Evolutionary Genetics of ESBL/pAmpC-producing *Escherichia coli* and *Salmonella enterica* from Poultry

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Evolutionaire genetica van ESBL/pAmpC-producerende *Escherichia coli* en *Salmonella enterica* uit pluimvee

(met een samenvatting in het Nederlands)

Proefschrift

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To one of the most abundant birds in the world,
the broiler chicken

Contents


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Chapter 1.
General Introduction

β-Lactam drugs

The β-lactam class of antimicrobials comprise all antimicrobials that contain a β-lactam ring in their molecular structure. The β-lactams with broad-spectrum antibacterial activity comprise several families, the main ones being aminopenicillins, cephalosporins, carbapenems and monobactams. Thousands of new β-lactam compounds followed the discovery of penicillin in 1928. The development of new β-lactam drugs was made to broaden the spectrum activity by targeting additional bacterial species or to overcome the appearance of resistance mechanisms in the bacteria of interest[1]. In general, the mode of action of β-lactam antimicrobials is to interrupt the formation of the bacterial cell wall by binding to the enzymes (transpeptidases) involved in the synthesis of peptidoglycans. These transpeptidases are also known as penicillin binding proteins or PBPs. Penicillins, cephalosporins, cephamycins and other β-lactam antimicrobials share this same mechanism of action[2].

Penicillin was discovered in 1928 by Alexander Fleming using a strain of *Penicillum notatum*[3,4]. Until 1942 Penicillin G became the first β-lactam to be used clinically to treat streptococcal infections[1,5]. In the 1950s, E. P. Abraham and G. G. F. Newton isolated the naturally occurring cephalosporin C from a strain of *Cephalosporium acremonium*[6,7]. Cephalosporin C was stable against penicillinase-producing *Staphylococcus aureus*[1]. The 7-aminocephalosporanic acid nucleus of this compound was used as precursor to further develop molecules with potent broad-spectrum activity (that is, activity against both Gram-positive and Gram-negative bacteria) which derived in the different generations of cephalosporins[8]. Other naturally-occurring compounds were obtained from bacteria. Carbapenem compounds were isolated from *Streptomyces spp*. and monobactam from *Pseudomonas acidophila*[9], *Agrobacterium radiobacter*[10] and *Chromobacterium violaceum*[11]. Nevertheless, the first carbapenem identified, thienamycin, had to be chemically stabilized adding N-formimidoyl, which resulted in the development of imipenem[12,13]. Aztreonam, the sole monobactam approved for therapeutical use, derives from the N1-sulfonic substitution in the compound isolated from *C. violaceum*[14]. Different spectrums of activity against Gram-positive and Gram-negative bacteria are observed within these families of β-lactams. For example: the narrow spectrum penicillin G and penicillin V, show activity towards Gram positive bacteria and only towards a few specific Gram-negative genera (e.g. *Pasteurellaceae*). In turn, carbapenems are characterized by their broad-spectrum activity. Being ertapenem, imipenem and doripemem effective against multiple Gram-negative bacteria such as several species of Enterobacteriaceae (e.g. *Escherichia coli*, *Klebsiella pneumoniae, Proteus mirabilis*), *Pseudomonas aeruginosa* and *Acinetobacter*. Carbapenems could also be used against methicillin-susceptible staphylococcal infections and penicillin-resistant streptococcal infections. As for monobactam agents, aztreonam has a narrow spectrum activity, being effective only against gram-negative bacilli such as *P. aeruginosa*. The spectrum of activity among cephalosporins is described next.

Classification of cephalosporins

The classification of cephalosporins into generations will be followed in the present chapter, in accordance with the Anatomic Therapeutic Chemical (ATC) and ATCVet index[15,16]. On the basis of their similar chemical structure, time of introduction and antibacterial spectrum, cephamycins and cephalosporins can be classified together[2].

First generation: Their spectrum of activity is the narrowest. Effective against Gram-positive cocci including penicillinase-producing, methicillin-susceptible staphylococci. Limited activity against Gram-negative but active against *Proteus mirabilis*, *K. pneumoniae* and *E. coli*. Examples of drugs allowed in Human (H) and/or Veterinary (V) medicine, including livestock and companion animals, are[2,17]: cefadroxil (H and V), cephalexin (H and V), cefazolin (H and V), cephradine (H), cefalonium (V), cephapirin (V), cephalothin (V) and cephacetrile (V). Second generation: increased activity against Gram-negative bacteria with a more extended spectrum than first generation cephalosporins. Examples of drugs include cefuroxime (H), cefaclor (H), cefprozil (H) and the cephamycins cefoxitin (H), cefotetan (H) and cefmetazole (H). Their use (e.g. cefoxitin) may occur in extra-label, or unapproved manner in companion animals. Third generation: active against Gram-negative and Gram-positive bacteria. More stable against β-lactamases affecting first and second generation cephalosporins. Examples are cefdinir (H), cefditoren (H), cefixime (H), cefotaxime (H), cefpodoxime (H), ceftazidime (H), ceftibuten (H), ceftriaxone (H) cefoperazone (V) cefovecin (V) and ceftiofur (V). Fourth generation: extended activity against Gram-negative bacteria and increased stability in comparison to third generation cephalosporins. Examples are: cefepime (H), cefpirome (H) and cefquinome (V). Other cephalosporins: some drugs in this category are often called 'fifth generation cephalosporins' though this terminology is not universally accepted. They have increased activity against Multi Drug Resistant (MDR) Gram-positive bacteria in general and specifically MRSA. They are not licensed for use in veterinary medicine. Examples are ceftobiprole (H), ceftaroline (H) and ceftolozane (H)[2,17].

Relevance of third and fourth generation cephalosporins in human and veterinary medicine

Extended-Spectrum Cephalosporins (ESC) from the third, fourth and fifth generation, are listed as critically important antimicrobials with the highest priority for human medicine by the World Health Organization (WHO)[18]. In human and veterinary medicine, Antimicrobial Resistance (AMR) in bacteria jeopardize the capacity of antimicrobial drugs to kill or control bacterial growth. Occurrence of AMR is associated to selective evolutionary pressure posed by the use or misuse

of antimicrobials. In this regard, ESC must be used with great care to avoid the development and transfer of resistance in humans and animals.

In human medicine, third and fourth generation cephalosporins (e.g ceftriaxone) are used in hospitals to treat severe *Salmonella* and *Shigella* infections[19]. They are relevant in the treatment of infections in children, where the use of fluoroquinolones is avoided due to their potential toxicity[20]. In addition, these drugs are used to treat septicaemia, meningitis, hospital-acquired pneumonia, intra-abdominal and complicated urinary tract infections[21].

In veterinary medicine, cephalosporins from the first generation cefapirin, cefalexin, cefalonium, cefazolin, cefacetrile, third generation ceftiofur and cefoperazone and fourth generation cefquinome are approved for use in livestock animals in the European Union (EU). Among these, only ceftiofur is authorized centrally among all member states. Injectable ceftiofur, is authorized in all EU states to treat respiratory tract infections, septicaemia and pathogen associated polyarthritis and polyserositis in pigs. In most countries, it is authorized to treat interdigital necrobacillosis in cattle and puerperal metritis. Currently, no cephalosporins are authorized for poultry species in the EU. Injectable cefquinome, is used in some EU states to treat mainly respiratory infections, interdigital necrobacillosis in cattle, streptococcal infections in horses and septicaemia caused by *E. coli* in calves and foals. Cefquinome and cefoperazone are authorized in products destined for intrammary use. In general cephalosporins are extensively used in lactating or dry cow therapy in many EU states[2].

In the USA, cephapirin and ceftiofur are the only cephalosporins approved for use in livestock. They can be used in treatment of individual animals and in herds or flocks for control of specified bacterial diseases. For example, in individual animals injectable ceftiofur can be used to treat respiratory infections in cattle, swine, sheep and goats, acute bovine interdigital necrobacillosis (footrot), and acute bovine metritis. Ceftiofur hydrochloride is approved as an intramammary infusion to treat clinical mastitis in lactating dairy cattle caused by coagulase-negative staphylococci, *Streptococcus dysgalactiae*, and *E. coli*[19]. In groups of animals, ceftiofur crystalline-free acid can be used to control diseases such as swine and bovine respiratory disease, and *E. coli* mortality in day-old chicks and poults. In 2012, many extra-label uses of cephalosporins were prohibited for cattle, swine, chickens, and turkeys in the USA. These include uses that do not conform the labels for dosage, duration of therapy and route of administration. For instance, injections into chicken eggs, use of human cephalosporins and the preventive use of these drugs are banned[19]. In Canada, although no cephalosporins are registered for use in poultry, the extra-label use of third generation cephalosporins occurred in hatcheries to prevent *E. coli* associated omphalitis. This practice was dismissed in 2014, when the poultry industry eliminated the use of antimicrobials with very high importance to human medicine. In 2015 this policy was also adopted by the broiler breeder sector[22,23].

Enzyme-mediated resistance to ESC

In member species of Enterobacteriaceae (e.g. *S. enterica* and *E. coli*) the main mechanism of resistance to b-lactam drugs is the production of b-lactamases[24]. These enzymes can be classified according to their substrate specificity into penicillinases, broad-spectrum, extended-spectrum and AmpC b-lactamases and carbapenemases[2,25]. Noteworthy, additional schemes to classify b-lactamases include biochemical classification based on the characteristic active site of the enzymes (Serine/Metallo b-lactamases) and a molecular classification based on molecular size and sequence homology of the amino acids present on active sites (Ambler classes A/B/C/D). A recent overview considering the different classification schemes was made available by K. Bush[26].

Following substrate specificity classification, penicillinases hydrolyze benzylpenicillin and aminopenicillins. They can be inhibited by clavulanic acid and have been mainly described in *S. aureus*[2,25]. The main broad-spectrum β-lactamases are TEM-1, TEM-2 and SHV-1. Their substrates are benzylpenicillin, aminopenicillin, ureidopenicillins, carboxypenicillins and 1st generation cephalosporins. Some broad-spectrum β-lactamases from the OXA family are additionally active against penicillinase-stable penicillins such as oxacillin. Most broad-spectrum β-lactamases can be inhibited by clavulanic acid except OXA-1, which is inhibitor resistant[2,25]. Extended Spectrum β-Lactamases (ESBLs) from the TEM and SHV families (different than TEM-1, TEM-2 and SHV-1) have the same substrates as broad-spectrum β-lactamases. Additionally they are active against oxymino-cephalosporins and monobactams. Some ESBLs from the CTX-M and OXA families are additionally active against fourth generation cephalosporins. In general, ESBLs can be inhibited by clavulanic acid[2,25]. AmpC β-lactamases have the same substrates as ESBLs, excluding fourth generation cephalosporins. In turn, they are active against cephamycins such as cefoxitin. ACC, ACT, CMY, DHA, FOX and LAT families, MIR and MOX are representatives. AmpC β-lactamases are not inhibited by clavulanic acid[2,25]. In addition, *E. coli* strains harbor a chromosomal ampC gene which has a weak promoter and which expression is normally repressed by a transcriptional attenuator[27,28]. Overexpression of ampC is mainly due to mutations that create a strong promoter and can result in resistance to ampicillin, cefoxitin and ESC[29,30]. Carbapenemases GIM, SPM, KPC, NDM, some from the OXA family (e.g. OXA-48) and those from the IMP and VIM families, have the same substrates as ESBLs plus cephamyicins and carbapenems[31]. Among the carbapenemases listed above, KPC can be inhibited by clavulanic acid[2,25]. Within the scope of the present thesis, further references will focus on ESBL and plasmid-encoded AmpC β-lactamases (pAmpC).

Epidemiology of ESBLs and pAmpC

ESBLs are mostly encoded in plasmids and are disseminated through a combination of horizontal gene transfer and clonal spread of ESBL/pAmpC-harboring bacteria. They are frequently reported in *E. coli*, *S. enterica* and *K. pneumoniae* from both human and animal origin. They are also present in other member species of Enterobacteriaceae21,25. ESBLs are mainly represented by the bla_{TEM}, bla_{SHV} and bla_{CTX-M} families of β-lactamase genes. Known ESBL variants from the bla_{TEM} and bla_{SHV} families derived from mutations in the broad spectrum bla_{TEM-1}, bla_{TEM-2} and bla_{SHV-1} enzymes[21,25,32,33]. Likewise, variants from bla_{CTX-M}, are grouped into several clusters based on their sequence homology[34]. Emergence of ESBLs in humans was initially detected in 1983 on bla_{SHV-2}-producing *K. pneumoniae* and *Serratia marcescens* isolated in West Germany[35]. In animals, one of the first observations occurred in 2000 in a dog from Spain with recurrent urinary tract infection from which a bla_{SHV-12}-producing *E. coli* was isolated[36]. In the same period of time, detection of *E. coli* carrying $bla_{CTX-M-14}$, bla_{SHV-12} and bla_{CMY-2} isolated between 2000-2001 was reported in healthy poultry from Spain[37]. Similarly, *E. coli* carrying $bla_{CTX-M-14,}$ $bla_{CTX-M-2}$ and bla_{CMY-2} was isolated between 1999-2002 in healthy poultry from Japan[38].

As for pAmpC, it has been indicated that AmpC genes were mobilized from the chromosome of bacteria into plasmids[20,39,40]. For instance, the most prevalent pAmpC gene $bla_{CMY-241}$, originated from the chromosomal DNA of *Citrobacter freundii*[42,43]. Transmission of AmpC through plasmids have become an important driver for the spread of these genes among *Enterobacteriaceae*[44,45]. Emergence of pAmpC in humans was described in an isolate of *S. Senftenberg* producing bla_{CMY-2} isolated in 1994 in Algeria[46]. A report from pAmpC-producing S. Heidelberg followed in 1999 in the USA[47]. The reports in animals followed and bla_{CMY-2} was found in multiple serovars of *Salmonella* isolated from animals and food in North America[48–58] and Europe[59,60]. Likewise, a bla_{ACC-1}-producing isolate of *Salmonella* was described in 2005 in the Netherlands[61]. Noteworthy, in humans, livestock and companion animals from multiple countries bla_{CMY-2} is the most commonly identified pAmpC gene[62].

Horizontal Gene Transfer and clonal spread of ESBL/pAmpC genes

In general, ESBL/pAmpC genes are frequently linked with Mobile Genetic Elements (MGEs) such as Insertion Sequences (ISs), transposons, phages and the above-mentioned plasmids[44,45,67–69]. In addition, ESBL/pAmpC genes associated to particular ISs can be transferred between distinct plasmid and bacterial lineages[70–72]. Accordingly, the transfer of ESBL/pAmpC-associated MGEs between bacterial cells is known as Horizontal Gene Transfer (HGT), whilst the propaga-

tion of bacterial clones/lineages carrying the resistance genes is known as clonal spread[73]. Since spread of ESBL/pAmpC-mediated ESC Resistance (ESC-R) in Enterobacteriaceae can occur via HGT and clonal spread. It is commonly accepted that association of different ESBL/pAmpC genes with a large variety of MGEs have contributed to the emergence and rapid dissemination of these enzymes across humans and animals worldwide.

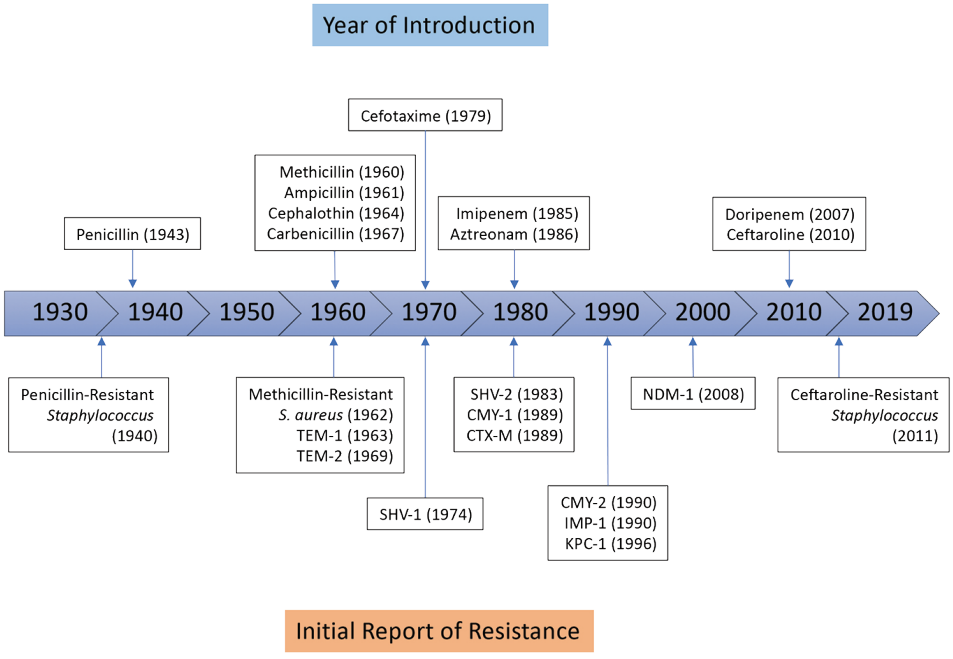

Figure 1. Introduction of β-lactam drugs and initial reports of mechanisms of resistance to β-lactams. Based on: Ventola, Jacoby, Rice and Walsh [63–66].

Occurrence of ESBL/pAmpC in poultry and potential transfer to humans

Strains of *S. enterica* and *E. coli* carrying ESBL/pAmpC genes are frequently reported in poultry and poultry products[62,74] and therefore poultry is considered a reservoir of ESC-R genes[61,75–79]. Occurrence of ESC-R bacteria in broilers is influenced by the pyramidal structure in which the poultry industry is organized. Vertical transmission of the genetic determinants of ESC-R may occur from breeding chickens at the top of the pyramid and disseminate throughout the different levels of poultry production and to the broiler chickens at the bottom[80–83]. In addition, transmission at hatcheries (from the hatchery environment or contaminated

eggs), horizontal transmission within and between broiler farms and transmission from the environment external to the farms have been reported[84].

Although no quantitative data is available to explain the levels of ESBL/pAmpC-producing Enterobacteriaceae that are transferred from the poultry pyramid into the broiler slaughter line[84], it is hypothesized that contamination during slaughter and subsequent contamination of poultry products is strongly influenced by contamination at farms. Thus, reducing contamination during slaughter and contamination of retail products requires interventions at the farm level as previously indicated for *Salmonella*[85] and ESBL/pAmpC-producing *E. coli*[80,86].

To date, transmission of ESBL/pAmpC genes, and ESBL/pAmpC-carrying plasmids and strains of *E. coli* throughout the poultry chain have been assessed using information of gene, plasmid and strain subtypes[80,86]. The potential transfer of these genetic determinants between poultry and humans have been assessed using sequence-based comparisons of genes, plasmids and strains originating from humans, chickens and retail meat[75,87,88]. Transmission between humans and poultry have been indicated when genetic similarity of these determinants was found. Some studies indicated clonal spread of bacteria with similar ESBL/pAmpC genes and plasmids[75,76,87] and others indicated HGT of ESBL/pAmpC-harboring plasmids[75,77,88]. These differences in study-outcome, indicate differences in particular settings of poultry production. Noteworthy, occurrence of particular genetic determinants and routes of transmission within poultry and between poultry and humans, can vary according to the geographical area of study[73].

Genetic determinants of resistance to ESC in poultry in Latin America

In Latin America, the molecular epidemiology of ESBL/pAmpC genes has been studied in *S. enterica* or *E. coli* from poultry[89–116]. However, the situation in Latin America is characterized by scarcity of studies from most countries. To date, the majority of studies originate from Brazil[89–104,110–116] and at least one study has been performed in Argentina[105], Ecuador[106], Mexico[107], Nicaragua[108] and Venezuela[109]. In general, the use of different, non-harmonized methodologies of sampling, isolate collection and molecular methods to characterize ESBL/pAmpC genetic determinants is observed and restrict comparisons of ESC-R determinants between countries. The majority of studies, have described the main ESBL/pAmpC genes mediating ESC-R. Some have characterized both ESBLs and pAmpC genes[89,92,95,96,99,102,103,105–107,112,115,116], whilst others either ESBL[90,91,93,94,97,101,108–111,113,114] or pAmpC genes[98,100,104]. Several of these studies have characterized the plasmids and strains carrying the resistance genes to a certain extent[90,91,94,95,98,100–102,104,105,107,110–112]. However, only a few have provided a sequence-based characterization of these genetic determinants, either based on plasmid and strain Multi Locus Sequence Typing (MLST) MLST, or Whole Genome Sequencing (WGS), that would allow general

comparisons[91,93–96,100,102,108,110,111]. In addition, some of the studies have assessed the potential transfer of ESBL/pAmpC-producing strains between poultry and humans[89,90,97,107–109]. For instance, two studies used collections of human and animal isolates gathered at national reference or research laboratories[90,97] and two other studies performed the collection of samples and isolates during the time of the study[107,108]. In general, these four studies included environmental samples and/or samples from other animals in addition to the ones from poultry. After phenotypic evaluation of ESC-R, all studies characterized the ESBL and/or pAmpC genes using PCR and sequencing and plasmids and/or strains were characterized with PFGE. As an outcome of these studies, genetically related ESBL-carrying isolates of *E. coli* were found among humans, poultry and wild birds in Nicaragua[108], and of *S.* Typhimurium and *S.* Muenchen among humans and poultry in Brazil[90,97]. In turn, pAmpC-carrying *S.* Typhimurim originating from swine and clinical samples from children in Mexico, were found genetically related.

In overview, the ESBL genes $bla_{\text{CTX-M-2}}$ (chromosomally and plasmid located) and $bla_{\text{CTX-M-8}}$ are the most frequently reported in poultry in the region[89–92,95–97,101–103,105,108–115] and $bla_{\text{CMY-2}}$ is the most frequent pAmpC gene[89,94–96,98–100,102–107,112,116]. To a lesser extent, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ are also prevalent in poultry[91–93,102,105,108,112]. These genes are being carried in a diversity of plasmid lineages, lineages of *E. coli* and different *S. enterica* serovars. In Argentina, $bla_{\text{CTXM-2}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CMY-2}}$ were reported in transferable plasmids from isolates of *E. coli* which also carried the colistin resistance gene *mcr-1*[117]. In Brazil, $bla_{\text{CTX-M-2}}$ was found chromosomally located[102,110] and also located on conjugative IncHI2 plasmids in *E. coli*[102]. Similarly, it was found in conjugative plasmids in *Salmonella*[90,101]. Moreover, conjugative IncI1 plasmid Sequence Types (ST) ST113, ST114, ST130-132, were identified as main carriers of $bla_{\text{CTX-M-8}}$ in diverse lineages of *E. coli*[91,111], and IncI1/ST12 plasmids as carriers of $bla_{\text{CMY-2}}$ in both *E. coli* and *Salmonella* in Brazil[94,95]. In Ecuador, $bla_{\text{CTX-M}}$ were reported in *S.* Infantis, in Mexico, $bla_{\text{CMY-2}}$ was reported in *S.* Typhimurium[106,107] and in Venezuela, $bla_{\text{CTX-M-2}}$ and $bla_{\text{SHV-12}}$ were reported in *S.* Give and *S.* Heidelberg[109]. In Guatemala $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-2}}$ were reported in *E. coli*[108]. A previous study of Colombian isolates, described $bla_{\text{CMY-2}}$ in different *Salmonella* serovars obtained from food samples. The samples collected in 2002, originated from fast-food stores and included chicken and beef products, sausages and cheese. However, the $bla_{\text{CMY-2}}$-carrying isolates were described in food types different than chicken[118]. In comparisons to other regions, the prevalence of $bla_{\text{CMY-2}}$ is common to samples from studies in poultry in other countries[62,119], whilst the prevalence of $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-8}}$ seems to be strongly associated to the region. Nevertheless, comparisons of these data of gene variants, plasmid and strain subtypes should be interpreted carefully due to different sampling strategies and scarcity of data suitable for general comparisons, as mentioned above.

Integrated Surveillance of AMR in Colombia

In a call to immediate and harmonize action against AMR, WHO member states adopted a Global Action Plan (GAP) on AMR in 2015. The 5-objective global plan serves as a guide to engage representatives from human and veterinary medicine, agriculture, finance, environment, consumer and political sectors in coordinated actions to tackle AMR, both at the national and international level[120]. The status of implementation of the GAP by individual states can be found in the WHO's library of National Action Plans (NAPs)[121] and the global database for AMR country self-assessment[122]. In countries associated to the Pan American Health Organization (PAHO), the regional Office of WHO for the Americas, only Argentina, Barbados, Brazil, Canada, Costa Rica, Peru and the USA, have officially submitted their NAP (Library of NAPs last accessed: 04 March 2019).

Although not yet available in the library of NAPs, a Colombian national strategic plan in response to AMR was prepared under the lead of the Colombian Ministry of Health and Social Protection[123]. The strategic plan also had the participation of the Ministry of Agriculture and Rural Development as well as representatives from the human and veterinary health and agricultural sectors, among other sectorial and extra sectorial actors[123]. In this document, the activities to date of the national agencies regarding surveillance of AMR are summarized. For its part, the National Institute of Health (*Instituto Nacional de Salud*, INS), started surveillance of AMR in humans in 1987 in the context of sexually transmitted gonococcal infections. In 1994, surveillance was implemented for bacterial pneumonia and meningitis. In 1997, surveillance of *Salmonella* sp., *Shigella* spp., *Campylobacter* spp. and *Listeria monocytogenes* was implemented for acute diarrhea and foodborne diseases. Most recently, in 2012, surveillance was implemented for relevant pathogens in the context of health care-associated infections[123]. In addition to the INS, the Colombian Institute for Agriculture and Livestock (*Instituto Colombiano Agropecuario*, ICA), monitors AMR in *Salmonella* sp., *Enterococcus* spp., *E. coli* and *Staphylococcus* spp. obtained from eggs and milk in primary production since 2014. In turn, for the National Institute for the Surveillance of Food and Medicines (*Instituto Nacional de Vigilancia de Medicamentos y Alimentos*, INVIMA) surveillance of AMR in enteropathogens isolated from food products is reported [123].

It is observed from this summary that the human health sector in Colombia has a large experience in terms of AMR surveillance in comparison to the animal and agricultural sectors. Notably, developments of AMR-surveillance by the INS have been accomplished with the support from PAHO/WHO.

One of the positive aspects from the Colombian NAP, is a clear call towards integrated surveillance of AMR across human, agricultural and environmental sectors. In addition to the Ministries of Health and Social Protection and Agriculture and Rural Development, the Ministry of Environment and Sustainable Development is implicated in the strategy to fulfill the 5-objective strategy as pro-

posed in the GAP. On the other side, data on Antimicrobial Use (AMU) in the country is scarce and a systematic collection of these data in the agricultural sector still needs to be consolidated[123]. The systematic collection of these data is central to measure the impact of interventions aiming to set up judicious AMU, such as education of professionals of human and veterinary health as outlined in the GAP and the Colombian NAP. These data are important since it allows the integrated analysis of AMR and AMU as collected from surveillance systems[124].

Scope of the thesis

In order to contribute to the epidemiological study of AMR in Colombia and Latin America, a pilot project for integrated surveillance of AMR in bacteria from poultry was initiated as a research-driven initiative in Colombia. In this project, the epidemiology of AMR in chickens and chicken meat, was used to assess potential exposure to AMR in humans through the food chain. The accomplishments of the project and results of AMR and AMU are presented in **Chapter 2**. The genetic determinants of ESC-R (ESBL/pAmpC gene variants, carrying plasmids and strains subtypes) in *E. coli* and *S. enterica* were determined using the isolates originating from the pilot project for integrated surveillance. The results of the molecular characterization based on PCR and sequencing for *E. coli* are presented in **Chapter 3**. In turn, the results for *S. enterica*, based on WGS and *in silico* characterization ESC-R genetic determinants are presented in **Chapter 4**. Dissemination and evolution of the most prevalent plasmid lineages carrying ESBL/pAmpC in both *E. coli* and *S. enterica* were assessed using WGS-based phylogenetic reconstruction (phylogenomics). The comparison of Colombian IncI1-Iγ plasmids was extended to other countries using sequences available in the public domain. These results are presented in **Chapter 5**. The recent evolutionary history of *S.* Paratyphi B var. Java ST28, a relevant *S. enterica* serovar-ST in poultry from both Europe and Latin America, was reconstructed using temporal and phylogenetic analysis with genomes from multiple countries. The results of these analysis are present in **Chapter 6**. The results are discussed in a broader context in **Chapter 7**.

In summary, the objectives of the present thesis were:

I. To describe and use a surveillance platform of AMR in Colombia to determine the ESBL/pAmpC gene variants, ESBL/pAmpC-carrying plasmids and strain subtypes of *E. coli* and *S. enterica* driving the spread of resistance to ESC in Colombian poultry.

II. To use genomic epidemiology of ESBL/pAmpC-carrying plasmids and strains to assess the dissemination and evolution of these genetic determinants within Colombia and between multiple countries.

III. To reflect on the impact of poultry farming and commerce in the dissemination and evolution of genetic determinants of AMR and reflect on opportunities to improve the detection of AMR in food production chains.

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Chapter 2.

The Establishment of the Colombian Integrated Program for Antimicrobial Resistance Surveillance (COIPARS): A Pilot Project on Poultry Farms, Slaughterhouses and Retail Market

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Abstract

The development of antimicrobial resistance among bacteria (AMR) is currently one of the world's most pressing public health problems. Use of antimicrobial agents in humans and animals has resulted in AMR which has narrowed the potential use of antibiotics for the treatment of infections in humans. To monitor AMR and to develop control measures, some countries, such as the USA, Canada and Denmark have established national integrated surveillance systems (FDA, 2012, Public Health Agency of Canada, 2007, DANMAP, 2012). The components of these programs monitor changes in susceptibility/resistance to antimicrobial agents of selected zoonotic pathogens and commensal organisms recovered from animals, retail meats, and humans. The rapid development of Colombia's animal production industry has raised food safety issues including the emergence of antibiotic resistance. The Colombian Integrated Surveillance Program for Antimicrobial Resistance (COIPARS) was established as a pilot project to monitor AMR on poultry farms, slaughter houses, and retail markets.

Impacts

Implementation of a National Integrated surveillance antimicrobial resistance program based on public-private partnership.

Description of good practices in building an integrated surveillance program on antimicrobial resistance that could be used by other countries.

Provide information to conduct risk assessment studies on antimicrobial resistance in Colombia to support policy making.

Introduction

The issue: Steady increase in antimicrobial resistance and the lack of development of new antimicrobial drugs

The use of antimicrobial drugs combined with improvements in sanitation, nutrition and immunization has led to a dramatic decrease in deaths and a major gain in human life expectancy (WHO, 2002). However, with the increased use of antimicrobials, antimicrobial resistance (AMR) has emerged as one of the greatest threats to human health security (WHO, 2007) and is an emerging issue of serious concern to public health, animal health and also food safety authorities (Courvalin, 2005, Marchese & Schito, 2007, O'Brien, 2002, Talbot *et al.*, 2006, Tenover, 2006).

The increase of bacterial pathogens exhibiting multiple resistance to current treatment has narrowed the potential uses of antimicrobial drugs for the treatment of infections in humans and animals (Angulo et al., 2004a). As a striking example, Latin America had among the highest extended-spectrum beta-lactamases (ESBL) rates among world regions. ESBL prevalence distribution in the United States, Europe, Latin America, Middle East/Africa, and Asia/Pacific regions among *Escherichia coli* was 2.8%, 6.4%, 12.0%, 10.0%, and 19.6%; among *Klebsiella* species was 5.3%, 8.8%, 27.6%, 27.4%, and 22.9%; and among *Enterobacter* species was 25.3%, 11.8%, 31.1%, 17.8%, and 36.4% (Rossi, 2011).

With *Salmonella* being an important cause of food-borne diarrheal disease in human beings (CDC, 2008, Hanning *et al.*, 2009, Kang *et al.*, 2009), the reduction in the number of antimicrobials available for effective treatment of *Salmonella*-related infectious diseases in humans and animals has become a serious health concern (Angulo et al., 2004b). The frequency and extent of the resistance to antimicrobials by *Salmonella* vary based on the antimicrobial usage in humans and animals and the ecological differences in the epidemiology of *Salmonella* infections (McDermott, 2006). Globally, *Salmonella* exhibits extensive resistance profiles which have been associated with higher rates of morbidity and mortality (Kariuki et al., 2005, Martin et al., 2004, McDermott, 2006), and the use of antimicrobials in food producing animals (Angulo et al., 2004a).

Similarly, in industrialized and developing countries, *Campylobacter* spp. has been described as one of the main bacterial agents of acute gastroenteritis in humans (Garin *et al.*, 2012). The thermophilic species *C. jejuni* and *C. coli*, are most frequently isolated from patients with diarrhea (WHO, 2001). Handling and consumption of poultry meat is considered to be the major risk factor for human campylobacteriosis(Dufrenne *et al.*, 2001, Jorgensen *et al.*, 2002). Antimicrobial treatment in severe *Campylobacter* infections in elderly and immune compromised patients includes macrolides, fluoroquinolones and tetracyclines (Engberg *et al.*, 2001). Since infections with AMR *Campylobacter* could lead to treatment failure and deaths in immune compromised patients (Engberg et al., 2001), mon-

itoring of resistant *Campylobacter* to these drugs in the meat production chains can help in prevention and control programs.

In similar ways, the commensal bacteria, *E. coli* (Gram-negative) and *Enterococcus* (Gram-positive) can also result in infections in humans. With their ability to transfer resistance genes to human pathogens (Poppe *et al.*, 2005, van den Bogaard & Stobberingh, 2000), these bacteria pose a more serious global threat for human health than previously recognized (Boerlin & Reid-Smith, 2008). Furthermore, the level and degree of resistance that occur in commensal bacteria is linked to the amount and class of antimicrobial agents used to produce a kilogram of meat (Parveen et al., 2007) which varies from country to country (Aarestrup, 2005). Finally, since *E. coli* and enterococci can be easily obtained from healthy animals, they are good indicator bacteria to monitor AMR.

The presence of AMR bacteria in primary animal production represents a risk for humans since AMR bacteria of animal origin can be transmitted from animals to humans through the food supply (food-borne pathogens), water or direct contact with animals (Funk et al., 2006, Ramchandani et al., 2005, Swartz, 2002). In farms, factors that can influence bacterial resistance vary depending on herd or flock health status, farm management and environment (Acar & Moulin, 2006). Over-prescription of antimicrobials by itself is an important factor for selection of resistant bacteria, regardless of the category to which the drugs belong. Over-prescription of antimicrobial drugs by veterinarians(Sarkar & Gould, 2006), feeding low doses of antimicrobials for growth promotion (Hammerum et al., 2007a, Hammerum et al., 2007b, Mathew et al., 2007, Prescott, 2008), and using non-approved drugs or drugs used in extra-label manner are believed to contribute to the development of antimicrobial resistance (Sharma et al., 2005, Weese, 2006). Although widespread use of antimicrobials in the primary sector has benefits for producers, it also contributes to the increasing emergence of AMR bacteria (Aarestrup & Pires, 2009).

An appropriate response to AMR: Integrated surveillance systems

An Integrated Surveillance System for AMR collects and analyses resistance data obtained from food animals, foods, environmental sources and clinically ill humans using harmonized methods for sampling and testing (Acar & Moulin, 2013).

In order to monitor the evolution of antimicrobial resistance and to derive appropriate measures to limit its development, some countries have set-up integrated monitoring systems such as the National Antimicrobial Resistance Monitoring System (NARMS) in the USA, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) and the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP). In accordance with The Codex Alimentarius Commission (CAC) guidelines for risk analysis of foodborne antimicrobial resistance (CAC, 2011), these systems have adopted the One Health approach to include human clinical cases, food producing animals, and retail meats. The CIPARS, for example, integrates data from the national microbiology laboratories on human cases, data

from the animal production sector at farm, abattoirs, and retail levels, and finally data on antimicrobial usage both in human and animal populations (CIPARS, 2007). Similarly, NARMS, which started in 1996, is a national collaborative network involving the FDA, CDC, and USDA (United States Department of Agriculture). The system was developed to monitor changes in susceptibility/resistance of select zoonotic bacterial pathogens and commensal organisms recovered from animals, some retail meats, and humans, to antimicrobial agents of public health and animal health significance (CDC, 2009).

The goals of these integrated surveillance programs could be summarized in the goals of NARMS, which include providing descriptive data and trends on antimicrobial susceptibility/resistance patterns in zoonotic, food-borne bacterial pathogens, and selected commensal organisms in order to identify unusual or high levels of antibacterial drug resistance in humans, animals, and retail meats as well as to contain it. The goals also include conducting epidemiological studies to better understand the phenomenon of resistance, promoting the prudent use of antimicrobials, and assisting the FDA in decision making for approving safe and effective drugs for humans and animals (NARMS, program review 2007).

The efficacy of such systems at curbing the development of AMR has been demonstrated on numerous occasions. As an example, DANMAP reported drastic reductions of vancomycin resistant *Enterococcus faecium* (VRE) in broiler and human isolates in Denmark as a result of the ban of avoparcin as a growth promoter . A reduction of VRE prevalence from 80 % to less than 5 % was achieved in 3 years among broiler isolates , and from 13% to 3 % among human isolates in 3 years (Hammerum et al., 2007a).

An important part of an AMR integrated surveillance system is the data on the consumption of antimicrobials in food producing animals. This data should be collected using harmonized standards to compare the use of antimicrobials at national and regional level (Grave *et al.*, 2012, Acar & Moulin, 2013). In 2012, Grave et al established the trends and patterns of sales of veterinary antimicrobial agents in nine European countries using harmonized data and a proxy unit for an animal biomass potentially treated with antimicrobials. It was observed that countries with lower sales reported a lower AMR prevalence and that the antimicrobial prescribing patterns and class priorization varied depending on the countries(Grave et al., 2012).

More recently, with the purpose of reinforcing national capacities for AMR surveillance, the implementation of integrated systems of foodborne bacteria in animal value chains has been identified by the tripartite approach proposed by WHO-AGISAR (Advisory Group on Integrated Surveillance of Antimicrobial Resistance), FAO and OIE as one of the priority actions (WHO, 2014). This approach will generate data to support the development of national policies and food animal husbandry guidelines for the food animal chains.

The Colombian situation

In early 2007, Colombia, decided to begin the organization of an integrated surveillance in AMR. At that time there was no formal integrated system at the national level for surveillance of AMR. Only, the Instituto Nacional de Salud (INS) was working with a passive surveillance and *ad hoc* studies on serotypes and trends of antimicrobial susceptibility, in pathogens of importance in human health such as *Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis, Neisseria gonorrhoeae*, *Salmonella* spp. and *Shigella* spp. (Agudelo *et al.*, 2006, INS, 2010, Moreno *et al.*, 2004, Ovalle *et al.*, 2003, Wiesner *et al.*, 2006).

