DOCTORAL THESIS

ZOONOTIC POTENTIAL OF *ESCHERICHIA COLI* AND OTHER ENTEROBACTERIACEAE ISOLATED FROM POULTRY MEAT: STUDY OF ANTIBIOTIC RESISTANCES AND DEFINITION OF CLONAL GROUPS PATHOGENIC FOR HUMANS

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ZOONOTIC POTENTIAL OF *ESCHERICHIA COLI* AND OTHER ENTEROBACTERIACEAE ISOLATED FROM POULTRY MEAT: STUDY OF ANTIBIOTIC RESISTANCES AND DEFINITION OF CLONAL GROUPS PATHOGENIC FOR HUMANS

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LIST OF PUBLICATIONS AND QUALITY INDEXES

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The present doctoral thesis includes three studies:

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SUMMARY

COVID-19 has been threatening the world for almost two years now. Fortunately, the care of many researchers has allowed the development of precise combat weapons in the form of vaccines in record time. But this pandemic will leave us many absences, and many consequences, such as those derived from the temporary eclipse of the greatest health challenge: the antimicrobial resistances (AMR). The increase of multidrug-resistant (MDR) bacteria to last-resorts antibiotics (i.e. to colistin, carbapenems, cephalosporins) is one of the most serious public health problems worldwide due to the lack of options for an adequate treatment, the increase of mortality rates and health costs. According to the European Centre for Disease Prevention and Control (ECDC), more than 670.000 bacterial infections can be attributed to MDR bacteria, which causes 33.000 deaths annually in Europe.

Considering the risk associated with the antimicrobial use in animals and potential impact on humans, the European Medicines Agency (EMA) has recently proposed a new categorization, including in Category A ("Avoid") those antibiotics not currently authorized in veterinary medicine in the EU, such as fosfomycin or monobactams; and Category B ("Restrict") for those antimicrobials that should be restricted in animals to mitigate the risk to public health, namely, quinolones, 3rd- and 4th-generation cephalosporins and polymyxins. Therefore, this is a critical moment when the reduction of antibiotic pressure by different approaches, makes necessary to track bacterial evolution in order to design new strategies.

Escherichia coli is part of the commensal microbiota of the digestive system in warm-blood vertebrates that can play diverse roles depending on its virulence profile. While intestinal pathogenic *E. coli* (InPEC) are accurately distinguished from the commensal gut microbiota based on certain virulence factors, this is not as simple with the extraintestinal pathogenic *E. coli* (ExPEC) since they behave as opportunistic pathogens that can colonize the intestinal environment without causing harm to the host. Thus, no set of genes can unequivocally define ExPEC strains or the different categories. So far, they have been categorized due to their isolation from infections located outside of the digestive system, and / or based on the presence of genes statistically associated with the extraintestinal pathogenic potential of the strains, which can be used predictively. Besides, certain extraintestinal lineages of *E. coli*, such as the pandemic ST131, have been worldwide recognized by their implication in human infections, and also, by their role in the spreading of antibiotic resistances. The hypothesis that food, particularly poultry products, can act as a reservoir for human extraintestinal pathogens like *E. coli* and other Enterobacteriaceae in humans is based on scientific evidence. Certain strains that cause avian pathology (avian pathogenic *E. coli*, APEC) show a high genetic similarity to those that cause extraintestinal pathology in humans, so several studies report that some human ExPEC strains have evolved from or are common to APEC strains. The evidence suggesting this hypothesis are, among others: A) The geographical and temporal grouping of ExPEC strains isolated from patients with extraintestinal infections, suggesting the appearance of an outbreak or a common source of exposure. B) The global distribution of lineages of identical ExPEC strains, which indicate the global spread of contamination carried through food. C) The detection of identical genotypes of ExPEC isolated from human infections, as well as from food products when strains from the same geographic area were analyzed. D) The disproportionate representation of pandemic or international ExPEC lineages among the hundreds of ST causing

extraintestinal infections in all regions of the world, indicating a greater biological or fitness advantage in different reservoirs, production animals or non-production animals (birds wild). E) The relatively recent appearance of the ST69, ST131 and ST393 genotypes as ExPEC, suggesting the recent introduction of these genotypes into the human intestinal niche from external sources.

Due to the high plasticity of the *E. coli* genome hybrid pathotypes are frequent and unpredictably emerging due to the important role played by different mobile genetic elements (MGEs) such as plasmids, bacteriophages, pathogenicity islands, transposons and insertion sequence elements, in the evolution of the bacteria. Furthermore, strains with complex hybrid pathotypes with combinations of two different groups of intestinal pathogenic *E. coli* (InPEC) (Shiga toxin-producing *E. coli;* STEC + enteroaggregative *E. coli*; EAEC) or InPEC and ExPEC (for example, atypical enteropathogenic *E. coli*; aEPEC + ExPEC and STEC + APEC) are increasingly reported in human clinical cases. Since 2011, when a novel Shiga-toxin-producing *E. coli* belonging to serotype O104:H4, with virulence features common to the EAEC and CTX-M-15 producer was identified as the one involved in the large German outbreak the concept of pathotype has been questioned and currently, classical and new approaches (WGS), are being used to enhance the understanding of the evolution of this highly adaptable species.

The use of antibiotic therapy in food production animals has been accepted as the main cause of the AMR increase, including resistance to colistin. A rapid spread of extended-spectrum β-lactamases (ESBL) has occurred in the last decades, mainly due to their presence in plasmids and expansion through successful clonal groups, such as the pandemic ST131 of *E. coli*. Presently, there is great concern about the *in vivo* acquisition of *mcr*- and bla_{ESBL}-bearing plasmids by human *E. coli* isolates following treatment with colistin, or via animal transmission through direct contact or the food chain. ST131 is the main pandemic clone responsible for the global spread of ESBLs. First identified in 2008, ST131 strains belong to phylogroup B2 and mainly to the serotypes O25b:H4 or, less frequently, O16:H5. Three years after its first isolation, it was already spread, being the bacterial agent involved in more than 50% of cases of UTIs caused by ESBL-producing strains in numerous hospitals in different countries. The prevalence of resistance to first-line oral antibiotics such as trimethoprim-sulfamethoxazole, amoxicillin, and amoxicillin-clavulanate has been steadily increasing during these years, making the treatment of infections very difficult and endangering the lives of patients. Although it is associated with ExPEC infections such as UTI, septicemia, surgical wound infections and meningitis, this clone is also frequently isolated from the digestive tract of healthy humans. That is why, the human intestinal tract was though the main reservoir of ST131. However, the growing scientific community interest towards this ST, found it within diverse sources such as companion, food-production and wild animals, rivers, sewage, even in the Antarctic region. The clades A and C of ST131 are mainly associated with human pathology, while the clade B is determined in strains isolated from different niches such as poultry and pigs, along with humans. An important challenge is to know which determinants make certain clones adapt to a specific host meanwhile others can be transmitted between different species, with jumps as important as between mammals and birds. In the case of ST131, this relationship between the different clades and their presence in different hosts has not be completed understood yet.

The present doctoral thesis comprises three studies, “Chicken and turkey meat: Consumer exposure to multidrug-resistant Enterobacteriaceae including *mcr*-carriers, uropathogenic *E. coli* and high-risk lineages such as ST131” (Díaz-Jiménez et al., 2020a), “Microbiological risk

assessment of turkey and chicken meat for consumer: Significant differences regarding multidrug resistance, *mcr* or presence of hybrid aEPEC/ExPEC pathotypes of *E. coli"* (Díaz-Jiménez et al., 2021) and "Genomic Characterization of *Escherichia coli* Isolates Belonging to a New Hybrid aEPEC/ExPEC Pathotype O153:H10-A-ST10 *eae*-beta1 Occurred in Meat, Poultry, Wildlife and Human Diarrheagenic Samples" (Díaz-Jiménez et al., 2020b).

The aim of the present doctoral thesis, developed in the frame of two national projects (PN AGL2016-79343-R and PID2019-104439RB-C21/AEI/10.13039/501100011033), were to analyse the zoonotic potential of Enterobacteriaceae isolated from poultry, with the characterization of antibiotic resistances and definition of clonal groups pathogenic for humans. Thus, we evaluated the consumer exposure to Enterobacteriaceae with capacity to develop problematic extraintestinal infections, either by their virulence and / or resistance traits, via chicken and turkey meat. The hypothesis of the present thesis was that poultry meat would act as a reservoir, and potentially transmitter, of pathogenic strains that might be implicated in human UTI. To demonstrate this hypothesis, the strategy was to analyze retail poultry meat directly acquired at points of sale with the idea that the final product provides data on what is happening on the farm, at the slaughterhouse, and what goes into the consumer's kitchen. The second strategy was to identify potential uropathogenic clonal groups of *E. coli* based on specific genetic markers. Finally, we considered "high-risk" strain that with the capacity to develop a serious extraintestinal infection in humans, due to either its virulence potential and / or its antibiotic resistance.

The specific goals of the present doctoral thesis were first to design an efficient protocol for the recovery of food-borne *E. coli* and other pathogenic and / or antimicrobial-resistant Enterobacteriaceae. The second objective was to acquire knowledge on the current situation regarding AMR in poultry farming, paying special attention to antimicrobial categories A and B of EMA. We also aimed to assess the consumer exposure, via poultry meat, to high-risk *E. coli* and other Enterobacteriaceae isolates with potential to develop severe infections by either bacterial virulence and / or antibiotic resistance traits. Finally, we aimed to explore the food transmission route of specific *E. coli* clones of human and animal origin through comparative genetic and genomic analysis.

We randomly sampled 100 retail fresh meat products (50 of chicken and 50 of turkey) in six Spanish supermarket chains and local butcher located in Lugo (northwest Spain). By conventional culture, 358 different Enterobacteriaceae isolates were recovered (170 isolates recovered from chicken samples and 188 isolates recovered from turkey samples) using MacConkey Lactose, MacConkey Sorbitol with tellurite and cefixime, CHROMID® ESBL and CHROMID®CARBA SMART. Bacterial identification revealed that 323 out of 358 isolates were *E. coli*, 28 *K. pneumoniae*, six *Serratia fonticola* and one *Enterobacter cloacae*. This collection was fully characterized including: phylogroup, serotype, ST and clonal complex, clonotype, virulence and resistance profile.

A second collection was obtained during the period of 2005 to 2015 from different surveillance studies performed at LREC, in Lugo, Spain, which aimed the detection of ESBL-producing *E. coli*. These studies included samples from chicken, beef and pork meat, as well as poultry farm environment and wildlife. Those isolates conforming the aEPEC pathotype of serotype O153 were further characterized.

In our first study we evaluated the consumer exposure via poultry meat to Enterobacteriaceae with capacity to develop severe extraintestinal infections by either bacterial virulence and / or antibiotic resistance traits. The characterization of 256 isolates (84 representative *E. coli* isolates, 137 ESBL-producing *E. coli* isolates, 28 ESBL-producing *Klebsiella pneumoniae* isolates, six ESBL-producing *Serratia fonticola* isolates and one ESBL-producing *Enterobacter cloacae* isolate) and the assessment of five parameters showed that 96 out of 100 poultry meat samples acquired in supermarkets of the northwest of Spain posed ≥ one potential risk. Specifically, i) 96% of the samples carried Enterobacteriaceae resistant to antimicrobials of categories A (64% with resistance to monobactams) or B (95% with resistance to cephalosporins of 3rd- and 4rd- generation, quinolones and / or polymyxins) of the new categorization of EMA. ii) More than one ESBL-producing Enterobacteriaceae species were recovered from 29% of samples, mostly *E. coli* and *K. pneumoniae*. iii) Characterization of *E. coli* isolates showed that extraintestinal and / or uropathogenic high-risk clonal groups (ST10, ST23, ST38, ST48, ST58, ST69, ST88, ST93, ST95, ST101, ST115, ST117, ST131, ST141, ST167, ST350, ST345, ST354, ST359, ST410, ST602, ST617, ST641, ST906, ST1485) were present in 62% samples. iv) *E. coli* isolates recovered from 25% samples conformed the ExPEC status v) *E. coli* isolates recovered from 17% samples conformed UPEC status. Regarding *K. pneumoniae*, at least eight of the 11 STs identified in our collection have been reported within human clinic isolates; specifically: ST15, ST45, ST111, ST147, ST307, ST627, ST966 and ST1086 (22 of the 28 *K. pneumoniae* belonged to these eight STs). The plasmid-mediated colistin resistance *mcr-1* gene was determined in 13 *E. coli* isolates from seven meat samples, however, the eleven *K. pneumoniae* phenotypically resistant to colistin were negative by PCR for the presence of *mcr-1* to *mcr-8* genes, probably indicating chromosomic-encoding resistance.

In our second study, we assessed the risk for consumers attending only to *E. coli* isolates, we proposed a laboratory workflow based on six virulence and / or antimicrobial resistance traits and included the development of a duplex PCR for the screening of ExPEC isolates. We characterized 323 isolates recovered from 100 poultry meat samples. This characterization revealed that poultry meat is a rich phylogenetic source of *E. coli* phylogroups (A to G) and *Escherichia* clade I. Non-susceptible *E. coli* isolates to monobactams, 3rd-generation cephalosporins and / or fluoroquinolones, were present in 71% of the samples. Besides, 47% carried ≥2 different *E. coli* positive for ESBL, pAmpC or mcr genes. Isolates from 78% of the poultry meat exhibited ExPEC status, and 53% were carriers of isolates positive for the UPEC status. The STs identified in 86% of the samples belonged to the so-called ExPEC high-risk lineages, being 73% carriers of clonal groups identified in human infections of the same Health Area. Moreover, different human-associated clones co-occurred in same meat sample: ST131-B2 (CH40-22), ST648-F (CH4-58), ST93-A (CH11-neg) or ST95-B2 (CH38-27), ST354-F (CH88-58), ST155-B1 (CH4-neg). Globally, 84% of the meat samples posed ≥ 3 risks factors, including resistance genes, successful clones and virulence traits. Turkey meat showed significant higher rates concerning *mcr*-carriage or MDR; while the ExPEC status rate, or the presence of hybrid pathotypes such as the aEPEC/ExPEC O153:H10-A-ST10 (CH11-54), were associated with chicken origin ($P < 0.05$).

In our third study we took as start point the different surveillance studies (2005–2015) in northwest Spain that revealed the presence of *eae*-positive isolates of *E. coli* O153:H10 in meat for human consumption, poultry farm, wildlife and human diarrheagenic samples. The aim of this study was to explore the genetic and genomic relatedness between human and animal/meat

isolates, as well as the mechanism of its persistence. We also wanted to know whether it was a geographically restricted lineage, or whether it was also reported elsewhere. Conventional typing showed that 32 isolates were O153:H10-A-ST10 *fimH*54, *fimAv*MT78, *traT* and *eae*-beta1. Amongst these, 21 were CTX-M-32 or SHV-12 producers. The PFGE XbaI - macrorestriction comparison showed high similarity (>85%) between the isolates of the collection. The plasmidome analysis revealed a stable combination of IncF (F2:A-:B-), IncI1 (ST unknown) and IncX1 plasmid types, together with non-conjugative Col-like plasmids. The core genome investigation based on the core genome multilocus sequence typing (cgMLST) scheme from EnteroBase proved close relatedness between isolates of human and animal origin.

From our results we concluded that poultry meat microbiota is a source of genetically diverse Enterobacteriaceae, resistant to relevant antimicrobials (categories A and B of EMA) and potentially pathogenic for humans, including hybrid pathotypes of *E. coli*, high-risk clonal groups of *E. coli* associated with human extraintestinal and / or uropathogenic pathologies, as well as *K. pneumoniae* clonal groups of clinical interest. Our results would indicate that the industrial production system for turkey meat seems to exert greater selection pressure of antibiotic resistant strains compared to chicken, which is reflected in significant higher rates of *mcr*-positive *E. coli* and MDR isolates, including ESBL-producing *K. pneumoniae*, in turkey meat.

With regard to the methods here investigated, we found that protocols I and II, based on MacConkey Lactose and MacConkey Sorbitol with telurite and cefixime agar incubated at 37 ºC, are the most effective for the recovery of isolates satisfying the ExPEC and UPEC status, as well as the *rbfO25b*-positive isolates associated with the clonal group STl31. The protocol V (CHROMID® ESBL agar plates 37 ºC) is key for the recovery of ESBL or pAmpC-producing Enterobacteriaceae. The duplex PCR based on *iutA* and *KpsM II* genes on MacConkey Lactose and MacConkey Sorbitol with telurite and cefixime agar is essential for the accurate screening of the isolates conforming ExPEC status, as well as for the recovery of those with UPEC status. Finally, we concluded that the microbiological method proposed here (pre-enrichment, enrichment in MacConkey Lactose broth, and inoculation onto MacConkey Lactose agar/ MacConkey Sorbitol with telurite and cefixime agar/CHROMID® ESBL), followed by the screening of six virulence/AMR traits (ExPEC status, UPEC status, ESBL/pAmpC producer, *mcr-1* carrier, MDR, *rfbO25b*), would help to elucidate the role of ExPEC as new extraintestinal food-borne pathogens.

