses, located in the Catalonia region (Spain). Broiler house capacities ranged from 15,000 to 46,000 birds per house. Only farm 2 presented cats at the premises. Minimum and maximum distances between farms were 15 km and 200 km, respectively. From May to November 2012, each broiler farm was visited twice per rearing cycle to capture flies (6 to 8 visits per farm in total). Overall, 23 broiler flocks were reared in the 5 study houses during the study period. Fly sampling was performed when chickens were ~14 and 28 days old. At each visit, up to 50 flies were collected, always outside the same broiler houses (within a 10-m periphery). Overall, 682 flies were captured individually, placed into disposable sterile plastic bags, and transported refrigerated (to be kept alive) to the laboratory. Once at the laboratory, flies were anesthetized with CO_2, identified to the genus or species level, and subsequently processed for *Campylobacter* isolation (our unpublished data) and thereafter for cephalosporin-resistant *E. coli* isolation. Each individual fly was aseptically macerated in plastic bags with 2.5 ml Bolton broth (CM0983 with selective supplement [SR0183] and laked horse blood [SR0048]; Oxoid, Basingstoke, United Kingdom) and incubated at 42°C for 24 h in 2-ml screw-cap tubes. A 10-μl loop of broth was plated onto MacConkey agar (Oxoid, Basingstoke, United Kingdom) supplemented with ceftriaxone (1 mg/liter). Three lactose-positive colonies from each plate were selected and confirmed to be *E. coli* by PCR (19). Subsequently, one representative was selected for further studies.

Phylogeny, pulsed-field gel electrophoresis, and multilocus sequence typing. Isolates were discriminated into phylogenetic groups by PCR (phylogroup A, B1, B2, C, D, E, or F), as previously described by Clermont et al. (20, 21).

Pulsed-field gel electrophoresis (PFGE) was performed to analyze the genomic relatedness among *E. coli* isolates. PFGE of chromosomal DNA digested with the restriction enzyme XbaI was carried out according to PulseNet protocols (22). *Salmonella enterica* serovar Braenderup H9812 was used as a size marker. The results were analyzed by Fingerprinting II Informatix software (Applied Maths, Sint-Martens-Latem, Belgium). Isolates were considered to have a different pattern when at least one band difference was detected. The analysis of the bands generated was performed by using the Dice coefficient and unweighted-pair group method with arithmetic averages (optimization of 1.25% and position tolerance of 1.25%).

Multilocus sequence typing (MLST) was performed to determine the potential evolutionary relatedness between isolates. MLST was carried out by gene amplification and sequencing of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*), according to protocols and primers specified on the *E. coli* MLST website (http://mlst.ucc.ie/mlst/dbs/Ecoli) and as previously described (23). Sequences were analyzed with Vector NTI Advance 11 (InforMax, Inc., Bethesda, MD). The allelic profiles of the gene sequences and the sequence types (STs) were obtained via the electronic database at the *E. coli* MLST website.

Antimicrobial susceptibility testing. Disc diffusion was performed according to CLSI guidelines, using the following discs (Oxoid, Basingstoke, United Kingdom): cefoxitin at 30 mg; cefepime at 30 mg; ceftazidime at 30 mg; cefotaxime at 30 mg; cefotaxime-clavulanic acid at 30 and 10 mg, respectively; and ceftazidime-clavulanic acid at 30 and 10 mg, respectively. The synergies between cefotaxime and cefotaxime-clavulanic acid and between ceftazidime and ceftazidime-clavulanic acid were used as suggestive evidence of ESBL production; cefoxitin was used for the detection of AmpC-type beta-lactamase (24). Additionally, all isolates were tested for antimicrobial susceptibility using a MIC-based broth microdilution (VetMIC GN-mo; National Veterinary Institute, Uppsala, Sweden) for the following antimicrobial agents: ampicillin (1 to 128 mg/liter), cefotaxime (0.016 to 2 mg/liter), ceftazidime (0.25 to 16 mg/liter), nalidixic acid (1 to 128 mg/liter), ciprofloxacin (0.008 to 1 mg/liter), gentamicin (0.12 to 16 mg/liter), streptomycin (2 to 256 mg/liter), kanamycin (8 to 16 mg/liter), chloramphenicol (2 to 64 mg/liter), florfenicol (4 to 32 mg/liter), trimethoprim (1 to 128 mg/liter), sulfamethoxazole (8 to 1,024 mg/liter), tetracycline (1 to 128 mg/liter), and colistin (0.5 to 4 mg/liter). *E. coli* ATCC 25922 was used as a control strain. Isolates were considered to be wild-type (WT) or non-WT isolates based on epidemiological cutoff values according to EUCAST guidelines (http://www.eucast.org/) (25).

Resistance genes. All strains were tested by PCR for the presence of the bla_{CTX-M}, bla_{SHV}, bla_{TEM}, and bla_{CMY-2} genes as described previously by Hasman et al. (26). Sequencing of both strands of amplicons was performed. The presence of the *aac(6′)-Ib-cr*, *qnrA*, *qnrB*, *qnrS*, *qepA*, and *oqxAB* genes conferring resistance to fluoroquinolones was also assessed (27).

Plasmid DNA analysis. One isolate from each PFGE clonal cluster was selected for plasmid characterization. The presence of plasmid replicons HI1, HI2, I1, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIA, and K was assessed by PCR-based replicon typing methods described previously (28, 29), including screening for the IncR replicon (30). The detection of plasmids and sizing were carried out on all the isolates by PFGE of total DNA digested with S1 nuclease (31). Restriction fragments from S1-PFGE gels (i.e., PFGE gels digested with S1 nuclease) were transferred onto positively charged nylon membranes and hybridized with specific probes for $bla_{CTX-M-1}$ and $bla_{CTX-M-9}$ and with specific probes for each previously identified replicon.

Detection of virulence-associated genes. All 42 strains were tested for a pool of 35 virulence-associated genes (see Table 2), including 7 adhesins, 4 siderophores, 9 toxins, 8 capsule synthesis-associated genes or protectins, and 7 miscellaneous genes, by PCR using primers described previously (32, 33). The five virulence factors for ExPEC detection (*pap*, *sfa/foc*, *afa/dra*, *iutA*, and *kpsM* II) (34) together with the five potential APEC virulence genes (*iutA*, *hlyF*, *iss*, *iroN*, and *ompT*) (35) were included in the PCR analysis. Virulence scores were calculated as the sum of all virulence genes detected for each isolate; *pap*, *sfa/foc*, *clbB-clbN*, and *kpsM* II were counted only once, regardless of the number of elements or subunits identified (maximum possible score of 27).

Statistical analysis. Differences in the prevalences of phylogroups and STs between the distinct groups were determined by Fisher's exact test. The associations between groups were assessed by calculation of the odds ratio (OR) with 95% confidence intervals (CIs). The null hypothesis was rejected for data with P values of <0.05. Statistical analyses were performed by using GraphPad Prism, version 3.1, software (GraphPad Software, Inc., San Diego, CA). Virulence scores were compared by the Mann-Whitney U test.

RESULTS

During the course of the study, a total of 682 flies were captured from the environments surrounding five different broiler farms. The 42 ESBL producers were collected from farm 1 (9%; $n = 193$), farm 2 (3%; $n = 138$), farm 3 (15%; $n = 109$), and farm 4 (4%; $n = 134$). Finally, all flies collected from farm 5 ($n = 108$) were negative for ESBL-producing *E. coli*. Most of the fly species were classified as *Musca domestica* ($n = 615$), followed by *Ophyra* spp. ($n = 33$), *Stomoxys calcitrans* ($n = 15$), *Muscina stabulans* ($n = 7$), *Fannia canicularis* ($n = 6$), and others ($n = 6$). A total of 42 ESBL-producing *E. coli* strains were isolated mainly from *M. domestica* ($n = 41$), and 1 was isolated from *Muscina stabulans*.