Also, for AMR in humans, four groups of researchers reported results in human hospitals and clinical cases (Arias *et al.*, 2003, Leal *et al.*, 2006, Miranda *et al.*, 2006, Pfaller *et al.*, 1999). The research of all these groups showed an increasing trend in the occurrence of antimicrobial resistance in several microorganisms that cause disease in human populations. For instance the Bogotá Bacterial Resistance Control Group's (GREBO – *Grupo para el Control de la Resistencia Bacteriana de Bogotá*) reported the frequencies and antimicrobial resistance patterns of *E. coli* and *E. faecalis* in intensive care units of hospitals of third-level that participates in a surveillance network in Colombia (Alvarez *et al.*, 2006, Cortes *et al.*, 2013).

However, in the case of food animals and food of animal origin, there were no surveillance reports, though the Instituto Colombiano Agropecuario (ICA) had information on AMR bacterial isolates from clinical cases in different animal species and regions of the country. Also the Instituto Nacional de Vigilancia de Medicamentos y Alimentos (INVIMA) had few analyses of AMR in some bacteria in food of animal origin.

Thus, there was a need for collaboration among the human, agricultural, and food sectors. In particular integrating the data in AMR that could yield information on trends in AMR and at the same time monitor the consumption of antimicrobials. The latter was considered highly beneficial to many stakeholders given the rapid increase in animal protein consumption in Colombia and the potential for food exports.

One of the limiting factors detected were inadequate knowledge of the baseline prevalence of food-borne pathogens like *Salmonella*, and their antimicrobial resistance profiles in retail meat of different origins in the country, that needed to be addressed to fully benefit from international commerce. A comprehensive prevalence survey of *Salmonella* in poultry in the previous 10 years was non-existent. Only fragmented reports from localized areas with limited sample sizes were available (INS, 2010). Also, there was a lack of knowledge regarding AMR in farms, in abattoirs, and in retail stores for any of the animal production systems.

For all the previous reasons, a Colombian Integrated Program for Antimicrobial Resistance Surveillance (namely COIPARS) was an appropriate response. The COIPARS could meet the animal health and welfare requirements of the animal industries and address the public health concerns about resistance that originate from

the use of antimicrobials. Also, an integrated AMR surveillance program would support the participation of Colombia in the World Trade Organization where the goal is to secure food safety of products of animal origin. Furthermore, The Colombian poultry industry has shown interest in improving the quality of the poultry-derived products, and in implementing Hazard Analysis Critical Control Point (HACCP) systems on farms and at abattoirs. In both cases, knowledge of the frequency, risk factors and resistance patterns of AMR in the country was fundamental to the development of HACCP programs.

Design of the pilot program of COIPARS

Since a comprehensive survey on the AMR situation in Colombia did not exist, this research project was designed as a pilot program before implementing a fully integrated system at the national level. The objective of this pilot program was twofold: 1) to establish baseline data, and 2) to adapt working processes between national institutes and future stakeholders of the COIPARS in AMR surveillance.

Conformation of an international advisory team

In order to incorporate the existing international expertise in AMR integrated surveillance systems, an international advisory board was formed, consisting of experts from Pan American Health Organization-World Health Organization-PAHO-WHO, CIPARS, DANMAP, CDC-NARMS and University of California, Davis.

Constitution of stakeholder consortium

A consortium of Colombian private and public organizations was firstly assembled to facilitate access to the sites of sampling and to adequate laboratory capacities. This consortium included: a) Corpoica, Corporacion Colombiana de Investigacion Agropecuaria, the National Institute of Agricultural Research; b) ICA, the Instituto Colombiano Agropecuario, National Institute for Agriculture; c) INVIMA, Instituto para la Vigilancia de Medicamentos y Alimentos, the Colombian Food and Drug Administration; d) INS, Instituto Nacional de Salud, the National Health Institute; e) LEMA, Laboratory of Microbiology and Food Ecology of Los Andes University; f) GREBO, Research group in Human AMR of the National University of Colombia; g) Carulla Vivero S.A., the largest retail chain in Colombia which is linked to the French group Casino; and h) the poultry industry. In this case, Corpoica provided scientific-based evidence intended to support the decisions regarding AMR made by policy makers, working in close relationship with agricultural producers under a non-coercive framework.

Selection of standard procedures

Following the recommendation of the advisory team, the standard procedures developed by CIPARS were established as reference approaches and were adapted to the Colombian situation as needed (CIPARS, 2008, CIPARS, 2007). One foodborne pathogen of importance for human health, *Salmonella* spp., was included in the pilot program, in addition to two commensal bacteria. Baseline studies on AMR of *Campylobacter* spp. was included in a later stage of the program.

Selection of the animal chain for the pilot program

To establish a baseline in the animal production sector, the poultry industry was selected because it was by far the most integrated and standardized animal production system in Colombia. It also offers an excellent tracking system of production from the farm level through to the retail sales outlets. Poultry production is a strategic sector in the economy of Colombia, having 11% of growth in 2007, and being the highest in the animal production sector. In 2007, a nationwide policy was launched about the health and safety status in the poultry chain by 5 ministries, namely CONPES Avicola (CONPES, 2007), resulting in favorable conditions to obtain collaboration of the various stakeholders of the poultry sector towards the COIPARS initiative. In order to achieve the general objectives of establishing baseline data and validating processes for the implementation of an integrated surveillance program for antimicrobial resistance (AMR) in Colombia, the implementation of the pilot program included three steps:

- **Engagement of stakeholders and planning.**
- **Rigorous implementation of a pilot program.**
- **Building on trust to consolidate COIPARS.**

Engagement of stakeholders and planning

Benchmark of what existed outside

The benchmark was defined in terms of accumulated experience, organization, working processes, human resources, budget and governance. CIPARS and DANMAP were used to resemble the technical and organizational methodologies, respectively. According to the epidemiological design of CIPARS, the pilot program was conducted by means of passive and active surveillance. Ideally, once adapted to the Colombian situation, the INS would provide AMR *Salmonella* isolates from clinical cases in humans and ICA from food animals. Commensal *Salmonella* spp*., E. coli,* and *Enterococcus* spp. isolates would be obtained from farms, abattoirs and retail points, and then screened for AMR with the help of INVIMA and other research institutes. Likewise, COIPARS would integrate data for the use of antimicrobials in humans and animals to further study the associations between the use

of antimicrobials and the occurrence of resistance.

Involvement of the private sector: a pre-requisite

Although stakeholders expressed their willingness to cooperate, the lack of experience in collaborative projects in Colombia was the principal obstacle to overcome. The solution encountered was to engage the private sector with a strategy that included the development of a proposal with added value from the economic, placeholder and scientific approaches. The economic benefits that were identified for the operation itself were the improvement of efficiency in the production and sanitary profile, inputs for in-depth investigation on the economic impact of the judicious use of antimicrobials, knowledge of the epidemiology of pathogenic and indicator bacteria, and capacity built for diffusion of the best international practices. The stakeholders acknowledged that with COIPARS they would improve the image of the poultry industry by helping to maintain the efficacy of antimicrobials and improving the safety conditions for operators in the chain, consumers, and public health.

Communication: Adapting a validated system to the Colombian situation

As a step toward the implementation of the pilot program, a workshop was organized gathering the constituted stakeholder consortium and the International Advisory Team. The value proposal, benefits, expectations, and obstacles to overcome were discussed. As a result, the participating institutions engaged in efforts to support the pilot program according to the extent of their official competences.

Pilot program in Poultry chain

At the moment of the implementation of an integrated AMR surveillance system, there was a lack of knowledge regarding AMR in farms, in abattoirs and in retail stores for any of the animal production systems. Since awareness of the prevalence of AMR in food animals was fundamental for the construction of the epidemiological baseline of AMR, the execution of a pilot program was offered to the private sector free of charge. Three steps were followed to build the pilot program for AMR surveillance in the poultry chain:

- Conduct a survey of AMR bacteria from chicken farms and abattoirs.
- Conduct a survey of AMR bacteria in chicken-derived products from retail stores.
- Compare the genetic diversity and antibiotic resistance patterns among AMR bacteria from human, poultry and retail chicken meat.

The pilot program for Integrated AMR surveillance was then conducted with the objectives to determine the prevalence, serovars, antimicrobial resistance profiles, and risk factors for *Salmonella* spp. isolated from poultry farms; determine the prevalence, resistance patterns, and risk factors for AMR *Salmonella* spp., generic *Escherichia coli* and *Enterococcus* spp. isolates from retail poultry meat; compare pulsed-field gel electrophoresis (PFGE) patterns among AMR *Salmonella*, *E. coli,* and enterococci isolates from feces of broilers between and within poultry farms and abattoirs; compare PFGE patterns among *Salmonella*, *E. coli,* and enterococci isolates from retail chicken meat; compare PFGE patterns among *Salmonella*, *E. coli,* and enterococci isolates from humans and animals of the same region.

Rigorous implementation of a pilot program

Initial Impetus

After the first meetings with the national consortium and the International Advisory Team, the initial financial support and laboratory facilities were provided by ICA, Corpoica, and PAHO-WHO. In kind contribution was received from the Public Health Agency of Canada (PHAC).

Initial expressions of interest were secured from the national authorities: INS, INVIMA, ICA, research entities such as Corpoica, National University of Colombia (GREBO), Los Andes University (LEMA and Microbiological Research Center -CIMIC), and Carulla.

Financial and human resources

For the implementation of the pilot program, PAHO and ICA provided the seed capital and Corpoica financed the epidemiologist leading position of the pilot program. For the development of the following projects the work team was financed according to the needs and budget of the projects. Facilitators for additional financial resources included the National Poultry Federation (Fenavi).

Work plan

The planning for the pilot program was forecast for 4 years. For each step of the pilot program a timeframe of 12 months was designated, with the feedback to stakeholders at the end of each year. The activities for the first year were concentrated in chicken farms and abattoirs: recruitment of integrator companies, contact and negotiation, discussion of planning and logistics of samplings, collection of samples and laboratory analyses and screening of antimicrobial resistance. For the second year, activities included creation of a census of retail stores in Bogotá, sampling and laboratory analyses and screening of antimicrobial resistance. For

the third year, main tasks included contacting INS and GREBO, database design, and final genotyping of isolates from human, food and animal origins. During the four years, the capacity building was integrated with the development of specific activities including writing reports for stakeholders and peer-reviewed articles.

Dynamics of the personalized approach to the public and private sector

The previously described stages aimed to assemble the strategy to address the private sector. Subsequently, the approach of the poultry companies was established through technical directors and top level managers. In order to achieve the support from Fenavi, 20 preparation meetings, 4 technical presentations, 1 technical seminar, 1 scientific seminar and 2 meetings with the president were conducted. As a result, access to farms, slaughterhouses, and meat retailers with 12 companies was granted.

Feedback and information diffusion

The implemented strategies to do feedback of the results to the private sector included individual reports to each company and meetings with technical teams and directors. In addition, results of the program relating AMR prevalence of microorganisms have been published through peer review papers (Donado-Godoy *et al.*, 2014, Donado-Godoy *et al.*, 2012a, Donado-Godoy *et al.*, 2012b) and technical presentations at regional meetings (PAHO-WHO), including the XVI Inter-Ministerial Health and Agriculture Meeting in 2009 and professional associations.

Validation of sampling scheme methodology, microbiological analysis, AMR, fingerprinting techniques (PFGE) and design of database and protocols to capture information.
Isolation of Salmonella spp.*, Campylobacter* spp.*, E. coli and Enterococcus* spp. *from farms, slaughterhouses and retail samples.*

A total of 3697 samples were collected in different times between 2008-2013, comprising 917 samples from 70 farms located in 2 of the most important Colombian poultry production departments, 1200 samples of caecal content and chicken carcasses from slaughterhouses, and 1580 samples from retail chicken purchased from markets, supermarkets and wet markets from 18 Colombian departments. All isolates of *Salmonella* spp., *E. coli* and *Enterococcus* spp., were recovered using the methodology previously described by CIPARS and validated for this study (CIPARS, 2008, CIPARS, 2007). Number of samples collected, positive samples and isolates analyzed for AMR are described in Table 1.

Isolation of *Campylobacter* spp. was performed using a modified version of the directive ISO 10272-1:2006 (ISO, 2006). Briefly, 400 ml of broiler carcass rinse or 20 ml of caecal content samples were inoculated into supplemented Preston broth (CM67, Oxoid, Basingstoke, England). The tubes were then incubated microaerobically for 6 hours at 37°C, followed by 42 hours at 42°C in anaerobic jars with gas-generating kits (BR56, Oxoid). Subsequently, 0.3 ml was streaked onto modified CCDA-Preston agar (CM739 and SR0155, Oxoid) with plates incubated for 48h at 42°C. Typical colonies were identified by catalase, oxidase and Gram-reaction and morphology by microscopy.

Antimicrobial susceptibility testing.

Isolates of *Salmonella* spp., *E. coli,* and *Enterococcus* spp. were tested for their resistance to 25 antimicrobials by using a BD Phoenix™ Automated Mycrobiology System NMIC/ID-121 or panels PMIC/ID-53 (BD, Sparks, MD), according to the manufacturer's instructions (Donado-Godoy et al., 2012b). Ceftiofur resistance analyses were performed according to an agar disk diffusion method (Donado-Godoy et al., 2014).For the purpose of this paper only the results for a few antimicrobials will be included, with Minimal Inhibitory Concentrations (MIC) values of ampicillin, cefotaxime, ceftiofur, ciprofloxacin, vancomycin, erythromycin and quinupristin/dalfopristin, estimated based on CLSI (Clinical and Laboratory Standards Institute) 2013 breakpoints (CLSI, 2013). Antimicrobial resistance analysis for *Campylobacter* spp., were performed using the agar dilution technique as described by the CLSI (CLSI, 2008). The MIC values for ciprofloxacin, erythromycin, and tetracycline were analyzed based on CLSI 2010 breakpoints (CLSI, 2010).

Consumption of antimicrobials

All veterinary used antimicrobials in Colombia are imported. Data are not available for different animal species and therefore a search on the total import of veterinary drugs was conducted. Data was collected using the Single Window of Foreign Trade – VUCE. This display is an internet-based platform where the procedures for foreign trade are registered before 21 official entities including ICA. This collection of data enables the registration of digital licenses of imported raw materials that requires entry permits from the official entities (MINCIT, 2013).

Key result examples (AMR and antimicrobial importation data)
Overview of antimicrobial resistance

Resistance to ampicillin, third-generation cephalosporin cefotaxime and ciprofloxacin was remarkably high in *Salmonella* spp. and *E. coli* isolates. Most enterococci showed high AMR to erythromycin and vancomycin resistance was observed in *Enterococcus faecalis* as well as resistance to quinupristin/dalfopristin in *E. faecium*.

Table 1. Key results on antimicrobial resistance prevalence among pathogenic and indicator bacteria through the poultry chain production in Colombia.

	Carcass rinse at retail	%R[a]	Carcass rinse at slaughterhouses	%R	Caecal content at slaughterhouses	%R
***Salmonella* spp.**	1375		600		600	
n samples	403		139		43	
n positive isolates						
n tested for AMR[b]						
Ampicillin	393	46	132	64	39	82
Cefotaxime	393	39	132	57	39	69
Ceftiofur	349	38	125	58	39	74
Ciprofloxacin	392	29	131	85	39	69
***Campylobacter* spp.**	165		600		600	
n samples	89		215		159	
n positive isolates						
n tested for AMR						
Erythromicin	27	48	76	11	29	21
Ciprofloxacin	27	70	76	92	29	97
Tetracycline	27	74	76	93	29	83
Escherichia coli	1003		600		733	
n samples	265		580		699	
n positive isolates						
n tested for AMR						
Ampicillin	263	61	88	49	164	55
Cefotaxime	263	28	57	32	164	24
Ciprofloxacin	262	28	87	30	164	23
Enterococcus faecalis	1003		600		600	
n samples	556		535		468	
n positive isolates						
n tested for AMR						
Ampicillin	556	0	134	0	121	0
Ciprofloxacin	547	28	107	38	110	32
Vancomycin	554	1	134	0	121	2
Erythromicin	556	77	134	76	121	86

	Carcass rinse at retail	%R[a]	Carcass rinse at slaughterhouses	%R	Caecal content at slaughterhouses	%R
Enterococcus faecium						
n samples	1003		600		600	
n tested for AMR						
Ampicillin	38	3	13	0	59	0
Ciprofloxacin	40	18	11	36	58	33
Vancomycin	40	0	13	0	60	0
Erythromicin	40	80	12	75	60	62
Quinupristin/ Dalfopristina	-	-	13	85	59	60

[a] %R = prevalence of resistance on tested isolates.
[b] AMR = antimicrobial resistance as tested by using automated system and agar disk diffusion method.

Table 2. Importation of antimicrobial agents, in kilograms of active ingredients, during 2009 – 2011 in Colombia.

Antimicrobial class	2009	2010	2011
Tetracyclines	1027931	1253701	4557712
Pleuromutilin	220349	68000	144400
Lincosamides	26000	21640	255125
Macrolides	52664	48751	44569
Penicillins	9046	3812	45626
Quinolones	8198	24858	14451
Trimethoprim-sulfamethoxazole	13900	7525	18650
Fosfomycin	4760	6902	16359
Tylosin-fosfomycin	[a]	10000	10000
Aminoglycosides	8136	3747	3300
Amphenicols	1265	1910	1880
Sulphonamides	2541	1677	59
Cephalosporins	5	18	17

[a]Data not available for 2009.

Campylobacter spp. exhibited high AMR levels to ciprofloxacin. Antimicrobial resistance to erythromycin, was observed to be higher in isolates from carcass retail. In terms of tetracycline the highest resistance prevalence was observed in isolates from carcass from slaughterhouses.

Description of the prevalence of resistance for selected antimicrobials are summarized in Table 1.

Data on antimicrobial Imports

Data of the importation of veterinary drugs (therapeutic and growth promoters) were collected from 2009 to 2011. All the information was compiled in Microsoft Access for the summary of data and queries building. A database was designed in Access software version 14.0.4760.1000 © 2010 Microsoft Corporation. The Access database allowed the consultation, query building and creation of reports. All the proportion of antimicrobials used in food animals in Colombia are imported, data is presented in Table 2.

Building on trust to consolidate COIPARS

Key success factors (threat free process and first phase free of charge)

The execution of the pilot program was offered to the private sector free of charge and aimed at consolidating a relationship based on trust. Because of high business sensitivity of the AMR issue, it was necessary to build a threat-free process based on confidentiality, transparency and, demonstration of the collaborative capacity (by the presentation of
results). This was made feasible due to the scientific and technical character of Corpoica in the country, which objective is to develop and execute activities of research, technology transfer, and promote technological innovation processes for the agricultural and livestock sectors.

In-depth organization benchmark (Significant accumulated experience and in-depth benchmark analysis)

The design and implementation of the pilot project of the COIPARS was endorsed by the sound scientific support provided by the international advisory team. After the adaptation of the methodologies of CIPARS and DANMAP, the accumulated experience of the benchmark strengthened the capabilities of the national consortium and provided proper conditions for the execution of the pilot project based on technical and organizational reliability. This was a key factor to build the confidence that allowed access to the poultry industry facilities and to the confidential information they had that would enable the traceability of the animals and animal samples in the whole food chain.

Logistic for COIPARS (Institutionalization, Coordination Unit, Process for contributions, Governance and Budget)

Funds provided by national and international stakeholders helped to establish a first coordination unit of the COIPARS headquartered in Corpoica´s facilities articulating the joint work of the national consortium.

Support of international organizations (impetus, benchmarks, technical assistance and networking)

Between the execution of the pilot project, and the implementation of the integrated surveillance program, COIPARS executed private contracts and international research alliances. In response to the relevancy and confidentiality of results, Fenavi financed a study to determine the prevalence and AMR profiles of *Salmonella* spp., *Campylobacter* spp., *E. coli* and *Enterococcus* spp. in chicken slaughterhouses in Colombia. The study included 67 slaughterhouses in 18 of the 32 departments of Colombia. Other studies in collaboration with the Center for Food Safety of the University of Georgia were conducted to determine the prevalence of *Salmonella* spp. on retail broiler carcasses in 23 departments of Colombia and the counts, serovars, and antimicrobial resistance phenotypes (Donado-Godoy et al., 2014, Donado-Godoy et al., 2012a). Additional projects financed by the Ministry of Agriculture and Rural Development (MADR - *Ministerio de Agricultura y Desarrollo Rural*) strengthened the implementation of the coordinator unit for the COIPARS program and helped to farther determine the baseline of *Salmonella* spp., *E. coli*, *Campylobacter* spp. and *Enterococcus* spp. in the swine meat chain in 2 departments of Colombia.

Feedback to each company, feedback to professional associations, publication of results, information diffusion, involvement of private sector. Private contracts, international contracts.

The diffusion of the results to the private and public stakeholders was done in a personalized manner. Feedback presentations were done in the facilities of each company to the top management boards of professional associations. Results presented included prevalence of pathogenic bacteria and AMR in both pathogens and indicator organisms. In response to the particular needs of the industry, analysis of risks factors along the poultry chain were provided. Regional committees were attended by the scientific board of COIPARS to provide support on private sector initiatives to reduce the prevalence of *Salmonella* and *Campylobacter*.

Lectures relating the strategy of COIPARS that involved the private and public sectors in common efforts to make a conclusive surveillance system of AMR have been presented in other Latin American countries like Argentina, Bolivia, Brazil, Chili, Costa, Rica, Mexico and Peru. In addition the results of the program have been published in international peer reviewed journals (Donado-Godoy et al., 2014, Donado-Godoy et al., 2012a, Donado-Godoy et al., 2012b).

Conclusions

This initiative demonstrates the importance of the logistic and financial support of international organizations as PAHO and WHO to move the public and private sectors as well as the academic institutions towards the implementation of an Integrated Antimicrobial Resistance Surveillance System in which the lead of Corpoica was a key. The starting point was the establishment of a scientific committee that recommended the adaptation of successful programs (CIPARS, DANMAP and NARMS) for the Colombian situation through a pilot study that facilitated the constitution of a public-private consortium in which all the stakeholders participated in an active way. The result is an integrated AMR surveillance system platform that can channel the collaboration between national and international stakeholders and enhance local initiatives to preserve the efficiency of antimicrobials.
This integrated program is the first of its kind in Colombia, and helped to validate the methodology in the poultry chain. Our recommendation is to continue the expansion of the surveillance program by inviting other animal production sectors to participate in the process and to develop similar plans that will help to improve the food safety and biosecurity of the animal production chains in Colombia. As a resource, COIPARS offers the experience and technical procedures to create new networks and to adapt this model for the use in other animal production systems such as bovine milk and meat, and aquaculture. This was the first program established in Latin America and has been used as an example in the region by the WHO Global Foodborne Infections Network (GFN) and The International Molecular Subtyping Network for Foodborne Disease Surveillance (PulseNet International). In addition, the WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) has used the example of COIPARS as a guide for the standardization of the methods for AMR monitoring and the use of antimicrobials in food animals in the Americas region.

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3

Chapter 3.

High Heterogeneity of *Escherichia coli* Sequence Types Harboring ESBL/AmpC Genes on IncI1 Plasmids in the Colombian Poultry Chain

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Abstract

Background

Escherichia coli producing ESBL/AmpC enzymes are unwanted in animal production chains as they may pose a risk to human and animal health. Molecular characterization of plasmids and strains carrying genes that encode these enzymes is essential to understand their local and global spread.

Objectives

To investigate the diversity of genes, plasmids and strains in ESBL/AmpC-producing *E. coli* from the Colombian poultry chain isolated within the Colombian Integrated Program for Antimicrobial Resistance Surveillance (Coipars).

Methods

A total of 541 non-clinical *E. coli* strains from epidemiologically independent samples and randomly isolated between 2008 and 2013 within the Coipars program, were tested for antimicrobial susceptibility. Poultry isolates resistant to cefotaxime (MIC ≥ 4 mg/L) were screened for ESBL/AmpC genes including $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, bla_{CMY} and bla_{OXA}. Plasmid and strain characterization was performed for a selection of the ESBL/AmpC-producing isolates. Plasmids were purified and transformed into *E. coli* DH10B cells or transferred by conjugation to *E. coli* W3110. When applicable, PCR Based Replicon Typing (PBRT), plasmid Multi Locus Sequence Typing (pMLST), plasmid Double Locus Sequence Typing (pDLST) and/or plasmid Replicon Sequence Typing (pRST) was performed on resulting transformants and conjugants. Multi Locus Sequence Typing (MLST) was used for strain characterization.

Results

In total, 132 of 541 isolates were resistant to cefotaxime and 122 were found to carry ESBL/AmpC genes. Ninety-two harboured $bla_{\text{CMY-2}}$ (75 %), fourteen $bla_{\text{SHV-12}}$ (11 %), three $bla_{\text{SHV-5}}$ (2 %), five $bla_{\text{CTX-M-2}}$ (4 %), one $bla_{\text{CTX-M-15}}$ (1 %), one $bla_{\text{CTX-M-8}}$ (1 %), four a combination of $bla_{\text{CMY-2}}$ and $bla_{\text{SHV-12}}$ (4 %) and two a combination of $bla_{\text{CMY-2}}$ and $bla_{\text{SHV-5}}$ (2 %). A selection of 39 ESBL/AmpC-producing isolates was characterized at the plasmid and strain level. ESBL/AmpC genes from 36 isolates were transferable by transformation or conjugation of which 22 were located on IncI1 plasmids. These IncI1 plasmids harboured predominantly $bla_{\text{CMY-2}}$ (16/22), and to a lesser extend $bla_{\text{SHV-12}}$ (5/22) and $bla_{\text{CTX-M-8}}$ (1/22). Other plasmid families associated with ESBL/AmpC-genes were IncK (4/33), IncHI2 (3/33), IncA/C (2/33), IncB/O (1/33) and a non-typeable replicon (1/33). Subtyping of IncI1 and IncHI2

demonstrated IncI1/ST12 was predominantly associated with bla_{CMY-2} (12/16) and IncHI2/ST7 with $bla_{CTX-M-2}$ (2/3). Finally, 31 different STs were detected among the 39 selected isolates.

Conclusions

Resistance to extended spectrum cephalosporins in *E. coli* from Colombian poultry is mainly caused by bla_{CMY-2} and bla_{SHV-12}. The high diversity of strain Sequence Types and the dissemination of homogeneous IncI1/ST12 plasmids suggest that spread of the resistance is mainly mediated by horizontal gene transfer.

Introduction

Infections caused by isolates of *Escherichia coli* resistant to extended spectrum cephalosporins (ESC), can result in antimicrobial treatment failure and increased health expenditures in humans[1–3], livestock[4] and companion animals[5]. Isolates resistant to ESC produce enzymes capable of hydrolyzing the β-lactam ring of these drugs, and exhibit reduced susceptibility to third generation cephalosporins and monobactams. These ESC hydrolyzing enzymes are mostly plasmid mediated and they include Extended Spectrum Beta-Lactamases (ESBLs), and AmpC beta-lactamases[6,7]. ESBL/AmpC-producing *E. coli* have been isolated from humans as well as from livestock, animal meat products, companion animals[8], and vegetables[9]. ESBL/AmpC-producing *E. coli* have been frequently reported in poultry and therefore poultry production and poultry meat are considered a reservoir of ESBL/AmpC-producing *E. coli* potentially causing infections in humans[10–15].

Previous studies have compared the occurrence and characteristics of genes, plasmids and strains of ESBL/AmpC-producing *E. coli* from humans, poultry and poultry products[11,16,17]. Some of these studies have found genetic relatedness of strains, genes and/or plasmids, suggesting transmission of ESBL/AmpC-producing *E. coli* between poultry and humans. Some studies suggest the transmission of bacterial strains, with plasmids and genes through clonal spread[12,16] while others suggest the transmission of plasmids conferring the resistance through horizontal gene transfer between different *E. coli* strains[13,17]. Whether this transmission happens by means of clonal spread or dissemination of mobile genetic elements, likely corresponds to different conditions within the analysed geographical areas[18].

In Colombia, available literature from human clinical isolates shows that the most frequent ESBL- gene variants are $bla_{\text{CTX-M-12}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-12}}$ and $bla_{\text{SHV-5}}$[19–24]. More recently, some of these studies included AmpC-genes on their genotypic screening, and demonstrated the association of $bla_{\text{CMY-2}}$ to community onset infections[22,23]. Nevertheless, reports that describe the genetic determinants of ESBL/AmpC-producing *E. coli* from Colombian poultry are not available. This information is essential to know whether a proportion of the mobile genetic elements harbouring ESBL/AmpC–producing *E.coli*, in particular plasmids, identified in isolates from poultry and humans are genetically related. Therefore, the aim of this study was to characterize the genes, plasmids and strains of a collection of ESBL/AmpC-producing *E. coli* from baseline studies in the Colombian poultry chain using sequence-based characterization that enables comparisons regardless of time, place and source of isolates.

Materials and methods

Origin of samples and isolation of *E. coli*

Non-clinical samples were obtained from three different sources from the Colombian poultry production chain: i) broiler farms ii) broilers at slaughter and iii) raw chicken meat at retail. The samples from farms (n=1097), slaughterhouses (n=1566), and retail (n=1203) were taken as part of a pilot project for the establishment of the Colombian Integrated Program for

Antimicrobial Resistance Surveillance (Coipars)[25]. The samples were collected between 2008 and 2013 and originated from i) feces, drag swabs and cloacal swabs from broiler farms ii) cecal content and carcass rinse from slaughterhouses iii) chicken thighs (homogenized with stomacher method) from independent stores and a large retail distribution centre. Carcass rinse samples were obtained from independent stores and supermarkets. Three out of 32 departments of Colombia (that is, provinces) responsible for more than 65% of Colombian chicken production, were represented at the farm level[26]. At the slaughterhouse and retail level, samples were collected from the most populated departments, 18/32 and 23/32, respectively. After collection, samples were stored in insulated containers at 4°C and transported to the laboratory within 24 hours.

Isolates were recovered from samples from the three sources using the methodology previously described by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS)[27]. Briefly, samples were mixed in a 1:10 ratio (w/v) with buffered peptone water (BD, USA) and one loop of the mixture was streaked on a MacConkey agar plate (BD, USA) and incubated overnight at 35°C. For chicken thighs samples, 50 ml of EC broth (7% w/v) (BD, USA) was added to the mixture and incubated overnight at 45°C for 24 hours. Next, one loop of the mixture was streaked on a MacConkey agar plate (BD, USA) and incubated overnight at 35°C.

From MacConkey agar plates, one typical lactose-fermenting colony was further plated on BHI agar and incubated overnight at 35°C. One colony of the pure culture was screened with Sulphur Indole and Motility medium (SIM) (Difco, USA). Subsequently, presumptive *E. coli* strains (negative H_2S production and positive tryptophan degradation) were stored at -80°C on sterile Skim Milk (20% w/w) (Difco, USA).

Analyzed strains, antimicrobial resistance screening and species confirmation

From the stored isolates from farms and slaughterhouses, a convenient selection was made. Given the country-wide scope of an integrated surveillance program like Coipars, and the absence of previous reports of ESC resistance from poultry

in Colombia, our strategy aimed to represent as much levels, years of production and departments as possible. A sum of 155 and 182 isolates representing 14% and 12% of the total number of samples, respectively, was made in a way that at least one isolate originated from every sampled department. In order to avoid overrepresentation of genes, plasmids and strains due to sample duplicity, repeated isolates from the same production flocks (that is, epidemiologically related isolates) were excluded. In contrast to samples from farms and slaughter, each isolate from retail originated from epidemiologically unrelated flocks and were therefore all included to assess the level of exposure to retail consumers and widen the comparisons with isolates from humans in Colombia. Based on this selection, 541 epidemiologically independent isolates were considered for this study, 45 from farms, 66 from slaughterhouses and 430 from retail.

Isolates were tested using the BD Phoenix automated system and the NMIC/ID-121 panels (BD, USA). Drugs included in the panel and the tested concentrations are available in S1 Table. Minimal Inhibitory concentrations (MIC) were interpreted using CLSI 2013 guidelines[28]. Strains resistant to cefotaxime (MIC ≥ 4 mg/L) were screened for the presence of ESBL/AmpC genes. Additional species confirmation was performed with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker, Delft, the Netherlands). The isolates originated from baseline studies that were financed for specific years only during the development of Coipars (Table 1). As a result for some years no samples were included.

ESBL/AmpC gene characterization

Polymerase chain reaction (PCR) with previously described primers was used to screen for ESBL[29] and plasmidic AmpC[30]. The genes analysed included $bla_{CTX\text{-}M}$, $bla_{C\text{-}MY}$, bla_{SHV}, bla_{TEM}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{OXA\text{-}2\text{-}like}$ and $bla_{OXA\text{-}10\text{-}like}$. PCR products were purified using ExoSAP-IT according to manufacturer's protocol (Affymetrix, USA) and sent to BaseClear (the Netherlands) for sequencing. Sequence results were analysed with BioNumerics v7.5 software (Applied Maths, Belgium) and compared with reference sequences deposited at http://lahey.org/studies/.

Plasmid and strain characterization

A selection of strains based on the distribution of ESBL/AmpC variants was made and further characterized. Due to its low prevalence, the selection included all isolates positive for $bla_{CTX\text{-}M}$ (n=7) and all combinations of bla_{CMY}-bla_{SHV} (n=6). For the highly prevalent bla_{CMY} and bla_{SHV} genes, a random selection was made using the Random function in Microsoft® Excel to assign random numbers to isolates positive for bla_{CMY} and bla_{SHV}. The size of selection was fixed to a maximum of 30% for both genes. Finally, strains with the largest 21 and 5 numbers, representing 23% and 29% of the isolates, were selected for bla_{CMY} and bla_{SHV} respectively.

Table 1. Distribution of cefotaxime-resistant *E. coli* in Colombian poultry (A) and ESBL/AmpC genes and isolates selected for plasmid and strain characterization (B).

	Source	Farms	Slaughter	Retail	Total
(A)	2008	8 / 29[a]	13 / 29	-	21 / 58
	2009	4 / 9	-	20 / 165	24 / 174
	2010	-	-	44 / 168	44 / 168
	2011	-	-	30 / 97	30 / 97
	2012	1 / 7	6 / 20	-	7 / 27
	2013	-	6 / 17	-	6 / 17
	2008-2013	13 / 45	25 / 66	94 / 430	132 / 541
	bla_{CMY-2}	8 (2)[b]	14 (6)	70 (13)	92 (21)
(B)	bla_{SHV-12}	2 (1)	5 (2)	7 (1)	14 (4)
	bla_{SHV-5}	- (0)	1 (1)	2 (0)	3 (1)
	bla_{CMY-2}-bla_{SHV-12}[c]	-	1	3	4
	bla_{CMY-2}-bla_{SHV-5}	-	-	2	2
	$bla_{CTX-M-15}$	-	-	1	1
	$bla_{CTX-M-2}$	1	2	2	5
	$bla_{CTX-M-8}$	-	1	-	1
	Total	11 (4)	24 (13)	87 (22)	122 (39)

[a] Cefotaxime-resistant / Tested isolates. [b] Numbers of isolates selected for plasmid and strain characterization are presented in brackets. [c] All isolates positive for combinations of bla_{CMY}-bla_{SHV} and bla_{CTX-M} were selected for plasmid and strain characterization.

Plasmids were purified from the original donor strain using the Qiagen Plasmid Midi-kit (Qiagen, Germany). Plasmid DNA was transformed into ElectroMAX DH10B cells through electroporation (Invitrogen, USA) by mixing 5µl of isolated plasmid DNA with 20µl of electro-competent cells. Electroporation conditions were 25 µF, 200 Ω, and 2.0 Kv. Cells were recovered for 1 hour at 37°C in SOC-medium (Invitrogen, USA). Subsequently, transformants were selected on Luria-Bertani agar (LB-agar) supplemented with 2 mg/L cefotaxime (Sigma, USA). Conjugation experiments were performed when electroporation did not yield transformants. Donor strain and recipient rifampicin-resistant strain *E. coli* W3110, were grown separately overnight in 5 ml of LB-Broth. Volumes of 0.1 ml of donor and 0.9 ml of recipient strains were added to 9 ml of LB broth and cultured overnight. Conjugants were selected on LB-agar supplemented with 2 mg/L cefotaxime (Sigma, USA) and 75 mg/L rifampicin (Sigma, USA). Lysates of DNA for confirmation of ESBL/AmpC genes, PCR Based Replicon Typing (PBRT), plasmid Multi Locus Sequence Typing (pMLST), plasmid Double Locus Sequence Typing (pDLST) and plasmid Replicon Sequence Typing (pRST) were obtained from transformants and transconjugants.

PBRT was performed using the PBRT-kit (Diatheva, Italy) following the manufacturer instructions. Noteworthy, the kit did not differentiate between IncI1 or IncIγ plasmids. For readability all IncI1-Iγ plasmids are here designated as IncI1. Plasmid MLST, pDLST and pRST were performed as previously described for IncI1, IncHI2 and IncF plasmids, respectively[31–33].

For Multi Locus Sequence Typing (MLST), bacterial lysates were obtained after boiling the original donor strain and PCR protocols were performed as previously described at http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/. PCR products were purified and sequenced as described above.

Allele and profile analysis for pMLST, pDLST and pRST was done at http://pubmlst.org/plasmid/. Sequences and minimal spanning trees for MLST were analysed with BioNumerics v7.5 software (Applied Maths, Belgium).

Results

Screening of resistance, ESBL/AmpC genes and selection of isolates for molecular characterization

In total 132 of 541 strains were phenotypically resistant to cefotaxime (MIC≥ 4mg/L), 13 from farms, 25 from slaughterhouses and 94 from retail (Table 1). From these, 122 were positive for ESBL and/or AmpC genes, 11 from farms, 24 from slaughterhouses and 87 from retail (Table 1).The most prevalent gene variant at the farm, slaughterhouse and retail level was bla_{CMY-2}, present in 73% (n=8/11), 58% (n=14/24) and 80% (n=70/87), respectively. Second most prevalent was bla_{SHV-12}, present in 18% (n=2/11), 21% (n=5/24) and 8% (n=7/87), respectively (Table 1). In addition to these gene variants we found bla_{SHV-5} (n=3), $bla_{CTX-M-15}$ (n=1), $bla_{CTX-M-2}$ (n=5), $bla_{CTX-M-8}$ (n=1) and combinations of bla_{CMY-2}-bla_{SHV-12} (n=4) and bla_{CMY-2}-bla_{SHV-5} (n=2) distributed in the three levels. After sequencing, all positive reactions for bla_{TEM} PCR, were identified as bla_{TEM-1} or bla_{TEM-1b} and were considered as wild-type beta-lactamase. None of the isolates were positive for $bla_{OXA-1-like}$, $_{-2-like}$ or $_{-10-like}$ genes.

Based on the distribution of gene variants, 39 isolates were selected and further characterized at the plasmid level using PBRT, pMLST, pDLST and pRST (if applicable) and at the strain level using MLST. The selection was aimed to investigate the diversity of genetic determinants and cover as much plasmid and strain STs as possible. Accordingly, all strains positive for bla_{CTX-M} (n=7) and all for bla_{CMY}-bla_{SHV} (n=6) were included to cover the diversity or homogeneity of plasmids and strains associated to their spread. In contrast, a random selection was performed for the strains positive for bla_{CMY} (n=92) and bla_{SHV} (n=17) (Table 1).