Our results prove that a hybrid MDR aEPEC/ExPEC belonging to the clonal group O153:H10-A-ST10 (CH11-54) *eae*-beta1 is circulating in our region within different hosts, including wildlife. It seems implicated in human diarrhea *via* food (meat) transmission, and in the spreading of ESBL genes (mainly of CTX-M-32 type). The concomitant presence of IncF (F2:A-:B-), IncI1 and IncX1, together with non-conjugative Col156-like plasmids might be implicated in the successful persistence of this hybrid pathotype.

Keywords: Enterobacteriaceae, *Escherichia coli, Klebsiella pneumoniae*, ExPEC, ST131, *mcr*, hybrid pathotype, antibioresistance, ESBL, EnteroBase, risk assessment, poultry meat, One Health, from farm to fork.

RESUMEN

La COVID-19 lleva amenazando al mundo durante ya más de dos años. Por suerte, el trabajo de muchos investigadores ha permitido el desarrollo en un tiempo récord de herramientas para combatir esta pandemia de forma precisa en forma de vacunas. Pero esta pandemia nos está dejando muchas ausencias y consecuencias, como las derivadas del eclipse temporal del mayor desafío de salud en la actualidad: las resistencias a los antibióticos (ABR). El aumento en el número de bacterias multirresistentes (MDR) a antibióticos de último recurso (como por ejemplo a la colistina, los carbapenémicos o las cefalosporinas) es uno de los problemas de salud pública más graves a nivel mundial debido a la falta de opciones terapéuticas alternativas adecuado, al aumento de las tasas de mortalidad y a los costes de salud derivados de los tratamientos no efectivos. Según el Centro Europeo para la Prevención y el Control de Enfermedades (ECDC), más de 670.000 infecciones bacterianas pueden atribuir a las bacterias MDR, siendo responsables de más de 33.000 muertes al año solo en Europa.

Debido al riesgo asociado al uso terapéutico de antibióticos en animales de producción y a su potencial impacto para el ser humano, la Agencia Europea de Medicamentos (EMA) ha propuesto recientemente una nueva categorización, en la cual incluye en la Categoría A ("Evitar") aquellos antibióticos no autorizados actualmente para su uso en medicina veterinaria en la Unión Europea (UE), como son la fosfomicina o los monobáctamicos; y en la Categoría B ("Restringir") se incluyen aquellos antimicrobianos en los que debe restringirse su uso en animales para así mitigar el riesgo para la salud pública, a saber, las quinolonas, las cefalosporinas de tercera y cuarta generación y las polimixinas. Por lo tanto, este es un momento crítico en el que la reducción de la presión de los antibióticos a través de diferentes enfoques hace necesario rastrear la evolución bacteriana para comprobar la evolución de las medidas tomadas y así diseñar nuevas estrategias.

La bacteria *Escherichia coli* forma parte de la microbiota comensal natural del sistema digestivo en vertebrados de sangre caliente y pueden desempeñar diversas funciones dependiendo de sus características y su perfil de virulencia. Si bien las cepas de *E. coli* patógenas intestinales (InPEC) se distinguen con precisión de la microbiota intestinal comensal debido a la presencia de factores de virulencia asociados a los diferentes patotipos, esto no es tan simple con las bacterias de *E. coli* patógenas extraintestinales (ExPEC), ya que se comportan como patógenos oportunistas pudiendo colonizar de forma indefinida el sistema digestivo sin causar daño al anfitrión. Por ahora no ha sido determinados ningún conjunto de genes pueda definir inequívocamente las cepas ExPEC o sus diferentes categorías. Actualmente se están categorizando en fusión de su aislamiento en infecciones localizadas fuera del sistema digestivo y / o en función de la presencia de genes asociados estadísticamente con el potencial patogénico extraintestinal de estas cepas, los cuales pueden usarse de manera predictiva. Además, ciertos linajes de *E. coli* extraintestinales, como el clon pandémico ST131, han sido reconocidos mundialmente por su implicación en infecciones humanas, así como por su papel en la propagación de resistencias a antibióticos de uso habitual. La hipótesis de que los alimentos, en particular los productos avícolas, pueden actuar como reservorio de patógenos extraintestinales humanos como *E. coli* y otras Enterobacteriaceae se basa en la evidencia científica. Cepas causantes de patología aviar (avian pathogenic *E coli*, APEC) muestran una alta similitud

genética con las que causan patología extraintestinal en humanos, por lo que la hipótesis que ha surgido en varios estudios es que algunas cepas humanas ExPEC pueden haber evolucionado a partir de cepas APEC o ser iguales a ellas. Las evidencias que apuntan a esta hipótesis son, entre otras, las siguientes: A) La existencia de una agrupación geográfica y temporal de las cepas ExPEC aisladas de pacientes con infecciones extraintestinales que sugiere la aparición de un brote o una fuente común de exposición. B) La distribución global de linajes de cepas ExPEC idénticas, que indican la propagación global de la contaminación transmitida a través de los alimentos. C) La detección de genotipos idénticos de ExPEC aislados de infecciones humanas, así como de productos alimenticios detectados y analizados en una misma área geográfica. D) La representación desproporcionada de ciertos linajes pandémicos de ExPEC entre cientos de ST diferentes que causan infecciones extraintestinales en todas las regiones del mundo, lo que sugiere una ventaja biológica o de aptitud para diferentes reservorios de estos linajes pandémicos, como pueden ser los animales de producción u otros animales como las aves silvestres. E) La aparición relativamente reciente de los clones ST69, ST131 y ST393 como cepas ExPEC, sugiriendo la reciente introducción de estos genotipos en el nicho intestinal humano a partir de fuentes externas.

Debido a la alta plasticidad del genoma de *E. coli*, los patotipos híbridos están siendo cada vez más frecuentes e impredecibles debido al importante papel que juegan los elementos genéticos móviles (EGM) como los plásmidos, los bacteriófagos, las islas de patogenicidad, los transposones y las secuencias de inserción en la evolución de las bacterias. Además, las cepas con patotipos híbridos complejos con combinaciones de dos grupos diferentes de cepas patógenas intestinales de *E. coli* (InPEC) (*E. coli* productores de toxinas Shiga; STEC + *E. coli* enteroagregativos; EAEC) o InPEC y ExPEC (por ejemplo, *E. coli* enteropatógeno atípica; aEPEC + ExPEC y STEC + APEC) se notifican cada vez más en casos clínicos humanos. Desde 2011, cuando se identificó una nueva cepa de *E. coli* productora de toxinas Shiga perteneciente al serotipo O104: H4, con características de virulencia comunes a la EAEC y productora del gen de resistencia CTX-M-15, como involucrada en el gran brote alemán, el concepto de patotipo ha sido cuestionado y actualmente se están utilizando enfoques clásicos y actuales como la secuenciación genómica completa (WGS) para mejorar la comprensión de la evolución de esta especie altamente adaptable.

Actualmente está aceptada la premisa de que el uso de terapia con antibióticos en animales destinados a la producción de alimentos para consumo humano es la principal causa del aumento de la ABR, incluida la resistencia a la colistina. En las últimas décadas se ha producido una rápida diseminación de cepas portadoras de betalactamasas de espectro extendido (BLEE), principalmente debido a su presencia en plásmidos y su expansión a través de grupos clonales exitosos, como el clon pandémico ST131 de *E. coli*. Hoy en día existe una gran preocupación ante la posibilidad de adquisición *in vivo* de plásmidos portadores del gen *mcr* de resistencia a colistina, así como de genes bla_{BLEE} por parte de cepas de *E. coli* causantes de patología humana después de tratamientos clínicos o por transmisión animal a través del contacto directo o la cadena alimentaria entre cepas portadoras y cepas de la microbiota humana. ST131 es considerado el principal clon pandémico responsable de la propagación global de genes BLEE. Identificado por primera vez en 2008, el grupo clonal ST131 pertenece al filogrupo B2 y principalmente a los serotipos O25b:H4 o, con menor frecuencia, al serotipo O16: H5. Tres años después de su primer aislamiento, ya se encontraba diseminado a nivel global, siendo el agente bacteriano involucrado en más del 50% de los casos de UTIs por cepas productoras de BLEE en numerosos hospitales de diferentes países. La prevalencia de resistencias a

antibióticos orales de primera línea como trimetoprim-sulfametoxazol, amoxicilina y amoxicilina-clavulánico ha ido aumentando constantemente durante estos años, dificultando cada vez más el tratamiento de infecciones y poniendo en peligro la vida de los pacientes. Aunque está asociado con infecciones ExPEC como UTIs, septicemias, infecciones de heridas quirúrgicas y meningitis, este clon también se encuentra con frecuencia en el sistema digestivo de humanos sanos. Por eso, se sospecha que el tracto intestinal humano conforma un posible nicho para el clon ST131. Sin embargo, el creciente interés de la comunidad científica hacia este clon ha hecho que haya sido detectado fuentes tan diversas como animales de compañía, animales de producción de alimentos y salvajes; o en el propio medio ambiente, como en ríos, playas o en el alcantarillado; incluso en la región antártica. Los clados A y C están asociados principalmente a patología humana, mientras que el clado B agrupa a cepas aisladas de diferentes nichos como aves y cerdos, junto con humanos. Un desafío importante es saber qué determinantes hacen que ciertos clones se adapten mejor a un huésped específico mientras que otros pueden transmitirse entre diferentes especies, con saltos tan importantes como entre mamíferos y aves. En el caso de ST131, esta relación entre los diferentes clados y su presencia en diferentes hospedadores aún no se ha entendido completamente.

La presente tesis doctoral incluye tres estudios, "Chicken and turkey meat: Consumer exposure to multidrug-resistant Enterobacteriaceae including *mcr*-carriers, uropathogenic *E. coli* and high-risk lineages such as ST131" (Díaz-Jiménez et al., 2020a), "Microbiological risk assessment of turkey and chicken meat for consumer: Significant differences regarding multidrug resistance, *mcr* or presence of hybrid aEPEC/ExPEC pathotypes of *E. coli"* (Díaz-Jiménez et al., 2021) y "Genomic Characterization of *Escherichia coli* Isolates Belonging to a New Hybrid aEPEC/ExPEC Pathotype O153:H10-A-ST10 *eae*-beta1 Occurred in Meat, Poultry, Wildlife and Human Diarrheagenic Samples" (Díaz-Jiménez et al., 2020b). El objetivo de la presente tesis doctoral, desarrollada en el marco de dos proyectos nacionales (PN AGL2016-79343-R y PID2019-104439RB-C21 / AEI / 10.13039 / 501100011033), fue analizar el potencial zoonótico de cepas de la familia Enterobacteriaceae aisladas de ave de corral para consumo humano, con caracterización de resistencias a antibióticos y definición de grupos clonales patógenos para el ser humano. De este modo, evaluamos la exposición del consumidor a cepas de la familia Enterobacteriaceae con capacidad para desarrollar infecciones extraintestinales de riesgo, ya sea por la virulencia de las cepas o por su patrón de resistencias a antibióticos adquiridos a través de la carne de pollo y pavo.

La hipótesis de la presente tesis plantea que la carne de ave de corral destinada a consumo humano estaría actuando como reservorio y potenciales agentes transmisores de cepas patógenas que podrían estar implicadas en infecciones extraintestinales humanas. Para demostrar esta hipótesis, la estrategia fue analizar carne de ave a la venta al por menor adquirida directamente en los puntos de venta con el objetivo de que el producto final nos proporcione datos sobre lo que sucede en la granja, en el matadero, así como la calidad del producto que entra en la cocina del consumidor. La segunda estrategia sería la identificación de grupos clonales potencialmente uropatógenos de *E. coli* basados en marcadores genéticos específicos. Y, por último, consideramos como cepa de "alto riesgo" aquella con capacidad de desarrollar una infección extraintestinal grave en el ser humano, ya sea por su potencial de virulencia y / o por su resistencia a los antibióticos.

Los objetivos específicos de la presente tesis doctoral fueron en primer lugar, el diseño de un protocolo eficaz para la recuperación de *E. coli* transmitido por los alimentos y otras

Enterobacteriaceae con potencial patógeno y / o resistentes a los antimicrobianos. Un segundo objetivo fue conocer la situación actual de las ABR en la producción avícola, prestando especial atención a las categorías de antimicrobianos A y B de la clasificación EMA. Como tercer objetivo presentamos la evaluación de la exposición del consumidor, a través de la carne de ave a cepas de *E. coli* de alto riesgo y otras Enterobacteriaceae con potencial para desarrollar infecciones graves debido a su perfil de virulencia y / o a su perfil de resistencia a antibióticos. Nuestro último objetivo fue explorar la ruta de transmisión alimentaria de clones específicos de *E. coli* de origen humano y animal mediante análisis comparativo de genomas.

Se tomaron al azar muestras de 100 productos cárnicos de origen aviar (50 muestras de pollo y 50 muestras de pavo) en seis cadenas de supermercados españolas y carnicerías locales ubicadas en Lugo (noroeste de España). Mediante un protocolo de cultivo convencional se recuperaron 358 cepas diferentes de especies pertenecientes a la familia Enterobacteriaceae (170 aislamientos recuperados de muestras de pollo y 188 aislamientos recuperados de muestras de pavo) utilizando los medios agar MacConkey Lactosa, agar MacConkey Sorbitol con telurito y cefixima, CHROMID® ESBL y CHROMID®CARBA SMART. La identificación bacteriana reveló que 323 de 358 aislamientos eran *E. coli*, 28 de *K. pneumoniae*, seis de *Serratia fonticola* y uno de *Enterobacter cloacae*. Esta colección se caracterizó por completo incluyendo: filogrupo, serotipo, ST y complejo clonal, clonotipo, perfil de virulencia y de resistencia.

Una segunda colección fue obtenida durante el período entre 2005 y 2015 a partir de diferentes estudios de vigilancia realizados en el LREC, Lugo, España, que tenían como objetivo la detección de cepas de *E. coli* productoras de BLEE. Estos estudios incluyeron muestras de carne de ave, vacuno y porcino, así como del ambiente de granjas de producción avícola y animales salvajes, y se seleccionaron y caracterizaron los aislamientos que presentaban un patotipo aEPEC y el serotipo O153.

El primer estudio evalúa la exposición del consumidor a través de la carne de ave a cepas de la familia Enterobacteriaceae con capacidad para provocar infecciones extraintestinales severas debido a sus características de virulencia y / o rasgos de resistencia a antibióticos. La caracterización de 256 cepas (84 aislamientos representativos de *E. coli*, 137 aislamientos de cepas de *E. coli* productores de BLEE, 28 aislamientos de cepas de *Klebsiella pneumoniae* productoras de BLEE, seis aislamientos de cepas de *Serratia fonticola* productoras de BLEE y un aislado de *Enterobacter cloacae* productor de BLEE) y la evaluación de cinco parámetros mostró que 96 de cada 100 muestras de carne de ave de corral adquiridas en supermercados del noroeste de España presentaban más de un factor de riesgo potencial. En concreto, i) el 96% de las muestras eran portadoras de cepas de Enterobacteriaceae resistentes a antimicrobianos de las categorías A (64% con resistencia a monobactamicos) o B (95% con resistencia a cefalosporinas de 3a y 4a generación, quinolonas y / o polimixinas) de la nueva categorización de EMA. ii) Se recuperó más de una especie de Enterobacteriaceae productoras de BLEE del 29% de las muestras, principalmente cepas de *E. coli* y *K. pneumoniae*. iii) La caracterización de los aislados de *E. coli* mostró que los grupos clonales extraintestinales de alto riesgo y / o potencialmente uropatógenos (ST10, ST23, ST38, ST48, ST58, ST69, ST88, ST93, ST95, ST101, ST115, ST117, ST131, ST141, ST167, ST350, ST345, ST354, ST359, ST410, ST602, ST617, ST641, ST906, ST1485) estaban presentes en el 62% de las muestras. iv) De las cepas recuperadas de *E. coli* el 25% satisficieron los criterios para ser denominadas según el criterio como ExPEC y v) el 25% satisficieron los criterios para ser denominadas según el criterio como UPEC. Con respecto a las cepas de *K. pneumoniae*, al menos ocho de las 11 ST identificadas

en nuestra colección han sido reportadas como aislamientos de cepas clínicas humanas; específicamente: ST15, ST45, ST111, ST147, ST307, ST627, ST966 y ST1086 (22 de las 28 *K. pneumoniae* pertenecían a alguno de estos ocho ST). El gen *mcr*-1 de resistencia a la colistina mediado por un plásmido se identificó en 13 aislamientos de *E. coli* de siete muestras de carne diferentes, sin embargo, las once *K. pneumoniae* que presentaron resistencia fenotípica la colistina fueron negativas por PCR para la presencia de genes *mcr*-1 a *mcr*-8, probablemente indicando una resistencia de tipo cromosómica a la colistina.