PFGE, phylogeny, and MLST. XbaI PFGE analysis revealed a total of 29 different PFGE restriction profiles among the 42 *E. coli* isolates (Fig. 1). The number of fragments generated ranged from 14 to 21, and their sizes varied from 20 to 1,135 kb. In almost all

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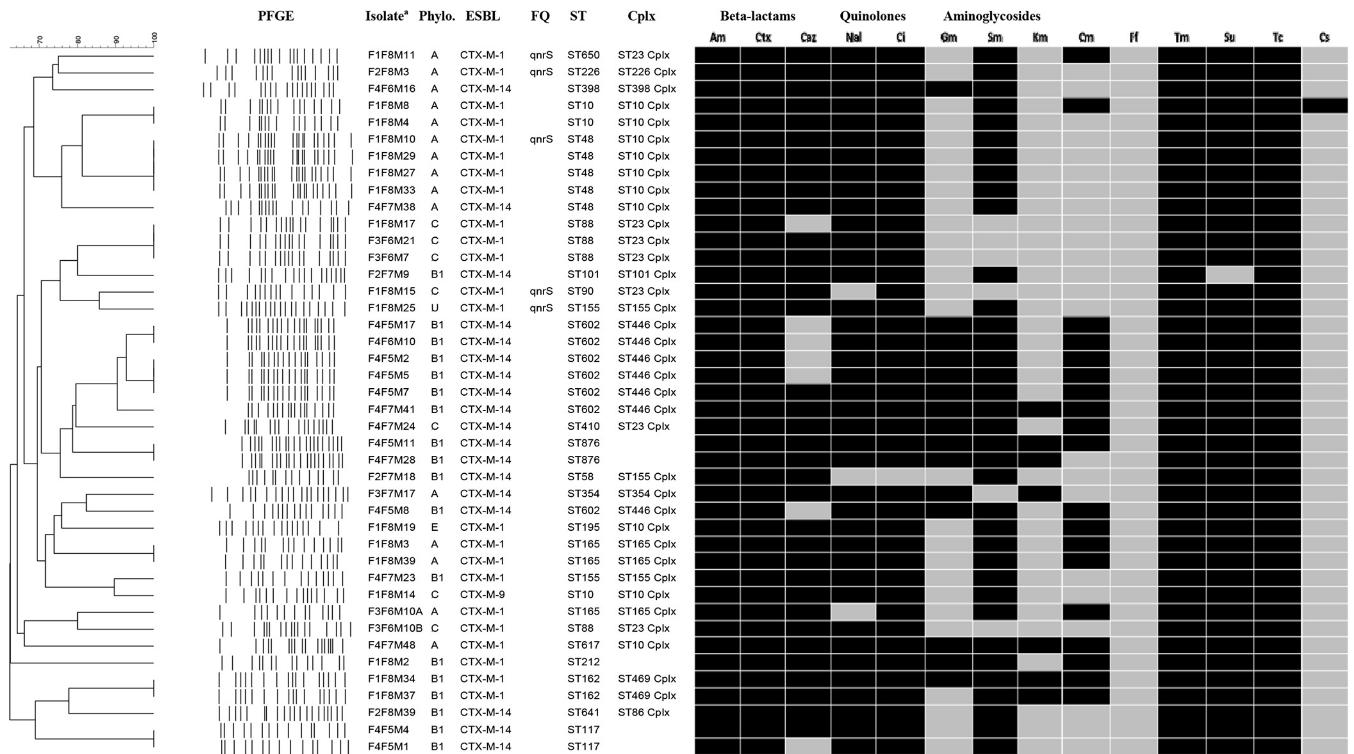

FIG 1 PFGE dendrogram illustrating the phenotypic and genotypic relationships of the strains, phylogenies, and cephalosporin resistance genes. PFGE, pulsed-field gel electrophoresis; Phylo., phylogroup; ESBL, extended-spectrum beta-lactamase gene; FQ, fluoroquinolone resistance genes; ST, sequence type; Cplx, clonal complex; U, unknown; Am, ampicillin (WT, ≤8 mg/liter); Ctx, cefotaxime (WT, ≤0.25 mg/liter); Caz, ceftazidime (WT, ≤0.5 mg/liter); Nal, nalidixic acid (WT, ≤16 mg/liter); Ci, ciprofloxacin (WT, ≤0.064 mg/liter); Gm, gentamicin (WT, ≤2 mg/liter); Sm, streptomycin (WT, ≤16 mg/liter); Km, kanamycin (WT, ≤8 mg/liter); Cm, chloramphenicol (WT, ≤16 mg/liter); Ff, florfenicol (WT, ≤16 mg/liter); Tm, trimethoprim (WT, ≤2 mg/liter); Su, sulfamethoxazole (WT, ≤64 mg/liter); Tc, tetracycline (WT, ≤8 mg/liter); Cs, colistin (WT, ≤2 mg/liter). [a], isolates were named based on the numbers assigned to the farm (F), flock (F), and fly (M).

cases, epidemiologically related isolates belonged to the same farm, except for three isolates (F1F8M17, F3F6M21, and F3F6M7) from farms 1 and 3 that presented identical fingerprints (Fig. 1).

Four different phylogroups were represented among the 42 isolates. Of these, 15 were of group A (36%), 18 were of group B1 (43%), 7 were of group C (17%), 1 was of group E (2%), and 1 was of an unknown group (2%) (Fig. 1).

MLST analyses identified 21 STs belonging to 11 different clonal complexes (Fig. 1). The most common clonal complex was the ST10 clonal complex ($n = 10$), containing four different ST types (ST10, ST48, ST195, and ST617), followed by the ST446 clonal complex ($n = 7$; all ST602) and the ST23 clonal complex ($n = 7$), comprising four different ST types (ST88, ST90, ST410, and ST650). Only two different complexes were represented by more than one phylogenetic group (phylogroups A and E for ST10 and phylogroups C and A for ST23).

Antimicrobial susceptibility testing and resistance genes. Disc diffusion demonstrated that all isolates presented the ESBL phenotype. All the strains were multiresistant (resistant to >3 antimicrobial families). Furthermore, 79% of the isolates had a non-WT phenotype for more than eight antimicrobials. MIC determination confirmed that all strains had a non-WT phenotype for cephalosporins (100% resistance to cefotaxime and 83% resistance to ceftazidime), with 23 isolates yielding amplicons for $bla_{CTX-M-1}$, 18 yielding amplicons for $bla_{CTX-M-14}$, and 1 yielding amplicons for $bla_{CTX-M-9}$. Out of the 42 isolates, 33 harbored the bla_{TEM} gene. None of the isolates were positive for bla_{SHV} or bla_{CMY-2}. In addition, 93% of the isolates had a non-WT phenotype for nalidixic acid, and 98% had a non-WT phenotype for ciprofloxacin. The presence of *qnrS* genes was detected in five isolates obtained from farms 1 and 2. The genes *aac(6′)-Ib-cr*, *qnrA*, *qnrB*, *qepA*, and *oqxAB* were not found in this collection. Additionally, 100% of the strains had a non-WT phenotype for ampicillin, trimethoprim, and tetracycline; 98% had a non-WT phenotype for sulfamethoxazole; 86% had a non-WT phenotype for streptomycin; 45% had a non-WT phenotype for chloramphenicol; 36% had a non-WT phenotype for gentamicin; 17% had a non-WT phenotype for kanamycin; and 2% had a non-WT phenotype for colistin. All isolates had a non-WT phenotype for florfenicol.

Localization of bla_{CTX-M}. The replicons IncFIB and IncI1 were detected in the majority of the isolates (90% and 83%, respectively), being associated or not with any of the CTX-M genes. However, IncP, IncK, IncY, Inc FIA, IncHI1, IncH12, and IncN were also detected (Table 1).