Plasmid Typing

ESBL/AmpC genes from 36 strains were transferable using electroporation or conjugation. In the remaining 3 strains (EC1.6, EC10PP62457 and UGCAR489EC) the genes were non-transferable (Table 2). The transferred genes of 33 strains were located on a single plasmid replicon in the recipient strain after selective isolation. For 1 strain the plasmids remained non-typeable (Fig 1). IncI1 plasmids were most abundant in isolates from all years, sources and department of origin of the samples (22/33), except for the department of Bolivar (Table 2). IncK (4/33), IncHI2 (3/33), IncA/C (2/33), IncB/O (1/33) and a non-typeable replicon (strain FBOG54) were also associated with ESBL/AmpC-genes at a low frequency (Fig 1 and Table 2). Overall, IncI1 plasmids were associated with carriage of bla_{CMY-2}, bla_{SHV-12} and $bla_{CTX-M-8}$ genes, IncHI2 with carriage of $bla_{CTX-M-2.}$ Further plasmid typing, pMLST for IncI1 and pDLST for IncHI2, demonstrated IncI1/ST12 (13/22) and the new IncHI2/ST7 (2/3) as the most abundant plasmid Sequence Types, respectively (Table 2). In addition to IncHI2/ST7, two new IncI1 STs were observed, designated as ST229 and ST230 (Table 2). Three IncI1 and one IncHI2 plasmid remained non-typeable due to missing amplification of one of the alleles in the pMLST and pDLST schemes (Table 2). Information relating to the year, source and origin of the strains with their respective gene, ST, incompatibility group (PBRT) and plasmid ST is shown in Table 2.

Strain Typing

Thirty-one different Sequence Types (STs) were detected among the 39 strains selected (Fig 1 and Table 2). Sequence Types ST38 (n=2), ST101 (n=3), ST155 (n=3), ST533 (n=2), ST624 (n=2) and ST4243 (n=2) were found more than once. ST155 and ST4243 were found in slaughterhouses and retail, respectively. ST533 and ST624 were found both in retail but ST533 in 2011 and ST624 in 2009. ST38 was encountered in different years and sources. Finally, ST101 was found in samples from farms (n=1), slaughterhouses (n=1) and retail (n=1) in different years.

Table 2. Strain and plasmid typing on selected strains after ESBL/AmpC genes characterization.

Source	Year	Location (Department)	Strain	ESBL/ AmpC enzymes in original isolates[a]	Plasmid replicons	Plasmid ST/FAB formula	Strain ST	Clonal Complex
Farm	2008	Santander	ECSLV.10.C1	SHV-12	I1[b]	Non-typeable[e]	366	-
		Santander	ECSXXXIV.1.C	CMY-2	I1[b]	12	57	350
	2009	Cundinamarca	EC1.6	CTX-M-2	-	-	1266	-
		Cundinamarca	EC4.5	CMY-2	I1[b]	Non-typeable[e]	101	101
Slaughter	2008	Santander	ECSIIIL.9.C1	CMY-2	I1-F[d]	12-F24:A-:B1	155	155
		Santander	ECSIIL.18.C2	SHV-12	I1[b]	12	3107	-
		Santander	ECSIVL.9	CTX-M-2	HI2[b]	Non-typeable[e]	101	101
		Santander	ECSLII.8.C3	CMY-2	I1[b]	12	155	155
		Santander	ECSLIV.27.C2	CMY-2	I1[b]	12	10	10
		Santander	ECSLIV.7.C1	CMY-2	I1[b]	Non-typeable[e]	3910	-
		Santander	ECSVIL.11.C1	CMY-2/ SHV-12	I1[b]	12	359	-
		Santander	ECSVIL.21.C3	CMY-2	I1[b]	12	212	-
		Santander	ECSXXXII.7	CTX-M-2	A/C[b]		38	38
	2012	Cundinamarca	FBOG54	SHV-5	Non-typeable[b]		155	155
		Santander	FSAN126	CMY-2	I1[b]	231	1775	-
	2013	Sucre	FSUC314	CTX-M-8	I1[b]	114	641	86
		Magdalena	FMAG347	SHV-12	I1[c]	230[g]	226	226
Retail	2009	Cundinamarca	EC102.1	CMY-2	I1[c]	229[g]	23	23
		Cundinamarca	EC107.1	CTX-M-2	HI2[b]	7[g]	624	648
		Cundinamarca	EC108.1	CTX-M-2	HI2[b]	7[g]	624	648
		Cundinamarca	EC114.1	CMY-2/ SHV-12	B/O[b]		4243	-
		Cundinamarca	EC10PP62457	CMY-2	-	-	101	101
		Cundinamarca	EC9PP62328	SHV-12	I1[b]	230[g]	973	-
	2010	Cundinamarca	UGBOG166EC	CMY-2	K[b]		2040	-
		Cundinamarca	UGBOG204EC	CMY-2/ SHV-5	I1[b]	12	189	165
		Cundinamarca	UGBOG301EC	CMY-2	I1[b]	12	201	469

Source	Year	Location (Department)	Strain	ESBL/ AmpC enzymes in original isolates[a]	Plasmid replicons	Plasmid ST/FAB formula	Strain ST	Clonal Complex
		Cundinamarca	UGBOG304EC	CMY-2/ SHV-12	K[b]/ I1[bf]	26	746	-
		Cundinamarca	UGBOG34EC	CMY-2	I1[b]	12	48	10
		Meta	UGVIL369EC	CMY-2/ SHV-12	I1[b]	12	1049	-
		Meta	UGVIL380EC	CMY-2	K[c]		135	-
	2011	Atlántico	UGBAR389EC	CMY-2	I1[b]	12	5416	-
		Atlántico	UGBAR425EC	CMY-2	I1[b]	12	533	-
		Atlántico	UGBAR428EC	CMY-2	I1-F[d]	12-F29:A-:B-	533	-
		Bolívar	UGCAR489EC	CTX-M-15	-	-	224	-
		Bolívar	UGCAR500EC	CMY-2	K[b]		1158	-
		Cundinamarca	UGCAR511EC	CMY-2	A/C[c]		4243	-
		Córdoba	UGMON457EC	CMY-2/ SHV-5	I1[b]	12	162	469
		Boyacá	UGSAN546EC	CMY-2	I1-F[d]	Non-typeable[e]-F36:A1:B1	2847	-
		Boyacá	UGTUN878EC	CMY-2	K[b]		38	38

[a] Underlined genes were transferable to recipient strains by electroporation or conjugation of plasmids [b] Single replicon transferred by electroporation or [c] by conjugation. [d] Both replicons were transferred together by conjugation. It is uncertain in which of the plasmids the ESBL/AmpC gene was transferred. [e] No PCR amplification product for one of the alleles. [f] bla_{CMY-2} and bla_{SHV-12} were transferred in IncK and IncI1 plasmids respectively. [g] New plasmid sequence types.

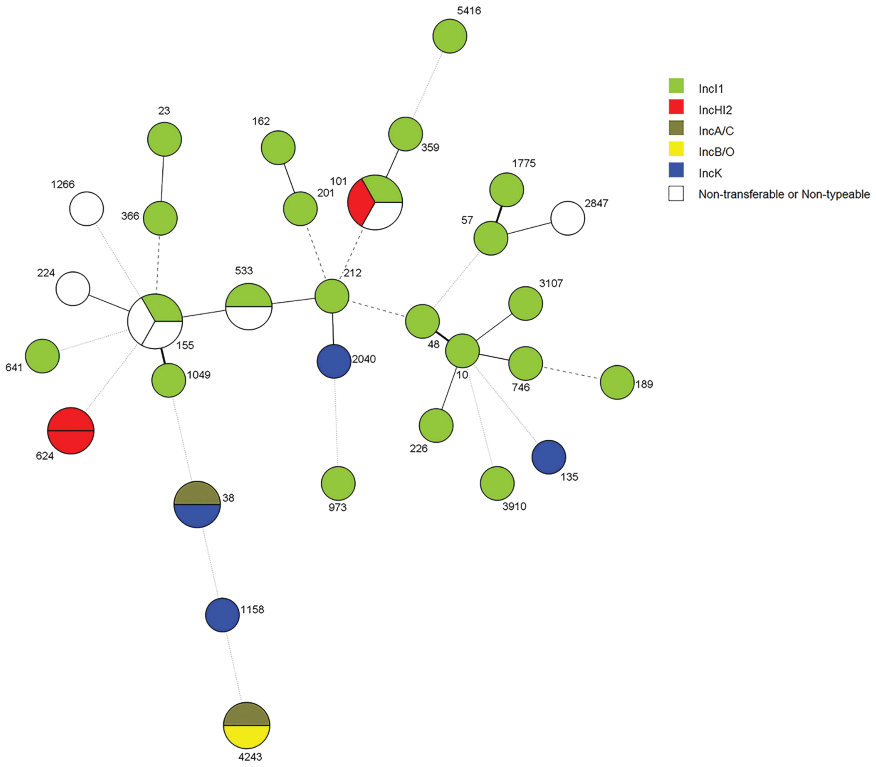

Figure 1. Distribution of plasmid families in ESBL/AmpC-producing *E. coli* from different Sequence Types in the Colombian poultry chain.

Discussion

We have provided the molecular characterization of ESBL/AmpC-producing *E. coli* from different base-line studies within the Colombian poultry production chain. Our selection aimed to reflect the characteristics of resistance in different Colombian departments in order to comply with the country-wide scope of an integrated program like Coipars. We have found that the most prevalent genes and plasmids were present in highly heterogeneous strain STs, which may well be indicative for non-clonal spread of resistance within the poultry chain. In addition, our results suggest that dissemination of the most prevalent genes, bla_{CMY-2} and bla_{SHV-12}, is mainly through horizontal transfer of plasmids belonging to the IncI1 group.

Among the systems providing automation to the phenotypic screening of ESC resistance, Vitek 2 (bioMérieux) and BD Phoenix have been used in the investigation of ESBL-producing *E. coli* in animal isolates[34–37]. Although Vitek 2 is most commonly used, no dedicated assessment of their performance for isolates

from animal origin is available in the literature. When testing human isolates in studies from Europe and the USA, Phoenix has been found to provide a better sensitivity and specifity in comparison with other commercial systems[38–41]. In our study, Phoenix was used for detection of resistance to cefotaxime regardless of its ESBL evaluation. In general, this drug is preferred among other cephalosporins given its advantage to cover both ESBL and plasmidic AmpC phenotypes[42]. In this respect, the automated phenotypic screening of cefotaxime resistance enabled the analysis of a large number of isolates with good discriminatory power. In total 122 out of 132 resistant strains were found to carry ESBL/AmpC genes, showing good performance of the automated system to screen large bacterial collections. The remaining isolates may be chromosomal *ampC*-promoter mutants or harbour a gene we did not screen for.

Our study has some limitations related to the limited number of isolates from farms and slaughterhouses from which a convenient selection of isolates was available. The exclusion of epidemiologically related isolates resulted in a marked reduction of samples, from 155 to 45 at farms and from 182 to 66 at slaughter. This exclusion may have influenced the prevalence of genes, plasmids and strains we encountered since it resulted in the uneven distribution of samples between the 3 levels of production. Additionally it may have resulted in loss of coverage of the diversity of genetic determinants within same production flocks. Nevertheless, this was a requisite to avoid general overrepresentation of genes, plasmids and strains due to sample duplicity. Thus, the characteristics of resistance were reported individually for the 3 different levels and our results served principally as a base line for the Colombian poultry chain. In addition, we included all isolates from retail and finally found that the proportion of the two most prevalent genes, $bla_{\text{CMY-2}}$ and $bla_{\text{SHV-12,}}$ was equal in the three sources. This strongly suggests that in our collection of *E. coli* the distribution of ESBL/AmpC genes in the 3 levels of production we sampled are comparable.

Gene frequencies

Other studies of ESBL/AmpC-producing *E. coli* from poultry in Latin America are scarce. However, available reports from Brazil have showed that together with $bla_{\text{CMY-2}}$[43], $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-8}}$ are important mediators of ESC-resistance in *E. coli* from poultry and chicken meat[44–48]. It is important to note that some of these studies only screened for $bla_{\text{CTX-M}}$ and did not include detection of plasmidic AmpC genes in their PCR screening. This will affect the accuracy of a comparison with the prevalences described in this study. Furthermore, studies from the USA and Canada demonstrated that resistance to third generation cephalosporins in samples from chicken meat is predominantly mediated by $bla_{\text{CMY-2}}$[49–54]. In our study, the prevalence of $bla_{\text{CMY-2}}$ was higher than other genes within the three sources. We found a proportion of over 58% in ESBL/AmpC positive strains from farms, slaughter and retail. In our case, the encountered prevalence was based on random

isolation of *E. coli* (that is, without selection using antimicrobials during enrichment). Therefore, the frequencies of other genes than bla_{CMY-2} may be underestimated and comparisons with reports using selective isolation with cephalosporins during enrichment have to be interpreted carefully. Our finding of this gene as the main mediator of ESC resistance, is in accordance with previous studies (using selective isolation) from European countries like Denmark, Sweden, and the Netherlands where bla_{CMY-2} was found to be highly prevalent at grandparent and parent level of the broiler production pyramid[55–57].

This is the first publication from Colombia in relation to ESBL/AmpC–producing *E coli* from poultry and chicken meat. In reports from human isolates, $bla_{CTX-M-12}$, $bla_{CTX-M-15}$, bla_{SHV-12} and bla_{SHV-5}[19–22] were most prevalent. More recently, bla_{CMY-2} has been included as part of the genotypic screening and associated to community onset infections caused by *E. coli* ST131[23]. Although previous studies did not characterize the plasmids mediating the resistance, our findings suggest a possible relationship with some of the ESBL/AmpC genes found in humans in Colombia, namely $bla_{CMY-2,}$ bla_{SHV-12}, bla_{SHV-5} and $bla_{CTX-M-15}$. Further studies using a one health approach, simultaneous collection of samples from human and animal sources and sequence-based molecular characterization of plasmids and strains are necessary to confirm this relationship.

ESBL/AmpC carrying plasmids

Various plasmid families, namely IncI1, IncK, IncF, IncHI2, IncA/C and IncB/O were found harbouring ESBL/AmpC genes. However, we have shown that the association of bla_{CMY-2} and bla_{SHV-12} was mainly to IncI1 plasmids. Both the diversity of the analysed strains (year, source and place of isolation) and the diversity in *E. coli* STs, in contrast to the homogeneity in plasmid types, support the hypothesis that spread of ESBL/AmpC in the Colombian poultry chain is mainly mediated by horizontal gene transfer of plasmids. A minimal spanning tree representing the genetic relatedness of *E. coli* STs and plasmid lineages is shown in Fig 1.

In contrast to strain Sequence Types, there is evidence that supports the association between specific plasmid Sequence Types and the spread of particular ESBL/AmpC genes. In a previous study, Smith and colleagues[58] reported the combination of IncI1/ST12 and bla_{CMY-2} in more than 80% of the plasmids they analysed. Among our isolates, bla_{CMY-2} was the most frequently encountered gene, and IncI1/ST12 was most frequently associated to its spread. In particular, we found 12 isolates carrying bla_{CMY-2} in IncI1/ST12 plasmids out of 16 carrying bla_{CMY-2} in overall IncI1 plasmids. It is likely that this combination of plasmid ST-gene is widespread in Colombian poultry since bla_{CMY-2} was the most prevalent gene in our study and IncI1 accounted for most of its occurrence (16/24). The features associated to this plasmid ST and its interactions with *E. coli* hosts are of further interest to assess the factors influencing the successful spread of bla_{CMY-2} in poultry.

Sequence Types of ESBL/AmpC producers

As a result of our selection, different samples from all years and levels of production were characterized. Some departments were represented with more than one sample, namely Cundinamarca (n=15) Santander (12), Atlántico (3), Meta (2), Bolívar (2) and Boyacá (2). In addition single samples from Sucre, Magdalena and Córdoba were included. In general, we observed high heterogeneity of strain STs harbouring same combinations of gene variants and plasmids (Fig 1 and Table 2). In addition, but to a lesser extent, samples originating from the same years, levels of production and departments carried the same genes, plasmids and strain STs, as observed for ST155 (Santander), ST624 (Cundinamarca) and ST533 (Atlántico).

From a plasmid perspective, IncI1 harbouring bla_{CMY} was distributed among highly heterogeneous *E. coli* STs from different years, levels of production and departments. However, it was also present among the closely related *E. coli* ST155 and ST533, in both cases carrying bla_{CMY-2} on an IncI1/ST12 plasmid (Table 2). In this respect, we conclude our selection of strains was sufficient to detect inter-department diversity and intra-department diversity to a limited level, and also the likelihood that in one geographical area *E. coli* belonging to the same ST exist. Nonetheless, an in-depth analysis for the spread within departments was out of the scope of the present study. This would require a much more intensive sampling scheme.

At a broader scale, seven out of 31 STs have been previously reported as ESBL/AmpC producers in samples from both humans and poultry, namely ST10, ST23, ST38, ST48, ST57, ST155 and ST624[11,14,59–64]. One has only been found as ESBL/AmpC producer in poultry, ST641[11,60,65,66] and 3 in humans, ST101, ST162 and ST746[63,67]. Additionally, ST224, ST359 and ST973 have been previously reported in other livestock and companion animals[65,68,69]. To our knowledge this is the first report in which ESBL/AmpC producers are associated with ST135, ST189, ST201, ST212, ST226, ST366, ST533, ST1049, ST1158, ST1266, ST1775, ST2040, ST2847, ST 3107, ST3910, ST4243 and ST5416. At the level of isolates from human clinical isolates in Colombia, only two strains belonging to ST38 coincide in our selection. Different than $bla_{CTX-M-15}$[24] our strains carried bla_{CMY-2} or $bla_{CTX-M-2}$ and were not considered as an evident link for the transmission of resistance.

In conclusion, the molecular characterization of ESBL/AmpC-producing *E. coli* has identified the genetic determinants mediating the spread of resistance to ESC in Colombian poultry and chicken meat. The differences in distribution of genes, plasmids and strains between our study and other reports may be related to different practices of farming and supply of chickens and chicken meat in the country. Further characterization of ESBL/AmpC-producing *E. coli* from human and poultry sources in Colombia is necessary to understand the potential transmission of resistance determinants. This approach could be enhanced by the use of next generation sequencing of isolates and plasmids.

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Supporting Information

S1 Table. Drugs and range of concentrations tested with the BD Phoenix panels NMIC-121.
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Chapter 4.

Genomic Characterization of Extended-Spectrum Cephalosporin-Resistant *Salmonella enterica* in the Colombian Poultry Chain

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Abstract

Salmonella enterica serovars have been isolated from Colombian broilers and broiler meat. The aim of this study was to investigate the diversity of ESBL/pAmpC genes in extended-spectrum cephalosporin resistant *Salmonella enterica* and the phylogeny of ESBL/pAmpC-carrying *Salmonella* using Whole Genome Sequencing (WGS).

A total of 260 cefotaxime resistant *Salmonella* isolates, obtained between 2008 and 2013 from broiler farms, slaughterhouses and retail, were included. Isolates were screened by PCR for ESBL/pAmpC genes. Gene and plasmid subtyping and strain Multi Locus Sequence Typing was performed *in silico* for a selection of fully sequenced isolates. Core-genome-based analyses were performed per ST encountered.

bla$_{\text{CMY-2-like}}$ was carried in 168 isolates, 52 carried *bla*$_{\text{CTX-M-2}}$ group, 7 *bla*$_{\text{SHV}}$, 5 a combination of *bla*$_{\text{CMY-2-like}}$-*bla*$_{\text{SHV}}$ and 3 a combination of *bla*$_{\text{CMY-2-like}}$-*bla*$_{\text{CTX-M-2}}$ group. In 25 isolates no ESBL/pAmpC genes that were screened for were found. WGS characterization of 36 selected strains showed plasmid-encoded *bla*$_{\text{CMY-2}}$ in 21, *bla*$_{\text{CTX-M-165}}$ in 11 and *bla*$_{\text{SHV-12}}$ in 7 strains. These genes were mostly carried on IncI1/ST12, IncQ1 and IncI1/ST231 plasmids, respectively. Finally, 17 strains belonged to *S.* Heidelberg ST15, 16 to *S.* Paratyphi B variant Java ST28, 1 to *S.* Enteritidis ST11, 1 to *S.* Kentucky ST152 and 1 to *S.* Albany ST292. Phylogenetic comparisons with publicly available genomes showed separate clustering of Colombian *S.* Heidelberg and *S.* Paratyphi B var. Java.

In conclusion, resistance to extended-spectrum cephalosporins in *Salmonella* from Colombian poultry is mainly encoded by *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{CTX-M-165}}$ genes. These genes are mostly associated with IncI1/ST12 and IncQ1 plasmids, respectively. Evolutionary divergence is observed between Colombian *S.* Heidelberg and *S.* Paratyphi B var. Java and those from other countries.

Introduction

Salmonella Enteritidis and *S.* Typhimurium have been reported as the most frequent serovars causing salmonellosis in humans worldwide (Hendriksen et al., 2011). According to data collected in the European Union (EU) in 2015 and the United States (USA) in 2013, *S.* Enteritidis accounted for 46% and 15% of *Salmonella* infections and *S.* Typhimurium for 16% and 13%, respectively (Centers for Disease Control and Prevention [CDC], 2016; European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2016). Likewise, *S.* Typhimurium and *S.* Enteritidis are the most isolated serovars from human cases in Colombia. Their overall prevalence among human isolates between 2005 and 2011 was 32% and 28%, respectively (Rodríguez et al., 2017).

Among foods of animal origin, poultry products (e.g. eggs) have been primarily associated with *S.* Enteritidis infections in humans while *S.* Typhimurium infections are associated with a wider range of sources including pork, beef and poultry products (Antunes et al., 2016; Mughini-Gras et al., 2014). Nevertheless, in broilers and chicken meat the prevalence of serotypes other than *S.* Enteritidis and *S.* Typhimurium have been on the rise over the last 2 decades (Foley et al., 2011; van Pelt et al., 2003; Waagenaar et al., 2013). Baseline studies of *Salmonella* performed between 2005 and 2006 in broiler chickens in the EU showed *S.* Infantis, *S.* Mbandaka and *S.* Hadar to be highly prevalent (European Food Safety Authority [EFSA], 2007). In the year 2006, *S.* Infantis, *S.* Enteritidis and *S.* Paratyphi B d-tartrate positive (here referred as *S*. Paratyphi B variant Java) were the most frequent serovars in broiler meat in the EU (European Food Safety Authority [EFSA], 2007). More recently, *S.* Infantis, *S.* Enteritidis, *S.* Mbandaka and *S.* Ohio were the most prevalent serovars in broilers and broiler meat in 2015 (European Food Safety Authority [EFSA] and European Centre for Disease Prevention and Control [ECDC], 2016). In North America, *S.* Kentucky, *S.* Enteritidis, *S.* Typhimurium, *S.* Heidelberg and *S.* Infantis are reported as the most prevalent serovars in broilers and ground chicken meat in the USA (United States Department of Agriculture [USDA] - Food Safety and Inspection Service [FSIS], 2013) while in Canada, *S.* Heidelberg, *S.* Kentucky and *S.* Enteritidis are the most prevalent in broilers and broiler meat (Public Health Agency of Canada, 2016). In Colombia, baseline studies performed by the Colombian integrated program for antimicrobial resistance surveillance (Coipars), demonstrated *S.* Paratyphi B var. Java and *S.* Heidelberg to be the most prevalent serovars in broiler farms and meat at retail. Serovar distribution at farm level was 76% and 23%, respectively (Donado-Godoy et al., 2012b), and at the retail level 45% and 19%, respectively (Donado-Godoy et al., 2014). A similar distribution of *S.* Paratyphi B var. Java and *S.* Heidelberg, has been reported in chicken meat at retail in Guatemala (35% and 16% respectively) (Jarquin et al., 2015) and broilers at slaughter in Venezuela (62% and 31% respectively) (Boscán-Duque et al., 2007).

Isolates of *S.* Paratyphi B var. Java and *S.* Heidelberg are often multi drug resistant (Antunes et al., 2016; Denny et al., 2007; Dutil et al., 2010; Liakopoulos et al., 2016) and have been associated with carriage of plasmid-mediated extended spectrum β-lactamases (ESBLs) and plasmid associated AmpC β-Lactamases in poultry (pAmpC) (Antunes et al., 2016). For instance, *S.* Paratyphi B var. Java isolates from poultry in Europe have been found carrying the ESBL gene $bla_{\text{CTX-M-1}}$ and the pAmpC gene $bla_{\text{CMY-2}}$ (Doublet et al., 2014; Mevius et al., 2015; Veldman et al., 2016). In turn, *S.* Heidelberg has been associated to $bla_{\text{CMY-2}}$ in North America (Andrysiak et al., 2008; Folster et al., 2012) and poultry products imported from South America into Europe (Liakopoulos et al., 2016). In South America *S.* Heidelberg has been associated with $bla_{\text{CTX-M-2}}$ (Antunes et al., 2016). To date, no data of the genetic determinants of Extended Spectrum Cephalosporin (ESC) resistance in *Salmonella* from broilers and chicken meat in Colombia are available. These data are highly relevant to understand the epidemic spread of ESBL/pAmpC-producing *Salmonella* in poultry resulting in frequent occurrence in chicken meat in multiple countries.

Serotyping has traditionally been used for the epidemiological investigation of *Salmonella,* but does not provide information about the evolutionary relatedness of strains. Sequence based methodologies such as Multi Locus Sequence Typing (MLST) and Whole Genome Sequencing (WGS) have been proposed as a replacement of serotyping to identify evolutionary and epidemiological relatedness (Achtman et al., 2012; Ashton et al., 2016; Nadon et al., 2017). Additionally, information on the genetic basis of antimicrobial resistance (AMR) and plasmids harboring AMR genes can be readily obtained from WGS assemblies (Carattoli et al., 2014; Zankari et al., 2012). Altogether, the objectives of this study were to investigate the diversity of ESBL/pAmpC genes and encoding plasmids found in ESC-resistant *S. enterica* from broilers and broiler meat in Colombia and to determine the genetic relatedness with *Salmonella* strains from other countries using MLST and core-genome alignments.

Materials and methods

Isolates of *Salmonella enterica*

The isolates included in this study originated from different cross-sectional baseline studies conducted between 2008 and 2013 at different stages during the development of Coipars. In these studies, non-clinical samples were obtained from three different levels of poultry production in Colombia: Broiler farms (Donado-Godoy et al., 2012b), broilers at slaughter (Donado-Godoy et al., 2015b) and broiler meat at retail (Donado-Godoy et al., 2012a, 2014, 2015a). The methodology for sampling, random isolation (i.e. without antimicrobials during enrichment) of *Salmonella* and antimicrobial susceptibility testing with the BD Phoenix automated system, was previously described in detail for the studies in broiler farms,

broilers at slaughter and broiler meat at retail mentioned above. Previous results of the prevalence of *Salmonella* and resistance to ESC at the different levels of poultry production, are summarized in Table 1.

For the present study, all available *S. enterica* isolates (n=673) were considered. The 673 isolates belonged to 578 production flocks, 28 flocks from broiler farms, 140 from slaughterhouses and 410 from retail (Table 1). Next, ESC-resistant isolates of *Salmonella* were selected based on suspected phenotypic resistance to cefotaxime, as previously measured with the BD Phoenix (Donado-Godoy et al., 2012b) and interpreted using the CLSI 2014 clinical breakpoints for Enterobacteriaceae (MIC≥4 μg/ml) (Clinical and Laboratory Standards Institute [CLSI], 2014). For all flocks with multiple ESC-resistant isolates, each first isolate was selected to make sure we included epidemiologically unrelated isolates only.

As a result, a total of 260 isolates were selected for ESC-resistance characterization. The selected isolates from farms originated from drag swabs of fecal material from the floor of broiler houses and fresh feces directly from the chicken. These isolates originated from 19 broiler farms (n=19) (Donado-Godoy et al., 2012b). Isolates at slaughter, originated from cecal content and carcass rinse from 32 slaughterhouses (n=84) (Donado-Godoy et al., 2015b). At retail, isolates originated from carcass rinse from 143 retail suppliers (Donado-Godoy et al., 2012a, 2014) and chicken thighs meat from 8 retail suppliers (n=157) (Donado-Godoy et al., 2015a). The isolates originated from 18 out of 32 departments (i.e. provinces) of Colombia. Together, these 18 departments are responsible for more than 90% of the chicken population in the country. A map of Colombia with the location of these departments is available in Supplementary Figure 1.
Information related to the origin of the samples and the prevalence of *Salmonella* is shown in Table 1.

PCR screening of ESBL/pAmpC genes

For the present investigation, the 260 isolates selected as mentioned above, were screened by PCR for the presence of ESBL/pAmpC genes families using previously described primers for bla_{TEM} and bla_{SHV} families, $bla_{\text{CMY-2-like}}$ and $bla_{\text{CTX-M}}$ family (Dierikx et al., 2010); $bla_{\text{CTX-M-1}}$ group (Carattoli et al., 2008); $bla_{\text{CTX-M-2}}$ group (Jiang et al., 2006); $bla_{\text{CTX-M-8}}$ group (Hopkins et al., 2006); $bla_{\text{CTX-M-9}}$ group (Paauw et al., 2006); $bla_{\text{OXA-1-like}}$, $bla_{\text{OXA-2-like}}$ and $bla_{\text{OXA-10-like}}$ (Voets et al., 2011).

Table 1. Prevalence and origin of *Salmonella* isolates used in this study, distribution of ESBL/pAmpC genes and selection of isolates for WGS.

	Number of samples	*Salmonella*-positive (%)	ESBL/ pAmpC-producing *Salmonella* (%) [a]	$bla_{CMY-2-like}$	$bla_{CTX-M-2}$ group	bla_{SHV} family	$bla_{CMY-2-like}$-$bla_{CTX-M-2}$ group	$bla_{CMY-2-like}$-bla_{SHV} family
Farms	74	28 (38)	17 (61)	16 / 2[b]	-	-	-	1 / 1[b]
2008	65	23	17	16	-	-	-	1
2009	9	5	-	-	-	-	-	-
Slaughter	644	140 (22)	82 (59)	54 / 4[b]	25 / 2[b]	-	2 / 2[b]	1 / 1[b]
2008	40	5	3	3	-	-	-	-
2012	251	76	39	31	8	-	-	-
2013	353	59	40	20	17	-	2	1
Retail	1554	410 (26)	136 (33)	98 / 7[b]	27 / 6[b]	7 / 7[b]	1 / 1[b]	3 / 3[b]
2009	179	38	11	11		-	-	-
2010	387	156	35	29	1	4	-	1
2011	823	170	76	49	22	2	1	2
2012	165	46	14	9	4	1	-	-
Total	2272	578 (25)	235 (41)	168 / 13 [b]	52 / 8[b]	7 / 7[b]	3 / 3[b]	5 / 5[b]

[a] Based on PCR screening of ESBL/pAmpC genes. [b] ESBL/pAmpC-positive samples / Selected for whole genome sequencing.

Selection of strains and WGS

Based on the diversity of gene families found after PCR screening, a representative selection of isolates was made and further subjected to WGS. Due to its high prevalence, a random selection was made using the Random function in Microsoft® Excel to assign random numbers to isolates positive for $bla_{CMY-2-like}$ and $bla_{CTX-M-2}$ group. The size of selection was fixed to the square root of the number of resulting positive strains for these genes. This resulted in 13 $bla_{CMY-2-like}$- and 8 $bla_{CTX-M-2}$ group-carrying isolates. In addition and due to their low prevalence, all positive isolates for bla_{SHV} family and combinations of $bla_{CMY-2-like}$-bla_{SHV} family and $bla_{CMY-2-like}$-$bla_{CTX-M-2}$ group were included. An overview of the selection of isolates is shown in Table 1.

Isolation of genomic DNA from selected isolates was performed using the UltraClean® Microbial DNA Isolation Kit (Mo Bio-Qiagen, USA). WGS was performed on Illumina MiSeq and NextSeq platforms (Illumina, USA) using 2 × 250-bp reads and 2 × 150-bp reads, respectively. Genomes were assembled with SPAdes v3.10.1 (Bankevich et al., 2012).

In silico characterization of ESBL/pAmpC gene variants

Subtyping of ESBL/pAmpC gene variants was performed using ResFinder 2.1 (Zankari et al., 2012). Investigation of resistance genes with an identity percentage < 100% was done using BLAST 2.6.0+ (Camacho et al., 2009).

In silico characterization of ESBL/pAmpC-carrying plasmids

Plasmid content of selected strains was investigated using PlasmidFinder 1.3 and pMLST 1.4 (Carattoli et al., 2014).

Identification of plasmids associated to the ESBL/pAmpC genes was based on co-localization on the same contig as resulted from ResFinder and PlasmidFinder analysis. Since this was not possible for all genomes, transformation of plasmids harboring each of the ESBL/pAmpC variants identified with ResFinder was performed together with selective culturing as described before (Castellanos et al., 2017). This was done to obtain the plasmid types identified with PlasmidFinder in transformed *Escherichia coli* DH10B harboring the different ESBL/pAmpC variants identified. Afterwards, the sequences of the transformed plasmids were used as reference to map against the genomes of the selected strains. To this purpose, transformants were sequenced with Illumina MiSeq and NextSeq sequencing as described above. Chromosomal contigs in transformants were detected and removed by mapping against the *E. coli* DH10B genome sequence (GenBank accession number: CP000948.1) using BLAST. The remaining contigs were considered to be part of the ESBL/pAmpC carrying plasmid and were used as a reference. Next, the contigs of the initially sequenced selected strains were aligned to the obtained reference plasmid sequences using MUSCLE (Edgar, 2004). Alignments were made on selected strains according to the ESBL/pAmpC gene variants they harbored. Resulting aligned contigs were selected and considered as newly inferred ESBL/pAmpC-carrying plasmids.

In silico MLST and serotype prediction

To determine the population structure of the selected ESC-resistant *Salmonella*, 7-gene MLST at the strain level was performed *in silico* with MLST 1.8 (Larsen et al., 2012). Serotype was predicted using the *Salmonella In Silico* Typing Resource (SISTR) (Yoshida et al., 2016). Whole genome phylogenetic analyses were performed for *S.* Heidelberg ST15 and *S.* Paratyphi B var. Java ST28. Given the limited number of isolates (n=1), this was not performed for *S.* Enteritidis ST11, *S.* Kentucky ST152 or *S.* Albany ST292.

Collection of publicly available genomes for phylogenetic comparisons

Genome sequences of *S.* Paratyphi B var. Java ST28, were downloaded from Enterobase (https://enterobase.warwick.ac.uk/; (last accessed: 12-Sept-2017) for comparison. Likewise, sequences of *S.* Heidelberg ST15 were obtained from Enterobase (last accessed: 11-Jan-2017). Only genomes with data available for their year of isolation, country of origin and source were considered for *S.* Heidelberg ST15. For both *S.* Paratyphi B var. Java ST28 and *S.* Heidelberg ST15, the genomes were collected disregarding their susceptibility to 3rd generation cephalosporins or to other antimicrobials. Additionally, the quality of genomes obtained in this study and the downloaded genomes was assessed with CheckM (Parks et al., 2015). Only genomes with >98% completeness score, when compared against the set of genomic markers for *S. enterica,* were included. MLST designation was amended using a custom BLAST-based tool (https://github.com/tseemann/mlst).

Core genome phylogenetic analysis

Whole genome analysis was performed by a core-genome alignment using Parsnp v1.2 (Treangen et al., 2014). Recombination regions in the core genome alignment were detected and filtered using Gubbins (Croucher et al., 2015). Phylogenetic maximum likelihood (mid-point rooted) trees were constructed with the recombination-filtered core genomes alignments using FastTree2 (http://meta.microbesonline.org/fasttree/) (Price et al., 2010) and visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Data availability

The obtained genome sequences of the *Salmonella* strains selected for WGS (Table 2) and those of the transformed *E. coli* DH10B strain with the ESBL/pAmpC-carrying plasmids used as reference have been deposited in the short-read archive of the ENA under Project Number: PRJEB23610.

Results

PCR screening of ESBL/pAmpC genes

After PCR screening of the 260 isolates, 235 isolates were positive for the genes screened for. 168 were positive for $bla_{\text{CMY-2-like}}$, 52 for $bla_{\text{CTX-M-2}}$ group, 7 for bla_{SHV} family, 5 harbored a combination of $bla_{\text{CMY-2-like}}$-bla_{SHV} family and 3 a combination of $bla_{\text{CMY-2-like}}$-$bla_{\text{CTX-M-2}}$ group. In 48 isolates, bla_{TEM} was co-located with $bla_{\text{CMY-2-like,}}$ $bla_{\text{CTX-M-2}}$ group or bla_{SHV}. In 25 isolates no ESBL/pAmpC genes that were screened for were encountered.

The distribution of positive samples for *Salmonella* according to their source, year and ESBL/pAmpC genes is shown in Table 1.

Selection of strains for WGS

After PCR characterization, a random selection of $bla_{\text{CMY-2-like}}$-positive isolates (n=13) and $bla_{\text{CTX-M-2}}$ group (n=8) together with all positive isolates for bla_{SHV} family (n=7), $bla_{\text{CMY-2-like}}$-bla_{SHV} family (n=5) and $bla_{\text{CMY-2-like}}$-$bla_{\text{CTX-M-2}}$ group (n=3) were included for WGS. In total, 36 isolates were selected and subjected to WGS. The list of selected isolates and the results of the characterization of ESBL/pAmpC genes, plasmid types, serotypes and strain MLST based on WGS is shown in Table 2.

Characterization of ESBL/pAmpC gene variants

After WGS characterization of resistance genes, 13 strains harbored $bla_{\text{CMY-2}}$, 8 $bla_{\text{CTX-M-165}}$, 6 $bla_{\text{SHV-12}}$ and 1 $bla_{\text{SHV-129}}$. Additionally, three harbored the combination of $bla_{\text{CMY-2}}$-$bla_{\text{CTX-M-165}}$, four $bla_{\text{CMY-2}}$-$bla_{\text{SHV-129}}$ and one $bla_{\text{CMY-2}}$-$bla_{\text{SHV-12}}$ (Table 2). All accompanying bla_{TEM} variants were identified as $bla_{\text{TEM-1A}}$ or $bla_{\text{TEM-1B}}$.