En nuestro segundo estudio, se evaluó el riesgo al que se exponían los consumidores de carne de ave atendiendo únicamente a las cepas aisladas de *E. coli.* Se propuso un flujo de trabajo de laboratorio basado en seis rasgos de virulencia y / o resistencia a los antimicrobianos e incluimos el desarrollo de una PCR doble para el cribado de cepas con genes asociados al criterio ExPEC. Caracterizamos 323 cepas recuperadas de 100 muestras de carne de ave de corral para consumo humano. Esta caracterización reveló que la carne de aves es una fuente de cepas con diversidad filogenética rica en filogrupos de *E. coli* (A a G) y *Escherichia* clado I. Además, el 47% de las muestras era portadora de 2 o más *E. coli* diferentes positivos para genes BLEE, pAmpC o *mcr*. Las cepas aisladas del 78% de las muestras carne de ave cumplieron los requerimientos del criterio del estatus ExPEC y el 53% fueron portadores de cepas positivas para el estatus UPEC. Las STs identificadas en el 86% de las muestras pertenecían a los llamados linajes ExPEC de alto riesgo, siendo el 73% portadores de grupos clonales identificados en infecciones humanas de la misma área de salud. Además, diferentes clones asociados con patología humana aparecieron en la misma muestra de carne: ST131-B2 (CH40-22), ST648-F (CH4-58), ST93-A (CH11-neg) o ST95-B2 (CH38-27), ST354-F (CH88-58), ST155-B1 (CH4-neg). De forma general, el 84% de las muestras de carne presentaban tres o más factores de riesgo, incluidos genes de resistencia, clones exitosos de riesgo y rasgos de virulencia. La carne de pavo mostró una presencia significativamente más alta de genes *mcr* o resistencia a múltiples fármacos; mientras que la tasa de cepas con estatus ExPEC, o la presencia de patotipos híbridos como el aEPEC / ExPEC O153: H10-A-ST10 (CH11-54), se asociaron con el origen del pollo ($P < 0.05$).

En nuestro tercer estudio tomamos como punto de partida los diferentes estudios de vigilancia (2005-2015) realizados en el noroeste de España. Estos revelaron la presencia de aislamientos *eae*-positivos de cepas de *E. coli* del serotipo O153: H10 en muestras de carne para consumo humano, granjas avícolas, fauna silvestre y casos de diarrea humana. El objetivo de este estudio fue explorar la relación genética entre los aislados humanos y animales / cárnicos, así como su mecanismo de persistencia. También era objetivo saber si se trataba de un linaje geográficamente restringido o si había sido reportado en otro lugar. La caracterización convencional mostró que 32 aislamientos eran O153: H10-A-ST10 *fim*H54, *fim*AvMT78, *traT* y *eae*-beta1. Entre ellas, 21 eran productoras de CTX-M-32 o SHV-12. La comparación empleando la técnica de PFGE XbaI - macrorrestricción mostró una alta similitud (> 85%) entre los aislamientos de diferentes orígenes de la colección. El análisis del plasmidoma reveló una combinación estable de los tipos de plásmidos IncF (F2: A-: B-), IncI1 (ST desconocido) e IncX1, junto con plásmidos de tipo Col no conjugativos. La investigación del core genome basada en el esquema de tipado de secuencias multilocus del core genoma (cgMLST) de EnteroBase demostró una estrecha relación entre los aislamientos de origen humano y animal.

A partir de nuestros resultados, llegamos a la conclusión de que la microbiota presenten en la carne de ave de corral es una fuente de enterobacterias genéticamente diversas, resistentes a

antimicrobianos relevantes (categorías A y B de EMA) y potencialmente patógenas para los seres humanos, incluyendo patotipos híbridos de *E. coli*, grupos clonales de *E. coli* de alto riesgo asociados a patologías humanas extraintestinales y / o cepas uropatógenas, así como grupos clonales de *K. pneumoniae* de interés clínico. Nuestros resultados indicarían también que el sistema de producción industrial de carne de pavo da como resultado una mayor presión de selección para cepas resistentes a los antibióticos en comparación con el sistema de producción de pollo, lo que se refleja en tasas significativamente más altas de cepas de *E. coli* MDR positivas para el gen *mcr*, y cepas de *K. pneumoniae* productora de BLEE, en carne de pavo.

Con respecto a los métodos aquí propuestos, encontramos que los protocolos I y II, basados en los medios MacConkey Lactosa y MacConkey Sorbitol con telurito y cefixima incubados a 37 ºC, son los más efectivos para la recuperación de cepas que cumplen el estatus ExPEC y UPEC, así como las cepas positivas al gen *rbfO25b*, asociado con el grupo clonal STl31. El protocolo V (placas de agar CHROMID® ESBL incubadas a 37 ºC) es clave para la recuperación de Enterobacteriaceae productoras de BLEE o pAmpC. La PCR dúplex basada en la detección de genes *iutA* y *KpsM* II en MacConkey Lactosa y MacConkey sorbitol con telurito y cefixima es esencial para el cribado preciso de cepas que cumplen el estatus ExPEC, así como para la recuperación de aquellos con estatus UPEC. Finalmente, concluimos que el método microbiológico propuesto aquí (pre-enriquecimiento, enriquecimiento en caldo ML e inoculación en MacConkey Lactosa / MacConkey sorbitol con telurito y cefixima / CHROMID® ESBL), seguido de la selección de seis rasgos de virulencia / ABR (estatus ExPEC, estatus UPEC, BLEE / productor de pAmpC, portador de *mcr*-1, MDR, *rfbO25b*), ayudaría a dilucidar el papel de ExPEC como nuevos patógenos extraintestinales transmitidos por los alimentos.

Nuestros resultados demuestran que un híbrido MDR aEPEC / ExPEC perteneciente al grupo clonal O153: H10-A-ST10 (CH11-54) *eae*-beta1 está circulando en nuestra región dentro de diferentes hospedadores, incluida la fauna silvestre. Parece estar implicado en la diarrea humana a través de transmisión alimentaria (carne) y en la propagación de genes BLEE (principalmente del tipo CTX-M-32). La presencia concomitante de IncF (F2: A-: B-), IncI1 e IncX1, junto con plásmidos de tipo Col156 no conjugativos podría estar implicada en la persistencia satisfactoria de este patotipo híbrido.

Palabras clave: Enterobacteriaceae, *Escherichia coli, Klebsiella pneumoniae*, ExPEC, ST131, *mcr*, patotipo híbrido, antibiorresistencia, BLEE, EnteroBase, evaluación de riesgo, carne de ave, Una Sola Salud, de la granja a la mesa.

RESUMO

A COVID-19 leva ameazando ó mundo durante máis de dous anos. Por sorte, o traballo de moitos investigadores permitiu o desenvolvemento nun tempo récord de ferramentas para combater esta pandemia de forma precisa en forma de vacinas. Pero esta pandemia está a deixarnos moitas ausencias e consecuencias, como as derivadas da eclipse temporal do maior desafío de saúde na actualidade: as resistencias aos antibióticos (ABR). O aumento no número de bacterias multirresistentes (MDR) a antibióticos de último recurso (por exemplo á colistina, os carbapenémicos ou as cefalosporinas) é un dos problemas de saúde pública máis graves a nivel mundial debido á falta de opcións terapéuticas alternativas adecuadas, ó aumento das taxas de mortalidade e aos custos de saúde derivados dos tratamentos non efectivos. Segundo o Centro Europeo para a Prevención e o Control de Enfermidades (ECDC), máis de 670.000 infeccións bacterianas podense atribuír ás bacterias MDR, sendo responsables de máis de 33.000 mortes ó ano só en Europa.

Debido ó risco asociado ó uso terapéutico de antibióticos en animais de produción e ó seu potencial impacto para o ser humano, a Axencia Europea de Medicamentos (EMA) propuxo recentemente unha nova categorización, na cal inclúe na Categoría A ("Evitar") aqueles antibióticos non autorizados actualmente para o seu uso en medicina veterinaria na Unión Europea (UE), como son a fosfomicina ou os monobáctamicos; e na Categoría B ("Restrinxir") inclúense aqueles antimicrobianos nos que debe restrinxirse o seu uso en animais para así mitigar o risco para a saúde pública, a saber, as quinolonas, as cefalosporinas de terceira e cuarta xeración e as polimixinas. Polo tanto, leste é un momento crítico no que a redución da presión dos antibióticos a través de diferentes enfoques fai necesario rastrexar a evolución bacteriana para comprobar a evolución das medidas tomadas e así deseñar novas estratexias.

A bacteria *Escherichia coli* forma parte da microbiota comensal natural do sistema dixestivo en vertebrados de sangue quente e poden desempeñar diversas funcións dependendo das súas características e o seu perfil de virulencia. Aínda que as cepas de *E. coli* patóxenas intestinais (InPEC) distínguense con precisión da microbiota intestinal comensal debido á presenza de factores de virulencia asociados aos diferentes patotipos, isto non é tan simple coas bacterias de *E. coli* patóxenas extraintestinais (ExPEC), xa que se comportan como patógenos oportunistas podendo colonizar de forma indefinida o sistema dixestivo sen causar dano ó anfitrión. Polo de agora non foi determinados ningún conxunto de xenes poida definir inequivocamente as cepas ExPEC ou as súas diferentes categorías. Na actualidade estanse categorizando en fusión do seu illamento en infeccións localizadas fóra do sistema dixestivo e / ou en función da presenza de xenes asociados estatisticamente co potencial patoxénico extraintestinal destas cepas, os cales poden usarse de maneira preditiva. Ademais, certas liñaxes de *E. coli* extraintestinais, como o clon pandémico ST131, foron recoñecidos mundialmente pola súa implicación en infeccións humanas así como polo seu papel na propagación de resistencias a antibióticos de uso habitual. A hipótese de que os alimentos, en particular os produtos avícolas, poden actuar como reservorio de patóxenos extraintestinais humanos como *E. coli* e outras Enterobacteriaceae, baséase na evidencia científica. Cepas causantes de patoloxía aviaria (APEC) mostran unha alta similitude xenética coas que causan patoloxía extraintestinal en humanos, polo que a hipótese que xurdiu en varios estudos é que algunhas

cepas humanas ExPEC poden evolucionar a partir de cepas APEC ou ser iguais a elas. As evidencias que apuntan a esta hipótese son, entre outras: A) A existencia dunha agrupación xeográfica e temporal das cepas ExPEC illadas de pacientes con infeccións extraintestinas que suxire a aparición dun brote ou unha fonte común de exposición. B) A distribución global de liñaxes de cepas ExPEC idénticas, que indican a propagación global da contaminación transmitida a través dos alimentos. C) A detección de xenotipos idénticos de ExPEC illados de infeccións humanas, así coma de produtos alimenticios detectados e analizados nunha mesma área xeográfica. D) A representación desproporcionada de certas liñaxes pandémicas de ExPEC entre centos de ST diferentes que causan infeccións extraintestinais en todas as rexións do mundo, o que suxire unha vantaxe biolóxica ou de aptitude para diferentes reservorios destas liñaxes pandémicas, como poden ser os animais de produción ou outros animais como as aves silvestres. E) A aparición relativamente recente dos clons ST69, ST131 e ST393 como cepas ExPEC, suxerindo a recente introdución destes xenotipos no nicho intestinal humano a partir de fontes externas.

Debido á alta plasticidade do xenoma de *E. coli*, os patotipos híbridos están a ser cada vez máis frecuentes e impredicibles debido ó importante papel que xogan os elementos xenéticos móbiles (EXM) como os plásmidos, os bacteriófagos, as illas de patoxenicidade, os transposons e as secuencias de inserción na evolución das bacterias. Ademais, as cepas con patotipos híbridos complexos con combinacións de dous grupos diferentes de cepas InPEC (*E. coli* produtores de toxinas Shiga; STEC + *E. coli* enteroagregativos; EAEC) ou InPEC e ExPEC (por exemplo, *E. coli* enteropatóxeno atípico; aEPEC + ExPEC e STEC + APEC) notifícanse cada vez máis en casos clínicos humanos. Desde 2011, cando se identificou unha nova cepa de *E. coli* produtora de toxinas Shiga pertencente ó serotipo O104:H4, con características de virulencia comúns á EAEC e produtora do xene de resistencia CTX- M-15, como involucrada no gran brote alemán, o concepto de patotipo foi cuestionado e actualmente estanse utilizando enfoques clásicos e actuais como a secuenciación xenómica completa (WGS) para mellorar a comprensión da evolución desta especie altamente adaptable.

Actualmente está aceptada a premisa de que o uso de terapia con antibióticos en animais destinados á produción de alimentos para consumo humano é a principal causa do aumento das ABR, incluída a resistencia á colistina. Nas últimas décadas produciuse unha rápida diseminación de cepas portadoras de betalactamasas de espectro estendido (BLEE), principalmente debido á súa presenza en plásmidos e a súa expansión a través de grupos clonais exitosos, como o clon pandemico ST131 de *E. coli*. Hoxe en día existe unha gran preocupación ante a posibilidade de adquisición *in vivo* de plásmidos portadores do xene *mcr* de resistencia a colistina, así como de xenes bla_{BLEE} por parte de cepas de *E. coli* causantes de patoloxía humana despois de tratamentos clínicos ou por transmisión animal a través do contacto directo ou a cadea alimentaria entre cepas portadoras e cepas da microbiota humana. ST131 é considerado o principal clon pandémico responsable da propagación global de xenes ESBL. Identificado por primeira vez en 2008, o grupo clonal ST131 pertence ó filogrupo B2 e principalmente aos serotipos O25b:H4 ou, con menor frecuencia, ó serotipo O16:H5. Tres anos despois do seu primeiro illamento, xa se atopaba diseminado a nivel global, sendo o axente bacteriano involucrado en máis do 50% dos casos de UTIs por cepas produtoras de BLEE en numerosos hospitais de diferentes países. A prevalencia de resistencias a antibióticos orais de primeira liña como trimetoprim-sulfametoxazol, amoxicilina e amoxicilina-clavulánico foi aumentando constantemente durante estes anos, dificultando cada vez máis o tratamento de infeccións e poñendo en perigo a vida dos pacientes. Aínda que está asociado con infeccións ExPEC como

UTIs, septicemias, infeccións de feridas cirúrxicas e meninxites, este clon tamén se atopa con frecuencia no sistema dixestivo de humanos sans. Por iso, sospéitase que o tracto intestinal humano conforma un posible nicho para o clon ST131. Con todo, o crecente interese da comunidade científica cara a este clon fixo que fose detectado en fontes tan diversas como animais de compañía, animais de produción de alimentos e salvaxes; ou no propio medio ambiente, como en ríos, praias ou na rede de sumidoiros; mesmo na rexión antártica. Cos datos actuais, apréciase o feito de que os clados A e C están asociados principalmente a patoloxía humana, mentres que o clado B agrupa a cepas illadas de diferentes nichos como aves e porcos, xunto con humanos. Un desafío importante é saber que determinantes fan que certos clons se adapten mellor a un hóspede específico mentres que outros poden transmitirse entre diferentes especies, con saltos tan importantes como entre mamíferos e aves. No caso de ST131, esta relación entre os diferente clados e a súa presenza en diferentes hospedadores aínda non se entendeu completamente.

A presente tese doutoral inclúe tres estudos, "Chicken and turkey meat: Consumer exposure to multidrug-resistant Enterobacteriaceae including *mcr*-carriers, uropathogenic *E. coli* and high-risk lineages such as ST131" (Díaz-Jiménez et al., 2020a), "Microbiological risk assessment of turkey and chicken meat for consumer: Significant differences regarding multidrug resistance, *mcr* or presence of hybrid aEPEC/ExPEC pathotypes of *E. coli"* (Díaz-Jiménez et al., 2021) e "Genomic Characterization of *Escherichia coli* Isolates Belonging to a New Hybrid aEPEC/ExPEC Pathotype O153:H10-A-ST10 *eae*-beta1 Occurred in Meat, Poultry, Wildlife and Human Diarrheagenic Samples" (Díaz-Jiménez et al., 2020b). O obxectivo da presente tese doutoral, desenvolta no marco de dous proxectos nacionais (PN AGL2016-79343- R e PID2019-104439 RB- C21 / AEI / 10.13039 / 501100011033), foi analizar o potencial zoonótico de cepas da familia Enterobacteriaceae illadas de ave de curral para consumo humano, con caracterización de resistencias a antibióticos e definición de grupos clonais patógenos para o ser humano. Deste xeito, avaliamos a exposición do consumidor a cepas da familia Enterobacteriaceae con capacidade para desenvolver infeccións extraintestinais de risco, xa sexa pola virulencia das cepas ou polo seu patrón de resistencias a antibióticos adquiridos a través da carne de polo e pavo.