All $bla_{CTX-M-1}$ isolates hybridized with a plasmid of ~110 kb containing an IncI1 replicon, except for the following exceptions: two isolates (F1F8M15 and F3F7M23) contained both IncI1 and IncFIB in a 120-kb plasmid, and one extra isolate (F1F8M25) contained IncI1 together with IncFIB in two plasmids of 120 and 190 kb (Table 1). Additionally, isolate F4F7M48 carried a second copy of the gene on a large plasmid of 300 kb (Table 1). The IncI1 and IncFIB replicons were identified on $bla_{CTX-M-14}$-carrying plasmids

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TABLE 1 Identification and characterization of the plasmid locations of $bla_{CTX-M-1}$, $bla_{CTX-M-14}$, and $bla_{CTX-M-9}$ among 29 CTX-M-producing *E. coli* isolates[e]

Gene and isolate[a]	ST	Clonal complex	Replicon(s)[b]	Plasmid[c]	Inc type(s)[d]	Plasmid size (kb)
$bla_{CTX-M-1}$						
F1F8M8	10	10	I1, FIB	pST10-1	I1	110
F1F8M10	48	10	I1, FIB	pST48-1	I1	110
F1F8M17	88	23	I1, FIB, P	pST88	I1	110
F1F8M15	90	23	I1, FIB	pST90	I1, FIB	120
F1F8M25	155	155	I1, FIB, P	pST155-1	I1, FIB	120
				pST155-2	I1, FIB	190
F1F8M34	162	469	I1, FIB	pST162	I1	110
F1F8M3	165	165	I1, FIB, P	pST165-1	I1	110
F1F8M19	195	10	I1, FIB, P, K	pST195	I1	110
F1F8M2	212		I1, FIB, Y, P	pST212	I1	110
F1F8M11	650	23	I1, FIB, P	pST650	I1	110
F3F6M10B	88	23	I1, FIB, P	pST88	I1	110
F3F6M10A	165	165	I1	pST165-2	I1	120
F4F7M23	155	155	I1, FIB	pST155-3	I1, FIB	110
F4F7M48	617	10	I1, FIA, FIB	pST617-1		50
				pST617-2		300
F2F8M3	226	226		pST226		50
$bla_{CTX-M-14}$						
F4F7M38	48	10	I1, FIB	pST48-2	I1	90
F4F5M4	117		FIB	pST117	FIB	145
F4F6M16	398	398	FIB	pST398		75
F4F7M24	410	23	HI1, HI2, I1, FIB, P	pST410	I1	110
F4F5M8	602	446	HI1, HI2, I1, FIB, P	pST602-1	I1	110
F4F5M2	602	446	HI1, HI2, I1, FIB, P	pST602-1	I1	110
F4F5M17	602	446	HI1, HI2, I1, FIB, P	pST602-1	I1	110
F4F7M41	602	446	HI1, HI2, I1, FIB, P	pST602-2	I1	90
F4F5M11	876		HI1, HI2, I1, FIB, P	pST876		90
F2F7M18	58	155	I1, FIB	pST58		90
F2F7M9	101	101	FIB	pST101		90
F2F8M39	641	86	I1, N	pST641	I1	100
F3F7M17	354	354	FIA, FIB	pST602-2	FIB	90
$bla_{CTX-M-9}$						
F1F8M14	10	10	I1, FIB, P	pST10-2	I1, FIB, P	120

[a] Isolates were named based on the numbers assigned to the farm (F), flock (F), and fly (M).
[b] Replicon identifications are based on positive amplifications from the PCR-based replicon typing method.
[c] Plasmids were named based on the source strain sequence type and plasmid size.
[d] In all *E. coli* isolates, replicons from plasmids containing the different *bla* genes were identified by PCR-positive amplification and by Southern hybridization of the S1-digested fragments.
[e] One representative for each PFGE cluster is shown. p(ST number), plasmid location; Inc, identified replicon.

of different sizes. The isolate carrying $bla_{CTX-M-9}$ exhibited three different replicons (IncI1, IncFIB, and IncP) on the same plasmid (Table 1).

Detection of virulence genes. The prevalences of 35 virulence-associated genes, including the genes associated with APEC and ExPEC, are illustrated in Table 2. The virulence genes detected with the highest prevalences were *fimH* (100%), *traT* (88%), *clbB* (76%), and *cvaC* (48%). The presence of *astA* (29%), *tsh* (29%), *papEF* (26%), and *kpsM* III (24%) was confirmed for an intermediate percentage of the isolates. In contrast, the presence of *fyuA* (14%), *ireA* (14%), *papC* (10%), *papA* (7%), *papG* (7%), *kpsM* II (5%), *sfa/focDE* (2%), *kpsM* II-K2 (2%), *kpsM* II-K5 (2%), *ibeA* (2%), *malX* (2%), *usp* (2%), and $fliC_{H7}$ (2%) was confirmed for a lower number of strains. None of the isolates were positive for *afa/draBC*, *cnf1*, *cdtB*, *sat*, *hlyD*, stx_1, stx_2, *kpsM* II-K1, and *clbN*. A total of 12 (29%) isolates from this study were identified as ExPEC according to the ExPEC definition. Additionally, 79%, 88%, 88%, 76%, and 86% of the strains yielded amplicons for *iroN*, *ompT*, *hlyF*, *iutA*, and *iss*, respectively; these genes have been described as the minimal predictors of APEC virulence. A total of 34 (81%) isolates were considered APEC, since they harbored between 4 and 5 of these genes. Moreover, 11 (26%) of the isolates were considered ExPEC and APEC at the same time.

Statistical analysis. No significant differences in the numbers of virulence genes found between phylogroups were observed. Phylogroups A, B1, and C exhibited virulence scores of between 7.8 and 9.7 (Table 2). Phylogroup C/ST23 clonal complex (mean, 9.8; range, 6 to 11), phylogroup A/ST10 clonal complex (mean, 10.6; range, 10 to 12), and phylogroup B1/ST446 complex (mean, 9.6; range, 9 to 12) isolates exhibited similar virulence scores but different gene contents (data not shown). Significant differences in virulence scores between phylogroup A/ST10 clonal complex isolates and phylogroup A/non-ST10 clonal complex isolates were

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TABLE 2 Distribution of virulence genes among the 42 isolates, the largest phylogenetic groups, and relevant ST clonal complexes[b]