In silico characterization of ESBL/pAmpC-carrying plasmids

Co-localization of ESBL/pAmpC and plasmid replicon genes in the same contig was observed for 17 out of 36 strains selected for WGS. For the remaining strains, co-localization was determined by analyzing selected contigs harboring ESBL/pAmpC and plasmid replicon genes using MUSCLE. Genes conferring resistance to other antimicrobials only co-localized with $bla_{\text{CTX-M-165}}$-harboring contigs, not with other ESBL/pAmpC genes (Supplementary Table 1). In detail, one transformant was obtained carrying either a $bla_{\text{CMY-2}}$-, $bla_{\text{CTX-M-165}}$- or $bla_{\text{SHV-12}}$-harboring plasmid. The plasmids isolated from strains UGBOG4 ($bla_{\text{CMY-2}}$), UGBAR1160 ($bla_{\text{CTX-M-165}}$) and UGBOG327 ($bla_{\text{SHV-12}}$) (Supplementary Table 1) were used as a reference to map against the genomes of all selected strains. The $bla_{\text{SHV-129}}$ gene present in five selected strains was not transferable by transformation from any of the strains, suggesting chromosomal localization of this gene.

After characterizing the plasmid contigs of the selected strains, 18 of 21 $bla_{\text{CMY-2}}$-carrying plasmids were found to belong to IncI1/ST12, 1 was non-typeable based on the PCR Based Replicon Typing (PBRT) scheme used in PlasmidFinder (Carattoli et al., 2014). Two were designated IncI1, but the plasmid from strain UGVAL515 lacked the *pilL* allele and the plasmid from FBOG7 lacked the *sogS* allele. Nonetheless, these two plasmids remained single-allele variants of IncI1/ST12. For $bla_{\text{CTX-M-165}}$, 9 of 11 plasmids harbored the IncQ1 plasmid-replicon and 2 remained non-typeable. Four of seven $bla_{\text{SHV-12}}$ plasmids belonged to IncI1/ST231 and three remained non-typeable (Table 2 and Supplementary Table 1).

Table 2. Origin of the *Salmonella* isolates selected for WGS and results of the characterization of their ESBL/pAmpC genes, plasmids and strains.

Source	Year	Strain	Location (Department)	Accession number ENA	β-lactam resistance genes[a]	ESBL/pAmpC-harboring plasmid[c]	*S. enterica* Serovar	*S. enterica* MLST
Farm	2008	SSXXXV.4.C1	Santander	ERS2017899	bla_{CMY-2}	IncI1/ST12	S. Java	ST28
		SSIII.4.C2	Santander	ERS2017900	bla_{CMY-2}-$bla_{SHV-129}$[b]-bla_{TEM-1A}	IncI1/ST12	S. Java	ST28
		SXXI.1.C5	Cundinamarca	ERS2017901	bla_{CMY-2}	IncI1/ST12	S. Java	ST28
Slaughter	2012	FBOG8	Bogotá	ERS2017931	bla_{CMY-2}	IncI1/ST12	S. Java	ST28
		FSAN161	Santander	ERS2017935	$bla_{CTX-M-16S}$-bla_{TEM-1B}	IncQ1	S. Heidelberg	ST15
		FSAN236	Santander	ERS2017936	$bla_{CTX-M-16S}$-bla_{TEM-1B}	IncQ1	S. Heidelberg	ST15
		FBOG7	Bogotá	ERS2017938	bla_{CMY-2}	IncI1[d]	S. Java	ST28
	2013	FSUC414	Sucre	ERS2017932	bla_{CMY-2}-$bla_{CTX-M-16S}$-bla_{TEM-1B}	IncI1/ST12-IncQ1[e]	S. Heidelberg	ST15
		FCAR509	Bolívar	ERS2017933	bla_{CMY-2}	IncI1/ST12	S. Heidelberg	ST15
		FANT596	Antioquia	ERS2017934	bla_{CMY-2}-$bla_{SHV-129}$-bla_{TEM-1B}	IncI1/ST12	S. Java	ST28
		FVAL369	Valle del Cauca	ERS2017937	bla_{CMY-2}	IncI1/ST12	S. Heidelberg	ST15
		FPAS506	Nariño	ERS2017939	bla_{CMY-2}-$bla_{CTX-M-16S}$	IncI1/ST12-Non-typeable[e]	S. Heidelberg	ST15
Retail	2010	UGBOG4	Bogotá	ERS2017904	bla_{CMY-2}-bla_{SHV-12}	IncI1/ST12-Non-typeable[e]	S. Java	ST28
		UGBOG316	Bogotá	ERS2017908	bla_{SHV-12}	Non-typeable	S. Heidelberg	ST15
		UGBOG327	Bogotá	ERS2017909	bla_{SHV-12}	IncI1/ST231	S. Java	ST28
		UGBOG339	Bogotá	ERS2017910	bla_{SHV-12}	IncI1/ST231	S. Java	ST28
		UGBOG340	Bogotá	ERS2017911	bla_{SHV-12}	IncI1/ST231	S. Java	ST28

2011	UGBAR394	Atlántico	ERS2017912	bla_{CMY-2}-$bla_{CTX-M-16S}$-bla_{TEM-1B}	IncI1/ST12-IncQ1[e]	*S.* Heidelberg	ST15
	UGBAR434	Atlántico	ERS2017913	bla_{CMY-2}	IncI1/ST12	*S.* Albany	ST292
	UGCAR507	Bolívar	ERS2017914	$bla_{CTX-M-16S}$-bla_{TEM-1B}	IncQ1	*S.* Heidelberg	ST15
	UGVAL515	Valle del Cauca	ERS2017915	bla_{CMY-2}-$bla_{SHV-129}$	IncI1[d]	*S.* Heidelberg	ST15
	UGBUC832	Santander	ERS2017916	$bla_{SHV-129}$	-	*S.* Heidelberg	ST15
	UGCUC851	Norte de Santander	ERS2017917	bla_{CMY-2}	IncI1/ST12	*S.* Java	ST28
	UGCUC867	Norte de Santander	ERS2017918	$bla_{CTX-M-16S}$-bla_{TEM-1B}	IncQ1	*S.* Heidelberg	ST15
	UGARA888	Arauca	ERS2017919	bla_{CMY-2}	IncI1/ST12	*S.* Enteritidis	ST11
	UGIBA933	Tolima	ERS2017920	bla_{SHV-12}	IncI1/ST231	*S.* Java	ST28
	UGPER971	Risaralda	ERS2017921	bla_{CMY-2}	IncI1/ST12	*S.* Java	ST28
	UGBOG1024	Bogotá	ERS2017922	bla_{CMY-2}	IncI1/ST12	*S.* Java	ST28
	UGPAS1097	Nariño	ERS2017923	bla_{CMY-2}	IncI1/ST12	*S.* Kentucky	ST152
	UGBUC1112	Santander	ERS2017924	$bla_{CTX-M-16S}$	Non-typeable	*S.* Heidelberg	ST15
	UGBUC1123	Santander	ERS2017925	$bla_{CTX-M-16S}$-bla_{TEM-1B}	IncQ1	*S.* Heidelberg	ST15
	UGBAR1160	Atlántico	ERS2017926	$bla_{CTX-M-16S}$	IncQ1	*S.* Heidelberg	ST15
	UGBAR1170	Atlántico	ERS2017927	bla_{CMY-2}-$bla_{SHV-129}$-bla_{TEM-1B}	IncI1/ST12	*S.* Java	ST28
	UGBAR1187	Atlántico	ERS2017928	$bla_{CTX-M-16S}$-bla_{TEM-1B}	IncQ1	*S.* Heidelberg	ST15
2012	UGBOG1279	Bogotá	ERS2017929	bla_{SHV-12}	Non-typeable	*S.* Heidelberg	ST15
	UGBOG1280	Bogotá	ERS2017930	bla_{CMY-2}	Non-typeable	*S.* Java	ST28

[a] The genes present in the contigs selected for plasmid characterization have been underlined. [b] $bla_{SHV-129}$ was non-transferable after electroporation experiments. [c] The plasmids carrying the β-lactam resistance genes have been underlined. [d] These plasmids missed one allele from the pMLST scheme. [e] Characterization of the two ESBL/pAmpC genes was performed in separate plasmid contigs.

Strain MLST, serotype characterization and core genome phylogeny

After using 7-gene MLST and the *Salmonella In Silico* Typing Resource (SISTR), 17 strains belonged to *S.* Heidelberg ST15, 16 to *S.* Paratyphi B var. Java ST28, 1 to *S.* Enteritidis ST11, 1 to *S.* Kentucky ST152 and 1 to *S.* Albany ST292 (Table 2). Further, whole genome analysis was performed for ST28 and ST15 isolates. For the phylogenetic analysis, additional genomes for ST28 (n=60) and ST15 (n=1221) were selected from Enterobase.warwick.ac.uk disregarding their characteristics of susceptibility to 3rd generation cephalosporins and used to construct the phylogenetic maximum likelihood trees. All Colombian genomes belonging to ST28 and ST15 formed a single cluster in the phylogenetic analysis. Phylogenetic trees for ST28 and ST15 with data regarding the source, year and country of the strains, are shown in Figures 1 and 2 respectively. No clustering related to the presence or absence of an ESBL/pAmpC gene is observed in Figure 1, suggesting the observed clustering is related to the geographical origin of *S.* Paratyphi B var. Java ST28 strains and not to the presence of an AMR gene. Likewise, in Figure 2, a cluster of *S.* Heidelberg ST15 strains originating from Colombian poultry is observed. Furthermore, the Colombian strains from ST28 and ST15 were disseminated in multiple departments within the country. A map of Colombia with the location of origin of ST28 and ST15 isolates selected for WGS is available in Supplementary Figure 2. An additional table with the metadata of strains selected for the construction of the ST28 and ST15 phylogenies is available as Supplementary Table 2.

Discussion

In summary, *bla*$_{CMY-2}$, *bla*$_{CTX-M-165}$, *bla*$_{SHV-12}$ and *bla*$_{SHV-129}$ are described as the most prevalent ESBL/pAmpC genes conferring resistance to ESC in *S. enterica* isolated from the Colombian poultry chain between 2008 and 2013. According to the objectives of the present study, the collection of isolates served to reflect maximum diversity of ESBL/pAmpC genes in different years, departments and levels of the poultry production chain in Colombia.

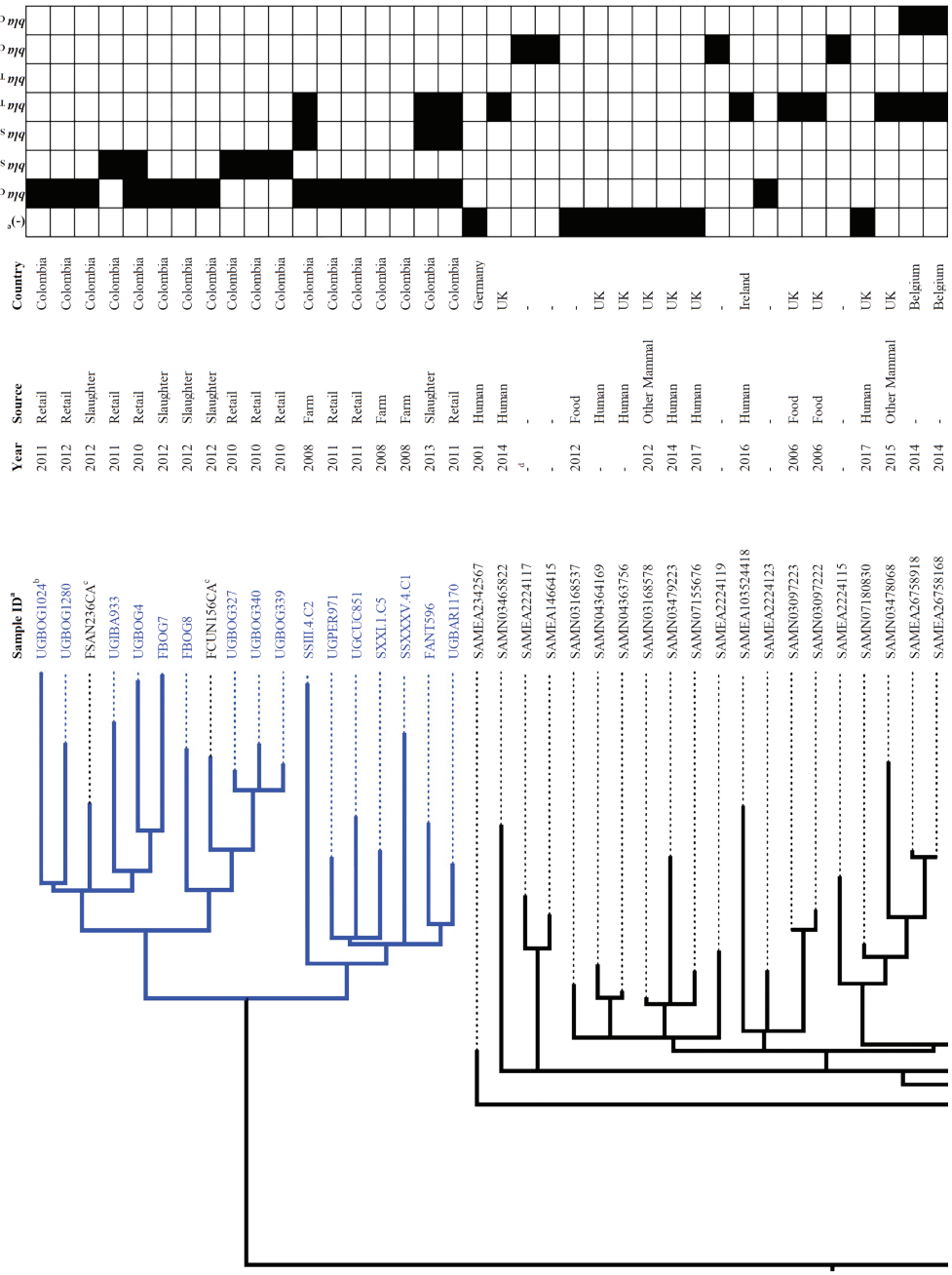
The finding of *bla*$_{CMY-2}$ as the main cause of ESC resistance is comparable to a previous report of ESBL/pAmpC-producing *E. coli* in Colombian poultry. In that study, it was encountered a prominent association of this gene with IncI1/ST12 plasmids (Castellanos et al., 2017). Those results suggested occurrence of horizontal gene transfer of this plasmid lineage between heterogeneous *E. coli* STs. The results from the present study, suggest that transfer of *bla*$_{CMY-2}$-IncI1/ST12 plasmids between *E. coli* and *Salmonella* is also likely to occur, and could be considered a driver of the frequent occurrence of this resistance gene along the poultry chain. This particular gene-plasmid association has been described in *E. coli* from Brazil (da Silva et al., 2017), *Salmonella* from the USA (Folster et al., 2010) and *E. coli* and *Salmonella* from Europe (Accogli et al., 2013; Smith et al., 2015), suggesting an epidemiological link between the presence of *bla*$_{CMY-2}$-IncI1/

ST12 and poultry from different countries. Nevertheless, WGS of these plasmids is necessary to assess the level of genetic relatedness among them and estimate the potential transmission of these plasmids between *E. coli* and *Salmonella*.

After PCR screening, $bla_{CTX\text{-}M\text{-}2}$ group was found to be the most prevalent ESBL gene among Colombian isolates. After subtyping a selection of these isolates with WGS, these genes were found to be $bla_{CTX\text{-}M\text{-}165}$. To date, this variant has been solely reported in an isolate of *Klebsiella pneumoniae* from a urine sample in Chile and reported in 2016 (Accession number: KP727572) without further epidemiological records. Noticeably, all $bla_{CTX\text{-}M\text{-}165}$-positive strains, alone or in combination with other *bla* genes, were identified in *S.* Heidelberg ST15. In our collection of isolates comprising the years 2008 to 2013, $bla_{CTX\text{-}M\text{-}165}$ is only detected from the year 2010 onwards (Table 1). Not taking into account the potential bias that could occur by having isolates comprising a period of time no longer than 5 years, this finding may suggest a recent introduction of this gene in Colombian poultry, and until 2013, is limited to *S.* Heidelberg ST15. However, analysis of recently collected isolates of *S. enterica* and other Enterobacteriaceae is necessary to confirm this hypothesis.

After electroporation experiments, $bla_{SHV\text{-}129}$ remained non-transferable and no plasmid markers were identified in its harboring contigs, which ranged in size between 2100 and 8594 bp. Therefore, it is likely that this gene is chromosomally located. Furthermore, the gene was found in two different serovars, *S.* Paratyphi B var. Java ST28 and *S.* Heidelberg ST15. It can be hypothesized that its transfer could be associated to an integrative or transposable element. Initial screening of transposases and Insertion Sequences (IS) using BLAST (Siguier, 2006) on the contigs harboring these genes detected several IS families flanking the sequences inside the contigs. Nevertheless, given the restricted size of the contigs no definite association with a unique IS element (e.g. AMR-associated IS) was possible. In such a case, complementing the short-read WGS data with additional data obtained through long-read sequencing is necessary to confirm the chromosomal location of this gene and its association to a particular mobile genetic element. This approach could also be used for further characterization of ESBL/AmpC-plasmids that were non-typeable according to the PBRT scheme used in PlasmidFinder, which could have also been affected by the limitations of genome and plasmid assembly of short-reads.

As mentioned previously, ESC and non-ESC *S.* Paratyphi B var. Java and *S.* Heidelberg were reported as the most prevalent serovars in the Colombian poultry chain (Donado-Godoy et al., 2012b, 2014). In the present study, investigation of resistance to cefotaxime showed a total of 235 (41%) resistant isolates from which, 17 (61%) originated from farms, 82 (59%) from slaughterhouses and 136 (33%) from retail (Table 1). Noteworthy, the prevalence of resistance diminishes from one level of production to the other. From previous studies, *S.* Paratyphi B var. Java and *S.* Heidelberg were the most prevalent serovars encountered at the farm level (Donado-Godoy et al., 2012b) and a larger diversity of serovars was


Sample ID[a]
Year
Source
Country
(-)[e]
blaCMY-2
blaSHV-12
blaSHV-129[f]
blaTEM-1[g]
blaTEM-52
blaCTX-M-1
blaCTX-M-2
UGBOG1024[b] 2011 Retail Colombia
UGBOG1280 2012 Retail Colombia
FSAN256CA[c] 2012 Slaughter Colombia
UGIBA933 2011 Retail Colombia
UGBOG4 2010 Retail Colombia
FBOG7 2012 Slaughter Colombia
FBOG8 2012 Slaughter Colombia
FCUN156CA[c] 2012 Slaughter Colombia
UGBOG327 2010 Retail Colombia
UGBOG340 2010 Retail Colombia
UGBOG339 2010 Retail Colombia
SSIII.4.C2 2008 Farm Colombia
UGPER971 2011 Retail Colombia
UGCUC851 2011 Retail Colombia
SXXL1.C5 2008 Farm Colombia
SSXXXV.4.C1 2008 Farm Colombia
FANT596 2013 Slaughter Colombia
UGBAR1170 2011 Retail Colombia
SAMEA2342567 2001 Human Germany
SAMN03465822 2014 Human UK
SAMEA2224117 [d] - -
SAMEA1466415 - - -
SAMN03168537 2012 Food -
SAMN04364169 - Human UK
SAMN04363756 - Human UK
SAMN03168578 2012 Other Mammal UK
SAMN03479223 2014 Human UK
SAMN07155676 2017 Human UK
SAMEA2224119 - - -
SAMEA103524418 2016 Human Ireland
SAMEA2224123 - - -
SAMN03097223 2006 Food UK
SAMN03097222 2006 Food UK
SAMEA2224115 - - -
SAMN07180830 2017 Human UK
SAMN03478068 2015 Other Mammal UK
SAMEA26758918 2014 - Belgium
SAMEA26758168 2014 - Belgium

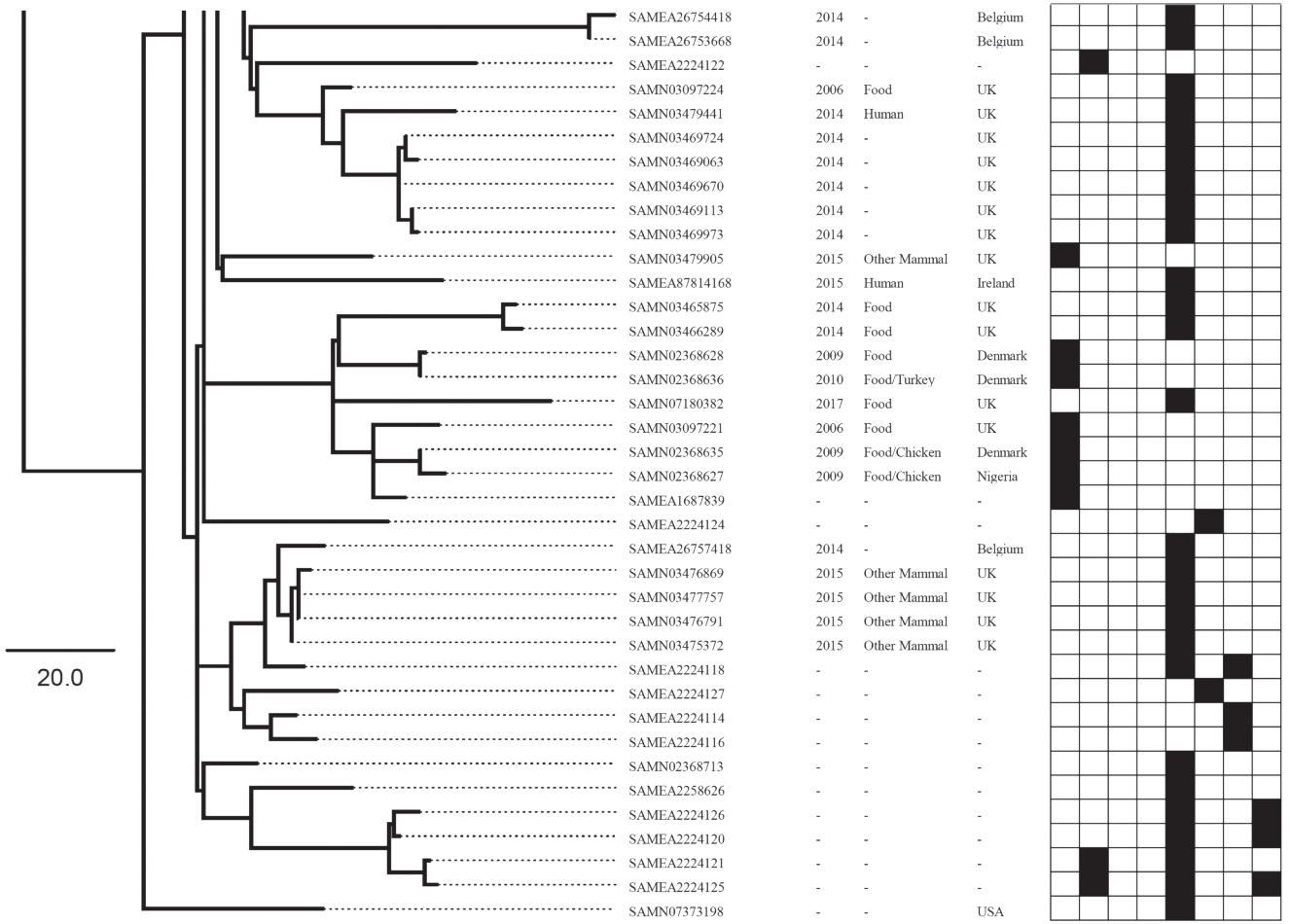
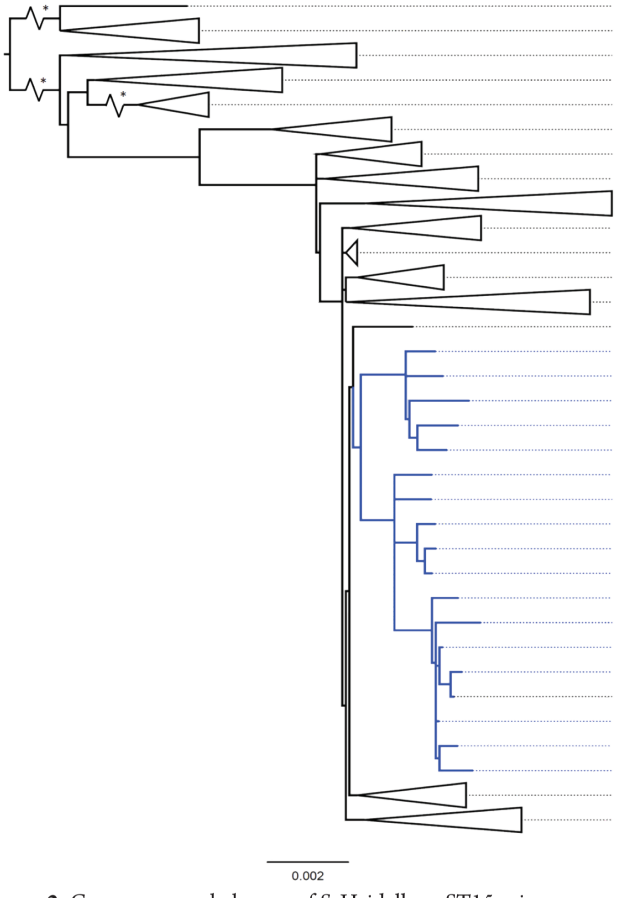


Figure 1. Core-genome phylogeny of *S.* Java ST28 using the genomes obtained in the present study and those available in Enterobase1 (last accessed:12-Sept-2017). [a] Sample ID for downloaded genomes is equivalent to the Biosample ID as available in Enterobase and NCBI. [b] Strains with their name in blue were sequenced in the present study. [c] Colombian strains with genomes sequenced prior to the present study and available in Enterobase. [d] No data available. [e] Negative for β-lactam resistance genes. [f] Non-transferable. [g] Wild-type β-lactamase.



Sample ID[a]/Number of strains	Years of isolation[e]	Source of isolation (%)[g]	Country of origin (%)[g]
SAMN02843731[b]	2002	Shellfish	Thailand
8	2002-2016	Human (37%)	UK (50%)
13	1961-2015	Human (69%)	UK (61%)
4	2014-2015	Macadamia nuts (75%)	USA (75%)
6	2012-2014	Human (83%)	UK (83%)
13	2009-2016	Bovine (85%)	USA (100%)
2	2007-2012	Human (100%)	France (50%); Canada (50%)
53	2003-2015	Poultry (92%)	USA (100%)
366	1998-2016	Poultry (87%)	USA (99%)
74	1997-2015	Poultry (85%)	USA (85%)
4	2006[f]	Bovine (100%)	USA (100%)
20	2003-2007	Human (45%); Manure (45%)	USA (95%)
186	2002-2016	Poultry (84%)	USA (99%)
SAMN03476166	2014	Human	UK
UGBOG316[c]	2010	Poultry	Colombia
UGVAL515	2011	Poultry	Colombia
UGBUC832	2011	Poultry	Colombia
FVAL369	2013	Poultry	Colombia
UGBOG1279	2012	Poultry	Colombia
UGCUC867	2011	Poultry	Colombia
UGBAR1160	2011	Poultry	Colombia
FPAS506	2013	Poultry	Colombia
UGBUC1123	2011	Poultry	Colombia
UGBUC1112	2011	Poultry	Colombia
UGCAR507	2011	Poultry	Colombia
FSUC414	2013	Poultry	Colombia
FSAN161	2012	Poultry	Colombia
FSAN236	2012	Poultry	Colombia
SAMN03842020[d]	2013	Poultry	Colombia
UGBAR394	2011	Poultry	Colombia
UGBAR1187	2011	Poultry	Colombia
FCAR509	2013	Poultry	Colombia
99	2004-2016	Poultry (97%)	USA (99%)
370	2000-2016	Poultry (83%)	USA (99%)

Figure 2. Core-genome phylogeny of *S.* Heidelberg ST15 using genomes obtained in this study and those with complete metadata selected from Enterobase1 (last accessed: 11-Jan-2017).

*Length of the branches have been reduced 10-fold for display purposes. [a] Sample ID for downloaded genomes is equivalent to the Biosample ID as available in Enterobase and NCBI. [b] Name of individual strains have been underlined. [c] Strains with their name in blue colour were sequenced in the present study. [d] Colombian strain with genomes sequenced prior to the present study and available in Enterobase. [e] A range of years is provided for multiple strains. [f] Multiple strains originated in the same year. [g] The main Source and main Country of origin of multiple strains is presented as percentage in brackets (%).

found at retail, with more than 10 different serovars isolated repeatedly (Donado-Godoy et al., 2014). As observed in the present study, strains belonging to *S.* Paratyphi B var. Java and *S.* Heidelberg had a higher prevalence of ESC-resistance in comparison to the other serovars. These results indicate that the higher prevalence of *S.* Paratyphi B var. Java and *S.* Heidelberg, accounted in large part for the higher prevalence of ESC–resistance at the farm level and the presence of different serovars resulted in the reduction of resistance along the production chain, which is reflected in the lower prevalence of ESC-resistance at retail.

As anticipated, the analysis of *Salmonella* strains using MLST in addition to the resolution provided by WGS data has proven to be very useful in showing clustering of Colombian strains belonging to *S.* Paratyphi B var. Java ST28. According to the phylogenetic analysis including ESBL/pAmpC-positive and –negative strains (Figure 1), the clustering seems to occur independently of ESC-susceptibility and may be related to the geographical origin of the strains. Whether the cluster of Colombian *S.* Paratyphi B var. Java ST28 represents a particular separate lineage circulating in the country, or is present elsewhere in Latin America is a question that requires further investigation. At the time of publication of the present study no genomes of ST28 from other Latin American countries were publicly available and the comparisons within the region were limited to the strains we sequenced and analyzed.

In conclusion, resistance to ESC in *S. enterica* from Colombian poultry is mainly caused by $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-165}}$ genes. These genes are mostly associated with IncI1/ST12 and IncQ1 plasmids, respectively. The resolution provided by WGS was appropriate to assess the evolutionary divergence of strains from Colombian poultry belonging to *S.* Paratyphi B var. Java ST28. Dissemination of ESBL/pAmpC genes in *Salmonella* is mainly due to the carriage of plasmids encoding these genes in strains belonging to *S.* Paratyphi B var. Java ST28 and *S.* Heidelberg ST15.

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Supplementary Material

The Supplementary Material for this article can be found online at:
https://www.frontiersin.org/articles/10.3389/fmicb.2018.02431/full#supplementary-material

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5

Chapter 5.

Phylogenomic Investigation of IncI1-Iγ Plasmids Harboring *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{SHV-12}}$ in *Salmonella enterica* and *Escherichia coli* in Multiple Countries

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Abstract

bla$_{CMY-2}$ and *bla*$_{SHV-12}$ are prevalent genes conferring resistance to Extended-Spectrum-Cephalosporins (ESC) in *Salmonella enterica* and *Escherichia coli* from Colombian poultry. The objective of this study was to investigate the genetic relatedness of *bla*$_{CMY-2}$ and *bla*$_{SHV-12}$-carrying IncI1-Iγ plasmids from animal and human sources originating from multiple countries.

Sequences of *bla*$_{CMY-2}$- and *bla*$_{SHV-12}$-carrying IncI1 plasmids from *S. enterica* and *E. coli* were extracted from whole genome sequence data and characterized by pMLST. Publicly available plasmid sequences with relevant pMLST types and/or resistance genes were collected from GenBank and the European Nucleotide Archive (ENA). Genes were identified and phylogenomic analysis based on core genome alignments and gene presence absence was performed.

Most IncI1/ST12 plasmids originated from poultry. IncI1/ST12 and its Single-Locus Variants (SLVs) in *Salmonella* and *E. coli* from American and European countries had near-identical plasmid core genomes. A sublineage of IncI1/ST231 plasmids was observed and grouped plasmids from *Salmonella* and *E. coli* from Colombian poultry and *E. coli* from the USA, originating from human samples. Plasmids belonging to SLVs of IncI1/ST231 were distributed in different clusters. In most *bla*$_{CMY-2}$-carrying IncI1/ST12 plasmids from *Salmonella* and *E. coli*, the genetic environment of *bla*$_{CMY-2}$ was identical, while it differed for *bla*$_{SHV-12}$-carrying IncI1/ST231 plasmids.

The high genomic relatedness and similarity of the genetic environment of *bla*$_{CMY-2}$ across IncI1/ST12 plasmids suggests that *bla*$_{CMY-2}$-carrying IncI1/ST12 plasmids originated from a common ancestor and share a common source of origin. In contrast, the differences in the genetic environment of *bla*$_{SHV-12}$ suggests the insertion of *bla*$_{SHV-12}$ has occurred multiple times independently.

Importance

Resistance to Extended Spectrum Cephalosporins (ESC) is often caused by enzymes encoded by the genes *bla*$_{CMY-2}$ and *bla*$_{SHV-12}$. These genes are frequently observed in *Salmonella enterica* and *Escherichia coli* isolated from Colombian poultry. ESC resistance genes are usually carried by plasmids, which can be classified in specific groups based on their incompatibility (Inc-type) and subclassified by Sequence Types (STs). In Europe and North and South America, *bla*$_{CMY-2}$ has been associated to the specific plasmid type IncI1/ST12. *bla*$_{SHV-12}$ has been reported on multiple IncI1 STs, however, in Colombian *S. enterica* it is associated to IncI1/ST231. In order to understand the epidemiology of plasmids and spread of these resistance genes, the aim of this study was to investigate the genetic relatedness of these specific plasmid types. The significance of this study is in reconstructing the potential origin of spread of these plasmids carrying these ESC resistance genes, and to determine any potential geographical effect.

Introduction

In Enterobacteriaceae, plasmid-encoded Extended-Spectrum and AmpC β-lactamases (ESBL/pAmpC) are the dominant causes of resistance to Extended Spectrum Cephalosporins (ESC) in a wide range of human, animal, and environmental reservoirs[1–3]. Poultry and poultry products have been considered a reservoir of ESBL/pAmpC-producing *Salmonella enterica* and *Escherichia coli*[2–8], and the presence of these bacteria in poultry products could be a potential risk for the health of human consumers[9].

In Enterobacteriaceae, genes encoding ESBL and pAmpC enzymes are mainly located on transferable plasmids which can be classified in different incompatibility groups (Inc-types), including IncI1-Iγ (here designated as IncI1), IncK, IncF, IncA/C, IncHI2[10,11]. Among these plasmids, IncI1 is dominant in *E. coli* and *S. enterica* originating from poultry in multiple countries[4,12–15]. In Colombia, bla_{SHV-12} and bla_{CMY-2} are the most prevalent ESBL/pAmpC-genes carried on IncI1 plasmids in *S. enterica* and *E. coli* in poultry[16,17]. Using plasmid Multi Locus Sequence Typing (pMLST) IncI1 plasmids can be subtyped into different Sequence Types (STs)[18,19]. Moreover, Specific ESBL/pAmpC variants were found to be associated to particular IncI1/STs[12,13,16,17]. bla_{CMY-2}-carriage has been associated to IncI1/ST12 in *E. coli* and *Salmonella* isolates from poultry in Europe[12,13,20] and North America[14]. Likewise, this combination was highly prevalent in *E. coli* and *Salmonella* from Colombian poultry[16,17]. In contrast to bla_{CMY-2}, carriage of bla_{SHV-12} has been described in multiple IncI/STs such as ST3, ST26, and ST29, in isolates originating from humans, animals (mainly poultry) and the environment[20–23]. In Colombian poultry, bla_{SHV-12} was found to be carried on IncI1/ST231 in *Salmonella*[17], however, in *E. coli* it was carried on IncI1/ST12, ST26, and ST230[16]. It has been suggested that horizontal gene transfer (HGT) is a potential driver of the spread of bla_{CMY-2} between *Salmonella* and *E. coli* in poultry. However, a resolution higher than the nucleotide sequences of the five house-keeping genes used in the IncI1 pMLST scheme is required to confirm this hypothesis[17]. Such resolution can be provided when Whole Genome Sequencing (WGS) of plasmids is performed[4].

In addition to the contribution of particular plasmid lineages to the spread and persistence of ESBL/pAmpC genes, the role of mobilizable elements such as Insertion Sequences (IS) and transposons should be considered when studying the transfer of ESBL/pAmpC genes between different plasmid lineages[21,24,25]. For instance, bla_{CMY-2} is frequently reported being bracketed by IS*Ecp1*, not only on IncI1 but also on IncK and IncA/C plasmids[13,25–27]. In turn, bla_{SHV-12} is reported frequently to be bracketed by IS*26*, on IncHI2, IncX3, IncP and IncI1 plasmids[21].

In these respects, the objective of the present study was to elucidate the evolutionary relatedness of bla_{CMY-2} and bla_{SHV-12}-carrying IncI1 plasmids within the same pMLSTs originating from *S. enterica* and *E. coli,* including the genomic environment of these genes. To this aim, plasmid sequences originating from *Salmonella* and *E. coli* from the Colombian poultry chain and of plasmids publicly

available on GenBank and the European Nucleotide Archive (ENA) from various countries and sources were used for comparisons.

Materials and Methods

Sequencing of plasmids from *Salmonella and E. coli*

For *S. enterica*, all plasmid sequences of *bla*$_{CMY-2}$- (n=20) and *bla*$_{SHV-12}$-carrying (n=4) IncI1 plasmids that originated from a previous characterization of ESBL/pAmpC-carrying *S. enterica* strains, collected in Colombia during baseline studies of COIPARS[17], were included. In the previous study, short-read WGS characterization of the ESBL/pAmpC gene variants and its harboring plasmids was performed using the assemblies of bacterial genomes obtained with Illumina MiSeq and NextSeq sequencing. To this aim, a set of reference plasmids was transformed into DH10B cells and chromosomal contigs in transformants were detected and removed by mapping against the *E. coli* DH10B genome sequence (GenBank accession number: CP000948.1) using BLAST. The reference transformed plasmids were used as a reference to obtain the sequences of the ESBL/pAmpC-carrying plasmids as previously described[17]. From the resulting plasmid sequences, ESBL/pAmpC gene variants and plasmids were characterized *in silico* with ResFinder 2.1[28], PlasmidFinder 1.3 and pMLST 1.4[19].

For *E. coli*, all available *bla*$_{CMY-2}$- (n=15) and *bla*$_{SHV-12}$-carrying (n=4) IncI1 plasmids from a previous characterization of ESBL/pAmpC-carrying *E. coli* strains originating from Colombian baseline studies of COIPARS[16] were included. In the previous study, PCR and sequencing were used to characterize ESBL/pAmpC variants. *bla*$_{CMY-2}$- and *bla*$_{SHV-12}$-carrying plasmids were transformed into *E. coli* DH10B cells using electroporation and selective culturing as previously described[16]. In addition, plasmid characterization was based on PCR Based Replicon Typing (PBRT) and pMLST[16]. For the present study, the transformed *E. coli* DH10B cells harboring *bla*$_{CMY-2}$ and *bla*$_{SHV-12}$ on IncI1 plasmids, were subjected to WGS with Illumina MiSeq and NextSeq sequencing. Genomes were assembled with SPAdes v3.11[29] and chromosomal contigs in transformants were detected and removed using BLAST as described above for *Salmonella*.