A hipótese da presente tese expón que a carne de ave de curral destinadas a consumo humano estarían a actuar como reservorio e potenciais axentes transmisores de cepas patóxenas que poderían estar implicadas en infeccións extraintestinais humanas. Para demostrar esta hipótese, a estratexia foi analizar carne de ave á venda polo miúdo adquirida directamente nos puntos de venda co obxectivo de que o produto final nos proporcione datos sobre o que sucede na granxa, no matadoiro, así como a calidade do produto que entra na cociña do consumidor. A segunda estratexia foi identificar os grupos clonais potencialmente uropatóxenos de *E. coli* baseados en marcadores xenéticos específicos. E finalmente, considerar como cepa de "risco" aquela con capacidade de desenvolver unha infección extraintestinal grave no ser humano, xa sexa polo seu potencial de virulencia e / ou pola súa resistencia aos antibióticos.

Os obxectivos específicos da presente tesis doutoral foron o deseño dun protocolo eficaz para a recuperación de *E. coli* transmitido polos alimentos e outras Enterobacteriaceae con potencial patógeno e / ou resistentes aos antimicrobianos. Un segundo obxetivo foi coñecer a situación actual das ABR na produción avícola, prestando especial atención ás categorías de antimicrobianos A e B da clasificación EMA. Tamén realizamos a avaliación da exposición do consumidor, a través da carne de ave a cepas de *E. coli* de alto risco e outras Enterobacteriaceae

con potencial para desenvolver infeccións graves debido ó seu perfil de virulencia e / ou ó seu perfil de resistencia a antibióticos. Por derradeiro, quixemos explorar o roteiro de transmisión alimentaria de clons específicos de *E. coli* de orixe humana e animal mediante análise comparativa de xenomas.

Tomáronse ó azar mostras de 100 produtos cárnicos de orixe aviaria (50 mostras de polo e 50 mostras de pavo) en seis cadeas de supermercados españolas e carnicerías locais situadas en Lugo (noroeste de España). Mediante un protocolo de cultivo convencional recuperáronse 358 cepas diferentes de especies pertencentes á familia Enterobacteriaceae (170 illamentos recuperados de mostras de polo e 188 illamentos recuperados de mostras de pavo) utilizando os medios agar MacConkey Lactosa agar MacConkey Sorbitol con telurito e cefixima, CHROMID® ESBL e CHROMID® CARBA SMART. A identificación bacteriana revelou que 323 de 358 illamentos eran *E. coli*, 28 de *K. pneumoniae*, seis de *Serratia fonticola* e un de *Enterobacter cloacae*. Esta colección caracterizouse por completo incluíndo: filogrupo, serotipo, ST e complexo clonal, clonotipo, perfil de virulencia e de resistencia.

Unha segunda colección foi obtida durante o período entre 2005 e 2015 a partir de diferentes estudos de vixilancia realizados no LREC, Lugo, España, que tiñan como obxectivo a detección de cepas de *E. coli* produtoras de BLEE en diferentes fontes na nosa rexión. Estes estudos incluíron mostras de carne de ave, vacún e porcino, así como do ambiente de granxas de produción avícola e animais salvaxes, e caracterizaronse os illamentos que presentaban un patotipo aEPEC e o serotipo O153.

O primeiro estudo evalua a exposición do consumidor a través da carne de ave a cepas da famila Enterobacteriaceae con capacidade para provocar infeccións extraintestinais severas debido ás súas características de virulencia e / ou trazos de resistencia a antibióticos. A caracterización de 256 cepas (84 illamentos representativos de *E. coli*, 137 illamentos de cepas de *E. coli* produtores de BLEE, 28 illamentos de cepas de *Klebsiella pneumoniae* produtoras de BLEE, seis illamentos de cepas de *Serratia fonticola* produtoras de BLEE e un illado de *Enterobacter cloacae* produtor de BLEE) e a avaliación de cinco parámetros mostrou que 96 de cada 100 mostras de carne de ave de curral adquiridas en supermercados do noroeste de España presentaban máis dun factor de risco potencial. En concreto, i) o 96% das mostras eran portadoras de cepas de Enterobacteriaceae resistentes a antimicrobianos das categorías A (64% con resistencia a monobactamicos) ou B (95% con resistencia a cefalosporinas de 3a e 4a xeración, quinolonas e / ou polimixinas) da nova categorización de EMA. ii) Recuperouse máis dunha especie de Enterobacteriaceae produtoras de BLEE do 29% das mostras, principalmente cepas de *E. coli* e *K. pneumoniae*. iii) A caracterización dos illados de *E. coli* mostrou que os grupos clonais extraintestinais de alto risco e / ou potencialmente uropatóxenos (ST10, ST23, ST38, ST48, ST58, ST69, ST88, ST93, ST95, ST101, ST115, ST117, ST131, ST141, ST167, ST350, ST345, ST354, ST359, ST410, ST602, ST617, ST641, ST906, ST1485) estaban presentes no 62% das mostras. iv) Das cepas recuperadas de *E. coli* o 25% satisfixeron os criterios para ser denominadas segundo o criterio como ExPEC e v) o 17% satisfixeron os criterios para ser denominadas segundo o criterio como UPEC. Con respecto ás cepas de *K. pneumoniae*, polo menos oito das 11 ST identificadas na nosa colección foron reportadas como illamentos de cepas clínicas humanas; especificamente: ST15, ST45, ST111, ST147, ST307, ST627, ST966 e ST1086 (22 das 28 *K. pneumoniae* pertencían a algún destes oito ST). O xene *mcr*-1 de resistencia á colistina mediado por un plásmido identificouse en 13 illamentos de *E. coli* de sete mostras de carne diferentes, con todo, as once *K. pneumoniae* que presentaron

resistencia fenotípica a colistina foron negativas por PCR para a presenza de xenes *mcr*-1 a *mcr*-8, probablemente indicando unha resistencia de tipo cromosómica á colistina.

No noso segundo estudo, avaliouse o risco ó que se expoñían os consumidores de carne de ave atendendo unicamente ás cepas illadas de *E. coli*. Propúxose un fluxo de traballo de laboratorio baseado en seis trazos de virulencia e / ou resistencia aos antimicrobianos e incluímos o desenvolvemento dunha PCR dobre para o cribado de cepas con xenes asociados ó criterio ExPEC. Caracterizamos 323 cepas recuperadas de 100 mostras de carne de ave de curral para consumo humano. Esta caracterización revelou que a carne de aves é unha fonte de cepas con diversidade filogenética rica en filogrupos de *E. coli* (Á - G) e *Escherichia* clado I. Ademais, o 47% das mostras eran portadoras de 2 ou máis *E. coli* diferentes positivos para xenes BLEE, pAmpC ou *mcr*. As cepas illadas do 78% de mostras de carne de ave cumpriron os requirimentos do criterio do status ExPEC e o 53% foron portadores de cepas positivas para o status UPEC. As STs identificadas no 86% das mostras pertencían ás chamadas liñaxes ExPEC de alto risco, sendo os 73% portadores de grupos clonais identificados en infeccións humanas da mesma área de saúde. Ademais, diferentes clons asociados con patoloxía humana apareceron na mesma mostra de carne: ST131-B2 (CH40-22), ST648-F (CH4-58), ST93-A (CH11-neg) ou ST95-B2 (CH38-27), ST354-F (CH88-58), ST155-B1 (CH4- neg). De forma xeral, o 84% das mostras de carne presentaban tres ou máis factores de risco, incluídos xenes de resistencia, clons exitosos de risco e trazos de virulencia. A carne de pavo mostrou unha presenza significativamente máis alta de xenes *mcr* ou resistencia a múltiples fármacos; mentres que a taxa de cepas con estatus ExPEC, ou a presenza de patotipos híbridos como o aEPEC / ExPEC O153:H10-A-ST10 (CH11-54), asociáronse coa orixe do polo ($P < 0.05$).

No noso terceiro estudo tomamos como punto de partida os diferentes estudos de vixilancia (2005-2015) realizados no noroeste de España. Estes revelaron a presenza de illamentos *eae*-positivos de cepas de *Escherichia coli* do serotipo O153:H10 en mostras de carne para consumo humano, granxas avícolas, fauna silvestre e casos de diarrea humana. O obxectivo deste estudo foi explorar a relación xenética entre os illados humanos e animais / cárnicos, así como o seu mecanismo de persistencia. Tamén era obxectivo saber se se trataba dunha liñaxe xeograficamente restrinxida ou se fora reportado noutro lugar. A caracterización convencional mostrou que 32 illamentos eran O153:H10-A- ST10 *fimH*54, *fimAv*MT78, *traT* e *eae*- beta1. Entre elas, 21 eran produtoras de CTX- M-32 ou SHV-12. A comparación empregando a técnica de PFGE XbaI - macrorrestricción mostrou unha alta similitude (> 85%) entre os illamentos de diferentes orixes da colección. A análise do plasmidoma revelou unha combinación estable dos tipos de plásmidos IncF (F2:A-:B-), IncI1 (ST descoñecido) e IncX1, xunto con plásmidos de tipo Col non conxugativos. A investigación do *core genome* baseada no esquema do tipado das secuencias multilocus do core genoma (cgMLST) de EnteroBase demostrou unha estreita relación entre os illamentos de orixe humana e animal.

A partir dos nosos resultados, chegamos á conclusión de que a microbiota presente na carne de ave de curral é unha fonte de enterobacterias xeneticamente diversas, resistentes a antimicrobianos relevantes (categorías A e B de EMA) e potencialmente patóxenas para os seres humanos, incluíndo patotipos híbridos de *E. coli*, grupos clonais de *E. coli* de alto risco asociados a patoloxías humanas extraintestinales e / ou cepas uropatógenas, así como grupos clonais de *K. pneumoniae* de interese clínico. Os nosos resultados indicarían tamén que o sistema de produción industrial de carne de pavo dá como resultado unha maior presión de selección para cepas resistentes aos antibióticos en comparación co sistema de produción de

polo, o que se reflicte en taxas significativamente máis altas de cepas de *E. coli* MDR positivas para o xene *mcr*, e cepas de *K. pneumoniae* produtoras de BLEE en carne de pavo.

Con respecto aos métodos aquí propostos, atopamos que os protocolos I e II, baseados nos medios ML e MLST incubados a 37 º C, son os máis efectivos para a recuperación de cepas que cumpren o estatus ExPEC e UPEC, así como as cepas positivas ó xene *rbfO25b*, asociado co grupo clonal STl31. O protocolo V (placas de agar CHROMID® ESBL incubadas a 37 º C) é clave para a recuperación de Enterobacteriaceae produtoras de BLEE ou pAmpC. A PCR dúplex baseada na detección de xenes *iutA* e *KpsM* II en MacConkey Lactosa e MacConkey sorbitol con telurio e cefixima é esencial para o cribado preciso de cepas que cumpren o estatus ExPEC, así como para a recuperación daqueles con status UPEC. Finalmente, concluímos que o método microbiológico proposto aquí (pre-enriquecemento, enriquecemento en caldo MacConkey inoculación en MacConkey Lactosa / MacConkey sorbitol con telurio e cefixima / CHROMID® ESBL), seguido da selección de seis trazos de virulencia / ABR (estatus ExPEC, estatus UPEC, BLEE / produtor de pAmpC, portador de *mcr*-1, MDR, *rfbO25b*), axudaría a dilucidar o papel dos ExPEC como novos patógenos extraintestinales transmitidos polos alimentos.

Os nosos resultados demostran que un híbrido MDR aEPEC / ExPEC pertencente ó grupo clonal O153:H10-A- ST10 (CH11-54) *eae*- beta1 está a circular na nosa rexión dentro de diferentes hospedadores, incluída a fauna silvestre. Parece estar implicado na diarrea humana a través de transmisión alimentaria (carne) e na propagación de xenes BLEE (principalmente do tipo CTX- M-32). A presenza concomitante de IncF (F2:A-:B-), IncI1 e IncX1, xunto con plásmidos de tipo Col156 non conxugativos podería estar implicada na persistencia satisfactoria deste patotipo híbrido.

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Palabras chave: Enterobacteriaceae, *Escherichia coli, Klebsiella pneumoniae*, ExPEC, ST131, *mcr*, patotipo híbrido, antibiorresistencia, BLEE, EnteroBase, evaluación do risco, carne de ave, Unha Soa Saúde, da granxa á mesa.

ABBREVIATIONS

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ABR: From Spanish “Cepas resistentes a antibióticos”
adk: Adenylate Kinase gene
aEPEC: Atypical enteropathogenic *E. coli*
AIEC: Disease-associated adherent-invasive *E. coli*
AMC: Amoxicillin-clavulanic acid
AMEG: Antimicrobial Advice Ad Hoc Expert Group
AMK: Amikacin
AMP: Ampicillin
AMR: Antimicrobial resistance
APEC: Avian pathogenic *E. coli*
AST: Antibiotic susceptibility testing
ATB: Antibiotic
ATM: Aztreonam

BFP: Bundle-forming pilus
BLAST: Basic Local Alignment Search Tool
BLEE: From Spanish “betalactamasas de espectro extendido”
bp: Base pairs
BPW: Buffered peptone water

CAZ: Ceftazidime
cba: Colicin B gene
CC: Clonal complex
celb: Endonuclease colicin E2 gene
CFA: Colonization factor antigens
CF3rd: Cephalosporine of 3rd generation
cfu: Colony forming units
CGE: Center for Genomic Epidemiology
cgMLST: Core genome Multilocus Sequence Typing
Ch: Chicken
CH: Clonotype
CHL: Chloramphenicol
CIP: Ciprofloxacin
CLSI: Clinical & Laboratory Standards Institute
cma: Colicin M gene
cnf: Cytotoxin necrotizing factor gene
CST: Colistin
CTX: Cefotaxime
CTX-M: Cefotaximases

DAEC: Diffusely adherent *E. coli*
DEC: Diarrheagenic *E. coli*
DNA: Desoxyribonucleic acid
DOX: Doxycycline
DPMT: Degenerate Prime MOB Typing

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E. coli: Escherichia coli
eae: Intimin gene
EAE: Attaching and effacing protein
EAEC: Enteroaggregative *E. coli*
EAST1: Heat-stable EAEC toxin
ECDC: European Centre for Disease Prevention and Control
EEA: European Economic Area
EIEC: Enteroinvasive *E. coli*
EMA: European Medicines Agency
EPEC: Enteropathogenic *E. coli*
ESBL: Extended Spectrum β-lactamases
ETEC: Enterotoxigenic *E. coli*
ExPEC: Extraintestinal pathogenic *E. coli*

fimH: Type 1 fimbrial adhesine gene
FQ: Fluoroquinolone
FOF: Fosfomycin
FOX: Cefoxitin
fumC: Type 1 fimbrial adhesin gene
FWD-Net: European Food and Waterborne Diseases and Zoonoses Network

GEN: Gentamicin
gyrB: DNA gyrase gene

HC: Hemorrhagic colitis
HierCC: Hierarchical Clustering
hly: Hemolysin gene
HNM: H non-motile isolate
HULA: Hospital Universitario Lucus Augusti
HUS: Hemolytic uremic syndrome

icd: Isocitrate/isopropylmalate dehydrogenase gene
IMP: Imipenem
Inc.: Incompatibility groups
InPEC: Intestinal pathogenic *E. coli*
iroN: Enterobactin siderophore receptor protein
IS: Insertion sequences
iss: Increased serum survival

LREC: From Spanish "Laboratorio de Referencia de *Escherichia coli*"
LT: Heat-labile toxin

MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization – Time of fly
MB: Monobactam
mchF: ABC transporter protein MchF gene
MCPs: Multicopy plasmids
mcr: Mobile colistin resistance gene
mdh: Malate dehydrogenase gene

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MDR: Multidrug-resistant
MEGA: Molecular Evolutionary Genetics Analysis
MGE: Mobile genetic element
MIC: Minimum inhibitory concentration
ML: MacConkey Lactose agar
MLEE: Multilocus Enzyme Electrophoresis
MLST: Multilocus Sequence Typing
MOB: Relaxase protein
Mpb: Mega Base Pairs
MSTC: MacConkey Sorbitol with Telurite and Cefixime

NAL: Nalidixic acid
NCBI: National Center for Biotechnology Information
NIT: Nitrofurantoin
NJ: Neighbor-Joining
NMEC: Neonatal meningitis-associated *E. coli*
nt: Non typable
NR: Not realized

ONT: O non-typeable isolate
ori: Replication initiator

PAI: Pathogenicity Island
PCR: Polymerase chain reaction
PDR: Pandrug resistance
PFGE: Pulsed field gel electrophoresis
PG: Phylogroup
pMLST: PCR-based replicon subtyping
PRAN: From Spanish "Plan Nacional Resistencia Antibióticos"
purA: Adenylosuccinate dehydrogenase gene

Q: Quinolone

R: Representative isolate
recA: ATP/GTP binding motif gene
Rep: Replication initiator

ShET2: Enteroinvasive toxin
SNP: Single Nucleotide Polymorphism
spp.: Species
ST: Sequence type
STa/STb: Heat-stable toxin
STEC: Shiga toxin-producing *E. coli*
Stx1/Stx2: Shiga toxins genes
SXT: Sulfamethoxazole-trimethoprim

T: Turkey

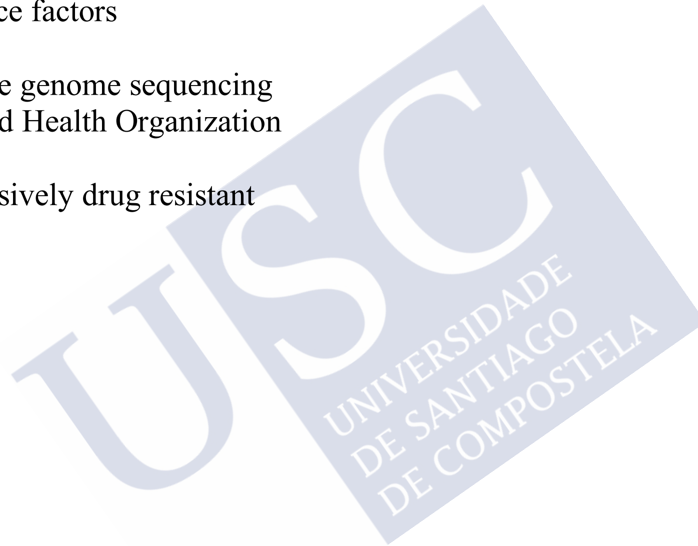
tEPEC: Typical enteropathogenic *E. coli*
TGC: Tigecycline
Tn: Transposons
tnp: Transposase genes
TOB: Tobramycin
Tra: Conjugative systems
TSA: Tryptone soy agar
TSB: Tryptone soy broth
tsh: Temperature-sensitive hemagglutinin gene

UPEC: Uropathogenic *E. coli*
UPGMA: Unweighted pair group method with arithmetic mean
UTI: Urinary tract infection
USC: Universidad de Santiago de Compostela

VF: Virulence factors

WGS: Whole genome sequencing
WHO: World Health Organization

XDR: Extensively drug resistant



1. INTRODUCTION

1.1. *ESCHERICHIA COLI*

1.1.1. General characteristics of *E. coli* and clinical relevance

This bacterium was first described by the German pediatrician Theodor Von Escherich in 1885 after isolating it from the feces of a healthy child. Initially was named *Bacterium coli* (Escherich, 1885).