Virulence gene or *E. coli* type	Product(s)	No. (%) of isolates: Total (n = 42)	Phylogroup A (n = 15)	Phylogroup B1 (n = 18)	Phylogroup C (n = 7)	Phylogroup A/ST10 clonal complex (n = 8)	Phylogroup A/non-ST10 clonal complex (n = 7)	*P* value[a] for phylogroup A/ST10 clonal complex vs phylogroup A/non-ST10 clonal complex
Virulence genes								
Adhesins								
fimH	D-Mannose-specific adhesin of type 1 fimbriae	42 (100)	15 (100)	18 (100)	7 (100)	8 (100)	7 (100)	
papEF	Pilus associated with pyelonephritis (P fimbriae)	11 (26)	5 (33)	4 (22)	0	5 (63)	0	0.0256
papG	P fimbriae carrying Gal(α1-4) Gal-specific PapG adhesin at its distal end	3 (7)	0	2 (11)	0	0	0	
papA	Major structural subunit of the P fimbrial shaft	3 (7)	0	3 (17)	0	0	0	
papC	Pilus assembly; central region of the *pap* operon	4 (10)	0	3 (17)	0	0	0	
sfa/focDE	Central region of the *sfa* and *foc* operons	1 (2)	0	1 (6)	0	0	0	
afa/draBC	Dr antigen-specific adhesin operons (AFA, Dr, F1845)	0	0	0	0	0	0	
Toxins								
cnf1	Cytotoxic necrotizing factor 1	0	0	0	0	0	0	
cdtB	Cytolethal distending toxin	0	0	0	0	0	0	
sat	Secreted autotransporter	0	0	0	0	0	0	
hlyD	Alpha-hemolysin	0	0	0	0	0	0	
hlyF	Hemolysin F	37 (88)	12 (80)	16 (89)	7 (100)	8 (100)	4 (57)	
astA	Enteroaggregative *E. coli* heat-stable toxin (EAST1)	12 (29)	7 (47)	4 (22)	0	7 (86)	0	0.0014
tsh	Temp-sensitive hemagglutinin-serine protease	12 (29)	2 (13)	4 (22)	6 (86)	2 (25)	0	
stx_1	Shiga toxin 1	0	0	0	0	0	0	
stx_2	Shiga toxin 2	0	0	0	0	0	0	
Siderophores								
fyuA	*Yersinia* siderophore receptor (ferric yersiniabactin uptake)	6 (14)	0	3 (17)	3 (43)	0	0	
iutA	Ferric aerobactin receptor (iron uptake; transport)	32 (76)	9 (60)	15 (83)	6 (86)	8 (100)	1 (14)	0.0014
iroN	Novel catecholate siderophore receptor	33 (79)	9 (60)	15 (83)	7 (100)	8 (100)	1 (14)	0.0014
ireA	Iron-regulated element (novel siderophore receptor)	6 (14)	0	5 (28)	0	0	0	
Protectins								
kpsM II	Group II capsule	2 (5)	1 (7)	0	0	0	1 (14)	
kpsM II-K2	K2 subgroup II capsule	1 (2)	1 (7)	0	0	0	1 (14)	
kpsM II-K5	K5 subgroup II capsule	1 (2)	0	1 (6)	0	0	0	
kpsM II-K1	K1 subgroup II capsule	0	0	0	0	0	0	
kpsM III	Group III capsule	10 (24)	7 (47)	2 (11)	0	7 (86)	0	0.0014
cvaC	Colicin V from serum resistance-associated plasmids	20 (48)	1 (7)	12 (67)	6 (86)	1 (13)	0	
iss	Increased serum survival (outer membrane protein)	36 (86)	10 (67)	17 (94)	7 (100)	8 (100)	2 (29)	0.0070
traT	Surface exclusion, serum survival (outer membrane protein)	37 (88)	12 (80)	17 (94)	6 (86)	8 (100)	4 (57)	
Miscellaneous								
ompT	Outer membrane protein (protease) T	37 (88)	12 (80)	16 (89)	7 (100)	8 (100)	4 (57)	
ibeA	Invasion of brain endothelium	1 (2)	1 (7)	0	0	0	1 (14)	
malX	Pathogenicity-associated island marker	1 (2)	1 (7)	0	0	1 (13)	0	
usp	Uropathogenic-specific protein (bacteriocin)	1 (2)	1 (7)	0	0	0	1 (14)	
clbB	Hybrid peptide-polyketide synthase (colibactin)	32 (76)	12 (80)	13 (72)	6 (86)	6 (75)	6 (86)	
clbN	Nonribosomal synthetase (colibactin)	0	0	0	0	0	0	
$fliC_{H7}$	H7 flagellin variant	1 (2)	0	1 (6)	0	0	0	
E. coli types								
APEC		34 (81)	9 (60)	17 (94)	7 (100)	8 (100)	1 (14)	0.0014
ExPEC		12 (29)	6 (40)	4 (22)	0	5 (63)	1 (14)	

[a] *P* values (by Fisher's exact test) are shown where the *P* value was <0.05.

[b] The mean virulence scores (number of virulence genes detected), adjusted for multiple detections of the *pap*, *sfa/foc*, *clbB-clbN*, and *kpsM* II operons, were 8.8 (range, 1 to 12) for the total number of isolates and 7.8 (1 to 12) for phylogroup A, 9.1 (4 to 12) for phylogroup B1, 9.7 (6 to 11) for phylogroup C, 10.6 (10 to 12) for phylogroup A/ST10 clonal complex, and 4.6 (1 to 7) for phylogroup A/non-ST10 clonal complex isolates. Virulence scores were compared by use of the Mann-Whitney U test ($P < 0.0001$).

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observed ($P < 0.0001$). The virulence factors that were significantly different were characteristic of APEC (*iss*, *iutA*, *iroN*, and *astA*) and ExPEC (*papEF* and *kpsM* III) (Table 2).

DISCUSSION

M. domestica is an arthropod distributed worldwide and the most abundant fly species in animal production and food at homes and restaurants. Flies are suspected reservoirs and vectors for human and animal pathogens due to their contact with animal manure, food, and humans. They can pick up bacteria present in those sites and transport them to the kitchen (36). Some studies have suggested that flies can also play an important role in the dissemination of antimicrobial resistance genes within the bacterial community (37, 38). In our study, the presence of multidrug-resistant *E. coli* isolated from flies, and in particular ESBL-producing *E. coli*, demonstrates the capacity of houseflies to disseminate and transport resistance genes located in mobile genetic elements. Additionally, five of the isolates also harbored plasmid-mediated quinolone resistance genes. *qnrS* genes were previously associated with the same plasmids harboring ESBL genes (39). The continuous increase in the prevalence of antimicrobial-resistant bacteria has been associated with the use of these drugs to treat human and animal infections, and the presence of ESBL-producing *E. coli* in flies suggests that animals and the farm environment are colonized and inhabited by these microorganisms. Flies are a reservoir of resistant bacteria and can contribute to the spread of resistance genes between different ecological niches.

Some studies have suggested that there is a relationship between different *E. coli* phylogenetic groups and the virulence capabilities of the strains (38, 40). Commensal isolates belong mainly to phylogenetic groups A, B1, and C (41, 42). In contrast, the most virulent phylogroups described in the literature are phylogroup B2 followed by phylogroup D, which are mainly responsible for extraintestinal infections (38, 40). None of the ESBL-producing isolates from this study belonged to the B2 and D phylogroups; most of them belonged to phylogroups A, B1, and C and possessed quite high virulence scores. Furthermore, the ESBL-producing *E. coli* A/ST10 clonal complex isolates from this study had significantly higher virulence scores than isolates of other STs from the same phylogroup. Similar results were obtained in other studies, where phylogroup A/ST10 isolates of APEC and ExPEC origins were described as emerging pathogens, suggesting that this ST complex may have relevant zoonotic potential (43, 44).

PFGE results demonstrated the same clonal groups in the same farms, suggesting dissemination of epidemiologically related clones within farm environments. An exception was the three strains from farm 1 and 3 belonging to the same PFGE. These farms were about 25 km apart. This observation would reinforce what has been previously reported, that flies can travel long distances, spreading resistant bacteria (14–16). Additionally, identical fingerprints have been recovered from different flies belonging to the same farm at different time points, including different broiler cycles, demonstrating the capacity of these bacteria to survive and persist in the environment for long periods of time.

This study also demonstrated the presence of *E. coli* isolates with virulence-associated genes characteristic of both APEC (81%) and ExPEC (29%) and the capacity of flies to transport them. Some of these virulence genes are also associated with mobile genetic elements, highlighting the relevance of flies in the transmission of virulence determinants in broiler farms and hospital settings (17).

In the present study, we found that $bla_{CTX-M-1}$ and $bla_{CTX-M-14}$ are the most prevalent ESBL genes detected in *E. coli* isolates obtained from flies captured in the areas surrounding broiler farms (55% and 43%, respectively). This result is in agreement with data from previous studies, which demonstrated that $bla_{CTX-M-1}$ is one of the most prevalent ESBL genes detected in *Enterobacteriaceae* of broiler origin (45–48). Also in line with studies performed on broiler farms, the most common replicons encountered in this study were IncI1 and IncFIB (49, 50). Moreover, we have found five isolates with the same ESBL gene harbored in two different plasmids. Having two or more copies of a resistance mechanism in different locations would ensure the maintenance and persistence of these genes even if selective pressure enforces the loss of one of these copies.

In conclusion, this study has demonstrated a very diverse population of multidrug-resistant *E. coli* recovered from flies at different broiler farms. ESBL-producing *E. coli* in flies reflects the colonization status of the farm environment. Flies are probably not the source but the result of the colonization of animals. These isolates contained a high number of virulence-associated genes and ESBL genes, which could be easily introduced and disseminated into farms through the flies and subsequently could potentially colonize animals. Additional biosecurity measures, aimed at blocking or reducing the entrance of flies into broiler houses, should be undertaken. Otherwise, zoonosis control and antimicrobial resistance reduction may be frustrated. Flies are also contributing to pathogen evolution since the transfer of resistance- and virulence-associated genes between different strains could be facilitated through flies.