Collection of publicly available sequence data

The allele sequences of IncI1 STs (available at: pubmlst.org/plasmid) encountered more than once in the *Salmonella* and *E. coli* selection described above were concatenated and used as a query for the Nucleotide database using BLAST with default settings (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome accessed on: 29 May 2018). From the BLAST output, all plasmid sequences with a Query Coverage value of 100% and Identity value ≥ 99% were selected for each queried IncI1 ST. In addition, the

plasmid sequences of two publications from Europe[12,13] were used to include additional IncI1/ST12 plasmids from the Netherlands and Denmark that were not available on GenBank. The sequences of two plasmids originating from poultry in the Netherlands[12] were made available by Wageningen Bioveterinary Research in the Netherlands. In addition, one plasmid originating from a human sample and nine from poultry from Denmark[13] were obtained from the sequence read archive at ENA (Supplementary Table S1) and assembled as described above.

Phylogenomic analysis based on core genome alignments and gene presence and absence

Core plasmid genome alignments were performed using Parsnp v1.2[30]. Phylogenetic maximum likelihood (mid-point rooted) trees were constructed with the core plasmid genome alignments using FastTree2 v2.1.8[31]. Colombian plasmids from *Salmonella* and *E. coli* belonging to these STs and its Single Locus Variants (SLVs), and the output plasmids from GenBank, the ENA and previous European publications were included. In these trees, plasmid R64 (NCBI Accession number: AP005147.1) was included as reference. Gene presence and absence maximum likelihood trees were built by annotating the plasmid genomes using Prokka v.1.13[32] followed by orthology predictions using Roary[33]. The resulting gene presence absence data was encoded as binary values and trees were constructed using RAxML v.8.2.4[34] with the BINCAT model. Visualization of the trees based on core genome and gene presence and absence was made with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Analysis of the genetic environment of bla_{CMY-2} and bla_{SHV-12}

Of the bla_{CMY-2} and bla_{SHV-12} genes, 10 kb upstream and 10 kb downstream were extracted to study the genetic environment of these genes. Comparison of the genetic environment in Colombian inferred plasmids was made using the bla_{CMY-2} and bla_{SHV-12}-harboring contigs alone. BLAST comparisons of the genetic environment were made and visualized using Easyfig 2.2.3[35].

Data availability

The genome sequences of the *Salmonella* strains previously selected for WGS and of the transformed *E. coli* DH10B strains harboring the reference ESBL/pAmpC-carrying plasmids from *Salmonella* were previously made available in the sequence read archive of the ENA under Project Number: PRJEB23610[17]. The transformed *E. coli* DH10B strains, sequenced for the present study and harboring the ESBL/pAmpC-carrying plasmids from *E. coli* from Colombian poultry, have been deposited in ENA under Project Number: PRJEB29690.

Results

Plasmid collection

The plasmid STs that were encountered more than once in *Salmonella* and *E. coli* from Colombian poultry, and therefore used as a query in GenBank, were IncI1/ST12 and ST231. A summary of the plasmids included in this study is given in Table 1.

From the Colombian plasmids found in *Salmonella* and *E. coli*, 32 of 35 plasmids carrying bla_{CMY-2} belonged to IncI1/ST12, two were SLVs of ST12 (pUGVAL515 lacked the *pilL* allele and pFBOG7 lacked the *sogS* allele) as previously reported[17] and one belonged to ST231. Four bla_{SHV-12}-carrying plasmids from *Salmonella* belonged to IncI1/ST231 and from *E. coli*, one belonged to ST12, one to ST26, one to ST230, and pECSLV.10.C1 was an SLV of ST26 because it lacked the *sogS* allele (Table 1). The information regarding the source and year of isolation of the *Salmonella* strains from Colombian poultry harboring these plasmids, together with their predicted serotype[36] and ST[37] characterization are shown in Table S1 in the supplemental material.

Table 1. Inventory of sequenced IncI1 plasmids from Colombian poultry and previous reports used for detailed phylogenetic comparisons and analysis of the genetic environment.

ESBL/pAmpC	From Colombian Poultry *n* (pMLST)	From GenBank or Previously Published *n* (pMLST) [d]
bla_{CMY-2}	32 (ST12); 2 (SLV ST12[c]); 1 (ST231)	29 (ST12); 8 (ST2[e]); 5 (SLV ST12[c]); 3 (ST23[e]); 1 (ST20[e]); 1 (ST265[e])
bla_{SHV-12}	4 (ST231); 1 (ST12); 1 (ST26); 1 (SLV ST26[c]); 1 (ST230[f])	1 (ST95[e]); 1 (ST178[g]); 1 (ST231)
Other *bla* genes [a]	-	5 (ST12); 1 (ST107[f]); 1 (ST131[f]); 1 (ST270[f])
No *bla* genes [b]	-	1 (ST12); 1 (SLV ST12[c]); 1 (ST230[f])
Total	43	61

[a] Other *bla* genes different than bla_{CMY-2} and bla_{SHV-12}. [b] No *bla* genes detected with ResFinder. [c] Single-Locus Variants (SLV) due to incomplete match or missing one allele from the pMLST scheme. [d] Sequences of plasmids listed in this column were obtained based on the allele sequences of the highly prevalent IncI1-Iγ/ST12 and ST231. [e] Selected for analysis of the genetic environment of bla_{CMY-2}/bla_{SHV-12}. [f] SLV of ST231. [g] SLV of ST12.

Sequences of 84 IncI1/ST12-related and of 75 IncI1/ST231-related plasmids were obtained from GenBank using BLAST. In addition, 12 IncI1/ST12 plasmids carrying bla_{CMY-2} were obtained from previous Dutch and Danish publications[12,13]. After characterization of the plasmids from GenBank with ResFinder, PlasmidFinder and pMLST, 23 plasmids belonged to IncI1/ST12. From these, 17 carried bla_{CMY-2}, 1 $bla_{CMY-2-like}$, 4 $bla_{TEM-1B-like}$ and 1 carried no ESBL/pAmpC genes. In addition, five plasmids carrying bla_{CMY-2} with unknown STs, one plasmid carrying bla_{SHV-12} from ST178 and one plasmid carrying no ESBL/pAmpC genes with unknown ST, were found to be SLVs of ST12 (Table 1 and Table S1). The plasmids from GenBank originated from isolates of different *S. enterica* serovars and *E. coli*. The bacterial isolates originated from multiple sources including human, swine, bovine and poultry and from countries of South and North America and Europe. The accession numbers of these plasmids and their metadata attributes are shown in Table S1 in the supplemental material.

One IncI1/ST231 plasmid carrying bla_{SHV-12} was found. This plasmid originated from an *E. coli* isolate of human origin collected in the USA. In addition, four plasmids belonging to SLVs of IncI1/ST231 (but not harboring bla_{SHV-12}) were found. These plasmids included an IncI1/ST107 plasmid carrying the ESBL gene $bla_{CTX-M-14}$, an ST131 plasmid carrying $bla_{CTX-M-8}$, an ST230 plasmid carrying no ESBL/pAmpC genes and an ST270 plasmid carrying bla_{TEM-1A}. These plasmids originated from isolates of *E. coli* and *Shigella sonnei*. The bacterial isolates harboring these plasmids originated from human samples from South and North America and Asia. The accession numbers of these plasmids and their metadata attributes are shown in Supplemental Table 1.

Additional bla_{CMY-2}- and bla_{SHV-12}-carrying plasmids from different STs than IncI1/ST12 and IncI1/ST231 were included in the analysis of the genetic environment. From GenBank, eight bla_{CMY-2}-carrying plasmids belonging to ST2, one to ST20, three to ST23 and one to ST265 were considered. These plasmids originated from *E. coli* and *S. enterica* isolates, obtained from human, animal and food samples in Asia, Australia, Europe and North America (Table 1 and Table S1). Likewise, two bla_{SHV-12}-carrying plasmids from IncI1/ST95 and ST178 were included (Table 1 and Table S1). These two plasmids originated from isolates of *E. coli*. The metadata from one of the bla_{SHV-12}-carrying plasmids indicate it originated from poultry in Europe, while for the other no metadata was available. When available, the information regarding the source, year and location of isolation, of the additional plasmids together with the characteristic of their bacterial hosts, is shown in Table S1.

Phylogenomic analysis of core genome and gene presence and absence

Phylogeny based on IncI1/ST12 plasmids

The core genome of the tree built based on IncI1/ST12-related plasmids was 40056 base pairs (~40% of the plasmid genome). Sequences of plasmids CP018104.1 (ST12), CP027409.1 (ST26) and pFBOG7 (SLV ST12) from Colombian *Salmonella*, were excluded prior phylogenomic analysis due to either missing sequence data or large discrepancies in their size in comparison with the average 100kbp of most collected IncI1 plasmids. Plasmids LT838204.1, LT838199.1 and LM996561.1 were excluded by the Parsnp program during phylogenomic reconstruction due to falling below the threshold of identity similarity when compared with the reference plasmid. The core genome phylogenetic tree was based on a sequence coverage of 100% and identity similarity ≥ 99% of the concatenated allele sequences of IncI1/ST12. It resulted in 129 plasmid sequences, of which 80 were ST12 or SLVs (see Figure S1 in the supplemental material). Plasmids pSSXXXV.4.C1-T, pSSIII.4.C2-T, pUGBOG4-T, and pUGBAR1170-T were transformants obtained from Colombian *Salmonella* and included as reference (Figure 1 and Figure S1). All plasmids, except CP014622.1, from IncI1/ST12 and SLVs, clustered together in a branch. CP014622.1 was found in a distant branch within the tree (Figure S1). The sublineage of IncI1/ST12 and its SLVs is shown in Figure 1. In total, 78 plasmids were displayed within this sublineage, of which 71 belonged to IncI1/ST12 and 7 were SLVs . Seventy carried bla_{CMY-2}, three $bla_{TEM1B-like}$, two $bla_{SHV-12,}$ one a combination of bla_{CMY-2} and $bla_{CTX-M-1}$, one $bla_{CMY-2-like}$ and one carried no *bla* genes. In general, a high level of similarity within the sublineage of IncI1/ST12 plasmids was shown. A few clusters, that is, plasmids with an identical core genome, were identified (Clusters I-IX in Figure 1A). A large cluster included plasmids from the Netherlands and Denmark, which showed high similarity between *E. coli* and *Salmonella* derived plasmids originating from American countries including Canada, Colombia, Uruguay and the USA (Cluster IX). Another distinct cluster that was observed, contained only plasmids originating from *E. coli* isolated in France (Cluster IV). At the source level, 63 plasmids (including 34 from Colombia) originated from samples from poultry. Sixteen bla_{CMY-2}-carrying non-Colombian plasmids from poultry, obtained from GenBank and the ENA, clustered together with Colombian plasmids (clusters V, VIII and IX). These were carried mainly in *E. coli* (Denmark and the Netherlands), but also *S. enterica* (USA and Uruguay), *S*. Heidelberg (Canada) and *S.* Typhimurium (USA). From the nine clusters identified, two clusters harbored plasmids from human and poultry (clusters VI and and IX), one cluster harbored plasmids from bovine and poultry (cluster VIII), one cluster carried only plasmids from humans (cluster III) and five clusters (I, II, IV, V and VII) carried only plasmids from poultry. One plasmid found in *E. coli* from swine in France (KR494248.1) car-

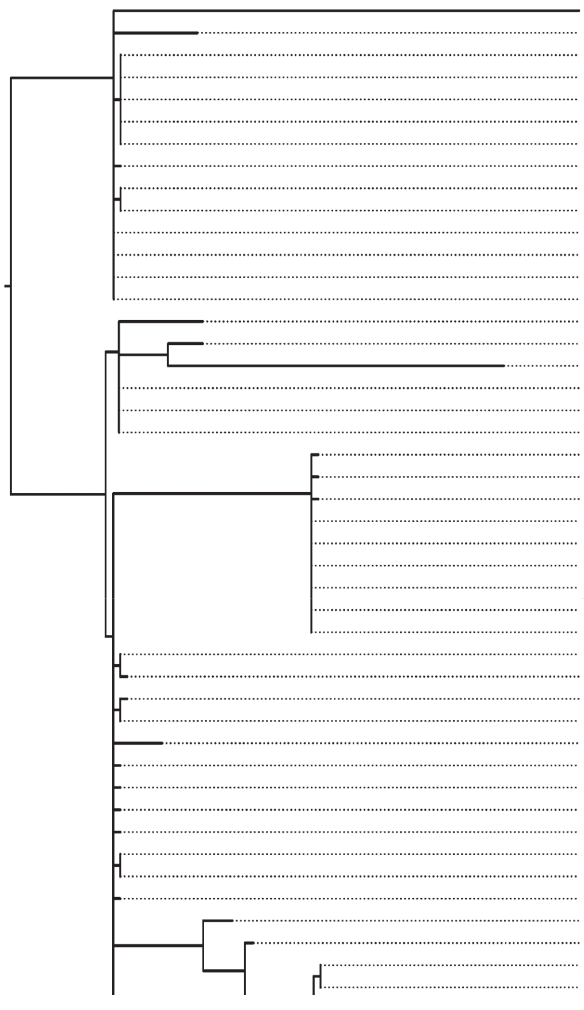
ried bla_{CMY-2} and $bla_{CTX-M-1}$ and was related to the group of plasmids that contained only plasmids originating from *E. coli* isolated in France (cluster IV). In addition to core genome analysis, also the gene presence and absence was used to assess the genetic relatedness of plasmids, including both their core and non-core genome (see Figure S2). In case of IncI1/ST12, the relatedness observed based on gene presence and absence was comparable to the one observed with core plasmid genome analysis only. Similar to core plasmid genome, the gene content phylogeny grouped most of the plasmids from ST12 and SLVs in a sublineage within the tree (MG825376.1-ERR915116). A close relatedness between bla_{CMY-2}-carrying IncI1/ST12 plasmids from *Salmonella* and *E. coli* originating from multiple sources (mostly poultry) and multiple American and European countries was observed. In addition, the plasmids originating from *E. coli* isolated in France (KR494248.1-MG692703.1) also formed a distinct cluster within the ST12-sublineage, suggesting they contain unique genetic content (Figure S2).

Phylogeny based on IncI1/ST231 plasmids

The core genome of the tree based on IncI1/ST231 plasmids was 32789 base pairs (~32% of the plasmid genome). Similar to the findings for IncI1/ST12, all plasmids from IncI1/ST231 and some from SLVs were grouped together in a branch and formed a sublineage within the tree. The sublineage of IncI1/ST231 and its SLVs is shown in Figure 1B. The full tree is shown in Figure S3.

In total, nine plasmids were displayed within the sublineage of IncI1/ST231 and SLVs. Seven belonged to IncI1/ST231 and two to SLVs of ST231 (ST230 and ST270) (Figure 1B). Seven carried bla_{SHV-12}, one bla_{CMY-2}, and one bla_{TEM1A}. The plasmids from *Salmonella* and *E. coli* from Colombia originated from poultry. The other two plasmids originated from human samples (CP018774.2 and CP019905.1). Within this sublineage, two groups of Colombian plasmids were observed. The first group contained one bla_{CMY-2}-carrying ST231 (pFSAN126) and one SLV plasmid (pEC9PP62328), both derived from *E. coli*. The second group includes five *Salmonella* derived IncI1/ST231 bla_{SHV-12}-carrying plasmids, of which four had an identical core (cluster X in Figure 1B). This group also included a highly similar plasmid of human origin, derived from *E. coli* in the USA (CP019905.1). The phylogeny based on gene content of ST231 and SLVs is available in Figure S4. In contrast to ST12, the phylogenetic tree for ST231, exhibit differences between the plasmids from Colombian *Salmonella* and *E. coli*.

A.



Plasmid [a j]	Pattern GE [k]	Country [l]	Source [m,n]	Year [l,n]	Bacterial Host (MLST) [o]	Cluster [p]
CP016865.1 [a]	●	USA	Human	2006	*S. enterica*	
pUGBOG34EC	❸	Colombia	Broiler	2010	*E. coli* (48)	
pFBOG8	❸	Colombia	Broiler	2012	*S.* Java (28)	I
pFANT596	❸	Colombia	Broiler	2013	*S.* Java (28)	
pUGBAR389EC	❸	Colombia	Broiler	2011	*E. coli* (5416)	
pUGBAR425EC	❸	Colombia	Broiler	2011	*E. coli* (533)	
pUGMON457EC	❸	Colombia	Broiler	2011	*E. coli* (162)	
pEC4.5	❸	Colombia	Broiler	2009	*E. coli* (101)	
pSSIII.4.C2	❸	Colombia	Broiler	2008	*S.* Java (28)	
pSSIII.4.C2-T [c]	❸					
pUGBAR434	❸	Colombia	Broiler	2011	*S.* Albany (292)	II
pUGCUC851	❸	Colombia	Broiler	2011	*S.* Java (28)	
pUGBOG1024	❸	Colombia	Broiler	2011	*S.* Java (28)	
pECSXXXIV.1.C	❸	Colombia	Broiler	2008	*E. coli* (57)	
pECSIII..18.C2 [d]	●	Colombia	Broiler	2008	*E. coli* (3107)	
CP027415.1 [j]	-	USA [l]	Human	2012	*S.* Typhimurium	
LT985231.1 [a,d]	◆	France [l]	N/A	N/A	*E. coli*	
CP018110.1 [e]	-	USA	Human	2016	*E. coli* (617)	III
CP018116.1 [e]	-	USA	Human	2016	*E. coli* (617)	
CP018122.1 [e]	-	USA	Human	2016	*E. coli* (617)	
MG692740.1 [a]	●	France	Broiler	2010-2011	*E. coli*	
KR494248.1 [f]	●	France	Pig	2005	*E. coli*	
MG692734.1	❶	France	Broiler	2010-2011	*E. coli*	
MG692703.1	❶	France	Broiler	2010-2011	*E. coli*	IV
MG692704.1	❶	France	Broiler	2010-2011	*E. coli*	
MG692706.1	❶	France	Broiler	2010-2011	*E. coli*	
MG692709.1	❶	France	Broiler	2010-2011	*E. coli*	
MG692735.1	❶	France	Broiler	2010-2011	*E. coli*	
MG692738.1 [a]	◆	France	Broiler	2010-2011	*E. coli*	
pFCAR509	●	Colombia	Broiler	2013	*S.* Heidelberg (15)	
KX452392.1 [a]	◆	Brazil	Turkey	2012 [l]	*E. coli*	
pUGBOG4	●	Colombia	Broiler	2010	*S.* Java (28)	
pUGBOG4-T [c]	❷					
pUGPAS1097	❷	Colombia	Broiler	2011	*S.* Kentucky (152)	
ERR915117	❷	Denmark	Broiler	2011	*E. coli* (745)	
CP012923.1	❷	Canada	Broiler	2010	*S.* Heidelberg (15)	
pUGVIL369EC	❷	Colombia	Broiler	2010	*E. coli* (1049)	
pSXXI.1.C5	❷	Colombia	Broiler	2008	*S.* Java (28)	
pUGBAR394	●	Colombia	Broiler	2011	*S.* Heidelberg (15)	V
CP022064.2 [g]	-	USA	Turkey	2011	*S.* Typhimurium	
pECSLIV.27.C2	❷	Colombia	Broiler	2008	*E. coli* (10)	
CP027127.1	◆	USA [l]	N/A	N/A	*E. coli*	
ERR915119	❷	Denmark	Broiler	2010	*E. coli* (410)	
pUGBAR1170	❹	Colombia	Broiler	2011	*S.* Java (28)	
pUGBAR1170-T [c]	❹					

CP012929.1 4 Canada Human 2012 S. Heidelberg (15) VI
MG825376.1 ◆ China[l] Broiler N/A E. coli
CP016568.1 2 Canada Broiler 2013 S. Heidelberg (15) VII
CP016572.1 2 Canada Broiler 2013 S. Heidelberg (15)
pFVAL369 ● Colombia Broiler 2013 S. Heidelberg (15) VIII
pFPAS506 ● Colombia Broiler 2013 S. Heidelberg (15)
CP016516.1 2 Canada Bovine 2011 S. Heidelberg (15)
pUGBOG301EC 2 Colombia Broiler 2010 E. coli (201)
pSSXXXV.4.C1 2 Colombia Broiler 2008 S. Java (28)
pSSXXXV.4.C1-T[c] 2
pUGVAL515[a] ◆ Colombia Broiler 2011 S. Heidelberg (15)
pUGARA888 2 Colombia Broiler 2011 S. Enteritidis (11)
pUGPER971 2 Colombia Broiler 2011 S. Java (28)
pFSUC414 ● Colombia Broiler 2013 S. Heidelberg (15)
CP001121.1 2 USA Broiler 2003 S. enterica
CP012936.1 2 Canada Turkey 2012 S. Heidelberg (15)
CP016522.1[a] 2 Canada Broiler 2009 S. Heidelberg (15)
ERR915115 2 Denmark Broiler 2010 E. coli (10)
ERR915116 ● Denmark Broiler 2010 E. coli (212)
ERR915118 2 Denmark Human 2011 E. coli (155) IX
ERR915120 ● Denmark Broiler 2011 E. coli (350)
pESBL-318 2 Netherlands Broiler 2008 E. coli
pESBL-355 2 Netherlands Broiler 2007 E. coli
HG428759.1 2 Uruguay N/A N/A S. enterica
pECSLIL8.C3 2 Colombia Broiler 2008 E. coli (155)
pECSLIV.7.C1 2 Colombia Broiler 2008 E. coli (3910)
pECSVIL.11.C1 2 Colombia Broiler 2008 E. coli (359)
pECSVIL.21.C3 2 Colombia Broiler 2008 E. coli (212)
pUGBOG204EC 2 Colombia Broiler 2010 E. coli (189)
ERR915121 2 Denmark Broiler 2011 E. coli (3272)
ERR915122 2 Denmark Broiler 2011 E. coli (88)
ERR915123 2 Denmark Broiler 2011 E. coli (206)
ERR915124 ● Denmark Broiler 2011 E. coli (1303)
0.005
B.
Plasmid[a,j] Pattern GE[k] Country Source[m] Year[n] Bacterial Host (MLST)[o] Cluster[p]
pEC9PP62328[b] ◆ Colombia Broiler 2009 E. coli (973)
pFSAN126[h] ◆ Colombia Broiler 2012 E. coli (1775)
CP018774.2[b,i] - USA Human N/A E. coli
pUGIBA933 ● Colombia Broiler 2011 S. Java (28)
CP019905.1 ◆ USA Human 2015 E. coli
pUGBOG327 5 Colombia Broiler 2010 S. Java (28) X
pUGBOG327-T[c] 5
pUGBOG339 5 Colombia Broiler 2010 S. Java (28)
pUGBOG340 5 Colombia Broiler 2010 S. Java (28)
0.02

Figure 1. Phylogenetic tree based on core genome of closely related (A) *bla*$_{CMY-2}$-carrying IncI1/ST12 plasmids and its SLVs and (B) *bla*$_{SHV-12}$-carrying IncI1/ST231 and its SLVs.

[a] Single-Locus Variants (SLVs) of IncI1/ST12. [b] SLVs of IncI1/ST231. [c] Transformed plasmids from Colombian Salmonella included as reference. [d] Carrying *bla*$_{SHV-12}$. [e] Carrying *bla*$_{TEM1B-like}$. [f] Carrying *bla*$_{CMY-2}$ together with *bla*$_{CTX-M-1}$. [g] Carrying *bla*$_{CMY-2}$-like. [h] Carrying *bla*$_{CMY-2}$. [i] Carrying *bla*$_{TEM1A}$. [j] Carrying no *bla* genes. *k* The patterns of the Genetic Environment (GE) with their designated numbers are to be found in Figure 2 and Figure 3; ◆= Unique pattern; •= Sequence was shorter than the 20kb used for comparisons; -= Carrying no *bla*$_{CMY-2}$ or *bla*$_{SHV-12}$ genes. [l] Data taken from information acccompanying the sequence submission in GenBank but not specifically found in the metadata fields. [m] Details of the Source listed in this column are available in supplemental Table S1. [n] N/A = Data is not available. [o] Strain MLST was added when information or complete sequence of strains was available. [p] Clusters I-X of plasmids referred in the manuscript are grouped in shaded boxes. Scale bar at the bottom of the phylogenetic trees represent nucleotide substitutions per site.

Genetic environment of *bla*$_{CMY-2}$ and *bla*$_{SHV-12}$

bla$_{CMY-2}$ *in IncI1/ST12 and SLVs of ST12*

The genetic environment of 85 *bla*$_{CMY-2}$ carrying plasmids, including the above-mentioned 4 transformed plasmids from Colombian Salmonella, was analyzed. From these, 71 belonged to IncI1/ST12 or SLVs and 14 to other STs. The genetic environment of most *bla*$_{CMY-2}$ in IncI1/ST12 plasmids and SLVs was similar and characterized upstream by insertion sequence ISEcp1 (NCBI RefSeq ID: WP_000608644.1), yagA (WP_001132032.1), yafB (WP_000517695.1), yaeA (WP_001334656.1), yadA (WP_001334658.1), yacC (WP_001057991.1), yacB (WP_032072246.1), yacA (WP_000079941.1) and multiple hypothetical proteins. The downstream sequence was characterized by blc (WP_001221666.1), sugE (P_000118520.1), yagA (partial sequences) (WP_001132034.1 and EFU56748.1), cib (colicin 1B) (WP_001283341.1), cib immunity protein (WP_000762570.1), ybaA (WP_000142436.1), ydeA (WP_000194550.1), ibfA (WP_000793307.1), ccdA (WP_000813630.1), ccdB (WP_001159871.1) and multiple hypothetical proteins. The arrangement of these genes and the patterns of the genetic environment found repeatedly among ST12 and SLVs plasmids are available in Figure 2A. Reference to the patterns (1-4) carried by the different ST12 and SLVs plasmids is made in Figure 1A.

The 4 patterns of genetic environment in Figure 2A were assigned to 54 plasmids from ST12 and SLVs described in Figure 1A. In addition, 5 plasmids showed unique patterns and the contigs of 12 plasmids were shorter than the 20kbp subregion designated for analysis and no pattern could be assigned (Figure 1A). The most common pattern of genetic environment was 2 (n=32), followed by 3 (n=13), 1 (n=6) and 4 (n=3). Most of the plasmids within the phylogenetic clusters in Figure 1A shared an identical pattern of the genetic environment (clusters I, II, IV, VII-IX). In all plasmids from ST12, SLVs and STs different than ST12, the

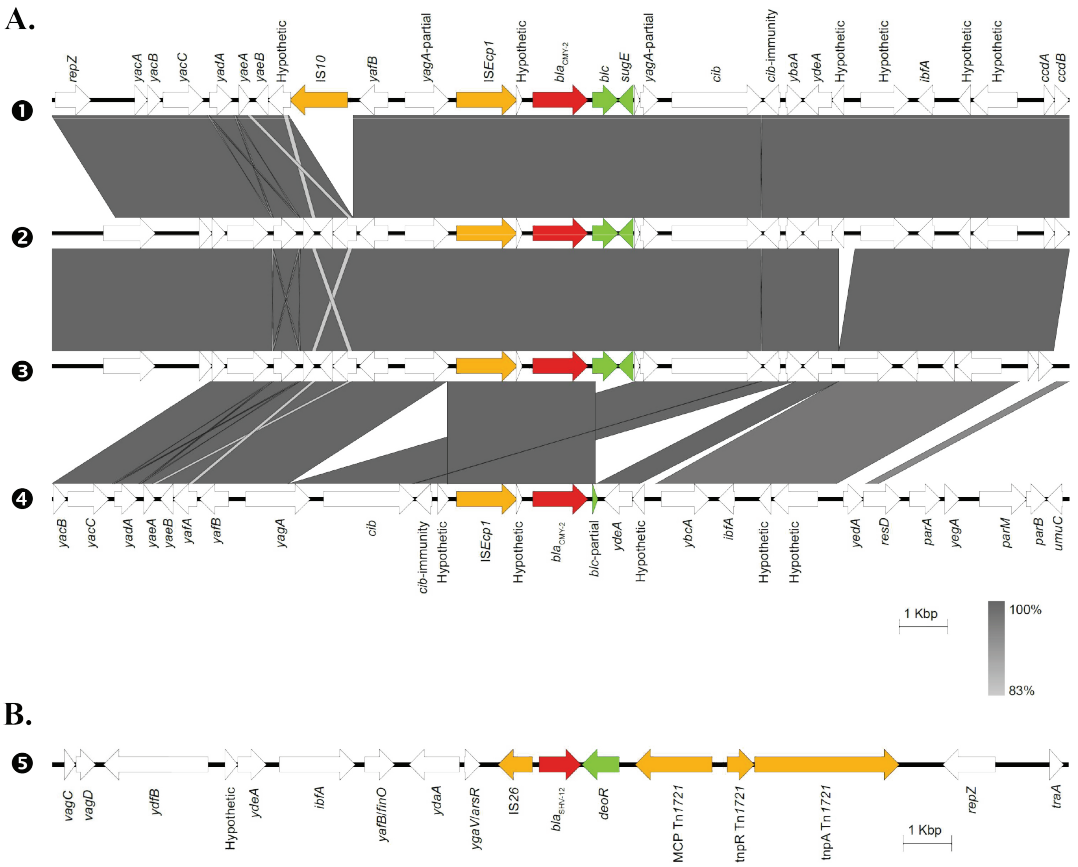


Figure 2. Patterns of genetic environment found repeatedly in (A) bla_{CMY-2}-carrying plasmids from ST12 and its SLVs and (B) bla_{SHV-12}-carrying plasmids from ST231.

nucleotide sequence of the *bla*$_{CMY-2}$-*blc-sugE* subregion was identical. However different genes were present in the genetic environment of *bla*$_{CMY-2}$ in non-ST12 plasmids. For instance, in plasmids CP009566.1 and KX058576.1 *bla*$_{CMY-2}$ was flanked upstream by IS*1294* (instead of IS*Ecp1*) and in plasmids CP023365.1, KF434766.1 and KT186369.1 by IS*26* (Figure S5).

bla$_{SHV-12}$ *in IncI1/ST231 and SLVs of ST231*

The genetic environment of 12 *bla*$_{SHV-12}$ carrying plasmids, including 1 transformed plasmid from Colombian *Salmonella*, was analyzed. From these, seven belonged to IncI1/ST231 or SLVs and five to other STs. Only one pattern of genetic environment was found repeatedly among ST231 and SLVs. This pattern was found in four plasmids from Colombian *Salmonella* that formed cluster X in the phylogenomic analysis (Figure 1B and Figure 2B). From one plasmid (pUGIBA933), the contig was shorter than the subregion designated for analysis and no pattern could be assigned (Figure 1B). The genetic environment of *bla*$_{SHV-12}$ in IncI1/ST231 plasmids was characterized upstream by IS*26* (NCBI RefSeq ID: WP_001067855.1), *ygaV*/*arsR* (WP_001175593.1), *ydaA* (WP_044502555.1), *yafB/finO* (WP_000521603.1), *ibfA* (WP_000818556.1), *ydeA* (WP_000194575.1), *ydfb* (WP_000350638.1), *vagD* (WP_001341418.1), *vagC* (WP_001261287.1) and one hypothetical protein. Downstream *bla*$_{SHV-12}$, only *deoR* (WP_002210513.1) was common (see Figure 3).

In Figure 3 can be observed that the *E. coli*-derived plasmids CP019905.1 (ST231) from the USA and pEC9PP62328 (ST230) from Colombia, showed unique patterns for which differences in gene content and/or gene arrangement were detected. In the plasmids from Colombian *Salmonella*, IS*26*-*bla*$_{SHV-12}$-*deoR*, was flanked downstream by MCP, *tnpR* and *tnpA* proteins of Tn*1721*whilst in CP019905.1, it was flanked by IS*26*, *intI1* (integron class I) and other AMR genes (Figure 3). In contrast, only the nucleotide sequence of the IS*26*-*bla*$_{SHV-12}$-MCPTn*1721* subregion was identical when compared to pEC9PP62328 (see Figure 3).

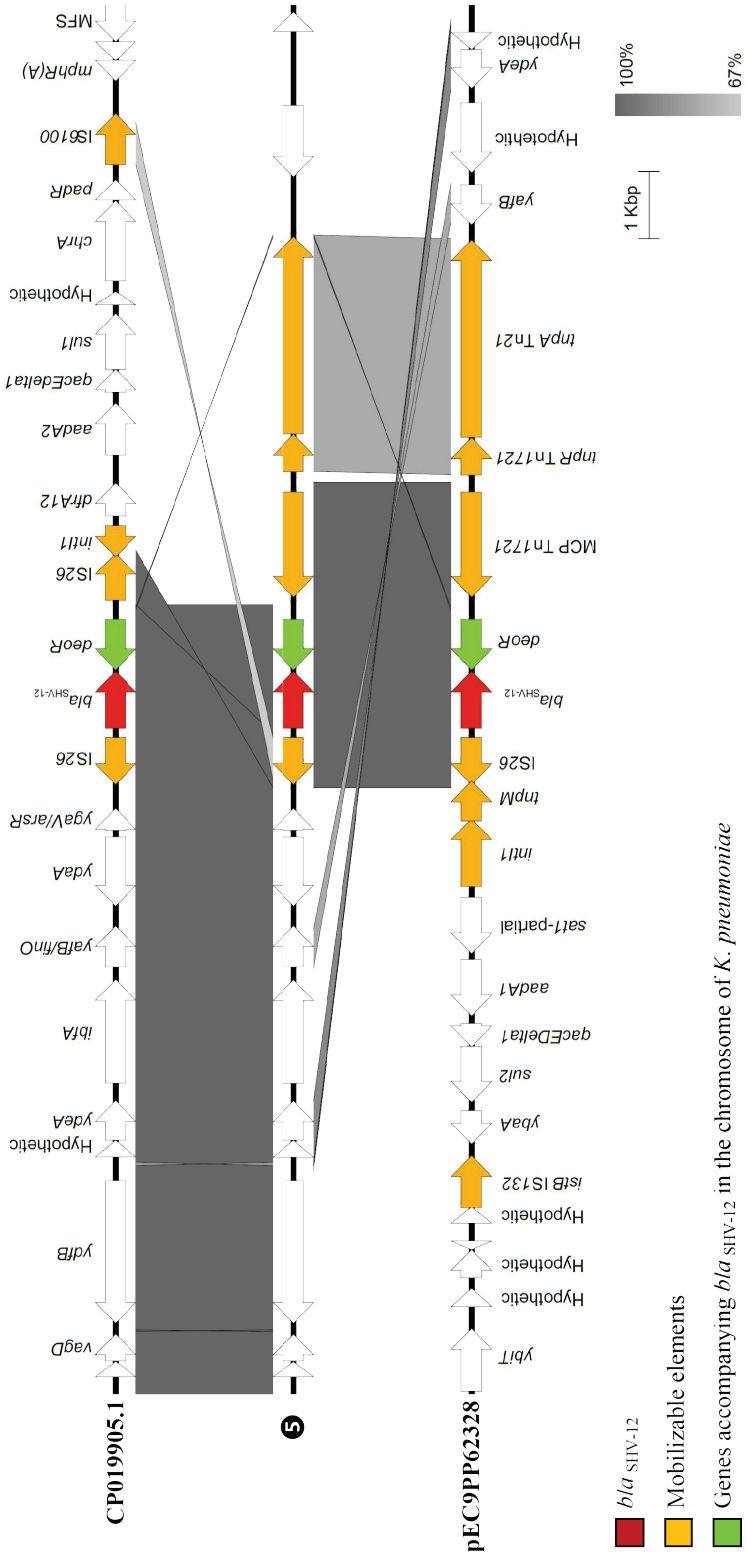


Figure 3. Differences in the genetic environment of bla_{SHV-12} in plasmids CP019905.1 (ST231) and pEC9PP62328 (ST230).

Discussion

In Colombian poultry, *bla*$_{CMY-2}$ is mainly carried by IncI1/ST12 plasmids in both *Salmonella* and *E. coli.* This gene-plasmid ST combination has been reported in studies from North America[14,38] and Europe[12,13,39]. Although frequently reported, the genomic relatedness of *bla*$_{CMY-2}$-carrying IncI1/ST12 plasmids originating from multiple countries has not been assessed previously. In this study, an extensive collection of IncI1/ST12 plasmids was analyzed and it was found that plasmids from different countries originating from poultry are genetically related. Most of the plasmids that were analyzed originated from North and South American and European countries. Interestingly, plasmids from multiple *E. coli* and *Salmonella* strain MLSTs/serovars, clustered together in both core plasmid genome and gene presence absence phylogenomic analysis. These observations indicate horizontal transfer of identical plasmids between *Salmonella* and *E. coli*. The plasmid exchange was evident in isolates from both enterobacteria originating from the same country, like the ones from Colombia, and also between plasmids originating from distant countries such as Canada, Denmark, Uruguay, the Netherlands and the USA (Figure 1A). Their close genomic relatedness indicates that poultry in multiple countries share a common source of the contamination with this lineage of plasmids. As previously suggested, the source of the contamination with *bla*$_{CMY-2}$ could be related to similar practices of poultry farming and chicken transport[40,41]. To a lesser extent, *bla*$_{CMY-2}$-carrying IncI1/ST12 plasmids from other sources such as other livestock animals and human were also found. In particular, plasmids ERR915118 and CP016516.1 originated from clinical human and bovine isolates respectively. These plasmids were closely related to the ones from poultry (see clusters VIII and IX in Figure 1A). These findings underscore the potential of IncI1 plasmids to be transferred in strains from *Salmonella* and *E. coli* outside the poultry environment. Examples being the report of a bloodstream infection in a human patient in Denmark[42], the high prevalence of *bla*$_{CMY-2}$-carrying IncI1 in *E. coli* from dogs in the same country[13] and multiple reports of invasive disease in Australia[43–45].

bla$_{SHV-12}$ was mainly carried in plasmids from IncI1/ST231 and its SLVs in *Salmonella* (n=5) and *E. coli* (n=1) strains from Colombia. In addition, one *bla*$_{CMY-2}$-carrying ST231 plasmid from *E. coli* from the USA was publicly available. Little *bla*$_{SHV-12}$-carrying plasmids from ST231 and SLVs were encountered in comparison to *bla*$_{CMY-2}$-carrying plasmids belonging to IncI1/ST12. At the core genome level, the plasmids from Colombian *E. coli* appeared related to the ones from *Salmonella* (Figure 1B) but at gene content level they were distant and distributed in separate branches (Figure S4). These findings indicate ST231 and SLV plasmids in Colombia from *E. coli* and *Salmonella* have different evolutionary backgrounds and represent no evidence to the exchange of *bla*$_{SHV-12}$-carrying plasmids between these enterobacteria in Colombia. In addition, the differences in the genetic environment of the *bla* gene, indicate IS*26*-mediated insertion of this gene occurred

independently in Colombian plasmids. In contrast, the plasmids from Colombian *Salmonella* originating from poultry and one from *E. coli* from a human in the USA were found closely related, both at the core genome and gene content level. In this case, these plasmids could originate from a common source. However, these results have to be interpreted carefully given the limited number of plasmids available for analysis.