Escherichia coli is a member of the family Enterobacteriaceae, which includes Gram-negative facultatively anaerobic rod-shaped bacteria (possessing both a fermentative and respiratory metabolism) and which do not produce the enzyme oxidase. *E. coli* cells are typically 1.1–1.5 µm wide, 2–6 µm long and occur as single straight rods. They can be either motile or nonmotile, and when motile produce lateral, rather than polar, flagella. In addition to flagella, many strains produce other appendages such as fimbriae or pili, which play a role in the attachment to other cells or host tissues. *E. coli* carry strain-specific O lipopolysaccharide antigens on their cell wall (at least 181 O antigens are currently recognized) and flagella or H antigens if present (53 H types are recognized). There are also 80 different capsular polysaccharide (K) antigens. *E. coli* are serotyped based on the combination of O, H, and K antigens, although generally only the O and H types are listed (Kaper et al., 2004; Desmarchelier and Fegan, 2011).

E. coli is part of the commensal microbiota of the digestive system in warm-blood vertebrates (Hartl and Dykhuizen, 1984). This bacterium fulfills physiological functions such as the acquisition of nutrients for the intestinal epithelium, plays a role in the vitamin K synthesis, processes waste, constantly stimulates the immune system response of the host organism or avoids the colonization of the intestine by other non-desired enteropathogens through competitive inhibition (Kruis, 2004). Due to its intestinal origin, the presence of *E. coli* in environment, food or water samples is used as an indicator of recent fecal contamination or unsanitary practices in food processing plants (Alonso et al., 2007; Odonkor and Ampofo, 2013).

Although most *E. coli* strains play a beneficial or harmless role for their hosts, there are also pathogenic members within this species. These have been classified into two main groups: intestinal pathogenic *E. coli* (InPEC), causing enteric pathologies, and extraintestinal pathogenic *E. coli* (ExPEC), causing pathology outside the digestive system (urinary tract infections, sepsis, meningitis, lung or wound infections, among others). ExPEC strains are the main agent responsible of urinary tract infections in humans (UTI) worldwide, accounting for between 75 and 85% of cases (Foxman, 2010). Pathogenic strains show specific virulence factors (VF) that provides the ability to produce a wide variety of infections in both humans and animals (Russo and Johnson, 2000; Kaper et al., 2004). Traditionally only enteric infections caused by InPEC have been accepted as food-borne pathogens. Within InPEC, seven main subgroups recognized on the basis of specific virulence mechanisms: Shiga toxin-producing or enterohaemorrhagic *E. coli* (STEC / EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and disease-associated adherent-invasive *E. coli* (AIEC) (Nataro and Kaper, 1998; Kaper et al., 2004; Denamur et al., 2021). On the other hand, the ExPEC group includes uropathogenic *E. coli* (UPEC), avian pathogenic *E. coli* (APEC) and neonatal meningitis-causing *E. coli* (NMEC) strains (Riley, 2014).

While InPEC are accurately distinguished from the commensal gut microbiota based on certain VF, this is not as simple with ExPEC since they behave as opportunistic pathogens that can colonize the intestinal environment without causing harm to the host (Riley, 2020). Thus, no set of genes can unequivocally define ExPEC strains or the different categories. So far, they are being categorized due to their isolation from infections located outside of the digestive system, and / or based on the presence of genes statistically associated with the extraintestinal pathogenic potential of the strains, which can be used predictively (Johnson et al., 2003c; Spurbeck et al., 2012). Besides, certain extraintestinal lineages of *E. coli*, such as the pandemic ST131, have been worldwide recognized by their implication in human infections, and also, by their role in the spreading of antibiotic resistances (Riley, 2014; Manges et al., 2019).

In industrial farming, colibacillosis is a highly frequent pathology affecting newborn animals. This syndrome causes significant economic losses due to mortality, weight loss and handling (Gyles and Fairbrother, 2010; Kazibwe et al., 2020). Its control and treatment have been largely based on antimicrobials. Currently, it is assumed that antibiotic used and misused in food producing animal has been playing an important role in the dissemination of multidrug-resistant (MDR) bacteria, which can reach human population through food chain (Mora et al., 2010; Hindermann et al., 2017; García-Meniño et al., 2021b). In fact, there is a great concern that extended-spectrum β-lactamase (ESBL) / AmpC- and carbapenemase-producing Enterobacteriaceae occurring in animals constitutes a public-health issue (EFSA, 2021). ESBL/AmpC genes are mostly located on mobile genetic elements (MGEs) such as plasmids, some of which are regarded as epidemic, and the size of the commensal ESBL/AmpC reservoir in non-human sources is dramatically rising (Viso, 2017). Even the companion animals are described as potential sources of acquisition (Abreu-Salinas et al., 2020).

1.1.2. Population structure of *E. coli*

E. coli presents great plasticity, genetic diversity and its population structure and evolution has been studied in depth over time, being the most extensively characterized prokaryotic model (Tenaillon et al., 2010). The genus *Escherichia* includes three species, *E. albertii, E. fergusonii*, and *E. coli,* and five clades named I to V. The clades are indistinguishable from *E. coli* at the phenotypic level, but divergent at various levels within the nucleotide profile. It has been proved that between the strains that belong to clade I and the ones that belong to *E. coli* there is an exchange of genes of the core genome that does not occur between them and the rest of the genus *Escherichia*. For that reason, it has been suggested that the strains of clade I change their name to *E. coli sensu lato* and those classically known as *E. coli,* to *E. coli sensu stricto* (Denamur et al., 2021).

E. coli sensu stricto has a very well defined and preserved phylogenetic structure, which includes eight phylogenetic groups, divided into two main clusters. The first cluster includes phylogroups B2, D, G, and F, usually associated with ExPEC strains, carriers of a higher number of virulence genes compared to other phylogroups. The second cluster groups the phylogroups A, B1, C and E, mainly including commensal strains and those that cause digestive pathology and. Phylogroup H, associated with phylogroup D, has been recently described (Clermont et al., 2019; Denamur et al., 2021). *E. coli* shows a clonal population structure within which, diversity is generated mainly by mutation events (vertical diversity). Although the species also experiments a very high number of small fragment recombination events, the vertical structure of the population is not affected. The recombination ratio is not the same all

through the chromosome, hence there are regions known as “bastions of polymorphism” where this ratio is higher (Tourret and Denamur, 2016).

The first studies on the *E. coli* population structure were based on the analysis of the serotypes and their global distribution, followed by Multilocus Enzyme Electrophoresis (MLEE), although the latter was quickly discarded because of its low phylogenetic resolution. With the implementation of new molecular typing tools such as Multilocus Sequence Typing (MLST) (Dale and Woodford, 2015), a better visualization of these relationships was achieved. The Achtman seven-gene MLST scheme (*adk, fumC, gyrA, icd, mdh, purA* and *recA*) has been adopted all over the world (Wirth et al., 2006). Currently, this scheme is implemented in EnteroBase, which is an integrated software environment that supports the identification of global population structures within several bacterial genera including *E. coli*. With the use of massive sequencing tools (Whole Genome Sequencing, WGS) and their availability at an affordable price, much more is known about the population structure of the species. The *E. coli* genome is generally formed by 3,900 - 5,800 genes, corresponding to 4,2 – 6,0 Mpb. All the strains of the species share a group of approximately 2,000 genes, known as the core genome, while the total number of genes found in the different strains of this species increases as more of them are sequenced, showing a continuously expanding pangenome. In one study, with the sequencing of 20 genomes, 15,000 genes were described, however, with the sequencing of 1,500 strains, the pangenome increased to 75,000 genes. It is estimated that for each new isolate sequenced, 26 new genes are identified (Denamur et al., 2021). EnteroBase assembles from Illumina short reads and genotypes those assemblies by core genome multilocus sequence typing (cgMLST). Hierarchical clustering of cgMLST sequence types allows mapping a new bacterial strain to predefined population structures at multiple levels. EnteroBase also supports single nucleotide polymorphism (SNP) calls and can also provide a global overview of the genomic diversity within an entire genus (Zhou *et al.*, 2020). Presently, there are recognized more than 10.700 sequences type profiles (ST) and more than 56 clonal complexes (CC) for *E. coli* (last access: 18/05/2021). It is remarkable that the STs most frequently identified within ExPEC strains represent 0.001% of the total STs (ST10, ST12, ST69, ST73, ST95, ST117, ST127, ST131, ST405), but the total number of strains associated with these STs exceeds 19% of the total, making it clear that there are successful linages of *E. coli* (Riley, 2020; Zhou et al., 2020). Within the *E. coli* species, the definition of a clone has been established as an organism descended from a common precursor strain, with similar phenotypic or genotypic traits, being grouped in the same ST (Riley, 2014). Similarly, according to the 2007 modification of the MLST database, CCs are defined as groups of, at least, three STs that share six alleles in a pair-wise comparisons (Wirth et al., 2006).

WGS allows the *in silico* prediction of the classical bacterial typing through computer simulation. For this, there are online tools available to researchers such as those integrated in the Center for Genomic Epidemiology (CGE). Examples of this can be SeroTypeFinder for typing the O and H antigens (Joensen et al., 2015), the MLST tool to determine the ST and CC (Larsen et al., 2012), or the FimTyper used for typing the *fimH* gene (Louise Roer et al., 2017). The typing carried out by these tools is based on the comparison of the problem sequence with the template sequences stored in databases, which present minor variations among themselves. *In silico* analysis tools also allow for a much deeper and powerful analysis such as the construction of phylogenetic trees based on the single nucleotide polymorphism (SNP) of the core genome, which gives us a more accurate information on the historical evolution of the isolates. This tool can be found in the EnteroBase database website (Zhou et al., 2018, 2020).

With this tool we can also see the divergence of the core genome between strains of the same ST and of different STs. An example of this would be the ST131 strains, which show great divergence between each other, being distributed in three clades (A, B, C) and in more than ten subclades. Therefore, even strains that share the same ST can differ substantially in their genetic repertoire (Denamur et al., 2021).

1.1.3. The emergence of virulence in *E. coli*

Virulence is the sum of different factors happening as variable combinations. The evolution of virulence in *E. coli* has exhibited three important mechanisms:

- The acquisition of genes and / or functions through horizontal transfer by MGEs such as plasmids, phages or conjugative and integrative elements. All these acquired elements have in common the presence of a modular mosaic structure, a compendium of various genes of different origins that can generate multiple combinations, potentially developing new phenotypes. The islands of pathogenicity seem to have their origin in the integration of some of these mobile elements in the chromosome. Virulence factors transmitted by mobile elements can be classified into 5 main groups: adhesins, toxins, iron acquisition systems, protectin-invasins and others (Table 1) (Dale and Woodford, 2015; Denamur et al., 2021).
- The inactivation of genes whose expression is incompatible with virulence (antivirulence genes). These genes are useful in a non-pathogenic context since it is an energy saving method, however it is limiting when the bacterium needs to express pathogenicity. Normally this phenomenon is seen in metabolic pathways (Bliven and Maurelli, 2012).
- Point mutations that cause changes in function. These patho-adaptive mutations are particularly well described in the type 1 fimbrial adhesive subunit (*fimH*). In some cases, a variation in amino acids can cause a change in their ability to adhere to different cells, for example, enhancing adherence into the urinary tract, in consequence increasing their urovirulence, while decreasing their intestinal colonization capacity (Sokurenko, 2016).

Table 1. Virulence factors in ExPEC (Dale and Woodford, 2015)

Adhesins	Gene (s)
Adhesion siderophore	*iha*
Dr binding adhesins	*afa/draBC*
E. coli common pilus	*ecpA*
F1C fimbriae	*foc* gene cluster
Heat-resistant haemagglutinin	*hra*
M fimbriae	*bmaE*
N-acetyl D-glucosamina-specific fimbriae	*gaf*
P fimbriae	*papACEFG*
S fimbriae	*sfa/sfaD*
Temperature sensitive haemagglutinin	*ths*
Type 1 fimbriae	*fimH*

Iron acquisition systems	Gene (s)
Aerobactin receptor	*iutA*
Peri-plasmic iron binding protein	*sitA*
Salmochelin receptor	*iroN*
Siderophore receptor	*ireA*
Yersiniabactin receptor	*fyuA*
Protectins and invasins	**Gene (s)**
Colicin V	*cva*
Conjugal transfer surface exclusion protein	*traT*
Group 3 capsule	*kpsMT II*
Increased serum survival	*iss*
Invasion of brain endothelium	*ibeA*
K1/K2/K5 grupo 2 capsule variants	*K1/K2/K5* genes
KpsM II group 2 capsule	*kpsM II*
Outer membrane protease T	*ompT*
Toxins	**Gene (s)**
A-haemolysin	*hlyID*
Cytolethal distending toxin	*cdtB*
Cytotoxic necrotising factor	*cnf1*
Enteroaggregative *E. coli* toxin	*astA*
α-Haemolysin	*hlyA*
Secreted autotransporter toxin	*sat*
Serine protease	*pic*
Vacuolating toxin	*vat*
Others	**Gene (s)**
Colibactin synthesis	*clb & clbB*
Uropathogenic-specific protein	*ups*
Pathogenicity island maker	*malX*
D-serine deaminase	*dsdA*

According to Denamur *et al.* (Denamur et al., 2021), two characteristics of the genetic history of the strains explains the emergence of these virulence mechanisms. First, the emergence of the same virulence mechanism is repeated several times throughout the evolutionary history of the bacterium until it is fixed, which is why it is considered that this convergent evolution is a strong sign of selection regarding certain characteristics. Secondly, the phylogeny of the strain plays an important role since strains of the phylogroups B2, D, F and G carry numerous virulence genes and are usually isolated in extraintestinal infections, while strains belonging to phylogroups A, B1, C and E are less virulent (Sannes et al., 2004; Clermont et al., 2019). Therefore, these mechanisms have a higher incidence in certain strains associated with specific phylogroups.

Virulence is also the result of the additive effect of different virulence factors present within the bacteria. For example, studies on the virulence of *wild type* strains, in which a specific pathogenicity island (PAI) was eliminated, the same level of virulence was observed when assayed in a murine model. Even knocking out islands known as "highly pathogenic" (PAI IV536 carrying the yersinabactin gene), strains did not show a decrease in the virulence of the modified strain. However, when several islands were knocked out, a decrease in virulence was observed, being able to conclude that the pathogenicity islands have an additive effect. This fact evidences the great genetic complexity of virulence in these strains (Tourret and Denamur, 2016). However, the number of virulence factors correlates with the intrinsic virulence of the strains. Those with a high content of virulence factors killed more than 80% of the mice in a murine septicemia model, while strains with a lower content of virulence factors caused less than 10% of casualties (Tourret and Denamur, 2016).