ACKNOWLEDGMENTS

This work was partially supported by projects AGL2011-28836 from the Ministerio de Economia y Competitividad of Spain, RTA2009-00117-00-00 from INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain), and CamCon (*Campylobacter* Control—Novel Approaches in Primary Poultry Production; funded by the European Community's Seventh Framework Programme, FP7/2007-2013, under grant agreement no. 244547).

We acknowledge the support received from farmers and poultry companies participating in the study as well as Marta Verdun and Sandra Talavera (CReSA) for their technical assistance in the identification of flies to the species level.

M.S.-G. is a Ph.D. student registered with the Universidad Autónoma de Barcelona.

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Impact of the Use of β-Lactam Antimicrobials on the Emergence of *Escherichia coli* Isolates Resistant to Cephalosporins under Standard Pig-Rearing Conditions

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The aim of this study was to evaluate if the treatments with ceftiofur and amoxicillin are risk factors for the emergence of cephalosporin resistant (CR) *E. coli* in a pig farm during the rearing period. One hundred 7-day-old piglets were divided into two groups, a control ($n = 50$) group and a group parenterally treated with ceftiofur ($n = 50$). During the fattening period, both groups were subdivided in two. A second treatment with amoxicillin was administered in feed to two of the four groups, as follows: group 1 (untreated, $n = 20$), group 2 (treated with amoxicillin, $n = 26$), group 3 (treated with ceftiofur, $n = 20$), and group 4 (treated with ceftiofur and amoxicillin, $n = 26$). During treatment with ceftiofur, fecal samples were collected before treatment (day 0) and at days 2, 7, 14, 21, and 42 posttreatment, whereas with amoxicillin, the sampling was extended 73 days posttreatment. CR *E. coli* bacteria were selected on MacConkey agar with ceftriaxone (1 mg/liter). Pulsed-field gel electrophoresis (PFGE), MICs of 14 antimicrobials, the presence of cephalosporin resistance genes, and replicon typing of plasmids were analyzed. Both treatments generated an increase in the prevalence of CR *E. coli*, which was statistically significant in the treated groups. Resistance diminished after treatment. A total of 47 CR *E. coli* isolates were recovered during the study period; of these, 15 contained $bla_{CTX-M-1}$, 10 contained $bla_{CTX-M-14}$, 4 contained $bla_{CTX-M-9}$, 2 contained $bla_{CTX-M-15}$, and 5 contained bla_{SHV-12}. The treatment with ceftiofur and amoxicillin was associated with the emergence of CR *E. coli* during the course of the treatment. However, by the time of finishing, CR *E. coli* bacteria were not recovered from the animals.

During the last decade, resistance to extended-spectrum beta-lactams (ESBLs), especially third- and fourth-generation cephalosporins and penems, has raised the concern of the scientific community. The World Health Organization has defined third- and fourth-generation cephalosporins as being "critically important" for use in humans (1) since the increased presence of resistance to these antimicrobials could seriously compromise the treatment of some life-threatening infections, including bacteremia and meningitis.

A third-generation cephalosporin, ceftiofur, and a fourth-generation cephalosporin, cefquinome, have been developed strictly for veterinary use (2). Ceftiofur is widely used in many different food animals to treat respiratory diseases. Cefquinome can also be used for the treatment of mastitis metritis agalaxia syndrome in sows, exudative epidermitis, and meningitis (3). The systemic use in food animals of cephalosporins that could potentially select for resistant organisms is worrisome due to the role that food-producing animals may play in the spread of extended-spectrum cephalosporinases into the community.

Previous studies have demonstrated a statistically significant association between the use of ceftiofur and reduced susceptibility to third-generation cephalosporins in *Escherichia coli* (4, 5). However, these studies did not find an association between ceftiofur usage and the presence of ESBL genes (bla_{CTX-M}), and, more importantly, none of these studies has examined other drug use practices that can cross- or coselect for cephalosporin resistance. To our understanding, there is a lack of comprehensive studies performed under standard pig-rearing conditions analyzing the presence and factors that can contribute to both the emergence and the increase in occurrence of cephalosporin-resistant (CR) *E. coli* in pig farms.

For this reason, this study intends to evaluate if the treatments with two different beta-lactams, ceftiofur and amoxicillin, are risk factors associated with the emergence of CR *E. coli* during two stages (preweaning-growing and finishing) of the rearing period and assess if there is enough selective pressure to maintain resistant strains during the lifetime of the animals.

MATERIALS AND METHODS

Study design. This study was conducted on a conventional commercial pig farm in the northeast of Spain. During the 6 months previous to the study, the site remained depopulated, and it was cleaned and disinfected using standard operation procedures under field conditions. Sixty-eight sows were housed in a climate-controlled house, and fecal samples were

Received 3 December 2014 **Accepted** 19 December 2014
Accepted manuscript posted online 29 December 2014
Citation Cameron-Veas K, Solà-Ginés M, Moreno MA, Fraile L, Migura-Garcia L. 2015. Impact of the use of β-lactam antimicrobials on the emergence of *Escherichia coli* isolates resistant to cephalosporins under standard pig-rearing conditions. Appl Environ Microbiol 81:1782–1787. doi:10.1128/AEM.03916-14.
Editor: H. Goodrich-Blair
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03916-14.
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doi:10.1128/AEM.03916-14

TABLE 1 Results obtained during the visits after treatment with ceftiofur

Sampling day[a]	Age (days)	No. of positive animals by group (%)	
		Control ($n = 50$)[b]	Treated ($n = 50$)
0	6–8	5 (10)	7 (12)
2	8–10	5 (10)	11 (26)
7	13–15	1 (2)	0
14	20–22	1 (2)	5 (8)
21	27–29	0	0
41	47–49	0	0

[a] Sampling on day 0 was performed prior to injecting the animals with ceftiofur.
[b] Three animals from the control group died after 7 days of treatment, decreasing the size of the group to 47 animals.

TABLE 2 Results obtained during the course of the study after treatment with amoxicillin

Sampling day[a]	Age (days)	No. of positive animals (%) in[b]:			
		Group 1 ($n = 20$)	Group 2 ($n = 26$)	Group 3 ($n = 20$)	Group 4 ($n = 26$)
0	70	0	0	0	0
2	72	0	2 (8)	0	0
7	77	0	7 (27)	0	0
14	84	0	1 (4)	0	0
21	115	0	0	0	1 (4)
45	138	0	1 (4)	0	0
73	155	0	0	0	0

[a] Sampling in day 0 was performed just before the beginning of the treatment.
[b] Group 1, untreated with antimicrobials; group 2, untreated with ceftiofur and treated with amoxicillin; group 3, treated with ceftiofur and not treated with amoxicillin; group 4, treated with ceftiofur and with amoxicillin.

collected to examine the presence of CR *E. coli*. After farrowing, a total of 100 7-day-old piglets from 10 different sows were spatially divided into two groups: untreated controls ($n = 50$) and animals parenterally treated ($n = 50$) with ceftiofur (5 mg/kg of body weight in one shot) according to the product characteristics of a commercial preparation (Naxcel; Zoetis SLU). Three animals from the control group died of noninfectious causes during the course of the study. Fecal samples were taken manually from the rectum of piglets on six occasions: before treatment (day 0) and at days 2, 7, 14, 21, and 42 posttreatment (Table 1).

During the fattening period (day 70), each of the original two groups was subdivided into two (Table 2). A treatment with amoxicillin (Maymoxi; Laboratorios Maymó) was administered in feed for 14 days to two of the four resulting groups (10 mg/kg of body weight/day), consisting of the following: group 1, untreated control group, or animals that did not receive any treatment with beta-lactams ($n = 20$); group 2, animals orally treated with amoxicillin during finishing ($n = 26$); group 3, animals parenterally treated with ceftiofur during preweaning ($n = 20$); and group 4, animals treated with ceftiofur and amoxicillin ($n = 26$). The four groups remained spatially separated until their departure to the abattoir. Fecal samples were taken from all animals before administration of amoxicillin (day 0) and on days 2, 7, 14, 21, 45, and 73 posttreatment. A final sampling was performed at the time of slaughter. During the course of the study, farm biosecurity was extreme. Animals of different groups were spatially separated in designated pens to avoid contact. Overshoes were used by investigators and replaced at the entrance of each pen. Sampling was always initiated from the control group to the treated group to minimize transmission of resistant bacteria from pen to pen.