In addition to the role plasmids play in the dissemination of resistance genes, IS elements contribute in their dissemination across multiple plasmids lineages[25]. These mobile elements also assist the transfer of other genes frequently reported in the genetic environment of these resistance genes and could contribute to their positive selection by conferring features such as co-resistance or co-selection to other antimicrobials or environmental compounds[46]. In the vast majority of encountered plasmids, bla_{CMY-2} was followed downstream by *blc* and *sugE* genes, both in IncI1/ST12 and non-ST12 plasmids. It has been suggested that the bla_{CMY-2}-*blc-sugE* subregion could have been originally transposed from the *ampC* chromosomal region of *Citrobacter freundii*[47,48] and mobilized by multiple IS elements into different plasmid lineages[25,26]. Upstream bla_{CMY-2}-*blc-sugE* genomic subregion, IS*Ecp1* was found in ST12 plasmids and its SLVs (Figure 2A), whilst in some plasmids from other STs IS*1294* and IS*26* were found (Figure S5 in supplemental material). In most ST12 and SLVs plasmids analyzed, IS*Ecp1*-bla_{CMY-2}-*blc-sugE* was found in identical positions in reference to the *repZ* gene (Figure 2A). In contrast, in plasmids from STs different than ST*12*, IS*26*-bla_{CMY-2}-*blc-sugE* was found in different positions in relation to *repZ* (Figure S5). The similar position of IS*Ecp1*-bla_{CMY-2}-*blc-sugE* in ST12 plasmids in contrast to the different positions of IS*26*-bla_{CMY-2}-*blc-sugE* in several non-ST12 plasmids, suggests IS*Ecp1*-mediated insertion of bla_{CMY-2} occurred once in the common progenitor of the bla_{CMY-2}-carrying ST12 plasmids analyzed in the present study. This hypothesis is further supported by the phylogenomic reconstruction of bla_{CMY-2}-carrying IncI1/ST12 carrying the same genetic environment (Figure 1A, Figure S1 and Figure S2). In addition, these findings underscore the contribution other ISs such as IS*1294*, could have in the dissemination of bla_{CMY-2} across multiple plasmid lineages as previously studied[25].

Despite the unrelatedness observed in the genomic content of ST231 and SLVs in supplemental Figure S4, the genomic subregion IS*26*-bla_{SHV-12}-*deoR* showed a high level of homology across analyzed plasmids (Figure 3). It has been suggested that the bla_{SHV-12}-*deoR* genes were originally translocated from the chromosome of *Klebsiella pneumoniae* and IS*26* accounted for is insertion into plasmids[21,24]. Notably, for the closely related plasmids pUGBOG327, pUGBOG339 and pUGBOG340 from Colombian *Salmonella* and CP019905.1 from *E. coli* from the USA, the genetic environment upstream bla_{SHV-12} was identical, however downstream, *deoR* was followed by Tn*1721* transposase in Colombian plasmids and by *intI1* integrase in CP019905.1 (Figure 3). The close relatedness at core and gene content level, indicates these plasmids share common ancestry but due to the insertion of these mobile genetic elements, they have possibly diverged at the

evolutionary level, as reflected in Figure 1B and Figure S4. However, as mentioned above, these results have to be interpreted carefully given the limited number of plasmids available for analysis.

Conclusions

The results of the phylogeny based on gene content supported the findings from the phylogeny based on core genome for bla_{CMY-2}-carrying IncI1/ST12 and SLV plasmids.
These plasmids likely originated from a common ancestor and form a sublineage within IncI1. Its occurrence in *Salmonella* and *E. coli* across multiple European and American countries could be related to common practices of farming and supply of chickens and chicken meat.

bla_{SHV-12}-carrying IncI1/ST231 and SLV plasmids originating from *Salmonella* and *E. coli* in Colombia were phylogenetically distant based on gene content analysis, in agreement with discrepancies in the genetic environment of bla_{SHV-12}. More observations are needed to better understand the transmission of bla_{SHV-12} in IncI1/ST231 plasmids in Colombia and other countries. Nevertheless, bla_{SHV-12} in association with IS*26* was likely introduced independently in different IncI1 plasmid lineages.

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Supplementary Material

Table S1 and **Figures S1-S5** can be found online in: https://doi.org/10.5281/zenodo.2577887

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6

Chapter 6.

Increased dissemination and parallel evolution of antimicrobial resistance in *Salmonella enterica* serovar Paratyphi B variant Java in poultry from Europe and Latin America

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Abstract

Previous reports from Latin American countries have identified *Salmonella enterica* Paratyphi B variant Java as prevalent in poultry and poultry products. In a previous report from Colombia, all isolates of *S.* Paratyphi B var. Java, analyzed by Multi-Locus Sequence Typing (MLST), belonged to ST28. Comparisons between strains of *S.* Paratyphi B var. Java ST28 from Colombia and Europe, showed a phylogenomic separation between Colombian and European isolates. In the present study the evolutionary separation between *S.* Java from Europe and Latin America was investigated using whole genome-based phylogenetic and temporal analysis.

A selection of *S*. Paratyphi B Java ST28 isolates from Colombia, Guatemala, Costa Rica and the Netherlands was sequenced. This was complemented with existing publicly available genomes. Phylogenetic time trees and effective population sizes (N_e) were determined. In addition, principal component analyses were performed on core-, non-core genomes and plasmid content.

Strains collected from Latin American countries formed a separate cluster from European strains in the phylogenetic tree. The separation is estimated to have occurred in the 1980's. Strains collected from the Netherlands clustered together with strains originating from other European countries. N_e of European strains increased sharply in the year 1995 (Confidence Interval [CI]: 1992-1998). In Latin America it increased in 2005 (CI: 2001-2007). A clear distinction in chromosomal accessory genes between European and Latin American clades was observed, while the plasmid content was relatively similar. The European and Latin American clades have acquired similar antimicrobial resistance genes and plasmids independently, which are indicative of parallel evolution of antimicrobial resistance in *S.* Paratyphi B var. Java ST28 in both regions.

Introduction

The d-Tartrate fermenting, non-paratyphoidal variant of *Salmonella enterica* serovar Paratyphi B, contemporarily known as variant Java, was first reported in 1935 by De Moor[1]. From that date until recently, *S.* Paratyphi B var. Java caused sporadic human gastrointestinal infections, which were known to be self-limiting[2–4]. From 1990 onwards, reports of an increasing number of human infections arose in Europe[5–10], North America[11–15] and Australia[16,17]. Likewise, a dramatic increase of the prevalence in European poultry and poultry products was reported[18,19]. This increase was accompanied by an increase in the features of Antimicrobial Resistance (AMR) of the strains[5,18]. More recently, isolates of *S.* Paratyphi B var. Java were identified as carriers of *mcr* gene variants conferring resistance to colistin[20,21].

Using a Multi-Locus Sequence Typing (MLST) scheme for *S. enterica*[22], strains of *S.* Paratyphi B var. Java, were classified into different Sequence Types (STs) and grouped into monophyletic groups using an eBurst (eBGs) evolutionary approach[22]. This evolutionary classification was later confirmed with Whole Genome Sequence (WGS)-data by Connor et al in 2016[23]. In a previous study from Germany, the use of both traditional serotyping and MLST, was instrumental to determine human infections caused by *S.* Java in Germany originated mainly from sources different than poultry[24]. Noteworthy, isolates originating from poultry were strongly associated to ST28, those from reptiles to ST88 and those from humans mainly to ST43 and ST149[24]. Hence, *S.* Paratyphi B var. Java originating from poultry has been rarely associated with human infections in Europe[6,24]. Human infections with this serovar are predominantly related to acquisition from fish or reptiles[16,17,24].

Previous reports from Latin American countries have identified *S.* Paratyphi B var. Java as highly prevalent in poultry and poultry products[25–29]. In a previous report from Colombia, all analyzed isolates of *S.* Paratyphi B var. Java belonged to ST28[30]. Comparisons between strains of *S.* Paratyphi B var. Java ST28 from Colombia and Europe, showed a phylogenomic separation between Colombian and European isolates. Strains from Belgium, Denmark, Germany, Ireland, Nigeria and the United Kingdom were grouped in the cluster of closely related strains originating from Europe. In contrast, all Colombian strains clustered together in a separate cluster[30]. Accordingly, it was hypothesized that the separate lineage of Colombian strains could be part of a larger lineage of *S.* Paratyphi B var. Java ST28 circulating in other Latin American countries. Further investigation of this hypothesis could help identify potential events in poultry management leading to the successful spread of *S.* Paratyphi B var. Java ST28 in both regions. Therefore, the objective of the present study was to investigate the evolutionary separation between *S.* Paratyphi B var. Java from Europe and Latin American using WGS-based phylogenomic and temporal analysis.

Materials and Methods

Strain and genome collection

Isolates from Latin American (LA) and European (EU) countries that were previously serotyped as *S.* Paratyphi B var. Java were included in the present study. Strain selection from different countries was made as follows.

Colombia: A total of 259 epidemiologically independent isolates from broilers and broiler meat were considered. The isolates were obtained between the years 2008 and 2013 and originated from farms (n=25), slaughterhouses (n=49) and retail meat (n=185) and represented 18 of the 32 departments (i.e. provinces) of Colombia. From these isolates a selection was made. As a rule of thumb, a random sampling of 20% of the isolates was made using the Select Cases option in SPSS v24. As a result, 52 isolates originating from farms (n=5), slaughterhouses (n=5) and retail (n=42), representing 8 Colombian departments were selected and subjected to WGS.

Costa Rica: All available strains from a previous study to determine the prevalence of *Salmonella* sp. in chickens at slaughter in the country in 2009[31], were included. A total of 15 isolates were made available by the Costa Rican Institute for Research and Training in Nutrition and Health (INCIENSA). In addition, three outgroup strains from human, reptile and swine origin were provided. The strains from human and reptile originated from an outbreak investigation in 2013 and the one from swine from a meat quality investigation in pig carcasses in 2014.

Guatemala: Available strains from a previous study to determine the prevalence of *Salmonella* in retail raw chicken carcasses in the country were considered[28]. In total, five isolates were made available by the Center for Health Studies from the Universidad del Valle de Guatemala.

The Netherlands: Since no genomes from the Netherlands were publicly available, isolates were provided by the Wageningen Bioveterinary Research institute in the Netherlands. These isolates were collected as part of the Monitoring of Antimicrobial Resistance and Antibiotic Usages in Animals program (MARAN)[32]. In total, 1279 isolates obtained from 2000 until 2016 were considered. The isolates originated from broilers, broiler meat and chicken products (n=1100), human enteric infections (n=159) and other animals and food items (n=20). A stratified random sampling was performed with the Transform, Rank and Select Cases options in SPSS v24 for every year within the samples from broilers and broiler meat. In addition, two randomly selected isolates from the entire pool of human isolates, and two from other animals and food items were selected as outgroup references. As a result, 21 Dutch isolates originating from broilers and broiler meat (n=17), human enteric infections (n=2) and other animals and food products (n=2) were selected and subjected to WGS.

Ancestral *S.* Paratyphi B var. Java ST28 strains: Two strains of human origin from Saudi Arabia isolated in 1987 and 1992, WS0777_6155_87 (WS0777) and WS0779_7734_92 (WS0799) respectively, and one strain from a turkey of Israeli origin isolated in Austria in 1988, WS0778_6395_88 (WS0778), were identified as the earliest *S.* Paratyphi B var. Java ST28 strains from the Enterobase database from their MLST profiles. The genomes of these strains were sequenced and provided by the Enteric Bacterial Pathogens unit from the Pasteur Institute in France.

Publicly available WGS sequences: Enterobase[33] was queried using the 'Experimental Data' search option for strains belonging to Sequence Type (ST) 'ST28' in the 'Achtman 7-gene MLST' scheme (Last accessed: 22 February 2018). Only the strains with metadata available for year, country and source of isolation were selected for phylogenomic and temporal analysis in the present study.

WGS and *in silico* screening of AMR genes and plasmid content

Isolation of genomic DNA from collected strains from the Netherlands, Colombia, Costa Rica and Guatemala was performed using the UltraClean® Microbial DNA Isolation Kit (Qiagen, USA). WGS was performed on Illumina MiSeq and NextSeq platforms (Illumina, USA) using 2 × 250-bp reads and 2 × 150-bp reads, respectively. Genomes were assembled with SPAdes v3.10.1[34]. Screening of Antimicrobial Resistance (AMR) genes and/or chromosomal mutations was performed using ResFinder 3.1[35]. Plasmid subtyping was made using PlasmidFinder 2.0 and plasmid MLST (pMLST) 2.0[36]. For newly sequenced genomes, 7-gene MLST at the strain level was performed with MLST 1.8[37].

Phylogenetic time-trees and effective population size (N_e) estimates

For phylogenetic single-nucleotide polymorphism (SNP) analysis of the core genome, whole genome sequences of all *S.* Paratyphi B. var. Java ST28 isolates were aligned using Parsnp v1.2[38]. Recombination regions in the core genome alignment were detected and visualized using Gubbins[39]. Time resolved phylogeny was performed on recombination-filtered SNPs of the *S.* Paratyphi B. var. Java ST28 isolates which were extracted from the Gubbins results and used for divergence dating in BEAST[40]. Only newly obtained genomes with coverage higher than 30 were included. Isolation dates were used as tip dates in the phylogenetic tree, as outlined in the BEAST manual with the following modifications: 10,000,000× sampling and a general time reversible (GTR) model plus gamma correction as the distance model. A strict clock, as the clock model and a Bayesian skyline plot with three groups as demographic models were used. All Effective Sample Size (ESS) values obtained exceeded 1000. To generate Bayesian skyline plots for the EU and LA populations, time resolved phylogeny analysis using BEAST was repeated on two subsets containing the ancestral isolates and the LA or the EU isolates.

Orthology prediction and plasmid/chromosome contig scoring

Genomes were annotated using Prokka v.1.13[41] followed by orthology predictions using Roary[42]. Chromosome and plasmid contigs were differentiated with an in-house built tool. Briefly, contigs were scored for the presence of known plasmid genes[43], single copy chromosomal marker genes[44] and kmer profiles. Their likely origin (plasmid or chromosomal) was inferred using a Random Forest model trained on known plasmid and chromosome assemblies.

Comparison of accessory genome

Principal Component Analysis (PCAs) were performed for all isolates on the gene presence/absence tables from the output of orthology predictions. Comparisons were made using the i) accessory (non-core) genome of chromosome contigs, ii) complete plasmid composition with all plasmid contigs and iii) plasmid composition with only plasmid contigs ≥ 50Kb. Additional characterization of prophage sequences was made using the PHAge Search Tool Enhanced Release (PHASTER)[45] and BLAST.

Data availability

Sequences of the newly sequenced strains from Colombia, Costa Rica, Guatemala and the Netherlands have been deposited in the short-read archive of the ENA under Project Number: PRJEB31547.

Results

MLST-based population structure of newly sequenced *S.* Paratyphi B var. Java

All strains from poultry sequenced in the present study from the Netherlands and Costa Rica belonged to ST28. From Colombia all but four isolates belonged to ST28 (two belonged to ST15, one to ST11 and one to ST22). From Guatemala, all but one belonged to ST28 (one isolate belonged to ST64). Two outgroup strains from a fish product and a turkey in the Netherlands, and one strain from swine in Costa Rica, belonged to ST28. In contrast, two isolates from human and reptile samples from Costa Rica belonged to ST88 and two isolates from humans in the Netherlands belonged to ST43. A summary of the used collection of *S.* Paratyphi B var. Java ST28 isolates is available in Table 1. Full information regarding the country, source and year of origin together with the *in silico* characterization of all collected isolates and genomes is available in Supplemental Table S1.

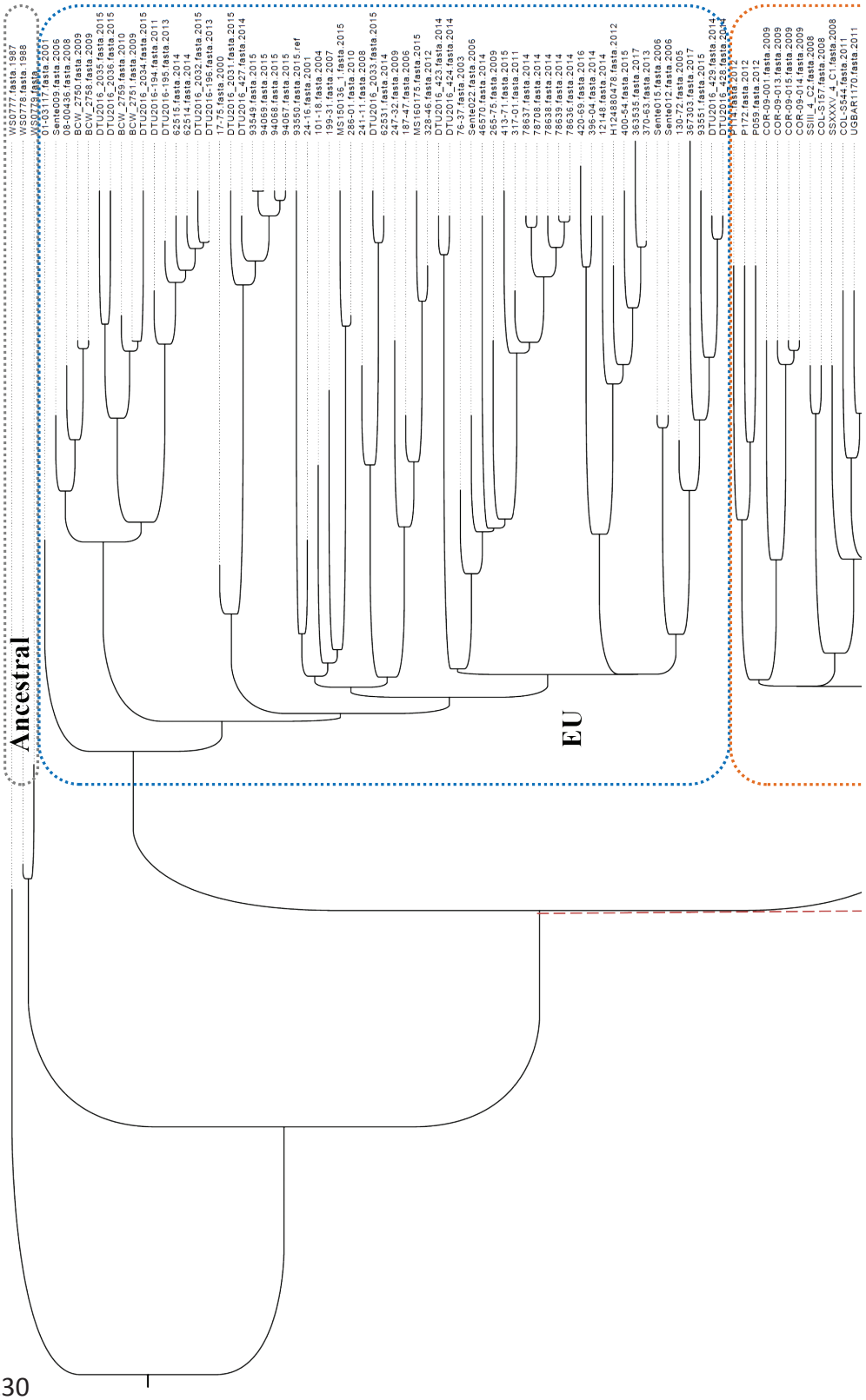
Phylogenetic time-tree and effective population size (N_e)

Time resolved phylogenetic reconstruction was performed exclusively with *S.* Paratyphi B. var. Java ST28 for which location and isolation dates were known. It was observed that the strains collected from Latin American countries (Guatemala, Costa Rica and Colombia) formed a separate cluster in the phylogenetic tree. In contrast, the strains collected from the Netherlands clustered with strains originating from other European countries (Figure 1). An additional cluster was formed by the ancestral strains, which were neither from Europe nor Latin America. The molecular clock was estimated at 3.5 x 107 *substitutions/site/year* (1.7 SNPs/genome/year Confidence Interval [CI]:1.44-2.0). The output of BEAST indicated the separation between strains from Europe and Latin America occurred in the year 1987 (Figure 1) with a confidence interval ranging from 1978-1988 (Supplemental Figure S1).

From the Bayesian Skyline plot, it can be inferred that the effective population size (N_e) of strains from Europe increased sharply in the year 1995 (CI: 1992-1998) (Figure 2A) and in Latin America in 2005 (CI: 2001-2007) (Figure 2B), 10 years later than in Europe.

In silico characterization of AMR genes and plasmid subtypes

A class 2 integron (with *dfrA1-sat1-aadA1* [GenBank Nucleotide accession number: AB188271.1]) was found in ancestral strain WS0777, collected in 1987 in Saudi Arabia. However, it was not found in the other two ancestral strains (WS0778 and WS0779). The integron was found in all EU and LA strains as well. Alignments of ~50Kb contigs revealed 100% identity within and between strains from EU and LA clades when compared to a complete contig carrying the integron. Thus, indicating it is integrated in the same position. In addition, 50% of strains from the EU clade, exhibited known gyrase mutations conferring resistance to fluoroquinolones.Whilst no strain from LA carried the chromosomal *gyrA* mutations, they did carry *qnrB19*-harboring plasmids, conferring reduced-susceptibility to quinolones in 98% of the cases. Resistance to β-lactams in strains from EU was mainly mediated by $bla_{\text{TEM-1B}}$, whilst in strains from LA, $bla_{\text{CMY-2}}$ was most prevalent. The *sul2* gene, conferring resistance to sulphonamides, was frequently encountered in both EU and LA strains. In EU it was mainly found co-localized with $bla_{\text{TEM-1B}}$. Interestingly, β-lactam and sulphonamide resistance genes in the LA clade were only observed in strains from Colombia and not in strains from Costa Rica or Guatemala (Table S1). Using the output from ResFinder and PlasmidFinder, *qnrB19* genes were co-localized with ColRNAI-like plasmids in LA strains and also in the two strains carrying this gene in EU from recent years (2015 and 2017) (Table S1). In EU strains, bla_{TEM1B} co-localized with *sul2* and was mostly associated with IncI/ST3 plasmids. To a lesser extent $bla_{\text{TEM-1B}}$ was also associated with IncX4 plasmids in EU. In LA strains, $bla_{\text{CMY-2}}$ was associated to IncI1/ST12 and *sul2* with COLRNAI-like plasmids.


Ancestral
EU
WS0777.fasta.1987
WS0778.fasta.1988
WS0779.fasta
01-03117.fasta.2007
Sente009.fasta.2006
08-00436.fasta.2008
BCW_2750.fasta.2009
BCW_2758.fasta.2009
DTU2016_2035.fasta.2015
DTU2016_2036.fasta.2015
BCW_2752.fasta.2010
BCW_2751.fasta.2009
DTU2016_2034.fasta.2015
DTU2016-194.fasta.2011
DTU2016-195.fasta.2013
62515.fasta.2014
62514.fasta.2014
DTU2016_2032.fasta.2015
DTU2016-196.fasta.2013
12-15.fasta.2012
DTU2016_2031.fasta.2015
DTU2016_427.fasta.2014
93549.fasta.2015
94069.fasta.2015
94068.fasta.2015
94067.fasta.2015
93550.fasta.2015.ref
24-16.fasta.2001
191-18.fasta.2004
189-18.fasta.2007
MS160135.1.fasta.2015
286-01.fasta.2010
241-11.fasta.2008
DTU2016_2033.fasta.2015
62531.fasta.2014
247-32.fasta.2009
187-47.fasta.2006
MS160175.fasta.2015
328-46.fasta.2012
DTU2016_423.fasta.2014
DTU2016_424.fasta.2014
76-37.fasta.2003
Sente022.fasta.2006
46570.fasta.2014
265-75.fasta.2009
413-71.fasta.2015
317-01.fasta.2011
78637.fasta.2014
78708.fasta.2014
78638.fasta.2014
78639.fasta.2014
78636.fasta.2014
420-69.fasta.2016
396-04.fasta.2014
12148.fasta.2014
H124880478.fasta.2012
400-54.fasta.2015
363533.fasta.2017
370-63.fasta.2013
Sente015.fasta.2006
Sente012.fasta.2006
130-72.fasta.2005
367303.fasta.2017
93551.fasta.2015
DTU2016_429.fasta.2014
DTU2016_428.fasta.2014
DH14.fasta.2012
P172.fasta.2012
P88.fasta.1973
COR-09-012.fasta.2009
COR-09-013.fasta.2009
COR-09-015.fasta.2009
COR-09-014.fasta.2009
SSIII_4_C2.fasta.2008
COL-5157.fasta.2008
SSXXXV_4_C1.fasta.2008
COL-5544.fasta.2011
UGBAR1170.fasta.2011

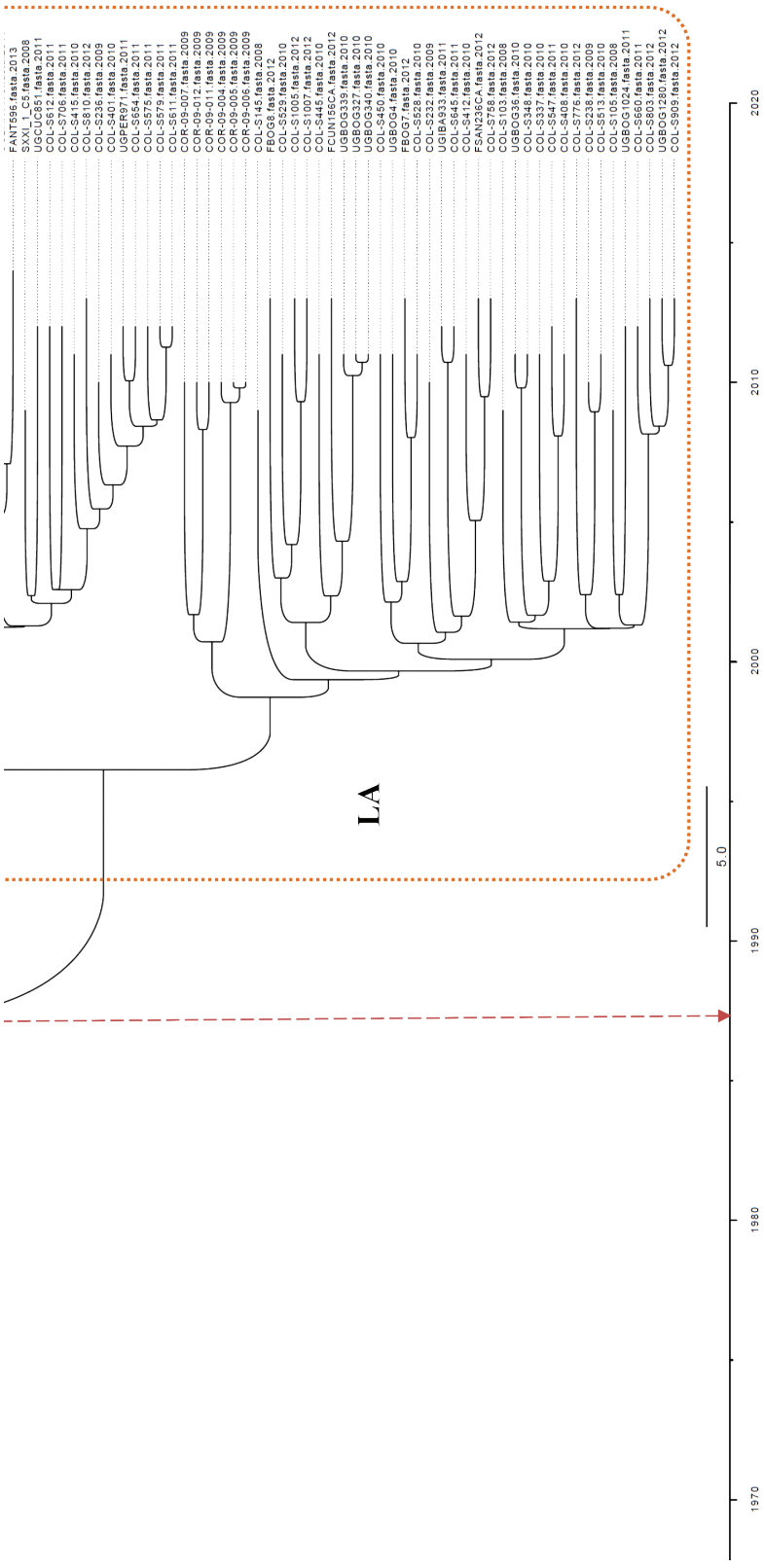


Figure 1. Phylogenetic time-tree showing the phylogenetic separation between the Ancestral, European (EU) and Latin American (LA) clades. Strains from Ancestral, EU and LA clades are circled in gray, blue and orange colored boxes respectively. X-axis represents the time in years (1970 to 2020). Scale bar represents the unit of time (years). Tips in the tree are aligned to the year of isolation of the strains. Nodes are dated in the x-axis as estimated by BEAST. The node and year of separation between EU and LA clades around 1987 are marked with an arrow.

Table 1. Summary of newly obtained and publicly available genomes of *S.* Paratyphi B var. Java ST28.

	n Total	n per Source [e]
Ancestral		
Saudi Arabia [a]	2	2 Human
Austria [a]	1	1 Poultry
Europe (EU)		
Belgium [b]	5	5 Unknown
Denmark [b]	9	8 Poultry, 1 Unknown
Germany [b]	5	3 Poultry, 1 Human, 1 Unknown
Ireland [b]	2	2 Human
Nigeria [b,c]	1	1 Poultry
The Netherlands [a]	19	18 Poultry, 1 Fish
United Kingdom [b]	24	18 Unknown, 5 Human, 1 Bovine
Latin America (LA)		
Colombia [a,d]	67	67 Poultry
Costa Rica [a]	16	15 Poultry, 1 Swine
Guatemala [a]	4	4 Poultry
Total	**155**	

[a] Newly obtained. [b] Publicly available. [c] Phylogenetically related to the European clade. [d] 19 genomes from previous reports in Colombia were publicly available in Enterobase as they were submitted in a previous study[30]. [e] Source metadata of publicly available genomes was obtained from Enterobase.

PCAs of accessory genomes

A marked distinction was observed for the accessory genes located on chromosome contigs between EU and LA clades (Figure 3). The separation in the PCA was associated with the presence of a prophage highly similar to the *Salmonella* phage SEN34 (NCBI Reference Sequence: NC_028699.1) in LA strains. This phage was also found in the genome of a *S.* Saintpaul strain submitted in Canada (NCBI Accession Number: CP022491.1). A few Colombian strains closer to the cluster of EU strains in the PCA (COLS415, COLS529, SSIII_4_C2 and COLS157) lacked the sequence of this phage. Plasmid composition was similar in strains from EU and LA as observed in Figure 4. The profiles of plasmid composition were characterized by the presence of IncI1 plasmids (Cluster I), IncHI2 (Cluster II), COLRNAI (Cluster III) and combinations of IncI1 and IncHI2 plasmids (Cluster IV). Although the IncI1 plasmids had different pMLST sequence types, their content appears to be remarkably similar as they are in proximity in the PCA plot (Cluster I). When

exploring this further using only plasmid contigs ≥50Kb in strains from both EU and LA clades it can be clearly oberved that the IncI1 plasmid contigs have near identical content (Cluster I in Supplemental Figure S2). In strains with multiple plasmid contigs, in addition to the cluster of IncI1-like plasmids, IncHI2-like plasmid contigs were differentiated in Cluster II (Figure S2).

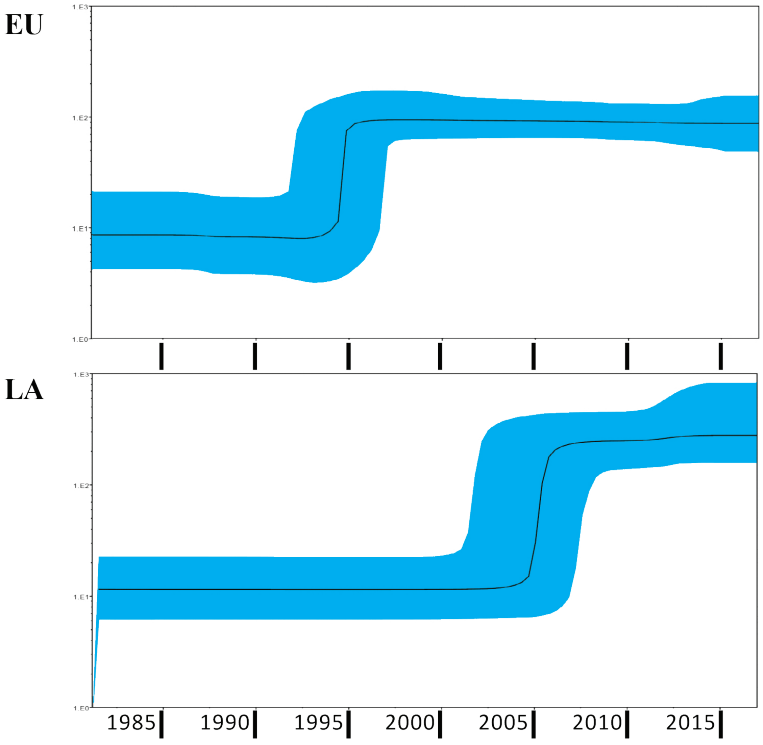


Figure 2. Bayesian skyline plots showing increase in effective population size (*Ne*) of *S.* Paratyphi B var. Java ST28. Plots were made separately with strains originating from Latin America (LA) or Europe (EU). *Ne* is represented in the y-axis and time from 1985 to 2015 in the x-axis. Increase in EU occurred around 1995 and in LA around 2005. The black lines represent estimates of the median population over time and the blue lines represent the upper and lower 95% confidence intervals.

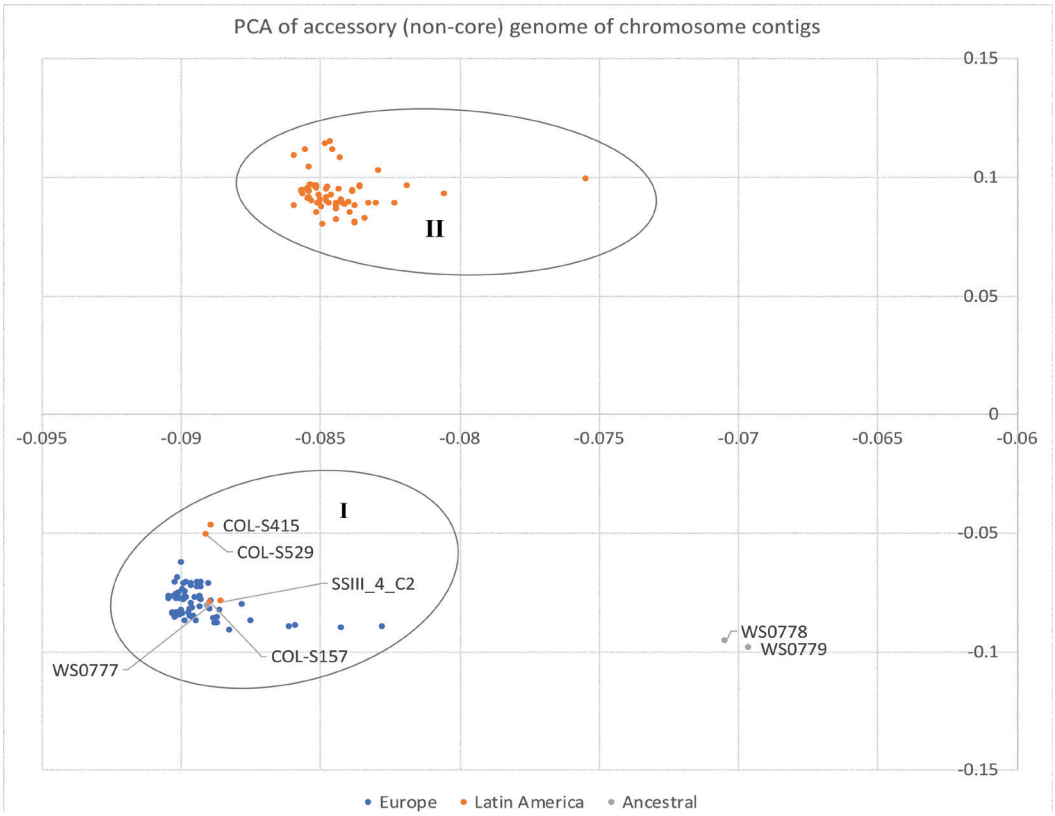


Figure 3. PCA plot comparing accessory (non-core) genome of chromosome contigs of strains of S. Paratyphi B var. Java ST28. Data points from European, Latin American and Ancestral strains are shown in blue, orange and gray colors respectively. Clusters I and II are indicated by oval rings. Cluster I grouped together Ancestral strain WS0777 with all EU strains and some LA strains. Cluster II grouped LA strains only. Cluster II was associated with a prophage sequence highly similar to the *Salmonella* phage SEN34 (NCBI Reference Sequence: NC_028699.1). The prophage sequence was absent in strains from Cluster I.

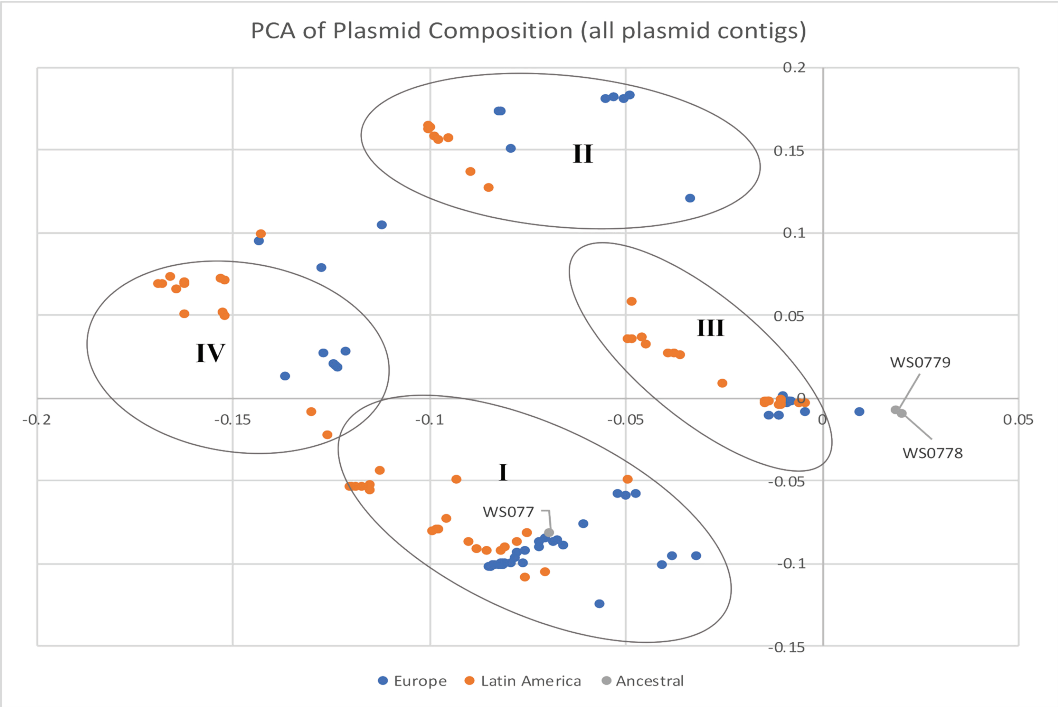


Figure 4. PCA plot comparing plasmid composition (all plasmid contigs) of strains of *S.* Paratyphi B var. Java ST28. Data points from European, Latin American and Ancestral strains are shown in blue, orange and gray colors respectively. Clusters I-IV are indicated by oval rings. All Clusters grouped European and Latin American strains and were associated with IncI1 plasmids (I), IncHI2 (II), COLRNAI (III) and combinations of IncI1 and IncHI2 plasmids (IV).