When the clinical relevance of virulence is considered, we cannot forget that infections are the result of the interaction between the pathogen and the host. Considering the hierarchy described by Tourret and Denamur (Tourret and Denamur, 2016) about the importance of the factors involved in extraintestinal clinical infections, we would first have to consider the factors related to the host such as the species, gender, age, previous and / or underlying pathologies, as well as the state of the immune system. The microbiota is also an important not fully understood. These factors condition the phylogenetic group of the strains responsible of the infection. Thus, in immunocompromised patients, the isolation of strains of phylogroups A and B1 is more frequent. On the other hand, in patients without predisposing factors who present pyelonephritis or urosepsis, strains of the phylogroup B2 with numerous virulence factors are the most frequently isolated. The most important risk factors for a patient decease in cases of *E. coli* septicemia are related with the patient, his/her age, possible cirrhosis, previous hospitalizations, immunosuppression or the cutaneous origin of the septicemia. Only one bacterial factor, the capsule, has been recognized as promoting the translocation of bacteria from the urinary tract to the bloodstream. When septicemias originate from the urinary tract, they seem to be associated with more virulent strains, frequently from the B2 phylogroup, although these have a better prognosis than infections caused by strains from the phylogroups A and B1, which usually have their origin mainly in the digestive tract (Lefort et al., 2011). Regarding antibiotic resistance, it is of note that the strains of phylogroup B2, although they tend to be more virulent, they are usually sensitive to antibiotics, except in some cases such as the high-risk pandemic clonal group ST131. In contrast, the strains of phylogroups A and B1 present a lower number of virulence factors and yet a higher resistance rate (Jauréguy et al., 2007; Krieger et al., 2011).

1.1.4. Intestinal *E. coli* pathotypes (InPEC)

As mentioned in section “1.1.1. General characteristics of *Escherichia coli* and clinical relevance”, three large groups can be differentiated according to their ability to cause pathology. First, the commensal strains, which do not normally cause harm on the host. On the other hand, InPEC strains that cause digestive or intestinal pathology present a set of virulence factors clearly defined and associated with each pathotype. And finally, the strains that cause extraintestinal pathology (ExPEC), which do not have specific virulence factors that allows their clear categorization, but are defined according to their pathogenicity mechanisms, the infections or syndromes that they cause or the point of isolation of the strains (Blanco et al., 2002). In the last decade, the increasing emergence of clones that fulfil the criteria of different

pathotypes has been reported, defining the new concept of hybrid pathotype. This situation is caused by the great plasticity of the genome of this bacterium. An example of it would be the outbreak that took place in Germany in 2011 where an *E. coli* strain with virulence factors of the enteroaggregative pathotype and producer of Shiga toxins caused an outbreak associated with the consumption of fenugreek seeds (Mora et al., 2011b).

Unlike commensal and ExPEC strains, InPEC strains rarely appear in the feces of healthy hosts and their presence is associated with digestive pathology if they are ingested in sufficient quantity (Russo and Johnson, 2000). Diarrheagenic pathologies are one of the major causes of mortality and morbidity among children under five years, with an annual death count of 718,000 and 1,731 million cases, the incidence of diarrheal processes in children was 2,7 episodes per year in 2011 (Walker et al., 2013). InPECs are classified into seven categories based on their clinical and epidemiological characteristics and virulence factors: STEC and / or EHEC, EPEC, ETEC, EIEC, EAEC, DAEC and AIEC (Table 2).

Shiga toxin-producing and / or enterohemorrhagic *E. coli* (STEC and / or EHEC): The group is characterized by the production of Shiga toxins (Stx1 and / or Stx2) (also called verotoxins, VT1 and / or VT2) transmitted by prophages, but only strains that also contain the pathogenicity island LEE "locus of enterocyte effacement" are considered EHEC (Kaper et al., 2004). The strains of this group were first described in 1977 by Konowalchuk *et al.* (Konowalchuk et al., 1977), and are the main cause of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Since its first description, its clinical importance has been increasing, and currently it is still among the main infectious agents responsible for gastroenteritis (CDC, 2019b). Verotoxins are produced in the colon and can travel through the bloodstream to the kidneys where they damage the endothelial cells and occlude the microvasculature through the combination of direct cytokine toxicity and the production of chemokines, which cause kidney inflammation. This damage is what triggers HUS, although the most common clinical presentation is HC, with a common combination of symptoms as abdominal pain and bloody diarrhea without fever. Only in approximately 10% of cases HC ends up developing HUS. This causes hemolytic anemia, thrombocytopenia and acute kidney failure, in some cases requiring dialysis or even transplantation. Its mortality rate is between 5 and 10% (Friedrich et al., 2002; Kaper et al., 2004).

This pathotype is characterized by the production of two important toxins that are cytotoxic to Vero cells and that is the reason they are known as verotoxins (VT1 and VT2), and as they are similar to toxins produced by *Shigella* spp. they are also known as Shiga toxins (Stx1 and Stx2). There are several subtypes, being the Stx2a variety the most powerful toxin, followed by Stx2d and Stx2c, commonly associated with HUS (Friedrich et al., 2002; Feng et al., 2011). These toxins are encoded in the genome of prophages integrated in the bacterial chromosome and are formed by two subunits, an enzyme subunit A of 33,000 d of molecular weight and 5 or 6 subunits B 7,500 d that bind the toxin to cellular receptors composed of glycolipids and inhibit cell protein synthesis by catalytically inactivating the 60S ribosomal subunit (Muniesa et al., 2003; Allison, 2007).

Most outbreaks have been associated with strains of the O157:H7 serotype. According to European Food Safety Authority / European Centre for Disease Prevention and Control (EFSA / ECDC) data from 2020, 38.4% of HUS cases, 60.4% of hospitalizations and 71.6% of bloody diarrhea cases caused by STEC between 2012 and 2017 were caused by this serotype. The

second serotype in order of clinical importance (9.8%) is O26:H11. Regarding sporadic cases, the prevalence between O157 and non-O157 ECVT is very similar, even being the predominant non-O157 (Koutsoumanis et al., 2020). Due to the prominent presence of serogroup O157 and its association with severe cases of HC and HUS, STEC are usually classified in O157 and non-O157 strains. It is clearly demonstrated that cattle act as the main reservoir of STEC. However, small ruminants, sheep and goats are also important carriers. Even wild ruminants can act as reservoir. During carcass processing operations in slaughterhouses, and especially during skinning and evisceration, strains of *E. coli* from the animal's intestinal microbiota reach the surfaces of the carcasses. The same occurs during milking, in which there is a risk of contamination of the milk with intestinal bacteria. The only way to avoid food poisoning is to heat the meat or pasteurize the milk to ensure bacterial inactivation. Especially dangerous are minced meat and derived products such as hamburgers, since microorganisms are found throughout their mass, it is not enough to heat the surface part. In fact, most of the outbreaks have been due to the consumption of hamburgers and therefore HC is known in Anglo-Saxon countries as "the disease of hamburgers". In addition, animal feces can contaminate the environment, especially irrigation water, which in turn contaminates vegetables and fruits (López Capón and Capon López, 2018)

Enteropathogenic *E. coli* (EPEC): This was first described in England in 1945, associated to large outbreaks of infant diarrhea (Kaper et al., 2004). EPEC is still a cause of infant mortality in developing countries. This pathotype is typically carrier of the *eae* gene as part of the pathogenicity island LEE, which encodes a protein called intimin. Intimin is responsible for the intimate adherence of the bacteria to the enterocyte membrane, and the attaching and effacing lesion (AE) of the borders of the intestinal microvilli (Caron et al., 2006). The protein encoded by the *eae* sequence has a C-terminal variable ending that defines more than 30 types and subtypes of intimins associated with tissue tropism (Blanco et al., 2004; Mora et al., 2009a). EPEC strains are classified as typical (tEPEC) when they carry a plasmid called pEAF (EPEC adherence factor) that encodes a type IV pilus, BFP (bundle-forming pilus) responsible for mediating distant adherence between bacteria and epithelial cells. Atypical strains (aEPEC) are those that produce the AE lesion but do not carry the *bfpA* gene and therefore do not express the adhesin BFP (Hernandes et al., 2009; Mora et al., 2009a). Some epidemiological and experimental studies support the hypothesis that some aEPEC could have evolved from STEC / ECVT that had lost their *stx* genes (Afset et al., 2008; Scaletsky et al., 2009; Horcajo et al., 2012).

Currently, aEPEC strains are one of the emerging enteropathogens detected worldwide in different niches (animal species, environment and food samples), meanwhile the main reservoir of tEPEC strains are humans (Otero et al., 2013; Alonso et al., 2017). tEPEC is clearly related to diarrhea in children under one year and they are the main cause of endemic diarrhea in developing countries (Regina et al., 1983; Gomes et al., 1991; Trabulsi et al., 2002). However, there are controversial reports regarding the epidemiologic association of aEPEC with diarrhea since they are also frequently isolated from healthy individuals. In Spain, aEPEC is routinely isolated in stool cultures of patients with diarrhea and other gastrointestinal disorders. In a LREC study carried out between the years 1996 and 1999, aEPEC strains accounted for 5.2% of the patients with diarrhea or digestive disorders, while tEPEC strains for 0.2% (Blanco et al., 2006). In a second study carried out in the period 2005-2013, a significant increase in infections caused by EPEC was detected (0.6% tEPEC and 11.5% aEPEC), supporting the hypothesis of the latter as an emerging cause of diarrhea in developed countries (Blanco et al., 2006).

Enterotoxigenic *E. coli* (ETEC): This pathotype was first reported in diarrhea associated with colibacillosis in neonatal and recently weaned pigs (Melkebeek et al., 2013). In 1971 DuPont *et al.* demonstrated that ETEC strains could cause diarrhea in humans by infecting adult human volunteers (DuPont et al., 1971). ETEC, together with EPEC and rotaviruses, are the pathogens most frequently reported as cause of childhood digestive disease and traveler's diarrhea in developing countries (World Health Organization (WHO), 2005; Denamur et al., 2021)(Denamur et al., 2021). ETEC strains colonize the mucosa of the small intestine by means of the fimbrial adhesins known as colonization factor antigens (CFA), and release two enterotoxins: heat-labile toxin (LT) and / or heat-stable toxin (STa/STb). The heat-labile toxin is related to the choleric enterotoxin expressed by the bacterium *Vibrio cholerae* (Sixma et al., 1993). There are two types of heat-stable toxins with different structure and mechanisms of action. One would be the STa toxin associated with human, porcine and ruminant strains and STb toxin associated with mainly pig strains. The ETEC strains that cause infections in humans possess intestinal colonization antigens (CFA / I, CFA / II, CFA / III, CFA / IV) different from those present in the ETEC strains that cause colibacillosis in pigs (K88, K99, F41, P987, F18) and in ruminants (K99, F41).

Enteroinvasive *E. coli* (EIEC): This pathotype is very similar to the members of the genus *Shigella* from the biochemical and genetic point of view and due to their pathogenicity mechanisms. Molecular evolution studies suggest that the different *Shigella* species arose from the acquisition of different virulence plasmids by *E. coli* strains (Yang et al., 2007). These strains invade the cells of the intestinal epithelium where they produce an enterotoxin (ShET2) encoded in a plasmid gene, *sen*. The pathogenicity mechanism follows several steps, firstly the bacterium penetrates the interior of the cells of the colon mucosa by endocytosis, then it produces the lysis of the enterocytic vacuole, to multiply intracellularly and moving through the interior of the cell cytoplasm until produce lateral spread to adjacent cells (Kaper et al., 2004). The symptoms of this type of infection include watery diarrhea, dysentery in some cases manifested with blood, mucus, leukocytes in the stool and fever. Ulcers can appear in severe cases (Nataro and Kaper, 1998).

Enteroaggregative *E. coli* (EAEC): The EAEC pathotype has been defined by its aggregative pattern of adherence to tissue culture cells. In developing countries, EAEC strains are associated with persistent childhood diarrhea, lasting up to 14 days. These strains present fimbriae that allow aggregative adherence to HEp-2 cells and the intestinal epithelium. They induce an increase in mucus secretion that leads to the formation of a biofilm where bacteria are trapped, allowing a more persistent colonization of the area and a worse absorption of nutrients at the intestinal level. EAEC strains produce a heat-stable enterotoxin known as EAST1 and a cytotoxin that is suspected of being responsible for diarrhea and characteristic histopathological lesions with shortening of the microvilli (Nataro and Kaper, 1998; Okeke and Nataro, 2001). The pCVD432 (pAA) gene associated with the enteroaggregative adherence pattern is usually targeted to investigate presence of EAEC (Nataro and Kaper, 1998; Piva et al., 2003; Blanco et al., 2005).

Diffuse adherent *E. coli* (DAEC): DAEC strains are defined based on the presence of a diffuse adherence pattern (DA) on HeLa and HEp-2 epithelial cells. In the DA pattern, bacteria uniformly cover the cell surface (Scaletsky et al., 2002). According to the adhesin expression, two groups of DAEC strains have been identified, Afa/Dr DAEC and AIDA-I DAEC. Afa/Dr DAEC strains are associated with acute diarrhea in children, especially in those 6 months and

older, with persistent diarrhea. The Afa/Dr family includes fimbrial and afimbrial adhesins: Afa-I, Afa-II, Afa-III, Afa-V, Afa-VII, Afa-VIII (afimbrial); plus Dr-2 as well as Dr and F1845 (fimbrial). Many of these adhesins have been identified in *E. coli* strains isolated from human UTIs or diarrhoea, except Afa-VII, which was only found in *E. coli* isolated from bovine faeces. F1845 adhesin was first identified in an *E. coli* strain (C1845) isolated from a child with chronic diarrhoea. DAEC could play an important role in the induction of inflammatory bowel disease (Lalioui et al., 1999; Kaper et al., 2004; Lozer et al., 2013; Servin, 2014; Denamur et al., 2021).

Adherent-invasive *E. coli* (AIEC): The main characteristics of AIEC are the ability to adhere to and invade intestinal epithelial cells, and the ability to survive and replicate expansively within macrophages without triggering host cell death and inducing the release of tumor necrosis factor alpha. AIEC strains, which are associated with Crohn's disease, share many genetic and phenotypic features with extraintestinal pathogenic *E. coli* (ExPEC) strains. However, the majority of ExPEC strains did not behave like AIEC strains, confirming that the AIEC pathovar possesses virulence-specific features that, to date, are detectable only phenotypically (Darfeuille-Michaud et al., 2004; Martinez-Medina et al., 2009; Denamur et al., 2021).

Table 2. Main characteristics of InPEC bacteria (Denamur et al., 2021).

Pathotype	Definition	Main strain host	Main virulence genes	Main PG asociated
STEC / EHEC	Verotoxin producing isolates	Human, cattle, sheep	*stx, eae, ehxA*	B1 and E
EPEC	*Attaching and effacing* lesions in intestinal epithelial cells	Humans, domestic mammals	*eae, bfpA*	A, B1, B2 and E
ETEC	Heat-labile and heat-stable enterotoxins producers	Humans, cattle, pig	Coding genes for LT and STa enterotoxins and colonization factors	A, B1, C and E
EIEC	Strains with intestinal invasion ability	Strictly humans	*ipa, isc, vir*. Inactivación de *nadA, nadB* y *cadA*	A, B1 and E
EAEC	Aggregative adhesion on enterocytes	Humans and domestic mammals	Aggregative adherence fimbriae (*aaf/agg*) and transcriptional genes (*aggR*)	A, B1, B2 and D
DAEC	Diffuse adhesion on enterocytes	Humans	Genes encoding adhesins (*afa* and *dra*)	All phylogroups
AIEC	Adhesion and invasion of intestinal epithelial cells	Humans	Unknown	All phylogroups with a majority of B2

1.1.5. Extraintestinal pathogenic *E. coli* (ExPEC)

E. coli is one of the predominant microorganisms in extraintestinal infections in both humans and animals, causing many types of infections. As mention previously, a great diversity of virulence genes associated with ExPEC strains that encode adhesins, toxins, siderophores, protectins, capsular antigens, invasins, etc. have been described (Table 1) (Kaper et al., 2004; Riley, 2014). ExPEC strains have been alternatively defined by the number and constellation of virulence genes they possess ("special pathogenicity" definition) and by their identification as predominant lineages in the gut prior to causing extraintestinal infections by mass action ("prevalence" definition) (Johnson et al., 2001). Johnson *et al.* (2003) determined 5 predictive virulence markers of the ExPEC status: *papA* and / or *papC* (encode P fimbria), *sfa / foc* (F1C and S fimbriae), *afa / dra* (Afa / Dr adhesins), *iutA* (aerobactin) and *kspMII* (group II capsule). *E. coli* strains conform the ExPEC status if carry two or more of these five markers (Johnson et al., 2003b). But despite the overrepresentation of classic ExPEC virulence genes in main lineages causing infection, there is still uncertainty about what defines or differentiates commensal *E. coli* and facultative ExPEC pathogens. Thus, Manges *et al.* (Manges et al., 2019) reviewed and meta-analyzed 217 studies (1995 to 2018) that performed multilocus sequence typing or whole-genome sequencing to genotype *E. coli* recovered from extraintestinal infections or the gut. As a conclusion, the authors found that a discrete set of ExPEC lineages contributes to the enormous burden of human extraintestinal infections. Twenty major ExPEC sequence types (STs) accounted for 85% of *E. coli* isolates from the included studies, including (by decreasing study positivity): ST131, ST69, ST10, ST405, ST38, ST95, ST648, ST73, ST410, ST393, ST354, ST12, ST127, ST167, ST58, ST617, ST88, ST23, ST117, and ST1193. ST131 was detected in over 90% of studies, however, it is important to take into consideration that emphasis on MDR ExPEC ST131 has unfortunately created a gap in our knowledge about other important ExPEC lineages, such as ST73 and ST95.