The study was performed in a commercial farm where the treatments, housing, and husbandry conditions conformed to the European Union (EU) Guidelines. In particular, the medicinal product used in this study (Naxcel) is EU registered (EU/2/05/053/001), and it was used according to veterinary rules without any additional requirement. Thus, it was not necessary to comply with additional ethical standards or approvals to carry out this experimental work since it did not require any invasive procedures (only collection of fecal samples) or management other than the field standard protocols set by the company.

***E. coli* isolation and identification.** Fecal samples were transported to the laboratory at 4°C on the same day of sampling. During the first two visits to the farm, a total of 268 fecal samples were collected from the sows ($n = 68$) and the piglets ($n = 200$), and a comparative study of isolation methods was performed. For each sample, direct plating of a loopful of homogenized feces onto MacConkey agar with ceftriaxone (1 mg/liter) was carried out in parallel to the following enrichment method. One gram of feces was suspended in 10 ml of MacConkey broth supplemented with ceftriaxone (1 mg/liter). After overnight enrichment at 37°C, 10 μl was plated onto MacConkey agar with ceftriaxone (1 mg/liter). Three colonies for each plate were stored, and one was confirmed as *E. coli* by Vitek-2 (bioMérieux) and further characterized.

PFGE and phylotyping. To assess the clonality of the isolates and their epidemiological relatedness, all isolates were analyzed for genetic relatedness by pulsed-field gel electrophoresis (PFGE) using XbaI according to the CDC PulseNet protocol (6). The *Salmonella* Braenderup H9812 strain was used as molecular standard. PFGE profiles were compared using Fingerprinting II Informatixe software (Applied Maths, Sint-Martens-Latem, Belgium). Isolates were considered to have a unique pattern when at least one band difference was detected. The analysis of the bands generated was performed using the Dice coefficient and unweighted pair group method with arithmetic averages (optimization of 1.5% and position tolerance of 1.5%).

The isolates were discriminated in phylogenetic groups (A, B1, B2, C, D, and E) according to the method previously described by Clermont et al. (7, 8).

Antimicrobial susceptibility testing. Disc diffusion was performed according to CLSI guidelines using the following discs (Oxoid, United Kingdom): cefoxitin, 30 mg; cefepime, 30 mg; ceftazidime, 30 mg; cefotaxime, 30 mg; cefotaxime-clavulanic acid, 30 plus 10 mg (30/10 mg), respectively; and ceftazidime-clavulanic acid, 30/10 mg, respectively. The disc combinations of cefotaxime and cefotaxime-clavulanic acid and of ceftazidime and ceftazidime-clavulanic acid were used for the identification of ESBLs; cefoxitin was used for the detection of *ampC*-type beta-lactamase (9). MICs of ampicillin, ciprofloxacin, nalidixic acid, gentamicin, streptomycin, tetracycline, florfenicol, colistin sulfate, sulfamethoxazole, trimethoprim, chloramphenicol, kanamycin, cefotaxime, and ceftazidime were determined by microdilution methods (VetMIC GN-mo; National Veterinary Institute, Uppsala, Sweden). Results were interpreted as epidemiological cutoff values following EUCAST recommendations (http://www.eucast.org/).

Detection of resistance genes. Resistance to third-generation cephalosporins was analyzed by PCR for the presence of the bla_{TEM}, bla_{CTX}, bla_{CMY-1}, bla_{CMY-2}, and bla_{SHV} genes as described previously (10). Detection of plasmid-mediated AmpC beta-lactamase genes was assessed by multiplex PCR (11). Sequence analysis was performed using Vector NTI Advance, version 11 (InforMax, Inc., Bethesda, MD). The amplified nucleotide sequences were compared to previously described sequences obtained from public databases (www.ncbi.nlm.nih.gov and http://www.lahey.org/Studies/).

Mating experiments and plasmid characterization. Filter mating experiments were performed to assess the capacity of the plasmids to conjugate. For this analysis, 14 isolates containing ESBL genes were selected. They comprised representative isolates from five PFGE clusters and nine PFGE types. Mating assays were performed as described elsewhere (12), using the isolates as donors and rifampin-resistant *E. coli* HB101 as a recipient. Transconjugants were selected on LB agar plates containing rifampin (50 mg/liter) and ceftriaxone (1 mg/liter) and were confirmed by PFGE.

Plasmidic DNA was purified from these 14 wild-type (WT) isolates and later from transformants using a Qiagen Plasmid Midi kit (Qiagen,

Hilden, Germany) according to the manufacturer's recommendations. Plasmids were introduced to electrocompetent plasmid-free *E. coli* cells by electroporation. Transformants were selected in brain heart infusion agar supplemented with ceftriaxone (1 mg/liter), and PCR for confirmation of the cephalosporin-resistant genes was performed. The presence of a unique plasmid in the transformants and their sizes were determined using S1 nuclease digestion followed by PFGE (S1-PFGE) (13). Finally, plasmids were classified by PCR-based replicon typing (14). Additionally, susceptibility testing was performed in all transformants to assess transferability of resistance genes unrelated to cephalosporins.

RESULTS

Emergence of cephalosporin resistance during treatment. In the first visit 168 samples were obtained (100 from piglets and 68 from sows). None of the samples was positive for CR *E. coli* by direct plating, in contrast with 11 positive piglets obtained with enrichment methods. Similar results (8 positive piglets versus 16, respectively) were obtained in the second visit ($n = 100$); furthermore, the 8 positive samples obtained by direct plating were also detected by the enrichment method. These results convinced the authors to continue the study using only the enrichment methodology.

All 68 sows were negative for CR *E. coli*. However, before administration of ceftiofur, five and seven of the 7-day-old piglets among the control and the treated groups, respectively, yielded CR *E. coli* (Table 1). During this first treatment, a total of 12 (4.1%) and 23 (8%) CR *E. coli* strains were isolated from the control ($n = 288$ samples) and the treated ($n = 300$ samples) groups, respectively. The difference in the proportion of CR *E. coli* recovered in the two groups was statistically significant ($P = 0.04$). The highest percentage of samples positive for CR *E. coli* was obtained within the treated group (22%) at 48 h posttreatment, showing a statistical tendency ($P = 0.1$) compared to the corresponding figure (10%) of the control group.

A total of 552 fecal swabs were collected during the second part of the study when animals were treated with amoxicillin in feed (Table 2). Previous to the treatment, all animals were negative for CR *E. coli*. CR *E. coli* isolates were recovered from group 2 (treated only with amoxicillin) after 2 (two isolates), 7 (seven isolates), 14 (one isolate), and 45 (one isolate) days posttreatment. One extra isolate was obtained from group 4 (treated with ceftiofur and amoxicillin) after 21 days posttreatment. No other positive samples were obtained in the rest of the groups during the study period. The highest percentage of samples positive for CR *E. coli* (27%) was obtained after 7 days of amoxicillin treatment within the group treated with amoxicillin and with no history of ceftiofur use. Significant differences were observed (Fisher test, $P = 0.02$) between the proportion of CR *E. coli* isolated from animals treated with amoxicillin and the rest of the groups after 7 days of treatment. By the finishing stage, all animals were negative for CR *E. coli*.

PFGE and phylogenetic analysis. Electrophoresis of XbaI-digested genomic DNA from the 47 CR *E. coli* isolates revealed 22 different profiles (Fig. 1). XbaI profiles typically had 14 to 21 restriction fragments between 20 and 1,135 kb (Fig. 1). Indistinguishable fingerprints were present in isolates from different animals and also in isolates obtained from the same animal at different sampling times (see Table S1 in the supplemental material), indicating the persistence of clones during the course of the treatment. None of the clones obtained during the treatment with ceftiofur was recovered during treatment with amoxicillin. Additionally, 10 of 12 isolates recovered during amoxicillin treatment presented identical PFGE patterns. A total of 66%, 25%, 4%, and 4% belonged to phylogroups A, B1, C, and E, respectively.