Discussion

MLST-based population structure of *S.* Paratyphi B var. Java

Overall in LA and EU, most of the strains from poultry belonged to ST28. However, five strains from Colombia and one from Guatemala belonged to STs associated to serotypes different than *S.* Paratyphi B var. Java. These results, unanticipated according to the MLST scheme, could be a result of mistaken serotyping of the strains, as previously discussed[22]. *S.* Paratyphi B var. Java ST28 was found in samples from a fish product and a turkey in the Netherlands, and one strain from swine in Costa Rica. In this case, cross contamination between food products with *S.* Java ST28 originating from poultry cannot be excluded.
In addition, a few isolates of *S.* Java belonged to ST43 and ST88 and were found in samples from humans and reptiles respectively (Supplemental Table S1). In this case, *S.* Java ST28 was not observed in humans. Nevertheless, the number of outgroup strains that were analyzed was too small to draw major conclusions.

Potential common ancestry of *S.* Paratyphi B var. Java ST28 and divergence into EU and LA clades

From the time resolved phylogeny, it was observed that strain WS0777 collected in 1987 was ancestral to all the other strains that were analyzed, including strains WS0778 and WS0779 isolated in 1988 and 1992 respectively. It could be hypothesized that WS0777 is the strain most closely related to the common ancestor of all analyzed strains, which according to the output from the time resolved phylogeny circulated in 1970 approximately (CI: 1962-1974). This hypothesis is supported by the high level of similarity between the contigs carrying the class 2 integron (with *dfrA1-sat1-aadA1* [GenBank: AB188271.1]) between ancestral strain WS0777 and strains from the EU and LA. In addition, contigs carrying the class 2 integron showed complete identity both between and within most strains from the EU and LA clades. Common ancestry of WS0777 was also reflected in the PCAs used to compare the accessory genome. At both chromosome and plasmid level, the accessory genome of this strain was closely related to strains from EU and/or LA (Figure 3 and Figure 4).

In comparison to previous estimations of mutation rates for *S.* Typhimurium DT104 (2.79 × 10−7 *substitutions/site/year*, corresponding to 1.38 *SNPs/genome/year*)[46], the molecular clock calibrated for *S.* Paratyphi B var. Java ST28 in the present study was estimated at 3.5 x 107 *substitutions/site/year* corresponding to 1.7 *snps/genome/year* CI:1.44-2.0) and was therefore slightly faster. The present results indicate differences in mutation rates for these two *S. enterica* serovars with distinct ecological niches.

Previously, genomic characterization of *S.* Paratyphi B var. Java ST28 from Colombia suggested a different clade than the one observed in EU could be circulating in LA countries[30]. In the present study, strains from other LA countries besides Colombia were sequenced. As an outcome, a distinct clade of *S.* Paratyphi B var. Java ST28 was found circulating in poultry from Costa Rica, Guatemala and Colombia. In Colombia, introduction of foreign technologies for poultry breeding, housing and processing occurred around the year 1960[47,48]. It was anticipated that import of this particular *S. enterica* serovar could have occurred around this period of time. Nevertheless, separation between the EU and LA clades in the present study was estimated with BEAST around the year 1987 (Figure 1 and S1). Furthermore, an increase in effective population size in LA was only observed in 2005 (CI: 2001-2007), 10 years after the known increase in Europe, reported in the literature[18] and observed with Bayesian skyline in the present study (Figure 2). Driving factors that led to the separation of clusters could not be determined based on the available data presented in this study.

Parallel evolution of *S.* Paratyphi B var. Java ST28 in EU and LA

The separation between the LA and EU clades comprised differences at both the core and non-core genome level. Notably, differences in AMR gene content, plasmid replicons and pMLSTs reflected the evolutionary separation of the two clades. Among these differences, gyrase mutations conferring resistance to fluoroquinolones and bla_{TEM-1B}-carrying IncI1/ST3 plasmids were characteristic in the EU clade. In contrast, *qnrB19*-carrying ColRNAI-like plasmids conferring reduced susceptibility to quinolones and bla_{CMY-2} carrying IncI1/ST12 plasmids were found in LA. In both clades, resistance to β-lactams was mainly carried on IncI1 plasmids, with near-identical gene content (Cluster I in Figure 4) but from different pMLST lineages (Figure 4). bla_{TEM-1B} in EU was associated with IncI/ST3 and bla_{CMY-2} with IncI1/ST12 in LA. It is remarkable that the genomic features mentioned above confer comparable AMR phenotypes in both EU and LA clades and thus are indicative of parallel evolution of *S.* Paratyphi B var. Java ST28 in both geographical regions. It could be hypothesized that acquisition of such AMR traits occurred as a consequence of selection pressure posed by the use of fluoroquinolones and β-lactam drugs in poultry production. In Europe the emergence of *S.* Paratyphi B var. Java was associated with an increase of AMR and the use of quinolones such as flumequine[18] or enrofloxacin, which could also explain the sharp increase in effective population size. For Latin America such data is not available.

In conclusion, *S.* Paratyphi B var. Java ST28 from poultry in EU and LA form two different clades. The separation is estimated to have occurred in the 1980's (CI:1978-1988). In EU, 1995 and in LA, 2005 are estimated as the years with sharp increase in effective population size, respectively. Previous reports on the emergence of *S.* Paratyphi B var. Java in European poultry supports these findings for Europe[6,18] whilst no historical data is available for LA. In spite of their evolutionary

divergence, the EU and LA clades have independently acquired AMR-associated genes on very similar plasmids. These genetic determinants confer resistance to fluoroquinolones and β-lactams, and thus are indicative of parallel evolution of *S.* Paratyphi B var. Java ST28 in both regions.

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Supplemental Material

Table S1 and **Figures S1-S2** can be found online in: https://doi.org/10.5281/zenodo.2588296

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7

Chapter 7.
General discussion

Benefits of National and Regional Integrated Surveillance Systems

Integrated surveillance systems collect and analyze data of AMR (Antimicrobial Resistance) and AMU (Antimicrobial Use) from human, animal and food origin in a centralized manner. Among the aims of these systems are guiding and measuring the effect of interventions to reduce AMU and AMR[1]. Countries such as Canada, Denmark, the Netherlands and the USA have such systems in place. One example is the Netherlands' Human Antimicrobial Resistance Surveillance (NethMap) and the Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN). Together, NethMap and MARAN provide a combined report on a yearly basis with the data of AMR and AMU in humans and food-producing animals in the country[2]. For its part, MARAN is the component dealing with data from animals and food. Data collection and analysis of MARAN are centralized in Wageningen Bioveterinary Research under the commission of the Dutch Ministry of Agriculture, Nature and Food Quality. MARAN also receives input from other partners such as the Netherlands Food and Consumer Product Safety Authority and the Dutch Authority of Veterinary Medicines. Historical data collected by MARAN date to 1998 and 1999 for some animal species, for AMR and AMU respectively. The reports from MARAN have documented the increase of sales of antimicrobials for food animals from 1998 until 2007 and a sustained decreasing trend since 2008, a year in which policies for major reductions in AMU in food animals were adopted in the country[3,4]. Likewise, a reduction of AMR, after the reduction of AMU, can be appreciated throughout the yearly reports[3]. Another example is the National Antimicrobial Resistance Monitoring System (NARMS) from the USA[5]. NARMS was established in 1996 and is sustained through the collaboration of three federal agencies, the Centers for Disease Control and Prevention (CDC), the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA). The surveillance activities of NARMS are carried throughout all 50 states in the USA. One of the main aims of NARMS is to inform the FDA to make decisions regarding the approval of safe and effective antimicrobials for animals[6]. For instance, after the approval of fluoroquinolone drugs in poultry, data from other sources and NARMS showed evidence of an increase in fluoroquinolone resistance in *Campylobacter* isolates originating from poultry and causing infections in humans. Likewise, the increase in resistance to third generation cephalosporins in *Salmonella* across animals at slaughter, retail meats and humans was documented[7–11]. Notably, these observations were also made across surveillance systems from other countries. In particular for the USA, the data from NARMS supported FDA decisions to withdraw approval for the use of fluoroquinolones in poultry and the ban of some extra-label use of cephalosporins in food-producing animals[6,7]. Unlike MARAN, each agency of NARMS provide a separate yearly report on AMR, and a single agency summarizes the data on AMU in food-producing animals. The latter, highlights the adjustability of integrated surveillance programs to local mandates and priorities[12].

Integrated surveillance of AMR in Latin America

In spite of international recommendations during the last two decades, systems with continuous integrated surveillance of AMR and AMU in humans, food-producing animals and food have been only implemented in some European countries and North America[13]. In this regard, integrated surveillance in Latin American countries is at an early stage. To date developments on AMR surveillance have focused only on the human health sector and data on AMU from food-production settings is largely absent. For instance, PAHO/WHO contributed at a regional level to initiate in 1993 the regional monitoring of important bacteria causing pneumonia and meningitis, (*Sistema de Redes de Vigilancia de los Agentes Responsables de Neumonias y Meningitis Bacterianas*, SIREVA II) and in 1996 the Latin American Antimicrobial Resistance Surveillance Network (ReLAVRA). Nevertheless, several Latin American countries have provided snapshots of the AMR situation in food-producing animals and food, following the examples of integrated surveillance systems as the ones mentioned above[14,15]. However more work is needed towards the harmonization of sampling, data collection and analysis in order to enable characterization of trends of AMR and AMU within countries. Much of these AMR snapshots have been provided by research groups, either working from the human health or the food-producing sectors. As a consequence, these data were provided in the absence of national guidelines for sampling and isolation of bacteria. These guidelines would ideally be provided by the national agencies performing routine activities of surveillance; these agencies should also use the generated data for publication. In the case of Colombia, and as presented in this thesis, a pilot project for a Colombian Integrated Program for Antimicrobial Resistance Surveillance (COIPARS) was initiated as a research-driven initiative at the Colombian Corporation for Agricultural Research (AGROSAVIA). In terms of AMR surveillance, COIPARS has provided baseline data of AMR at the farm[16] and retail level[17–19] in poultry production in the country. Furthermore, baseline data on slaughterhouses and antimicrobial sales in the country are provided in this thesis **(Chapter 2)**. In addition, a methodology for a thorough genomic and evolutionary characterization of resistance to Extended-Spectrum Cephalosporins (ESC-R) in *Escherichia coli* and *Salmonella* from poultry is also available **(Chapter 3-6)**. This methodology is applicable to determinants of resistance different than ESC-R. For the Colombian situation, the experience of COIPARS in AMR surveillance in food-producing animals would be an asset to establish a national surveillance program in the country. The methodologies and the data provided by COIPARS, have the potential to be exploited by the human and veterinary medicine and food-producing sectors.

After the announcement of WHO's Global Action Plan (GAP) in 2015, it is apparent that some surveillance initiatives in food-producing animals and food have gained visibility in Latin American countries. This could probably be a consequence of the call to coordinated actions between human health and agricultural

sectors presented in WHO's GAP. Notably, in Colombia, recent activities to bring together the experience of AMU and AMR surveillance in human and animal sectors have taken place[20].

In Colombia, the National Institute of Health (*Instituto Nacional de Salud*, INS), the Colombian Institute for Agriculture and Livestock (*Instituto Colombiano Agropecuario*, ICA) and the National Institute for the Surveillance of Food and Medicines (*Instituto Nacional de Vigilancia de Medicamentos y Alimentos*, INVIMA), are the official agencies designated to safeguard public health at the human, agricultural and food sectors, respectively. Following the model of NethMap-MARAN and NARMS, the use of harmonized methodologies for systematic sampling of AMR and collection of AMU-data by these agencies would enable the integrated analysis to guide the interventions needed in the country. As mentioned in **Chapter 1** of this thesis, both AMR and AMU data is key to measure the impact of interventions aiming to mitigate the impact of AMR and set up judicious AMU in the country. These aspects are foreseen in the Colombian national strategic plan in response to AMR[21]. Success in surveillance needs official governmental endorsement to be sustainable. One group either inside or outside the national agencies, could be designated under national mandate to manage and analyze the datasets originating from different sectors. A group like COIPARS, among others in Colombia, could play a significant role to bring together the data and safeguard its viability. It is desirable to implement a data-exchange approach for integrated analysis across different sectors. For this purpose, sampling and data collection should be harmonized. Collection and processing of samples can be centralized in one laboratory or performed in multiple laboratories once the methodology is standardized and proficiency testing is performed. In the same way, data collection can be gathered and analyzed by the designated group. Such group should also play a role in safeguarding the quality of the data, and guaranteeing its sustainability for longitudinal analysis, since continuous analysis of harmonized data is the basis to keep track of trends in AMU and AMR as mentioned above. Recommendations from WHO's Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) and external experts could be followed. Such recommendations can be used as a basis for sampling, data collection analysis in Colombia[1].

Communication of the output of national surveillance systems is an additional aspect to consider. As exemplified by surveillance systems such as NETHMAP/MARAN and NARMS, surveillance findings should be reported in a nation-wide manner and made accessible to the general public according to national mandates. Noteworthy, sensitive data of emerging pathogens and emerging mechanisms of resistance that could pose a threat to public health and/or industrial activities within the country should be dealt and reported with care. Misinterpretation of sensitive data by the general public could have negative repercussions in the commercial activities of the food-producing industry at national and international levels. The conditions to treat sensitive data should be discussed with anticipation among the participants in the surveillance activities.

Agreements should exist to guarantee mutual-trust between the official agencies performing the surveillance and the participants from food-producing sectors. In this regard, guidelines should be designed and implemented with anticipation to respect the reputation of the national industry and also enable action to mitigate potential threats to public health. However, safeguarding public health should be superior to economic values of the industry when it comes to reporting and implementation of preventive/corrective measures.

Sharing output data from national surveillance systems

Ideally, data analysis and reporting from integrated surveillance systems could be extended to regional and international comparisons. In this case, data collected from future national systems in Latin America should be conceived to be suitable for such comparisons. An example of regional reporting is the Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) report. The JIACRA is a joint effort by the European Centers for Disease Prevention and Control (ECDC), the European Food Safety Authority (EFSA), and the European Medicines Agency (EMA). In the JIACRA report, data of AMU and AMR across human, animals and food from member states of the European Union and Switzerland, are analyzed to identify potential relationships between AMU and occurrence of AMR in humans and food animals[22]. Another on-going initiative aiming to harmonize surveillance of AMR at an international level is the ESBL *E. coli* Tricycle AMR Surveillance Project coordinated by WHO-AGISAR[23]. This initiative aims to implement systematic integrated surveillance with high feasibility in both high-income and Low and Middle-Income Countries (LMICs). For this purpose, ESBL-producing *E. coli* is set to be the single indicator organism to be collected from three different sources (thus 'Tricycle') namely human sources, the food-chain and the environment. Although at a pilot phase, the characteristics of this project and the methodology that would derive from it could also serve as a basis to increase feasibility of harmonized surveillance in Latin America. Separate from the supporting activities of international agencies such as WHO, FAO, OIE and other groups such as the Codex Alimentarius in Latin America, no regulatory body exists with the capacity to measure and regulate risks to public health and food safety at a regional level. Such risks could be trade-associated, and could include for instance the introduction and dissemination of *Salmonella* serovars carrying AMR determinants through poultry farming, as reported in **Chapter 5** of the present thesis. In the absence of such regulatory entities, disseminated and harmonized integrated surveillance systems within countries could serve as means to trace and report the occurrence of such risks in the region. In this regard, the JIACRA report can be seen as a guide on how harmonized collection of data on AMU and AMR could be made at a regional level. Detailed information regarding data sources, data gaps and the methodology employed in the analysis are described in the report. This information is valuable and can be used to guide future harmonized collection of

AMU and AMR data across Latin American countries. Notably, the JIACRA report is based on data collected with the technical support for harmonization of the European Union surveillance networks and the National public health reference laboratories. This guarantees comparability of collected data. Concerning AMR surveillance, and as outlined in this thesis, the choice for Whole Genome Sequencing (WGS) as a central measure for the integrated analysis of AMR would facilitate the exchange of data within and between countries and allow analysis of genes, plasmids and bacteria in a standardized manner.

Opportunities to Improve Detection of AMR in Surveillance Systems

Characterization of AMR in isolates collected from surveillance systems can be made using different techniques. In this thesis, the characterization of genetic determinants of ESC-R was made using different approaches. In **Chapter 2**, the initial phenotypic characterization of resistance was described. In **Chapter 3,** PCR detection and sequencing of ESBL/pAmpC genes, and of the genes of the pMLST and MLST schemes were used to characterize *E. coli* isolates. In **Chapter 4**, PCR detection of ESC-R was followed by WGS and *in silico* characterization of AMR genes, plasmids and strains of *Salmonella*. In **Chapters 5 and 6**, WGS was the initial step prior to detailed phylogenetic and evolutionary characterization of plasmids and strains, respectively. PCR-based detection and sequencing as used in **Chapter 3** was economic in terms of the costs of the laboratory materials needed. However, the multiplicity of steps of PCR and sequencing for both the pMLST and MLST schemes made the PCR-based characterization complex. In contrast, the output of WGS could be analyzed in an automatized manner and in a shorter period of time. Once the sequence reads were assembled, characterization of AMR genes, plasmid and strain STs were readily obtained using the platform from the Center for Genomic Epidemiology (CGE). This approach was described in **Chapter 4**. In addition, departing from the same input data (that is, genome assemblies), WGS sequences of plasmids and strains could be used for thorough phylogenetic and evolutionary analysis as described in **Chapter 5 and 6**. An additional asset of WGS, was the availability of genomes in the public domain which enabled the comparison of plasmid and strain STs with reports from additional countries and different sources of isolation.

The advantages of WGS in laboratory-based surveillance of food-borne pathogens and AMR detection have been indicated by several agencies and research groups who advocate for the application of WGS[24–27]. In addition, new approaches are being developed to increase the efficiency of WGS. For instance, Nanopore sequencing is seen as a potential rapid diagnostic tool for bacterial infections and prediction of AMR in clinical cases[28,29]. Nanopore can also be adapted to low resource-settings, long-distanced or remote areas. In this case,

a relatively inexpensive sequencing station composed of a simplified DNA isolation component and a sequencer could be enough to obtain microbial genomic data[30–32]. With the use of an internet connection, sequences could be provided to reference laboratories with the capacity to store and analyze these data. However, the quality of WGS data obtained with Nanopore sequencing still needs to be optimized for robust genomic comparisons and work is being made to increase the level of quality of Nanopore sequences[33,34]. For use in LMICs, the costs should be reduced as, although the investment for the device is low, the running costs are still considerable. Besides, initiatives are being taken to standardize protocols for WGS in food microbiology. For instance, the FDA and the International Standard Organization (ISO) are part of a collaborative effort to develop standards for characterizing foodborne pathogens using WGS[35]. In addition, analysis of WGS data is becoming more accessible through web-based platforms intended to provide automatization of bioinformatic analysis and a friendlier experience for the non-bioinformatician[36–38]. WGS data is increasingly available in open-access databases[39,40]. The collection of genomic data within surveillance systems and comparisons with data from the public domain has enhanced the epidemiological study of AMR genes, plasmids and bacteria across countries, sources of isolation and years. This approach was exploited during development of this thesis and methodologies for future comparisons were described for plasmids **(Chapter 5)** and *Salmonella* strains **(Chapters 3 and 6)**. In addition, availability of historical WGS data facilitates analysis in post-outbreak investigations and was for instance useful to assess the dissemination of the colistin resistance gene *mcr-1* after its description[41–44]. However, as mentioned above, it is important to consider that WGS data related to emerging pathogens and mechanisms of resistance is sensitive data, and should be treated with care and as agreed between national agencies of human and veterinary health, agriculture and food-safety and representatives of the national food-producing industries.

Prioritization of AMR mechanisms to survey

In spite of the advantages of WGS, it is important to note that WGS should not be used in full replacement of routine phenotypic surveillance of AMR. If gene families causing previously unobserved resistances haven't been described, WGS-based analysis wouldn't be able to detect them. Systematic phenotypic characterization of AMR allows to identify trends of unknown resistance in surveyed bacteria and to prioritize the characterization of such trends. These AMR observations should be analyzed to identify the genetic causes for the phenotype. In settings where AMR surveillance systems have restricted research capabilities, strains could be sent to reference or research laboratories where characterization of unknown resistance genes could be performed and reported. In practical terms, future WGS-driven surveillance systems could perform a sampling among collected isolates for phenotypic susceptibility testing, with a sound statistical approach.

Relevance of multi-level analysis of AMR

There is a vast diversity of AMR genes, mobile genetic elements, plasmid families and bacterial species carrying AMR determinants[45–47]. The frequencies of occurrence and consequences for the epidemiology of these genetic determinants of AMR in human and animal health vary per country, source of isolation, animal host, among others. AMR is a phenomenon influenced by multiple factors which warrant the complexity of its epidemiological study. Nevertheless, the single most important determinant for its increase is selection by AMU[48,49]. From the vast multi-factorial dimension of AMR, this thesis focused particularly on ESC-R-conferring genes ESBL/pAmpC, ESBL/pAmpC-carrying IncI1 plasmids and strains of *E. coli* and *Salmonella* from poultry in Colombia. ESBL/pAmpCs are studied due to the negative impact in morbidity and mortality of ESC-R infections in humans worldwide[50–53] and because poultry and poultry products are a large reservoir of ESBL/pAmpC genes leading to exposure of human consumers[54–57].

The departing point for the characterization of genes, plasmids and bacterial strains provided in this thesis, were ESC-R isolates of *E. coli* and *Salmonella*. As a result of the characterization, a high level of similarity of ESBL/pAmpC genes and carrying plasmids was observed within *E. coli* **(Chapter 3)** and *Salmonella* **(Chapter 4)** in Colombian poultry. Some gene-plasmid combinations were common between *E. coli* and *Salmonella* (e.g. bla_{CMY-2}-IncI1 and bla_{SHV-12}-IncI1) **(Chapter 5)**, some remained restricted to *E. coli* (E.g. $bla_{CTX-M-2}$-IncHI2 and bla_{CMY-2}-IncK) and some restricted to *Salmonella* (e.g. $bla_{CTX-M-165}$-IncQ1).

As an initial characterization of ESC-R in Colombian poultry, this thesis focused on the most prevalent genetic determinants of ESC-R. The combination of bla_{CMY-2} and IncI1/ST12 plasmids was the most prevalent, and a high genetic relatedness was found among these plasmids within and between *E. coli* and *Salmonella* from Colombia and from other countries, such as South and North American and European countries **(Chapter 5)**. Nevertheless, different patterns of dissemination were observed between these two bacterial species. ESC-R genes were carried in heterogeneous *E. coli* Sequence Types (STs), indicating dissemination of ESC-R in *E. coli* through horizontal gene transfer **(Chapter 3).** The epidemiology of *Salmonella* in poultry is determined by clonal distribution of successful serovars-STs, such as *S.* Paratyphi B variant Java ST28 (here referred to as *S.* Java) or *S.* Heidelberg ST15. These clones have acquired ESBL/pAmpC-genes **(Chapter 4)**. Comparison with *Salmonella* STs from other European and North American countries, showed a marked geographical clustering in Colombia **(Chapter 4)**. The clustering was indicative of a geographically-related dissemination of the strains. Subsequent analysis including *S.* Java ST28 genomes from other Latin American countries, showed high level of genomic relatedness across Latin American countries, indicating the circulation of a conserved clone of *S.* Java ST28 within the region **(Chapter 6)**. Interestingly, in spite of the high level of genomic relatedness across Latin America, only strains from Colombia harbored ESBL/pAmpC genes on

plasmids. From this multi-level perspective on ESC-R genes, plasmids and strains, and the comparisons within and outside Colombia, the results of the present thesis indicate that occurrence of the genetic determinants of ESC-R in Colombian poultry are homogenous for *E. coli* and *Salmonella* and apparently driven by a strong within-country effect. National control programs of *Salmonella* in poultry will affect its epidemiology including the ESC-R variants[58] compared to *E. coli* where clonality in ESC-R variants was not observed. In this regard, high rates of recombination across a large diversity of *E. coli* lineages and the ubiquity of this bacterial species in a large number of ecological niches, could explain the high levels of diversity at the strain level[59]. In contrast, narrow association of some *Salmonella* lineages to particular hosts and ecological niches, in addition to *Salmonella* control programs, could explain the conserved genomic features of *Salmonella* strains[58,60]. Nevertheless, it is apparent that when both bacterial species co-exist in the chicken gut or the chicken environment, the exchange of identical plasmids such as bla_{CMY-2}-IncI1/ST12 occurs.

The drivers of a country effect on the occurrence of AMR genes, plasmids and strains in poultry could be numerous. To name a few, national policies of AMU in food-producing animals, poultry management (farming and trade practices), *Salmonella* control programs, geographical conditions in which the chickens are raised (e.g. weather, ecosystem characteristics) should be considered. In particular for the Colombian situation, poultry farming is centralized in three departments (i.e. provinces) where more than 60% of poultry farming is carried out[61]. The samples from farms analyzed in this thesis originated from two departments with the larger poultry population: Santander (26%) and Cundinamarca (22%). Altogether, ESC-R isolates of *Salmonella* analyzed in the present thesis from farms, slaughterhouses and retail, originated from 18 out of the 32 departments of Colombia holding more than 90% of the chicken population in the country **(**Supplementary Figure 1 in **Chapter 4)**. Notably, the high similarity in genes, plasmids and strains of *Salmonella* mentioned above, was observed not only in isolates from Santander and Cundinamarca, but throughout the whole collection of ESC-R isolates **(Chapter 4)**. In essence, a high similarity in ESC-R genetic determinants was observed in samples from different levels of poultry production (farm, slaughter and retail) and multiple departments in the country. These similarities are a reflection of the pyramidal structure of the poultry industry. As described in **Chapter 1**, interventions at the farm level, targeted to reduce the occurrence of *Salmonella* and AMR in the departments with the larger poultry population, can very likely help to reduce the prevalence of *Salmonella* and ESC-R Enterobacteriaceae in poultry and poultry products throughout the whole country. As previously reviewed, on-farm interventions should be aimed to reduce the level of contamination originating from vertical transmission from breeding chickens at the top of the pyramid, transmission at hatcheries, transmission within and between broiler farms and transmission from the environment external to the farms[62].

Noteworthy, a strong within-country effect on AMR in poultry and swine was observed in a study where samples from nine European countries were analyzed[63]. Some of these countries, such as the Netherlands and Denmark, have been successful in reducing AMR levels in bacteria from livestock by major reductions in AMU in food-producing animals[3,48]. In addition, reductions in ESC-R in poultry after restricted use of third-generation cephalosporins in poultry have been documented in the Netherlands[2], Canada after voluntary withdrawal of ceftiofur by the poultry industry[54,64], the USA[6] and more recently Japan[65].

Consequences for public health

Besides the factors influencing the geographical distribution of ESC-R genes, plasmids and strains as discussed above, an additional factor to consider is the occurrence of these determinants across multiple sources (e.g. poultry, human, environment). As mentioned in **Chapter 1**, one of the objectives of this thesis was to evaluate the exposure to humans of ESBL/pAmpC through poultry and poultry products. The most prevalent genes in Colombian poultry were bla_{CMY-2} in both *E. coli* and *Salmonella*, bla_{SHV-12} in *E. coli* and $bla_{CTX-M-165}$ exclusively in *Salmonella*. Within Colombia, bla_{SHV-12} and bla_{CMY-2} were found in poultry as well as humans. It was only possible to determine that these gene variants were similarly found. Further analysis of ESBL/pAmpC-carrying plasmids and strains were hindered due to unavailability of such sequences from humans in the country. Outside of Colombia, bla_{CMY-2} or bla_{SHV-12} have been reported in different sources (e.g. humans, poultry, swine, environment)[66–70]. As for $bla_{CTX-M-165}$, a single report from an isolate of *Klebsiella pneumoniae* from a human urine sample from Chile was available in the public domain (NCBI Nucleotide accession number: KP727572).

Transfer of MGEs between poultry and humans

After several years of refinement of the molecular characterization of ESBL/pAmpC genes, plasmids and strains, particular combinations of ESBL/pAmpC genes and plasmid subtypes have been found associated to particular sources[67,71]. Although bla_{CMY-2} and bla_{SHV-12} were both carried on IncI1 plasmids in Colombian poultry, only the association of bla_{CMY-2} with the particular IncI1/ST12 plasmid lineage has been frequently reported in poultry and poultry products outside the country[67,71]. The lineage of IncI1/ST12 plasmids carrying bla_{CMY-2} was investigated in detail in **Chapter 5**. As a result, bla_{CMY-2}-IncI1/ST12 plasmids originating from multiple countries were found highly related at the genomic level. The vast majority of these plasmids originated from poultry samples and some of them originated from clinical samples from human and animals. In this regard, bla_{CMY-2}-IncI1/ST12 is a poultry-associated plasmid which can be found carried in species of Enterobacteriaceae infecting other animals. It is apparent that the chance to found these plasmids and other AMR determinants in human infections increases once

the plasmid is carried on poultry-associated lineages of *E. coli* and *Salmonella* known to cause severe infections in humans. Examples being *E. coli* ST131 carrying allele 22 of the *fimH* type 1 fimbrial adhesin gene[72,73] or *S.* Heidelberg ST15[74]. In addition to associations with plasmids, association of ESBL/pAmpC genes with Insertion Sequences (IS) also play an important role in the transfer and evolution of ESBL/pAmpC genes. In **Chapter 5**, strong associations between ISEcp1 and bla_{CMY-2} and between IS26 and bla_{SHV-12} were reported. However, taking into account the multiple levels of analysis of AMR already described, it is likely that in order for an IS-gene association to become highly prevalent, the association with an epidemic plasmid and/or bacterial clone is required. As seems to be the case in Colombian poultry. In a recent One Health WGS-based study, identical sequences of $bla_{CTX-M-15}$ flanked by a Tn3 transposable element were found within isolates from livestock and between isolates from livestock and humans in the UK. However, the identical transposon was found in a large proportion of $bla_{CTX-M-15}$-carrying isolates from livestock (22/32) in comparison to human isolates (3/87). These findings indicate that IS mediated transfer of ESBL/pAmpC genes between different sources can occur, but alone the association of ESBL/pAmpC genes with particular ISs wouldn't contribute in large measure to their epidemic spread[75].

Co-resistance

Co-resistance could also contribute to the occurrence of ESBL/pAmpC genes. Co-carriage of genes conferring resistance to other classes of antimicrobials different than β-lactams were extensively found in *Salmonella* in this thesis and have been previously reported in ESC-R Enterobacteriaceae from different sources[76–79]. Co-resistance to fluoroquinolones, trimethoprim sulfamethoxazole and/or aminoglycosides were previously described[76–79]. As observed for the *Salmonella* strains analyzed in **Chapter 4**, a large diversity of AMR genes were described in addition to the ESBL/pAmpC genes after *in silico* characterization of the genomes **(**Supplementary Table 1 in **Chapter 4)**. Genes conferring resistance to aminoglycosides were found associated in larger measure and represented by a large diversity of genes including *aac(3)Iva, aph(3')Ia, aph(3')Iia, aph(4)Ia, aph(6)Ic, aph(6)Id, aadA1, aadA2, aadA16, strA* and *strB*. Specifically, for $bla_{CTX-M-165}$ found in *S.* Heidelberg, co-localization with genes conferring resistance to aminoglycosides (*strB* and *strA*), sulphonamides (*sul1*) and trimethoprim (*dfrA7*), was frequently observed. In this case, the successive co-localization of these genes could be a result of the horizontal transfer of an integron-like MGE. From an evolutionary perspective, once an MGE grouping all these genes was integrated into IncQ1 plasmids **(**associated to carriage of $bla_{CTX-M-165}$ in **Chapter 4)**, cassettes harboring the other genes found co-localized with $bla_{CTX-M-165}$ could be easily incorporated. This hypothesis could explain how other genes such as *aph(6)-Id*, *aph(3'')-Ib*, *sul2*

and/or *tet(A)* were found next to $bla_{CTX\text{-}M\text{-}165}$ in some but not all isolates of $bla_{CTX\text{-}M\text{-}165}$-carrying *S.* Heidelberg. Further phylogenomic characterization of the IncQ1 plasmids carrying $bla_{CTX\text{-}M\text{-}165}$ is necessary to elucidate the evolutionary trajectory of this plasmid lineage and the MGE elements driving the gain or loss of *aph(6)-Id*, *aph(3'')-Ib*, *sul2* and *tet(A)*. In terms of resistance to quinolones, virtually all sequenced Latin American isolates of *S.* Java ST28 carried the plasmid-harbored *qnrB19* gene (n=85/87). This gene confers reduced susceptibility to quinolones **(Chapter 6)**. Very few sequenced isolates of *S.* Heidelberg ST15 from Colombia (n=2/17) carried this gene **(Chapter 4)**. When analyzed with PointFinder (a built-in programme in ResFinder 3.1) no mutations in the quinolone resistance determining region (QRDR) of the topoisomerase type II and IV enzymes were found in genomes of *S.* Java from Latin America. In turn, all genomes from *S.* Heidelberg in Colombia exhibited mutations S83F and T57S in the QRDR *gyrA* and *parC* respectively. Noteworthy, previously unknown or uncharacterized mutations in *gyrB* and/or *parC* were also detected with PointFinder in all genomes of *S.* Heidelberg and some genomes of *S.* Java. The presence of *qnrB19* and QRDR mutations in both *S.* Java and *S.* Heidelberg respectively, is indicative of selection pressure pose by the use of quinolones in poultry. In fact, the use of fluoroquinolones such as enrofloxacin is a common practice in poultry production in Colombia[80,81]. Taking into account the presence of *qnrB19* and the absence of QRDR mutations in *S.* Java, it could be hypothesized that *qnrB19* and other resistance mechanisms such as efflux pumps and/or porin alterations in the outer membrane could be mediating additive resistance to quinolones in *S.* Java. Alternatively, the uncharacterized mutations detected with PointFinder as mentioned above, could be responsible for quinolone resistance, in addition to the presence of *qnrB19*. Nevertheless, functional studies to characterize the effect of such mutations would be needed to confirm this hypothesis.

In addition to positive co-selection effected by the use of antimicrobial drugs, the use of biocides could also play a role in the selection ESC-R genetic determinants. For instance, in the genetic environment of $bla_{CMY\text{-}2}$ the Quaternary Ammonium Compound (QAC)-resistance protein SugE was commonly found **(Chapter 5)**. In fact, in addition to IncI1, the mobile genomic subregion IS*Ecp1*-$bla_{CMY\text{-}2}$-*blc-sugE* is found in other plasmid lineages such as IncK and IncA/C[68,82]. The genomic subregion bla_{CMY}-*blc-sugE* is found in the chromosome of *Citrobacter freundii* where it seems to have originated and translocated into plasmids[83,84]. The plasmid-encoded SugE protein confers efflux-mediated resistance to QAC. A relationship between resistance to QAC compounds and other antimicrobials have been previously indicated in the food-industry and clinical settings[85,86]. Although frequently observed[87], the correlation between $bla_{CMY\text{-}2}$ and SugE have not been confirmed. Measurements of resistance to QAC still need to be standardized to facilitate the interpretation of conclusions from multiple studies assessing the effectiveness of these compounds[88,89]. The latter is necessary to guide the effective use of QAC, i.e. use above sub-lethal concentrations. This is an important aspect

to consider because in addition to the use of antimicrobial drugs, improper use of cleaning and disinfection products in the poultry environment could select for resistant bacteria[89]. Therefore, measures aiming to reduce AMU and AMR in the poultry industry should also consider the impact of biocides such as QAC could have in the emergence of AMR.

The burden of ESC-R Enterobacteriaceae

Overall, the high prevalence of ESBL/pAmpC genes in *E. coli* and *Salmonella* in poultry in several countries indicates high level of exposure to human consumers. Further comparative analysis of genes, plasmids and strains, indicate that the transfer of ESBL/pAmpC between poultry and human can occur[72]. However, most reports documenting this potential transfer are not quantitative. As previously mentioned, the transfer can occur in association with ISs, plasmids and/or poultry-associated lineages of bacteria known to cause infections in humans. There is large variation in the prevalence and consequences for public health among particular ESC-R genes, plasmids and strain subtypes. Thus, the molecular characterization of these determinants should be considered when performing risk analysis and attribution studies. In general, the burden of infections caused by ESC-R Enterobacteriaceae include larger health expenditures due to antimicrobial treatment failure[51–53]. Recently, Cassini and colleagues reported that the median number of attributable deaths to third-generation cephalosporin-resistant *E. coli* in Europe, increased from 2139 (95% Uncertainty Interval (UI): 1901–2420) cases in 2007 to 8750 (95% UI: 7505–10262) cases in 2017[50]. Nevertheless, in the report from Cassini and colleagues no differentiation of the genetic determinants of ESC-R, or between poultry- or non-poultry associated ESC-R infections was available. Previous estimates of the transfer of ESBL/pAmpC genetic determinants between poultry and humans in the Netherlands, range from low similarity of source-associated ESC-R genetic determinants (genes and plasmids)[90] and low exposure through poultry meat consumption (<20% of total meat consumption)[91] to high rates of chicken-associated ESC-R *E. coli* infections and deaths (56% of total ESC-R *E. coli* infections)[92,93]. The first estimate by Dorado-García and others was made using pooled analysis of primary/raw data extracted from all studies available in the Netherlands[90], whilst the latter by Collignon and others[92] was based on estimations from previous reports[93]. To date no global estimate of this burden has been made available since the data required for risk analysis is still lacking from many countries worldwide[48]. Besides, a large variation of burden estimates across European countries was observed in the report by Cassini and colleagues. It is then important to consider that burden estimates may vary in large measure according to the geographical area of study. In Colombia, no risk analysis or burden estimates of poultry-associated infections of *Salmonella* in humans is yet available. A risk profile of non-typhoidal *Salmonella* from chicken and chicken meat was provided in 2011 by the Unit of Food-Safety Risk Assessment of the INS

(*Unidad de Evaluación de Riesgos para la Inocuidad de los Alimentos*, UERIA). In this report, a lack of studies characterizing the distribution of *Salmonella* serovars in Colombian poultry was found. This aspect restricted estimations of the contribution of *Salmonella* from poultry to human cases in the country[94]. Initial characterization of *Salmonella* serovars was made publicly available as a result of the baseline studies of COIPARS until 2012[16,17]. From these initial reports and as detailed in this thesis, *S.* Heidelberg and *S.* Java, are the most prevalent serovars in broilers and chicken meat in most of the country. Data collected from passive surveillance in humans by the INS indicate that these two serovars are unfrequently found in clinical cases[95–97]. For instance, between 2000 and 2013, 12 cases (0.2%) of *Salmonella* clinical isolates belonged to *S.* Heidelberg in comparison to 2431 of *S.* Typhimurium (33.7%), 2064 of *S.* Enteritidis (28,6%) and 661 of *S.* Typhi (9,2%). Human clinical cases of *S.* Java weren't reported or were reported in the category 'Other serovars', which included serovars with one to nine cases between 2000-2013[96]. In these regards, it is apparent that the contribution of ESC-R *Salmonella* isolates from broilers and chicken meat causing infections in humans in Colombia is little. Nevertheless, it is of interest to assess the level of genetic similarity between isolates of *S.* Heidelberg originating from humans and those from poultry in Colombia. Although not assessed in this thesis, given the unavailability of sequences from human isolates, it can be anticipated that a high level of genetic similarity among these isolates exists. Regardless of their source, isolates of *S.* Heidelberg exhibit high levels of genomic identity and the vast majority belong to ST15. As for *S.* Java, it could be anticipated that clinical cases would originate from sources different than the poultry associated ST28. Human infections with this serovar are predominantly related to acquisition from fish or reptiles[98–100].