Most of the global ExPEC lineages are frequently determined in isolates from animal sources (farming, companion an also wildlife) (Mora et al., 2009b, 2010, 2013; Cortés et al., 2010; Jørgensen et al., 2019; Abreu-Salinas et al., 2020) . Such is the case of the recent study on three pinniped species (*Leptonychotes weddellii*, *Mirounga leonina* and *Arctocephalus gazella*) from the west coast of the Antarctic Peninsula, which were analyzed for the presence of *Escherichia* spp. As a result, 62 of the 158 *E. coli* isolates (39.2%) exhibited the ExPEC status and 27 (17.1%) belonged to top STs frequently occurring among urinary/bacteremia ExPEC clones: ST12, ST73, ST95, ST131 and ST141 (Mora et al., 2018)). There is a division in opinion regarding the idea of transmission of ExPEC strains between animals and humans. Most authors support this idea due to the genetic similarity that can be appreciated between animal strains, mainly those of avian origin, with strains that cause extraintestinal infections in humans (Johnson and Russo, 2005; Moulin-Schouleur et al., 2006; Jørgensen et al., 2019), however other authors have doubts about the clarity of these indications and argue that the most important transmission route is human-human and that strains of zoonotic origin would be minor (Schwarz et al., 2017). What it is becoming increasingly clear is that we cannot continue to work at the level of a single species anymore. A recent reformulation of the classic One Health approach emphasizes the role of interconnected (and hence geographically close) ecosystems in the emergence and dissemination of traits that influence local human, animal, plant, and integrated environmental health, such as antibiotic resistance. The increasing anthropogenic effects on the biosphere (such as globalization) might reduce the diversity of

niches and bacterial individuals, with the potential emergence of highly transmissible multispecialists (Baquero et al., 2019, 2021).

As stated in 1.1.1., the classical classification of the ExPEC group includes UPEC, NMEC and APEC, and they exhibit a considerable genomic diversity and a wide range of virulence-associated factors which do not allow a clear categorization. Nevertheless, these subgroups are defined according to their pathogenicity mechanisms, the infections or syndromes that they cause or the point of isolation of the strains:

Uropathogenic *E. coli* (UPEC): *E. coli* is recognized as the main cause of UTIs in humans and is commonly caused by autoinfections from the host itself (Yamamoto et al., 1997; Nataro and Kaper, 1998). The pathogenesis of a UTI involves several steps, first the periurethral and vaginal colonization for UPEC isolates, followed by the ascension into the bladder lumen and growth in the urine. The bacteria interact and adheres to the surface of the bladder epithelium and forms biofilm. At this point, the bacteria grow forming communities who will stay in the underlying urothelium. From here is where the UPEC isolates can colonize the kidney, cause tissue damage and generate great risk of septicemia (Terlizzi et al., 2017).

It has been accepted that the main source of these isolates is the human gut microbiota, nevertheless, external reservoirs and host to host infections need to be studied. An important part of the prevention of the spread of these pathotype is to be able to identity the reservoirs in order to reduce the risk of disease transmission. Spurbeck *et al.* (Spurbeck et al., 2012) described for it a set of four genes (*yfcV, vat, fyuA* and *chuA*) that present a statistical association with the strains causing urinary pathology and consider that the strains that possess at least three of these virulence genes as UPEC. Whit this knowledge the aim is to identify the carriers and, to investigate why some individuals are more susceptible to the colonization and recolonization of UPEC isolates than others.

Some authors pointed out the similarity between virulence profiles from both UPEC and APEC isolates, highlighting the potential of the latter to cause a zoonotic infection mainly when associated with plasmids and pathogenicity islands of UPEC, also pointing out the possibility of the APEC isolates to act as possible reservoirs of urovirulence genes for humans. Nevertheless, there are still some significant differences in the prevalence of virulence traits between the two groups that suggest that not all APEC would be potentially involved in human infections (Mora et al., 2009b; Jørgensen et al., 2019).

Avian pathogenic *E. coli (*APEC): These isolates are responsible for avian colibacillosis in wild birds as well as domesticated ones. The clinical presentation of the infection usually starts with respiratory symptomatology but also is associated with non-specific signs as weakness, depression, reduced appetite, poor growth that could evolve to a systemic infection that affects internal organs, presenting with fibrinous lesions as airsacculitis, pericarditis or perihepatitis, usually associated with septicemia (Solà-Ginés et al., 2015; Kazibwe et al., 2020).

There is no unique virulence profile for this pathotype, nevertheless is frequently associated with virulence genes that allow their extraintestinal survival and colonization. This genes are usually harbored by plasmids and by its acquisition, an avian commensal strain can enhance its abilities to kill chicken embryos, grow in human urine and colonize the murine kidney on in vivo assays (Skyberg et al., 2006; Mora et al., 2013).

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As mentioned before, this pathotype has been hypothesized numerous times with the possibility that they constitute a zoonotic risk, since this strains even though they show a higher virulence in poultry, are able to cause infections in mammals. Jorgensen *et al.* demonstrated the existence of multiple lineages belonging to the ExPEC lineage ST95, where the majority may cause infection in humans, only a part of the ST95 cluster was able to cause avian infection, supporting the zoonotic hypothesis (Ron, 2006; Skyberg et al., 2006; Mora et al., 2013; Maluta et al., 2014; Jørgensen et al., 2019).

Neonatal meningitis-causing *E. coli* (NMEC): These isolates can cause bacterial meningitis in newborns (NMEC) in different hosts. The mortality rate of these infections is found between 15 and 40%, leaving neurological sequelae in 50% of cases. (Nataro and Kaper, 1998; Wang and Kim, 2013). As well as the previous ExPEC subgroups, these isolates can be found in meat-source samples and are proved to have overlapping traits shared between them (Mellata et al., 2018). In order to cause meningitis, the bacteria have to invade the blood-brain barrier and penetrate into the brain, and so as to it the cytotoxin necrotizing factor 1 (CNF1) has been described as an associated virulence factor to this subgroup of ExPEC.

1.1.6. ST131 and other high-risk clones

As stated above, the rapid dissemination of ESBLs seems to be largely associated with the so-called successful ExPEC lineages such as the ST131, ST38, ST69, ST405, ST648 or ST1193 (Shaik et al., 2017; Yamaji et al., 2018a; Manges et al., 2019). For a clone to be considered high-risk, it must meet the next six criteria (Mathers et al., 2015; Pitout and Finn, 2020):

- To exhibit global distribution.
- To be associated with multiple determinants of resistance.
- To have the ability to colonize and persist in a host for at least six months.
- To be able to effectively spread between different hosts.
- To have an improved pathogenicity and aptitude compared to other clones.
- To have the ability to cause severe or recurrent infections.

ST131 is the main pandemic clone responsible for the global spread of ESBLs. First identified in 2008, ST131 strains belong to phylogroup B2 and mainly to the serotypes O25b:H4 or, less frequently, O16:H5 (Coque et al., 2008; Nicolas-Chanoine et al., 2008). Three years after its first isolation, it was already spread, being the bacterial agent involved in more than 50% of cases of UTIs caused by ESBL-producing strains in numerous hospitals in different countries. Normally, phylogroup B2 strains are characterized by being associated with a significant load of virulence genes, however, in addition in the case of this clone, its association with genes of resistance to both ESBL and fluoroquinolones is remarkable. The prevalence of resistance to first-line oral antibiotics such as trimethoprim-sulfamethoxazole, amoxicillin, and amoxicillin-clavulanate has been steadily increasing during these years, making the treatment of infections very difficult and endangering the lives of patients (Mora et al., 2011a, 2014; Dahbi et al., 2013, 2014; Nicolas-Chanoine et al., 2014, 2017; Ghizlane Dahbi Zbiti, 2015; Mamani et al., 2019).

Although it is associated with ExPEC infections such as UTI, septicemia, surgical wound infections and meningitis, this clone is also frequently found in the digestive system of healthy humans (Leflon-Guibout et al., 2008; Zhong et al., 2015; Rodrigues et al., 2016; Cortés-Cortés

et al., 2017)That is why, it was thought that human intestinal tract was ST131 only niche. However, the growing scientific community interest towards this ST, detected it in all diverse sources such as companion, food-production and wild animals; or the environment itself in rivers, beaches or sewage; even in the Antarctic region (Coelho et al., 2011; Colomer-Lluch et al., 2013; García-Meniño et al., 2018; Mora et al., 2018; Pitout and Finn, 2020).

WGS analysis had revealed that ST131 consists of three different clades (A, B, and C) characterized by different alleles of the *fimH* gene that is implicated in the colonization abilities, i.e., *fimH41*, *fimH22*, and *fimH30*, respectively (Petty et al., 2014; Ben Zakour et al., 2016). The first expansion of clone ST131 was described in the United States and the predominant subclade was H30. This clone is defined by the presence of fimbrial adhesin *fimH*, allele 30 (H30). Within this, new subclades emerged as H30R, which includes point mutations in the *gyrA* and *parC* genes that confers them resistance to fluoroquinolones. Other subclade emerged from H30 is H30Rx, where in addition to point mutations in *gyrA* and *parC*, it is associated with the presence of the $bla_{CTX\text{-}M\text{-}15}$ gene (Stoesser et al., 2016).

Clade A, associated with the *fimH41* allele and the O16:H5 serotype, arose in Southeast Asia around 1880 and this is usually an antibiotic-sensitive clade. This clade is located on the longest branch of the ST131 phylogeny and because of it, this clade has been the one with more evolutionary changes accumulated. When compared with clade B/C a different plasmid and phage collection can be seen between them, and the hypothesis for it is that rarely these clades share ecological niches at the same time, making them have differentiated accessory genome elements (McNally et al., 2019; Pitout and Finn, 2020). Clade B, associated with strains predominantly carriers of the *fimH22* allele and of serotype O25b:H4, is suspected of having its origin in the 1900s in North America. Like clade A, this is typically an antibiotic-sensitive clade. This fact means that due to the bias of most studies designed to detect ESBL-producing strains, it could be underrepresented within the entire ST131 population. The studies by Zakout *et al.* and Flament-Simon *et al.* describe subclades within B between B0 and B9 (Ben Zakour et al., 2016; Flament-Simon et al., 2020c). Dean and Downing suggest that the subclade B0 was the one that led the evolution towards clade C (Decano and Downing, 2019). Clade C shows three clearly defined subclades. Clade C0, associated with *fimH30* and O25b:H4, evolved in the 1980s from strains from clade B in North America. After seven years, this clade separated, giving rise to the subclades C0, C1 and C2. The biggest difference between the original clade C0 and its derivatives C1 and C2 is in the susceptibility to fluoroquinolones, since C0 arose before they began to be used regularly in both human and veterinary clinical practice, therefore, is sensitive to this antibiotic. Clades C1 and C2 are considered successful globally due to their rapid and widespread. Both are resistant to fluoroquinolones due to two-point mutations in the *gyrA* and *parC* gene that, once introduced via recombination, were vertically transmitted, getting integrated into the clade. The main difference between clade C1 and C2 is that C2 (also known as subclone *H*30Rx) is also strongly associated with the production of extended spectrum β-lactamases (ESBL), normally $bla_{CTX\text{-}M\text{-}15}$, and seems to be the most expanded and successful ST131 so far (Banerjee et al., 2013; Price et al., 2013; Dahbi et al., 2014; Peirano et al., 2014; Sauget et al., 2016; Pitout and Finn, 2020). However, cluster C1-M27 of subclade C1, which produces CTX-M-27, has recently expanded, first in Japan (Matsumura et al., 2016, 2017), then in other countries (Thailand, Australia, Canada, USA, France, Italy, Germany, The Netherlands and Spain) (Blanc et al., 2014; Birgy et al., 2016; Bevan et al., 2017; Merino et al., 2018; Peirano and Pitout, 2019).

In 2013, Blanco *et al.* (Blanco et al., 2013) described for the first time a classification of the strains of the clone ST131 in four clusters regarding their virulence genes. This clusters where named virotypes, were found internationally distributed and that corresponded with pulsed-field electrophoresis profiles (PFGE). Later on, on their study Dahbi *et al.* (Dahbi et al., 2014) analyzed a total of 154 *E. coli* isolates from ST131 recovered between 2005 and 2012 from 5 different Spanish hospitals and studied the presence and absence of 32 genes coding for virulence factors typically associated with extraintestinal pathology as well as their PFGE profile. From the results, 12 virotypes and subtypes were described, A, B, C1, C2, C3, D1, D2, D3, D4, D5, E and F. The association between each virotype and virulence profile can be found in Table 3.

Table 3. Virulence-gene scheme for defining ST131 *E. coli* virotypes (Dahbi et al., 2014)

Virotype	*afa / draBC*	*afa* operón FM955459	*iroN*	*sat*	*ibeA*	*papG II*	*papG III*	*cnf1*	*hlyA*	*cdtB*	*neuC-K1*	*kpsM II-K2*	*kpsM II-K5*
A	+	+	-	+/- [a]	-	-	-	-	-	-	-	+	-
B	-	-	+	+/- [a]	-	+/-	-	-	-	-	-	-	+/-
C1	-	-	-	+	-	-	-	-	-	-	-	+	-
C2	-	-	-	+	-	-	-	-	-	-	-	-	+
C3	-	-	-	+	-	-	-	-	-	-	-	-	-
D1	-	-	+/-	-	+	-	-	-	-	+	-	-	+
D2	-	-	+/-	-	+	-	+	-	-	+	-	-	+
D3	+/- [b]	+/- [b]	+/-	+/- [b]	+	-	-	-	-	-	-	-	+
D4	-	-	+/-	-	+	-	-	-	-	-	+	-	-
D5	-	-	+/-	-	+	-	+	+	+	-	-	-	+
E	-	-	-	+	-	+	-	+	+	-	-	-	+
F	-	-	-	+	-	+	-	-	-	-	-	-	+

[a] Most strains of virotypes A (97%) and B (75%) are positive for the *sat* gene [b] Virotype D3 strains carry the *sat* and *afa/draBC* genes, or at least one of them. Furthermore, some *afa/draBC* positive strains are also positive for *afa* operon FM955459

An important challenge is to know which determinants make certain clones adapt to a specific host meanwhile others can be transmitted between different species, with jumps as important as between mammals and birds. In the case of ST131, this relationship between the different clades and their presence in different hosts has yet to be completed understood. With the current data, is noticeable that clades A and C are mainly associated with human pathology, while clade B groups strains isolated from different niches such as poultry and pigs, along with humans (Flament-Simon *et al.*, 2020). In fact, we recently proved the genomic identity of porcine (meat and animal origin) and clinical human ST131-*H22* isolates belonging to the new subclades B6 and B7 (Flament-Simon et al., 2020c). With a different approach, Liu *et al.* (Liu et al., 2018) combined detection of poultry-associated ColV plasmids with high-resolution phylogenetics to quantify the proportion of human extraintestinal infections. From their results, the authors stated that sub lineage ST131-*H22* has become established in poultry populations around the world and that meat may serve as a vehicle for human exposure and infection. According to the authors, ST131-*H22* would be just one of many *E. coli* lineages that may be transmitted from food animals to humans. Accordingly, a growing number of studies suggests

APEC strains as an external reservoir for human ExPEC strains, including UPEC (Jørgensen et al., 2019). The results of the study conducted by Jørgensen *et al.* (Jørgensen et al., 2019) demonstrate, via data collected through WGS that there are multiple ST95 lineages, most of which cause infection in humans while only a part of them cause avian pathology. Within these branches, there is overlap between strains of both origins. This overlap can be understood as evidence of a zoonotic capacity of a group of strains from this ST. Regarding the association between genes and the origin of isolation of the strain, only the *iss, papC, vat* and *sitA* genes seem to be associated with the APEC strains, while *cgsA* and *fimH* appear to have a strong association with the human ExPEC strains.