MIC determination. All 47 CR *E. coli* isolates (Fig. 1) were resistant to ampicillin (for the WT, MIC $\leq$ 8 mg/liter) and cefotaxime (for the WT, MIC $\leq$ 0.25 mg/liter), and all but four (belonging to the ceftiofur study) were resistant to ceftazidime (for the WT, MIC $\leq$ 0.5 mg/liter). Regarding the remaining antimicrobial families tested (tetracyclines, sulfamides, trimethoprim, aminoglycosides, quinolones, phenicols, and polymyxins), all isolates but two were multiresistant (15), ranging from resistance to three families of antimicrobials to resistance to six. MIC differences were detected among isolates according to treatment and sow. Higher levels of resistance were found during the ceftiofur treatment against phenicols (both chloramphenicol [for the WT, MIC $\leq$ 16 mg/liter] and florfenicol [for the WT, MIC $\leq$ 16 mg/liter]) and gentamicin (for the WT, MIC $\leq$ 2 mg/liter) than during the amoxicillin treatment, whereas levels of resistance were lower against ciprofloxacin (for the WT, MIC $\leq$ 0.064 mg/liter), nalidixic acid (for the WT, MIC $\leq$ 16 mg/liter), trimethoprim (for the WT, MIC $\leq$ 2 mg/liter), and kanamycin (for the WT, MIC $\leq$ 8 mg/liter). In the litter from sow number 25, all 10 positive CR isolates except 1 had the same resistance phenotype (beta-lactams, quinolones, and trimethoprim), whereas the remaining isolates obtained from the rest of the sows exhibited higher diversity of resistance traits. One isolate was resistant to colistin (for the WT, MIC $\leq$ 2 mg/liter).

Detection of genes responsible for ESBL resistance. ESBL genes were detected in 36 of these 47 CR *E. coli* isolates and in most cases were combined with the $bla_{\text{TEM-1}}$ gene. Fifteen isolates were confirmed to contained $bla_{\text{CTX-M-1}}$ (GenBank accession number X92506), 10 contained $bla_{\text{CTX-M-14}}$ (GenBank accession number AF252622), 4 contained $bla_{\text{CTX-M-9}}$ (GenBank accession number AF174129), 2 contained $bla_{\text{CTX-M-15}}$ (GenBank accession number AY044436), and 5 contained $bla_{\text{SHV-12}}$ (GenBank accession number AJ920369). Four isolates were resistant to cefoxitin, and the genotype could not be determined. Seven isolates with MICs of 0.5 mg/liter and 2 mg/liter for cefotaxime and ceftazidime, respectively, were negative for all PCRs tested, suggesting low susceptibility to cephalosporins, probably by upregulation of the AmpC promoter.

Conjugation and transformation. Eight of the 14 selected isolates were able to transfer the cephalosporin-resistant genes by conjugation. Additionally, 11 out of 14 isolates transferred cephalosporin-resistant genes to the electrocompetent strain. The 11 transformants together with the three transconjugants resulting from the wild-type strains were subjected to S1 nuclease, and the presence of one unique plasmid was confirmed. Sizes of plasmids varied between approximately 33.4 kb and 173.4 kb (Table 3). IncI1 was the most common replicon, followed by IncN. Four of the isolates presented two different replicons on the same plasmid, and no replicons were detected in one of the transformants.

The transformants/transconjugants were also resistant to streptomycin ($n = 10$), tetracycline ($n = 9$), sulfamethoxazole ($n = 8$), trimethoprim ($n = 4$), ciprofloxacin ($n = 2$), and kanamycin ($n = 1$).

DISCUSSION

Cephalosporin-resistant *E. coli* isolates were found in samples from 7-day-old piglets prior to the administration of any medica-

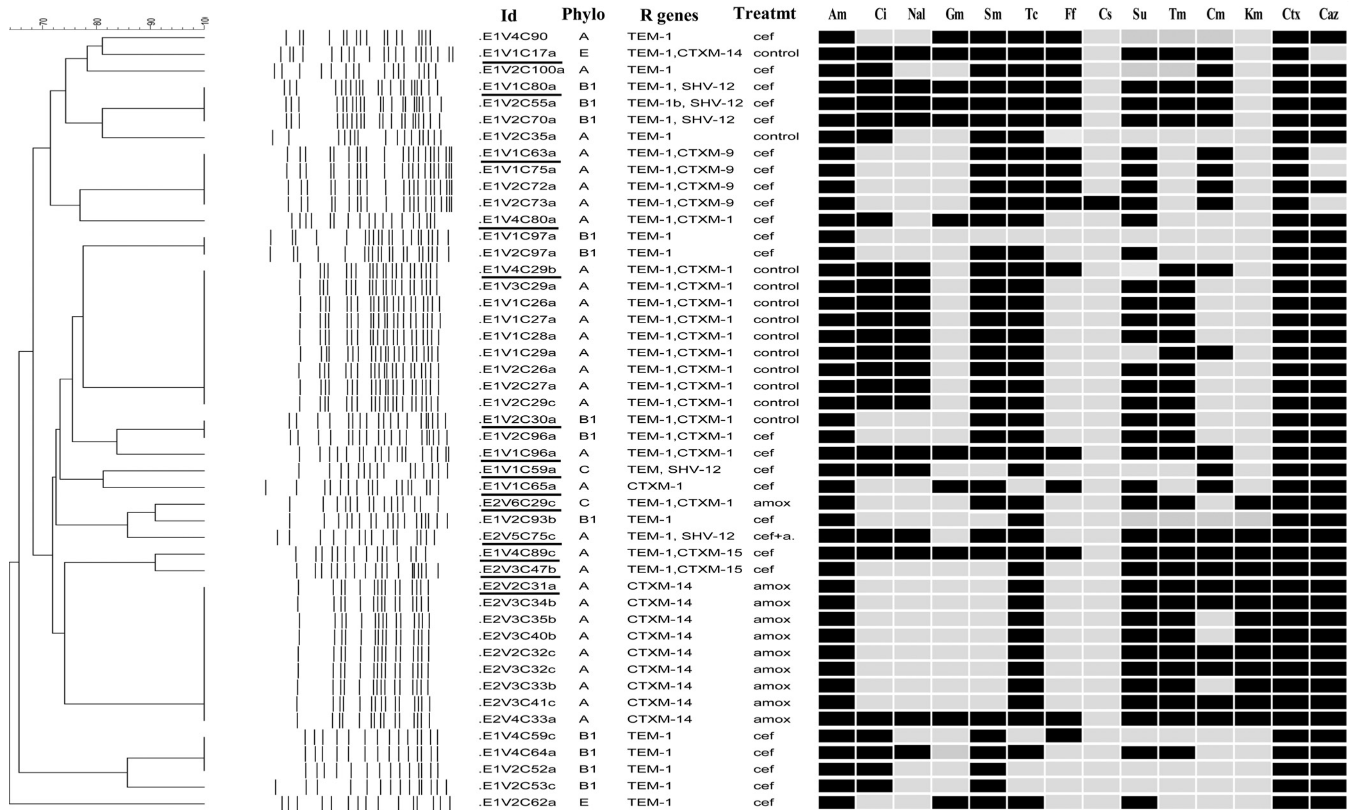

FIG 1 Dendrogram showing the genotypic relatedness of the CR *E. coli* bacteria isolated during the course of the study, the phylogeny, cephalosporin resistance genes, treatment, and phenotypic diversity. Am, ampicillin; Ci, ciprofloxacin; Nal, nalidixic acid; Gm, gentamicin; Sm, streptomycin; Tc, tetracycline; Ff, florfenicol; Cs, colistin; Su, sulfamethoxazole; Tm, trimethoprim; Cm, chloramphenicol; Km, kanamycin; Ctx, cefotaxime; Caz, ceftazidime. Strains selected for transformation and conjugation experiments are underlined. Phylo, phylogroup; Id, identification number; cef, ceftiofur; amox, amoxicillin; cef+a, ceftiofur and amoxicillin.