WGS-based comparative analysis from the Netherlands[101] and other countries such as the UK[75], have indicated that the transfer of ESBL/pAmpC-producing *E. coli* between livestock and humans is smaller than previously estimated[57]. With the recent advent of WGS for epidemiological comparisons, a possible explanation to the previously reported high burden of poultry-associated ESBL/pAmpC-producing *E. coli* in the Netherlands, could be the low discriminatory resolution of MLST for *E. coli* in contrast to WGS comparisons[92,93]. All in all, estimating the burden of AMR morbidity and mortality is a challenging task[50] and sometimes leading to controversial positions[102]. It is apparent, that the data needed for a global estimate to the burden of infections caused by ESC-R Enterobacteriaceae associated to poultry causing infections in humans is still lacking in quantity and quality from many countries. Furthermore, it is desirable to include the genomic characterization of genes, plasmids and strains, to better understand the transmission of these genetic determinants, alone or in combination between sources. As already described throughout this chapter, there is no doubt that WGS-based comparisons have enough phylogenetic resolution to indicate whether the transfer between livestock and humans occurs or not[72,73,75,101].

The way forward

The data needed to study the attribution and burden of AMR, originate from surveillance activities of AMR across the human-animal-food-environment interphase under a one health approach. The output of attribution and burden studies, are used to indicate priorities for public health representatives and guide interventions to lower the negative impacts associated to AMR. From the one health perspective it is expected that interventions to lower AMU and AMR in human health, animal health, agriculture and/or the environment, would aid in the mitigation of the burden of AMR across all the one health areas. Approaches proposed in the human health sector, indicate that infection control measures are essential to reduce the burden of AMR[103,104]. In animal husbandry, disease prevention/biosecurity measures are expected to reduce the need of AMU, the main factor influencing the emergence and spread of AMR[105]. It is anticipated that such measures will reduce not only the emergence and spread of AMR but also reduce the burden of food-borne pathogens such as *Salmonella* and *Campylobacter* paving the way to an overall reduction of the burden associated to food-borne infections.

Conclusions

In the analyzed geographical areas, the poultry-associated determinants of ESC-R circulate within poultry and its environment. Its dissemination within and between countries can occur due to common practices of poultry farming and management. Within *E. coli*, transfer is mainly mediated through horizontal transfer of genes in combination with plasmid lineages **(Chapters 3 and Chapter 5)** and genes and other MGEs such as ISs **(Chapter 5).** Within *Salmonella*, spread is mainly mediated by clonal spread of homogeneous genes, MGEs and epidemic lineages of *Salmonella* strains combined **(Chapters 4 and 6)**. The risk of transfer of poultry-associated AMR to humans seems to occur in association with pathogenic *E. coli* and *Salmonella* lineages, such as *E. coli* ST131 *fimH*-22 and *S.* Heidelberg. However, quantitative data of the burden of poultry-associated infections is lacking in most geographical areas. In the future, burden analysis should include WGS-based comparisons of AMR genes, MGEs and bacterial isolates. The prevalence and consequences for public health of ESC-R is associated to a multiplicity of factors that are particular to every country. It is recommended to follow the immediate-call-to-action presented by WHO on its GAP on AMR. In order to measure the success of the activities outlined in the five objectives of the strategic plan of the GAP and Colombian NAP, the utmost priority is to implement integrated surveillance systems of AMU and AMR at a national scale. The data collected in these systems should be conceived suitable for regional and international comparisons.

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Appendix

English summary

Background

Antimicrobial chemotherapy relies in large measure on the effectiveness of β-lactam drugs. The main families of β-lactams are penicillins, cephalosporins, carpabenems and monobactams. Since their introduction in human and veterinary medicine, resistance to each of these families have been described. Among β-lactams, third and fourth generation cephalosporins are listed as critically important antimicrobials with the highest priority for human medicine by the World Health Organization (WHO). After indications of increased levels of resistance to these drugs in Enterobacteriaceae from human and animal origin, the use of third and fourth generation cephalosporins in food-producing animals was restricted in some European countries. Similarly, some extra-label uses of third generation cephalosporins were restricted in the USA.

The main mechanism of resistance to third and fourth generation cephalosporins, also referred as Extended Spectrum Cephalosporins (ESC), is enzyme-mediated degradation. In species of Enterobacteriaceae, different families of Extended Spectrum β-Lactamases (ESBL) and plasmid-mediated AmpC β-lactamases (pAmpC) are the most predominant enzymes. In terms of substrate specificity, ESBLs are active against almost all penicillins, cephalosporins (except cephamycins) and monobactams, and can be inhibited by clavulanic acid. pAmpC have the same substrates as ESBLs, excluding 4th generation cephalosporins. In turn, they are active against cephamycins such as cefoxitin. ESBLs and pAmpC are mostly encoded on plasmids. Their dissemination occurs through a combination of horizontal transfer of plasmids and clonal spread of bacteria carrying these genes.
In poultry and poultry products, occurrence of ESBL/pAmpC in isolates of *Escherichia coli* and *Salmonella enterica* has been extensively described. The pyramidal structure of poultry production favors vertical and horizontal transmission of ESBL/pAmpC genetic determinants (i.e. genes, plasmids and strains) at different levels of poultry production. Poultry has been suggested as a reservoir of ESBL/pAmpC-producing *E. coli* and *Salmonella* causing infections in humans.

The epidemiological studies of the genetic determinants conferring resistance to ESC (ESC-R) rely on sequence-based characterization of ESBL/pAmpC gene variants, and ESBL/pAmpC-carrying plasmids and bacterial strains. Previous studies have described the transfer between poultry and humans of whole bacteria, carrying identical sequences of genes, plasmids and strains combined (clonal spread), whilst others described the horizontal transfer of related plasmid lineages across different lineages of *E. coli*. With the advent of Whole Genome Sequencing (WGS) and high-resolution differentiation between ESBL/pAmpC-carrying plasmid and bacterial lineages, it is apparent that horizontal transfer of related plasmids between different lineages of *E. coli* mainly mediates the transfer between humans and poultry. However, no quantitative measures of this potential transfer

are available to date. Overall, the factors influencing the epidemiology of ESBL/pAmpC in humans and animals vary according to the geographical area of study.

Data of ESBL/pAmpC-producing *E. coli* and *Salmonella* from poultry is largely scarce in Latin America. Available reports are characterized by differences in methodologies of sampling and characterization of the genetic determinants of ESC-R. These differences complicate comparisons across countries in the region. A recent call to action against Antimicrobial Resistance (AMR) was made by the WHO. WHO's global action plan against AMR strongly advocates the establishment of harmonized surveillance initiatives that integrate data on Antimicrobial Use (AMU) and AMR across human and veterinary medicine, agricultural and environmental sectors. With the support of members of the Advisory Group on Integrated Surveillance on Antimicrobial Resistance (AGISAR) for WHO, a research-driven initiative for integrated surveillance of AMU and AMR was started in Colombian poultry production. Before this initiative, public data on the status of AMU and AMR in agricultural settings was absent in the country.

Aims of the research

The aims of the present thesis were i) to describe an initiative to develop an integrated program on AMR surveillance in Colombia, a Latin American country; ii) to provide a highly-detailed characterization of ESBL/pAmpC genetic determinants in isolates of *E. coli* and *S. enterica* from poultry; iii) to reflect on the impact of poultry farming and commerce in the dissemination of ESBL/pAmpC and reflect on opportunities to improve the detection of AMR.

Results

The description of an initial consortium of stakeholders for the initiative of an integrated surveillance system in Colombia was provided **(Chapter 2)**. This consortium consisted of representatives from the public and private sectors and included participants from the national public health, agricultural and food authorities, research and academic groups, the food industry and retailers. Methodologies of sampling, microbiology and AMR characterization were adapted from mature integrated surveillance systems represented in the international advisory team of the pilot surveillance program**.** As a result, a pilot for a surveillance platform was adopted in poultry production with the support of the poultry industry in different geographical areas. Baseline data of AMR of food-borne pathogens *Salmonella* and *Campylobacter* spp. and commensal *E. coli* and *Enterococcus* spp. was provided. Likewise, baseline data on AMU was provided as imported antimicrobials for use in animals. Resistance to third generation cephalosporins was found to be remarkably high in *Salmonella* and *E. coli* **(Chapter 2)**.

Characterization of ESC-R in *Salmonella* and *E. coli* was performed using different approaches. These approaches included: PCR detection of ESBL/pAm-

pC genes and plasmid replicon types followed by sequencing-based characterization of genes, plasmids and strains, using Multi Locus Sequence Typing (MLST) schemes in isolates of *E. coli* **(Chapter 3)**; PCR detection of ESBL/pAmpC genes followed by WGS and *in silico* characterization of AMR genes, plasmids and strains in isolates of *Salmonella* **(Chapter 4)**; WGS as an initial step prior to detailed phylogenetic and evolutionary characterization of plasmids of *E. coli* and *Salmonella* and of strains of *Salmonella* **(Chapters 5 and 6).** As a result, high level of similarity of ESBL/pAmpC genes and carrying plasmids was observed within *E. coli* **(Chapter 3)** and *Salmonella* **(Chapter 4)** in the country. Some gene-plasmid combinations were common between *E. coli* and *Salmonella* (e.g. bla_{CMY-2}-IncI1 and bla_{SHV-12}-IncI1) **(Chapter 5)**, some remained restricted to *E. coli* (e.g. $bla_{CTX-M-2}$-IncHI2 and bla_{CMY-2}-IncK) and some restricted to *Salmonella* (e.g. $bla_{CTX-M-165}$-IncQ1). Overall, the combination of bla_{CMY-2} and IncI1/Sequence Type (ST) 12 plasmids was the most prevalent, and a high genomic relatedness was found among these plasmids within and between *E. coli* and *Salmonella* from Colombia and from other countries **(Chapter 5)**. Nevertheless, different patterns of dissemination were observed between the two bacterial species at the strain level. ESBL/pAmpC genes were carried in heterogeneous *E. coli* STs, indicating dissemination of ESC-R through horizontal transfer of plasmids carrying these genes **(Chapter 3)**. In contrast, the genes were carried in homogenous serovars-STs of *Salmonella* strains, indicating clonal spread of resistance **(Chapter 4)**. A marked geographical clustering within the most prevalent *Salmonella* serovar-STs in Colombia, *S.* Paratyphi B variant Java (here referred as *S.* Java) ST28 and *S.* Heidelberg ST15 was observed **(Chapter 4)**. Subsequent analysis including *S.* Java ST28 genomes from other Latin American countries, showed high level of genomic relatedness across Latin American countries, indicating the circulation of a conserved clone of *S.* Java ST28 within the region **(Chapter 6)**. Using temporal phylogenetic analysis, it was found that a European lineage of *S.* Java emerged in Europe around 1995, and the Latin American one around 2005. Interestingly, and in spite of the high level of genomic relatedness across Latin American *S.* Java ST28, only strains from Colombia harbored ESBL/pAmpC genes and plasmids **(Chapter 6)**.

Conclusions

In *E. coli* in poultry from Colombia and other countries, dissemination of ESBL/pAmpC is mainly mediated by both horizontal transfer of epidemic genes and plasmids. In *S. enterica* from Colombia, dissemination is mainly mediated by a combination of clonal spread of epidemic genes, plasmids and strains. Nevertheless, the prevalence of ESBL/pAmpC genes seems associated to a multiplicity of factors that are particular to every country.

The dissemination within and between countries of poultry-associated ESBL/pAmpC can occur due to common practices of poultry farming and commerce. Quantitative data of the burden of poultry-associated infections in hu-

mans is lacking in most geographical areas. In the future, burden analysis of ESBL/pAmpC should include WGS-based comparisons of genes, plasmids and bacterial strains.

It is recommended to follow the immediate-call-to-action presented by WHO on its GAP on AMR. In order to measure the success of interventions outlined in the GAP, it is necessary to implement integrated surveillance systems at national scale in Latin American countries before future intervention takes place. The data collected in these systems should be conceived suitable for international comparisons.

Samenvatting in het Nederlands

Achtergrond

De β-lactam-antibiotica zijn van groot belang voor de effectieve behandeling van bacteriële infecties bij mens en dier. De belangrijkste β-lactam-groepen zijn penicillines, cefalosporines, carpabenems en monobactams. Sinds hun introductie in de humane geneeskunde en diergeneeskunde is resistentie voor elk van deze groepen beschreven. De derde en vierde generatie cefalosporinen worden door de Wereldgezondheidsorganisatie (WHO) geclassificeerd met de hoogste prioritering binnen de antibiotica die van kritisch belang zijn voor de volksgezondheid. Toenemende resistentie tegen deze antibiotica in Enterobacteriaceae van humane en dierlijke oorsprong heeft geleid tot beperkingen in gebruik van deze cefalosporines bij voedselproducerende dieren in sommige Europese landen. Ook zijn in de Verenigde Staten enkele *extra-label* toepassingen van derde generatie cefalosporines beperkt.

Het belangrijkste resistentiemechanisme tegen de derde en vierde generatie cefalosporinen (Extended Spectrum Cefalosporines (ESC)) is enzym gemedieerde afbraak. De meest voorkomende enzymen in Enterobacteriaceae zijn verschillende soorten Extended Spectrum β-Lactamases (ESBL) en plasmide-gemedieerde AmpC β-lactamases (pAmpC). ESBL's zijn actief tegen bijna alle penicillines, cefalosporines (behalve cephamycines) en monobactams. Activiteit van ESBL's kan worden geremd door clavulaanzuur. pAmpC heeft dezelfde substraten als ESBL's, met uitzondering van vierde generatie cefalosporinen. Op hun beurt zijn deze minder actief tegen cephamycines zoals cefoxitin. ESBL's en pAmpC's zijn meestal gecodeerd op plasmiden. Hun verspreiding vindt plaats door een combinatie van horizontale overdracht van plasmiden en klonale verspreiding van bacteriën die deze genen dragen.

De aanwezigheid van ESBL/pAmpC in *Escherichia coli* en *Salmonella enterica* afkomstig van pluimvee en pluimveeproducten is uitgebreid beschreven. De piramidale structuur van de pluimveeketen bevordert zowel verticale als horizontale overdracht van ESBL/pAmpC determinanten (d.w.z. genen, plasmiden en stammen) op verschillende niveaus binnen de pluimveeketen. Pluimvee wordt gezien als een reservoir van ESBL/pAmpC-producerende *E. coli* en *Salmonella* die infecties bij de mens kunnen veroorzaken.

Moleculair-epidemiologische studies van resistentie tegen ESC (ESC-R), zijn gebaseerd op de genetische karakterisering van ESBL/pAmpC-genvarianten, alsmede van de ESBL/pAmpC-dragende plasmiden en bacteriestammen. Eerdere studies hebben de overdracht beschreven van resistente bacteriën tussen pluimvee en mensen, waarbij beiden identieke genetische informatie bevatten op zowel gen, plasmide als chromosomaal DNA niveau (klonale verspreiding), terwijl ook overdracht van verwante plasmiden in verschillende lijnen van *E. coli* tussen pluimvee en mensen zijn beschreven, waar horizontale gen overdracht aan ten grond-

slag ligt. Het gebruik van Whole Genome Sequencing (WGS) waarmee ESBL/pAmpC-genen, plasmiden en bacteriën met een hoge resolutie onderscheiden kunnen worden, laat zien dat vooral horizontale overdracht van gerelateerde plasmiden tussen verschillende *E. coli lineages* bepalend is voor de overdracht van ESBLs/pAmpCs tussen mens en pluimvee. Kwantitatieve gegevens van deze overdracht ontbreken echter nog. Er wordt verondersteld dat de factoren die van invloed zijn op de epidemiologie van ESBL/pAmpC bij mens en dier variëren, afhankelijk de geografische locatie.

Er zijn nauwelijks gegevens van ESBL/pAmpC-producerende *E. coli* en *Salmonella* uit pluimvee in Latijns-Amerika. De beschikbare studies verschillen sterk qua methodologie van bemonstering en de genetische karakterisering van ESC-R. Deze verschillen bemoeilijken vergelijkingen tussen landen in de regio. De WHO heeft recent het *Global Action Plan on antimicrobial resistance* gepubliceerd. Hierin pleit de WHO tot het geharmoniseerd en geïntegreerd verzamelen van gegevens over gebruik van antimicrobiële middelen (AMU) en AMR vanuit de humane geneeskunde en diergeneeskunde, landbouw en milieu. Met steun vanuit de WHO-adviesgroep AGISAR (*WHO-Advisory Group on integrated surveillance of antimicrobial resistance*) werd een initiatief voor geïntegreerde monitoring van AMU en AMR gestart in de Colombiaanse pluimveeproductie.

Doel van het onderzoek

De doelstellingen van dit proefschrift waren i) het beschrijven van een geïntegreerd programma voor AMR-monitoring in Colombia; ii) het karakteriseren van ESBL/pAmpC –producerende *E. coli* en *S. enterica* uit pluimvee; iii) het verkennen van de impact van pluimveehouderij en handel van pluimvee en pluimveeproducten op de verspreiding van ESBL/pAmpC en na te gaan of er mogelijkheden zijn om de detectie van AMR te verbeteren.

Resultaten

De beschrijving van het opzetten van een geïntegreerd monitoringssysteem in Colombia met betrokkenheid van stakeholders wordt gegeven in **Hoofdstuk 2**. De betrokkenen voor het opzetten van het systeem waren afkomstig van de publieke en private sector: de nationale volksgezondheid-, landbouw- en voedselautoriteiten, onderzoeksinstituten, academische groepen, de voedingsindustrie en retailers. Methoden voor bemonstering, microbiologische analyse en AMR-bepaling werden overgenomen, en waar nodig aangepast, van reeds lopende monitoringsprogramma's in andere landen. Betrokken experts uit die landen vormden het internationale adviesteam. Dit resulteerde in een pilot-monitoring met ondersteuning vanuit de pluimveeindustrie. Gegevens van AMR van de voedselpathogenen *Salmonella* en *Campylobacter* spp. en commensale *E. coli* en *Enterococcus* spp.

werden in deze pilot verzameld. Ook werden gegevens over geïmporteerde antimicrobiële middelen voor gebruik bij dieren verzameld. Resistentie tegen derde generatie cefalosporinen bleek opmerkelijk vaak voor de te komen in *Salmonella* en *E. coli* uit pluimvee in Colombia **(Hoofdstuk 2)**.

ESC-R van *Salmonella* en *E. coli* werd met verschillende methoden gekarakteriseerd: PCR-detectie van ESBL/pAmpC-genen en van plasmiden, gevolgd door sequentie-analyse van genen, plasmiden en stammen. Typering van plasmiden en *E. coli* stammen vond plaats met Multi Locus Sequence Typing (MLST) **(Hoofdstuk 3)**. Voor *Salmonella* werd PCR-detectie van ESBL/pAmpC-genen gevolgd door WGS en *in silico*-karakterisering van AMR-genen, plasmiden en stammen **(Hoofdstuk 4)**. Uiteindelijk werd WGS uitgevoerd om gedetailleerde fylogenetische en evolutionaire karakterisering van plasmiden van *E. coli* en *Salmonella* en van *Salmonella* isolaten uit te voeren **(Hoofdstuk 5 en 6)**. Er werd een grote overeenkomst van ESBL/pAmpC-genen en dragende plasmiden gevonden binnen *E. coli* **(Hoofdstuk 3)** en *Salmonella* **(Hoofdstuk 4)** uit Colombia. Sommige gen-plasmide-combinaties kwamen zowel in *E. coli* als in *Salmonella* voor (bijv. bla_{CMY-2}-IncI1 en bla_{SHV-12}-IncI1) **(Hoofdstuk 5)**, sommige bleven beperkt tot *E. coli* (bijv. $bla_{CTX-M-2}$-IncHI2 en bla_{CMY-2}-IncK) en sommige beperkt tot *Salmonella* (bijv. $bla_{CTX-M-165}$-IncQ1). De combinatie van bla_{CMY-2} en IncII/Sequence Type (ST)12 plasmiden was de meest voorkomende en er werd een sterke genetische verwantschap gevonden tussen deze plasmiden, zowel binnen als tussen *E. coli* en *Salmonella* uit Colombia en uit andere landen **(Hoofdstuk 5)**. Er waren echter duidelijke verschillen in patronen van *E. coli* and *Salmonella*. ESBL/pAmpC-genen werden gevonden in verschillende *E. coli* ST's, wat duidt op de verspreiding van ESC-R door horizontale overdracht van plasmiden **(Hoofdstuk 3)**. In *Salmonella* kwamen de genen voor in dezelfde serovar-ST's van *Salmonella* stammen, wat wijst op klonale spreiding van de resistentie in *Salmonella* **(Hoofdstuk 4)**. Er werd een duidelijke geografische clustering binnen de meest voorkomende *Salmonella*-serovar-ST's in Colombia, *S.* Paratyphi B variant Java (hier aangeduid als *S.* Java) ST28 en *S.* Heidelberg ST15 waargenomen **(Hoofdstuk 4)**. Wanneer *S.* Java ST28-genomen uit andere Latijns-Amerikaanse landen toegevoegd werden in de analyse bleek er een grote genetische verwantschap te zijn tussen stammen afkomstig van verschillende Latijns-Amerikaanse landen, wat wijst op de circulatie van een geconserveerde kloon van *S.* Java ST28 in de regio **(Hoofdstuk 6)**. Met behulp van temporale fylogenetische analyse bleek dat in Europa rond 1995 een Europese *lineage* van *S.* Java opkwam, en in Latijns-Amerika rond 2005 **(Hoofdstuk 6)**.

Conclusies

In *E. coli* uit pluimvee in Colombia en andere landen wordt de verspreiding van ESBL/pAmpC voornamelijk gemedieerd door horizontale overdracht van plasmiden. In *S. enterica* uit Colombia vindt verspreiding voornamelijk plaats door een combinatie van horizontale overdracht van genen en plasmiden en klonale verspreiding van genen, plasmiden en stammen.

De verspreiding binnen en tussen landen van pluimveegerelateerde ESBL/pAmpC is het gevolg van de gangbare praktijken van pluimveehouderij en handel. Kwantitatieve gegevens over het voorkomen van pluimvee-geassocieerde infecties met ESC-R *E. coli* bij de mens ontbreken in veel landen en regio's. Voor wat betreft de karakterisering wordt geadviseerd dit in de toekomst te baseren op WGS, waarbij genen, plasmiden en bacteriestammen goed vergeleken kunnen worden.

Als landen de roep en noodzaak voor interventies volgen, kan het succes van die interventies alleen maar gemeten worden als er een geïntegreerd monitoringssysteem geïmplementeerd is in de betreffende Latijns-Amerikaanse landen. De gegevensverzameling moet geharmoniseerd plaatsvinden zodat deze geschikt is voor internationale vergelijkingen.

Resumen en español

Marco teórico

La terapia antimicrobiana depende en gran medida en la eficacia de los antibióticos β-lactámicos. Las principales familias de estos antibióticos son las penicilinas, cefalosporinas, antibióticos carpabenémicos y monobactámicos. Desde su introducción en la medicina humana y veterinaria se ha descrito resistencia para cada una de estas familias en bacterias. Entre los antibióticos β-lactámicos, las cefalosporinas de tercera y cuarta generación están clasificadas como antimicrobianos de importancia crítica con la mayor prioridad para la salud humana por la Organización Mundial de la Salud (OMS). Después del aumento en los niveles de resistencia en enterobacterias de origen humano y animal, el uso de cefalosporinas de tercera y cuarta generación fue restringido en animales destinados para el consumo en algunos países europeos. De manera similar, algunos usos afuera de las indicaciones prescritas para las cefalosporinas de tercera generación fueron restringidos en los EE. UU.

La degradación enzimática, es el principal mecanismo de resistencia a las cefalosporinas de tercera y cuarta generación, también denominadas Cefalosporinas de Espectro Extendido (CES). En especies de Enterobacteriaceae, las enzimas predominantes son las diferentes β-lactamasas de Espectro Extendido (BLEE) y β-lactamasas AmpC mediadas por plásmidos (pAmpC). En términos de especificidad de sustrato, las BLEE son activas contra casi todas las penicilinas, cefalosporinas (excepto las cefamicinas) y antibióticos monobactámicos, y pueden ser inhibidas por el ácido clavulánico. Las pAmpC tienen los mismos sustratos que las BLEE, excluyendo las cefalosporinas de cuarta generación. A su vez, las pAmpC son activas contra las cefamicinas como por ejemplo la cefoxitina. Las BLEE y pAmpC están codificadas principalmente en plásmidos. Su diseminación ocurre a través de la combinación de transferencia horizontal de plásmidos y diseminación clonal de bacterias portadores de estos genes.

En aves de corral y productos cárnicos avícolas, la presencia de BLEE/pAmpC en aislamientos de *Escherichia coli* y *Salmonella enterica* ha sido reportada ampliamente. La estructura piramidal de la cadena de producción avícola favorece la transmisión vertical y horizontal de los determinantes genéticos de BLEE/pAmpC (es decir, genes, plásmidos y aislamientos/cepas) a diferentes niveles de producción. Las aves de corral han sido propuestas como un reservorio de *E. coli* y *Salmonella* productoras de BLEE/pAmpC que causan infecciones en los seres humanos.

Los estudios epidemiológicos de los determinantes genéticos que confieren Resistencia a las CES (R-CES) se basan en la caracterización de las secuencias de las variantes de genes de BLEE/pAmpC, y de los plásmidos y aislamientos de bacterias portadores de estos genes. Estudios previos, han descrito la transferencia de dichos determinantes entre aves de corral y humanos a través de

secuencias idénticas de genes, plásmidos y aislamientos (diseminación clonal), mientras que otros han descrito la transferencia horizontal de plásmidos con una composición genética muy similar entre diferentes linajes de *E. coli*. Con la llegada de la secuenciación del genoma completo ó *Whole Genome Sequencing* (WGS) y la posibilidad de diferenciar con una alta resolución los linajes de plásmidos y bacterias portadoras de BLEE/pAmpC, se ha hecho evidente que la transferencia horizontal de plásmidos es el principal mecanismo para la transferencia de BLEE/pAmpC entre humanos y aves de corral en aislamientos de *E. coli*. Sin embargo, al día de hoy, no se disponen suficientes datos sobre las medidas cuantitativas de esta posible transferencia.

En general, los factores que influyen sobre la epidemiología de BLEE/pAmpC en humanos y animales varían según el área geográfica de estudio. Los datos de *E. coli* y *Salmonella* que producen BLEE/pAmpC en aves de corral son en gran medida escasos en América Latina. Los estudios disponibles se caracterizan por diferentes metodologías de muestreo y caracterización de los determinantes genéticos de la R-CES. Estas diferencias dificultan las comparaciones entre los países de la región. Recientemente, la OMS ha realizado un llamado a la acción contra la Resistencia a los Antimicrobianos (RAM). El Plan de Acción Global (PAG) de la OMS contra la RAM aboga firmemente por el establecimiento de iniciativas de vigilancia armonizadas que integren datos sobre el uso de antimicrobianos (UAM) y RAM en los sectores de medicina humana y veterinaria, agricultura y medio ambiente. Con el apoyo de los miembros del Grupo Asesor para la Vigilancia Integrada de la RAM (AGISAR) de la OMS, se llevo a cabo una iniciativa para la vigilancia integrada del UAM y la RAM en la producción avícola colombiana. Previo a esta iniciativa, datos públicos sobre el estado de UAM y RAM en el sector agropecuario eran ausentes en el país.

Objetivos de la investigación

Los objetivos de la presente tesis fueron: i) describir una iniciativa para desarrollar un programa integrado de vigilancia de la RAM en Colombia; ii) proporcionar una caracterización detallada de los determinantes genéticos de BLEE/pAmpC en aislamientos de *E. coli* y *S. enterica* en aves de corral y productos cárnicos avícolas; iii) reflexionar sobre el impacto de la avicultura y el comercio de aves en la diseminación de BLEE/pAmpC y discutir oportunidades para mejorar la detección de la RAM.

Resultados

La descripción de un consorcio inicial de las partes interesadas en la iniciativa de un sistema de vigilancia integrado de la RAM en Colombia ha sido provista **(Capítulo 2)**. Este consorcio estuvo formado por representantes de los sectores público y privado e incluyó participantes de las autoridades de salud pública, agricultura y

alimentos, grupos académicos y de investigación, la industria alimentaria y principales cadenas de supermercados. Las metodologías de muestreo, microbiología y caracterización de la RAM se adaptaron de sistemas de vigilancia desarrollados en otros países. Representantes de sistemas de vigilancia de diferentes países hicieron parte del equipo asesor internacional del programa piloto de vigilancia integrada en Colombia. Como resultado, se adoptó un piloto de vigilancia de la RAM en la cadena de producción avícola con el apoyo de la industria en diferentes áreas geográficas. Datos de línea base de RAM en aislamientos de *Salmonella*, *Campylobacter* spp., *E. coli* y *Enterococcus* spp. fueron provistos. Asimismo, se proporcionaron datos sobre el UAM usando como referencia las cantidades de antimicrobianos importados para su uso en animales. También se encontró que los niveles de resistencia a las cefalosporinas de tercera generación fueron notablemente altos en *Salmonella* y *E. coli* **(Capítulo 2)**.

La caracterización de la R-CES en *Salmonella* y *E. coli* se realizó utilizando diferentes metodologías. Estas metodologías incluyeron: detección por PCR de los genes de BLEE/pAmpC y subtipos de plásmidos, seguidos por la caracterización basada en las secuencias de los genes, plásmidos y aislamientos utilizando *Multi-Locus Sequence Typing* (MLST) en *E. coli* **(Capítulo 3)**; Detección por PCR de genes BLEE/pAmpC seguido de WGS y caracterización *in silico* de genes, plásmidos y aislamientos de *Salmonella* **(Capítulo 4)**; WGS como paso inicial para la caracterización filogenética y evolutiva de plásmidos provenientes de *E. coli* y *Salmonella* y de diferentes linajes de *Salmonella* **(Capítulos 5 y 6)**. Como resultado, se observó un alto nivel de similitud entre los genes BLEE/pAmpC y los plásmidos portadores de estos genes en *E. coli* **(Capítulo 3)** y *Salmonella* **(Capítulo 4)** en el país. Algunas combinaciones de genes y plásmidos fueron comunes en *E. coli* y *Salmonella* (por ejemplo, bla_{CMY-2}-IncI1 y bla_{SHV-12}-IncI1) **(Capítulo 5)**, algunas solo se encontraron en *E. coli* (por ejemplo, $bla_{CTX-M-2}$-IncHI2 y bla_{CMY-2}-IncK) y algunos solo en *Salmonella* (por ejemplo, $bla_{CTX-M-165}$-IncQ1). En general, la combinación del gene bla_{CMY-2} y los plásmidos IncI1/*Sequence Type* (ST) 12 fue la más prevalente. Además, se encontró una alta similitud genómica entre estos plásmidos en aislamientos de *E. coli* y *Salmonella* de Colombia y de otros países **(Capítulo 5)**. Sin embargo, se observaron diferentes patrones de diseminación entre las dos especies bacterianas. Genes idénticos de BLEE/pAmpC fueron observados en plásmidos con alta similitud genética en STs de *E. coli* heterogéneos, lo que indica diseminación de la R-CES a través de la transferencia horizontal de plásmidos **(Capítulo 3)**. Por el contrario, en aislamientos de *Salmonella*, genes idénticos fueron observados en serovares-STs más homogéneos, lo que indica una diseminación clonal de la resistencia **(Capítulo 4)**. Adicionalmente, se observó una marcada agrupación filogenética en los serovares-ST de *Salmonella* más prevalentes en Colombia, en este caso *S.* Paratyphi B variante Java (aquí referida como *S.* Java) ST28 y *S.* Heidelberg ST15, en comparación con aislamientos de otros países **(Capítulo 4)**. El análisis posterior de los genomas de *S.* Java ST28, mostró un alto nivel de similitud genómica en países de América Latina, lo que indica la circulación de un clon conservado

de *S.* Java ST28 en la región **(Capítulo 6)**. Utilizando un análisis filogenético con inferencia bayesiana, se encontró que un linaje europeo de *S.* Java ST28 emergió en Europa alrededor del año 1995, y un linaje latinoamericano alrededor de 2005 **(Capítulo 6)**.

Conclusiones

En aves de corral en Colombia y otros países, la diseminación de BLEE/pAmpC en *E. coli* es principalmente mediada por la transferencia horizontal de genes y plásmidos. En *S. enterica* en Colombia, la diseminación ocurre a través de una combinación de transferencia horizontal de genes y plásmidos y la diseminación clonal de genes, plasmidos y aislamientos con una alta similitud genética. Adicionalmente, la prevalencia de los genes BLEE/pAmpC parece estar asociada a una multiplicidad de factores específicos para cada país.

La diseminación en aves de corral de BLEE/pAmpC a nivel nacional e internacional puede ocurrir debido a prácticas comunes de avicultura y comercio de aves. En la mayoría de países hacen falta medidas cuantitativas sobre el impacto en la salud pública de las infecciones asociadas a las aves de corral y el consumo de productos cárnicos avícolas. En el futuro, el análisis del impacto de BLEE/pAmpC en la salud pública debe incluir comparaciones de genes, plásmidos y aislamientos bacterianos basadas en WGS.

Es altamente recomendado seguir el llamado a la acción presentado por la OMS en su PAG contra la RAM. Para medir el éxito de las intervenciones descritas en el PAG, es indispensable implementar sistemas de vigilancia integrados a escala nacional en los países de América Latina antes de que tenga lugar cualquier intervención. Los datos recopilados en estos sistemas deben ser concebidos aptos para comparaciones internacionales.

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Dear reader, the output of this thesis is the result of the efforts and companionship of different persons. This part of the thesis is my opportunity to recognize their valuable contribution to this work and my personal development.

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With these words, a very important chapter of my life ends. Thank you all for being part of it.

Best wishes from my heart,

Ricardo
June 2019, Utrecht

Curriculum Vitae

Luis Ricardo Castellanos Tang was born on the 23rd of May 1987 in Barrancabermeja, Colombia. He graduated from high school in 2003 at the Liceo de Cervantes el Retiro from Bogotá. From 2004 until 2009 he studied Microbiology at the Universidad de Los Andes in Bogotá. In 2009 he conducted a six-month internship at the Food Microbial Ecology Laboratory (LEMA) of the same university, supervised by MSc. Maria Consuelo Vanegas. Among his responsibilities at LEMA was the isolation of *Salmonella enterica*, *Escherichia coli* and *Enterococcus* spp. from poultry meat for the pilot phase of a Colombian Integrated Program for Antimicrobial Resistance Surveillance (COIPARS), led by Dr. Pilar Donado from AGROSAVIA (formally CORPOICA). Luis Ricardo graduated as a microbiologist in September 2009. Following a strong interest in food processing, he completed the Technology in Gastronomy program between 2010 and 2011 at the Servicio Nacional de Aprendizaje (SENA), Bogotá. In late 2011, Luis Ricardo joined AGROSAVIA as a professional research assistant. Together with his colleagues from COIPARS, he worked on projects to assess the levels of antimicrobial resistance in *S. enterica*, *E. coli*, *Enterococcus* spp. and *Campylobacter* spp. in the Colombian poultry chain. In September 2013, he helped co-organize a meeting of the Advisory Group on Integrated Surveillance of Antimicrobial Resistance for WHO (AGISAR) in Bogotá. Soon after this conference, Luis Ricardo travelled to France to study French at L'Université de la Rochelle. Once in Europe, he had the opportunity to visit Utrecht in January 2014 for a two-week internship at the laboratory of Prof. Jaap Wagenaar at Utrecht University. Later in 2014, a PhD research project for the molecular characterization of antimicrobial resistance in isolates originating from the baseline studies of COIPARS was conceived. During his project, he was supervised by Prof. Wagenaar, Prof. Dik Mevius, Dr. Joost Hordijk and Dr. Aldert Zomer. The results from his PhD project are presented in this thesis.

List of publications

Related to this thesis:

Castellanos LR, Van der Graaf-van Bloois L, Donado-Godoy P, Mevius DJ, Wagenaar JA, Hordijk J. and Zomer A. Phylogenomic Investigation of IncI1-Iγ Plasmids Harboring bla_{CMY-2} and bla_{SHV-12} in *Salmonella enterica* and *Escherichia coli* in Multiple Countries. Antimicrobial Agents and Chemotherapy 2019. doi:10.1128/AAC.02546-18.

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Oral presentations in scientific meetings

Castellanos LR, Donado-Godoy P, Wagenaar JA, Hordijk J. Genetics and Spread of Resistance to Third Generation Cephalosporins in *Salmonella enterica*. Seminar on Bacterial Antimicrobial Resistance and Antimicrobial Use in Animals. AGROSAVIA-Universidad de los Andes, 3 September 2018. Bogotá, Colombia. *Speaker and member of the organizing committee*.

Castellanos LR, Donado-Godoy P, Wagenaar JA, Hordijk J. Genetics and the Spread of Resistance to Cephalosporins in *E. coli*. APEC-Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile. Workshop: Coordinated Research Initiative for the Implementation of Antimicrobial Resistance Control Strategies, 28-30 October 2015. Santiago, Chile. *Invited oral presentation.*

Castellanos LR, Donado-Godoy P, León M, Clavijo V, Arevalo A, Bernal J, Timmerman AJ, Mevius DJ, Wagenaar JA, Hordijk J. Characterization of ESBL/AmpC-producing *Escherichia coli* from the Colombian Poultry Chain: A Descriptive Study of Gene Variants and Strains. 4th ASM Conference on Antimicrobial Resistance in Zoonotic Bacteria and Foodborne Pathogens, 8–11 May 2015. Washington DC, USA. *Oral presentation, ASM student travel grant awarded.*