tion. Moreover, we could not detect the isolates from the sows even though we used an enrichment step for isolation of the specific resistance trait. The high clonality of the isolates demonstrated by PFGE does not argue for vertical transmission but, rather, for multiple acquisitions of isolates with limited colonization properties, perhaps from an external origin (personnel working at the farm, the food source, or the presence of rodents or other vectors). Other studies have also detected high diversity of CR isolates in newborn piglets, especially when enrichment methods were used for isolation due to the ability of low-prevalence strains

TABLE 3 Results of the conjugation and transformation experiments together with plasmid replicons and plasmid sizes obtained

Strain	Resistance gene	Conjugation result[a]	Transformation result[a]	Inc family member found[b]					Mol size (kb)
				I1	N	FIA	FIB	A/C	
E1V1C17a	CTXM-14	TC1b	**TF1a**	+					120
E1V1C80a	SHV-12	**TC2a**		+					138.9
E1V1C63a	CTXM-9	**TC3b**		+					138.9
E1V4C80a	CTXM-1	TC4a	**TF4a**		+				40
E1V4C29b	CTXM-1		**TF5a**		+		+		140
E1V2C30a	CTXM-1		**TF6a**	+					138
E1V1C96a	CTXM-1	TC7a	**TF7a**		+				40
E1V1C59a	SHV-12	TC8c	**TF8**	+	+				180
E1V1C65a	CTXM-1		**TF9a**		+				50
E2V6C29c	CTXM-1	TC10a	**TF10a**					+	180
E2V5C75c	SHV-12		**TF11a**						140
E1V4C89c	CTXM-15		**TF12a**			+	+		150
E2V3C47b	CTXM-15		**TF13a**			+	+		150
E2V2C31a	CTXM-14	**TC14a**		+					120

[a] Transconjugants and transformants used for replicon typing are in boldface.
[b] Inc family members are those present in the wild type and in the corresponding transformant or transconjugant.

to overgrow high-prevalence strains during enrichment (16). Additionally, weaning poses enough stress that it may contribute to *E. coli* overgrowth in pigs (17, 18). On the other hand, in some cases, PFGE results suggest that some of the clones were shared among piglets of the same pen (for example, the litters from sows numbers 25 and 11) (see Table S1 in the supplemental material), indicating a common source within the pen. Several studies have demonstrated a short-lived increase in the *E. coli* population after antimicrobial treatment or a stressful event. Since the sows were far from these events, they may carry undetectable amounts of CR *E. coli* (17, 18), and the limitations of the bacteriological techniques did not allow their detection. Hence, the farm was cleaned and depopulated during the 6 months prior to the study; incorrect cleaning and disinfection of the premises may play a role in the persistence of these organisms. Since environmental samples of the barn were not taken prior to the study, this option cannot be ruled out. Thus, a further visit to this farm, after 1 year of finishing this trial and applying a cleaning and disinfection protocol, demonstrated the presence of CTX-M-producing *E. coli* in the environment with a PFGE profile different from the profiles isolated from feces (data not shown).

After 48 h of the parenteral treatment with ceftiofur, an increase in the prevalence of CR *E. coli* was detected. These levels decreased after the first week of treatment. In the case of in-feed amoxicillin treatment, a similar increase was observed after 7 days of treatment. In the last visit, prior to departure to the abattoir, all the animals were negative for CR *E. coli*. Results from this study are in agreement with those of other studies performed in calves (19, 20), in which CR *E. coli* emerged for a short time while in the course of treatment and diminished shortly after treatment. Perhaps the resistant population could not compete well with the sensitive population after withdrawal of the antibiotic (19). However, during treatment with beta-lactam antimicrobials, animal feces could become a source of resistant bacteria. Biosecurity measures should be undertaken during treatment, such as feces removal or isolation of animals under medication, to avoid transfer of resistance. Additionally, farmers are at potential risk of contamination during exposure to animals shedding CR bacteria. Studies have demonstrated that ESBL genes and plasmids obtained from *E. coli* of farmers exhibited genetic similarity to those obtained from *E. coli* bacteria isolated from animals belonging to their farms (21).

It appears that both treatments with beta-lactams have selected for a wide range of cephalosporin resistance genes from different families, and these genes were recovered during both treatments. Previous studies analyzing the presence of cephalosporin resistance genes in pig farms in Spain described the presence of different *bla* genes, with SHV-12 being the most frequent (22), which is a completely different picture from what is found in other European countries, where SHV-12 is associated with human infections (23). Results from this study have shown the coexistence of many different resistance genes within one farm. The most frequent CTX-M variants in ESBL producers in animals and food of animal origin are currently CTX-M-1 and CTX-M-14, while CTX-M-15 ESBL-producing *E. coli* isolates have only exceptionally been observed in the veterinary context (24). However, this study has demonstrated in healthy pigs the presence of CTX-M-15 genes harbored in high-molecular-weight plasmids of approximately 150 kb containing two replicons, FIA and FIB. Are we seeing a change in the evolution of resistance similar to that we have perceived on the human side (25), where it was a shift in occurrence from CTX-M-14 and CTX-M1 toward CTX-M15?

Transformation experiments and replicon typing revealed the presence of a great variety of plasmids of many different sizes harboring the same resistance genes, with the most common replicons being IncI1 and IncN. However, further studies should be performed at the animal level and at the farm level to assess both the occurrence and spread of plasmids within the pig bacterial population in a particular farm and the persistence and transmission of these plasmids from herd to herd.

Additionally, CR *E. coli* isolates recovered during the course of the study were phenotypically resistant to different families of antimicrobials, and half of them were resistant to ciprofloxacin even though fluoroquinolones were never used to treat these animals. These results are in line with a high background of antibiotic resistance genes in the gut bacteria of livestock after over 60 years of antibiotic use (26). Although fluoroquinolone resistance is mostly conferred via a *gyrA* or *parC* mutation in the bacterial chromosome, two of the transformants exhibited resistance to fluoroquinolones. Plasmid-mediated quinolone resistance in some cases has been associated to the same plasmids as those harboring cephalosporin resistance genes (27). Furthermore, as demonstrated by the phenotype of the transformants exhibiting resistance to several antimicrobial families, coselection by plasmids bearing resistance genes for different antimicrobial families probably plays an important role in the maintenance of resistance mechanisms, as demonstrated via metagenomics in the gut bacteria of swine (28). In-depth studies should be performed to avoid the transmission of these resistance genes from farm to fork since several studies have demonstrated the presence of resistant *E. coli* and, in particular, CR *E. coli* of pig origin in the abattoir (29–31). Although animals from this study departed to the abattoir free of CR *E. coli*, it should be noted that this study was conducted under control conditions, and no extra medication apart from ceftiofur and amoxicillin was applied during the course of the study. However, conventional farming could also require the administration of macrolides, polymyxins, and tetracyclines during the fattening period, which could coselect for CR *E. coli* (32, 33). Currently, there is a scarcity of data linking antimicrobial consumption in veterinary medicine and the generation of antimicrobial-resistant bacteria; hence, it seems clear that the use of different families of antimicrobials in the same population could be a risk factor for the development of antimicrobial resistance in several microorganisms under field conditions (26, 34).

Taken together, these results suggest that the use of ceftiofur and amoxicillin at different stages of the rearing cycle are independent risk factors for the selection of CR *E. coli*. Both beta-lactam antimicrobials select for resistant *E. coli* during the course of treatment. However, CR *E. coli* bacteria were not detected in the absence of the selective pressure or when the animals departed to the abattoir. Further studies should be designed to identify other risk factors associated with the persistence of resistance determinants to minimize the recirculation of isolates and/or plasmids within farms.

ACKNOWLEDGMENTS

This work was supported by project AGL2011-28836 from the Ministerio de Economia y Competitividad of Spain.

K.C.-V. is a Ph.D. student registered with the Universidad Autonoma de Barcelona.

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