In recent years, an emerging high-risk lineage belonging to phylogroup F, ST648, has been described. A particular characteristic of this clonal complex CC648 is the absence of the *uidA* (β-glucuronidase) gene, which has made this CC to be underrepresented in all the samplings in which the techniques for the detection of *E. coli* were based on the β-glucuronidase detection (Johnson et al., 2017a). ST648 together with ST131 represented 44% of the ESBL strains isolated in a water treatment plants, a very high percentage of the total of strains recovered. Through faeces, since humans are a known reservoir of ExPEC strains, and urine of patients with UTIs caused by UPEC strains, the presence of bacteria in the wastewater of populations has increased significantly and the water processing plants and the environment have begun to be considered an adequate sampling point to be representative of the presence of these bacteria in a population (Paulshus et al., 2019). The presence of the high-risk clonal groups ST131 and ST648, both carriers of resistance, has been described in aquatic environments in different countries such as Brazil (Furlan et al., 2020), or in water purification systems in Norway (Paulshus et al., 2019) it makes clear the importance of the dissemination of these resistances worldwide through environmental niches. Wastewater, wastewater treatment plants and canalization systems are considered reservoirs for bacteria that potentially carry resistance genes, which can benefit from this scenario to carry out the horizontal transfer of resistance genes to both antibiotics and metals or disinfectants. All stated above, makes the One Health approach a priority.

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1.1.7. Hybrid clones of *E. coli*

Hybrid pathotypes of *E. coli* are frequent and unpredictably emerging due to the important role played by MGEs such as plasmids, bacteriophages, pathogenicity islands, transposons and insertion sequence elements in the evolution of the bacteria (Stokes and Gillings, 2011; Robins-Browne et al., 2016). Furthermore, strains with complex hybrid pathotypes with combinations of two different groups of InPEC (STEC + EAEC) or InPEC and ExPEC (for example aEPEC + ExPEC and STEC + APEC) are increasingly reported in human clinical cases (Denamur et al., 2021)

Since 2011, when a novel Shiga-toxin-producing *E. coli* (STEC) belonging to serotype O104:H4, with virulence features common to the EAEC and CTX-M-15 producer was identified as the one involved in the large German outbreak (Mora et al., 2011b), the concept of pathotype has been questioned. More recently, Lindstedt *et al.* (Lindstedt et al., 2018) reported that a high frequency (> 93%) of routinely submitted faecal *E. coli* strains from Norwegian hospitals, previously characterized as IPEC, also harbored ExPEC virulence factors. It is of note the EPEC/STEC O80:H2-ST301 clone, which emerged in France and spread within Europe. This emerging hybrid, associated with severe cases of CH and HUS, combines intestinal VFs (*stx2d*, *eae*-xi and *ehxA* genes) and extraintestinal genes characteristic

of the plasmid pS88. It is to highlight the location of MDR and pS88 genes in the same plasmid, as well as the presence of two additional plasmids (a carrier of *ehxA* gene and a cryptic one) (Cointe et al., 2018, 2020). Gati *et al.* (Gati et al., 2019) hypothesized that specific *E. coli* lineages, such as ST141, would serve as a melting pot for pathogroup conversion between IPEC and ExPEC, contrasting the classical theory of pathogen emergence from nonpathogens. Currently, classical and new approaches (WGS), are being used to enhance the understanding of the evolution of this highly adaptable species (Scheutz, 2014; Robins-Browne et al., 2016).

1.1.8. Mobile Genetic Elements (MGEs)

Mobile Genetic Elements (MGEs) is a term that refers to the elements that promote DNA mobility. The movement of the DNA can happen within the bacteria, as in the case of genes from the chromosome that integrates into a plasmid or parts shared from one plasmid to another, and is described as intracellular mobility, but also can take place between different bacteria, known in this case as intercellular mobility (Partridge et al., 2018).

This genetic movement contribute to the inter and intra species variations, makes possible to for the bacteria to achieve evolutionary genetic advantages against other bacteria as virulence or resistance genes, often associated with MGEs (Hacker and Kaper, 2000; Dobrindt, 2005).

1.1.8.1. Intracellular mobility

Insertion sequences (IS) and transposons (Tn) are discrete segments of DNA that are able to move by themselves and also associated resistance genes in a nonspecific way withing a single cell, meanwhile integrons use site-specific recombination to move resistance genes between defined sites (Partridge et al., 2018).

Insertion sequences are arguably the smallest and most numerous autonomous transportable elements, usually they carry little more than one, or sometimes two, transposase (*tnp*) genes (Partridge et al., 2018). There are classified into different families using a variety of mechanisms as the length and sequence of the short imperfect terminal inverted repeat sequences, the length and sequence of the short flanking direct target DNA repeats often generated on insertions, the organization of their open reading frames or the target sequences into which their insert. Nevertheless, the principal characteristic used for its classification is the similarity, at the primary sequence level, of the enzymes which catalyze their movement, the transposases (Siguier et al., 2014).

The replicative events can occur by copy-and-paste mechanisms, where the IS is preserved in the donor as well as in the recipient, and by copy-and-paste-in, where the IS integrates into the recipient (Partridge et al., 2018).

Transposons, like IS, are transposable elements with nonspecific movement and are usually integrated into the chromosome. They are distinguished from IS because they carry passenger or cargo genes not involved in catalyzing or regulating their movement, and therefore they are usually larger than ISs. These passenger genes may encode antibiotic resistance as in the case of the Tn3 family transposons or the superfamily Tn7-like transposons (Dobrindt, 2005; Partridge et al., 2018).

Gene cassettes are small mobile elements consisting in a single gene, or sporadically, two, that can exist in a free circular form as non-replicative, but usually they are found inserted into an. **integron**. The structure of an integron comprises a site-specific integrase (*int*I), an integron-associated attachment site (*att*I), an integron-carrier promoter (Pc) and a gene cassette as described previously, with a downstream attachment site (*att*C). These elements are ancient, diverse and widely spread. They possess mechanisms for creating genetic diversity and trigger adaptive responses in the bacteria due to their facility for the acquisition of antibiotic resistance genes and uses site-specific recombination mechanisms (Gillings, 2014; Partridge et al., 2018).

1.1.8.2. Intercellular mobility

In this case, the MGEs move from a doner cell to a recipient cell through different exchange mechanisms. Intercellular mechanisms of genetic exchange include conjugation or mobilization, mediated by plasmids and integrative conjugative elements, transduction, mediated by bacteriophages and transformation, when occurs the uptake of extracellular DNA.

As mentioned earlier, **plasmids** play an important role in the dissemination of antibiotic resistance since they promote their horizontal transfer through **conjugation or mobilization** processes. These processes involve the transfer of genetic material from a donor cell to a recipient cell. Plasmids are extrachromosomal elements that contain their own replication initiation (*ori*), genes encoding specific replication initiators (Rep) and internal systems to control de number of copies of the plasmid inside the cell to not affect their stability. They can be classified as conjugative plasmids when containing a conjugative system (Tra) and the coding genes for the functions necessary for this transfer to occur to the new host via conjugation, or as mobilizable plasmids if they must use those functions from other plasmids to be transferred, helper plasmids (Carattoli, 2011).

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In many cases, resistance and /or virulence genes are encoded in plasmids and provide the host bacteria with a selective advantage against non-carriers. Strains with traits that confer these advantages will disperse more successfully in the environment, producing a positive selection. The transmission of plasmids can occur even between bacteria that are not related, making possible to find indistinguishable plasmids in different bacterial species in very distant and diverse places. In this fact lies the importance of mobile elements, since they have the capability to perform conjugative event, the worldwide spread of genes associated with antibiotic resistance of high virulence profiles put at risk clinical treatments of patients infected with these strains and in consequence, the health of the whole population (Carattoli, 2011; Alvarado et al., 2012).

In 2013, the NCBI database had catalogued 580 complete circular DNA sequences from plasmids identified in different genera of the Enterobacteriaceae family and 60 from strains of *Acinetobacter* spp. In 2017, this number increased to 9,351 plasmid sequences in the same database (Carattoli, 2013; Roosaare et al., 2018).

The first typing schemes for these mobile elements emerged in 1971 designed by Datta and Hedges (Datta and Hedges, 1971) and was based on the stability of the plasmids throughout conjugation. This phenomenon, known as plasmid incompatibility (Inc), is defined as the situation whereby two plasmids from the same incompatibility group cannot stably propagate in the same cell. With WGS tools, 28 different replicons are presently described (Carattoli et

al., 2014; Rozwandowicz et al., 2018). Another typing scheme developed by Alvarado *et al.* (Alvarado et al., 2012) is based on the classification of the relaxases or MOBs, the only common component among all transmissible plasmids (both conjugative and mobilizable). This classification includes 6 families and for its classification the Degenerate Prime MOB Typing (DPMT) technique is applied.

The classification of the relaxases or MOBs shows a high correlation with the incompatibility scheme, which means that the plasmids of each type of Inc have relaxases from a single subfamily of MOB (Table 4) (Rozwandowicz et al., 2018).

Table 4. Correlation between relaxase classification (MOB) and the incompatibility scheme (Inc) (Rozwandowicz et al., 2018).

Replicon type	Relaxase type	Size (kb)	Copy number	Tranferability	Host range
IncF	MOB_F	45-200	Low	Conjugative	Enterobacteriaceae
IncI	MOB_P	50-250	Low	Conjugative	Narrow
IncK, InckB/O, IncZ	MOB_P	80-150	Low	Conjugative	Narrow
IncA/C	MOB_H	18-230	Low	Conjugative	Narrow
IncH	MOB_H	75-400	Low	Conjugative	Wide host rage
IncP	MOB_P	70-275	Low	Conjugative	Broad
IncL/M	MOB_P	50-80	Low	Conjugative	Broad
IncN	MOB_F	30-70	Low	Conjugative	Broad
Col	MOB_P	6-40	1-20	Mobilizable	
IncX	MOB_P	30-50			Narrow
IncR	Not included	40-160		Mobilizable	Broad
IncW	MOB_F	Up to 40	Low	Conjugative	Broad
IncQ	MOB_Q	8-14	Medium (4-12)	Mobilizable	Broad
IncT	MOB_H	~217	Low	Conjugative	Narrow
IncU	MOB_P	29-60	Low	Conjugative	Broad

The low copy number conjugative type plasmids IncF or MOBF are the most frequently described in humans and animals, mainly associated with *E. coli* (Rozwandowicz et al., 2018). This type of plasmid is prevalent within clone ST131 and is usually associated with ESBL resistance, especially with the production of the β-lactamase enzyme CTX-M, though it can undergo frequent recombination resulting in new genetic repertoires. In the plasmidome of this group we not only find antibiotic resistance, but also virulence factors and little-studied functions such as metabolic genes, colicins and other cryptic functions, all of which result in frequent and rapid adaptations to the environment that guarantee their survival and global expansion (Carattoli, 2013; Lanza et al., 2014; Rozwandowicz et al., 2018).

The integrative conjugative elements (ICE), also called conjugative transposons, do not contain an origin of replication, so it is necessary that they be integrated into a replicon to be able to maintain themselves in the host cell, this trait gives them an adaptive advantage over plasmids because the genetic load they require is lower. These elements are highly heterogeneous and usually have a modular organization (Roberts et al., 2008; Van Hoek et al., 2011).

Another type of transmission that can occur are **transduction** processes, where **bacteriophages**, instead of the own phage DNA, carry bacterial DNA with genes of interest, which is injected into a recipient bacterium. There are two types of transduction, the generalized, where any segment of DNA can be encapsulated in the phage, or specialized, where only a set of genes restricted to the points adjacent to the insertion point of the prophage in the chromosome is packaged. These elements play a central role in moderating bacterial populations as well as mediating horizontal gene transfer as mention previously (Chiang et al., 2019). And finally, **transformation** processes, which take place when the DNA is free in the environment and a competent bacterium captures it from there. In this process, the naked DNA is incorporated into the recipient's genome through homologous recombination or rearrangement (Van Hoek et al., 2011).

All the described MGEs can be identified in the same bacterial cell, so at this point, interactions between them have been studied. Rodríguez-Rubio *et al.* (Rodríguez-Rubio et al., 2020) performed a comprehensive study regarding the relevance of the multicopy plasmids carriers of antimicrobial resistance (AMR) genes and their relationship with phage particles, due to the fact that recently fragments of phages had been identified in small multicopy plasmids (MCPs) from Enterobacteriaceae. The fact of finding these fragments is what made them hypothesize about the possibility of this MCPs being transferred between bacteria using phage transduction. They suggest that this transduction phenomenon could be an extremely efficient mean of AMR genes mobilization. They propose a model where MCPs transduction is a major powerful route for AMR gene disseminations in nature because these genes, borne on small MCPs are encapsidated up to 1000 times more efficiently than when borne on large low-copy plasmids and have the possibility to be disseminated over distance to transduce resistance into susceptible bacteria (Figure 1).

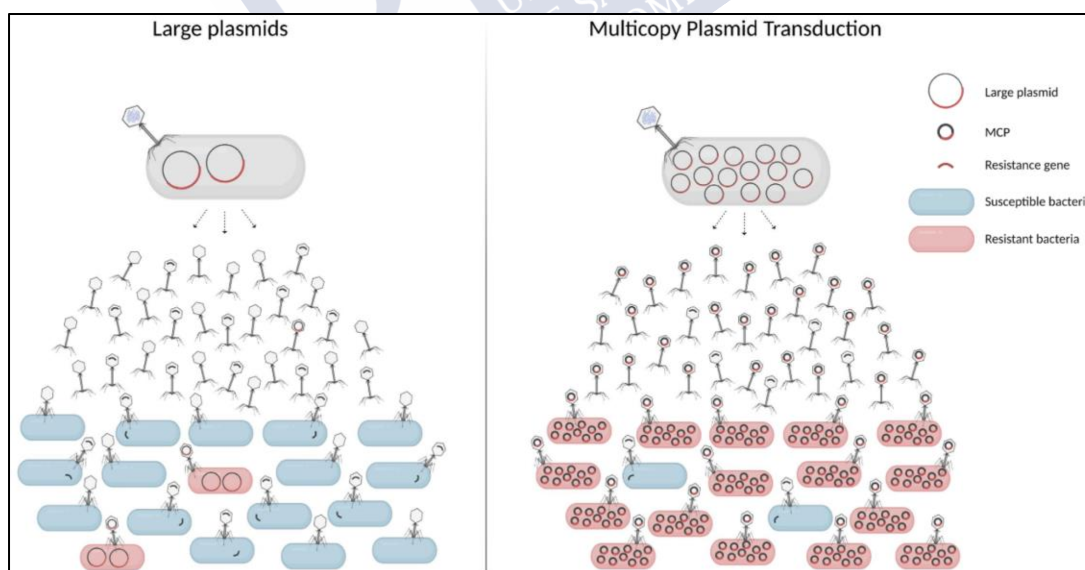

Figure 1. Efficiency of AMR spread through multicopy plasmid transduction (Rodríguez-Rubio et al., 2020).

1.2. THE SILENT PANDEMIC: ANTIBIOTIC RESISTANCES

The World Health Organization (WHO) currently identifies the spread of multiresistance, together with the decrease in the available antimicrobial treatments, as a main threat to the global health. WHO, together with ECDC and Center for Disease Control and Prevention (CDC), urge to implement a One Health approach, involving human and veterinary medicine (World Health Organization (WHO), 2019). The magnitude of the problem is now accepted, and the estimation is that by 2050, 10 million lives a year and a cumulative 100 trillion USD of economic output are at risk due to the rise of drug-resistant infections if we do not find proactive solutions to slow down the rise of drug resistance. Even today, 700,000 people die of resistant infections every year. Antibiotics are a special category of antimicrobial drugs that underpin modern medicine as we know it (O'Neill, 2016).

Overusing of antibiotics in the human and veterinary medicine has led to the development of multidrug-resistant (MDR; at least one antimicrobial agent in three or more antibiotic classes), extensively drug-resistant (XDR; species are only susceptible to two antimicrobial drug classes), and pandrug-resistant (PDR; resistant to almost all commercially available antimicrobials Gram-negative bacteria (Magiorakos et al., 2012). Rising AMR causes difficult-to-treat infections, longer hospital stays, therapeutic complications, and increased mortality. Especially, extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, such as *E. coli*, as well as carbapenem-resistant Enterobacteriaceae, particularly *Klebsiella* spp., have been increasingly associated with high morbidity rates due to limited treatment options (ECDC, 2019).

According to the ECDC, more than 670.000 bacterial infections can be attributed to MDR bacteria, which causes 33.000 death annually in Europe (ECDC, 2019). As a result, it is estimated that MDR infections and complications cost the healthcare system 1.1 billion annually in Europe. The enormous lack of novel antimicrobials active against these MDR Gram-negative bacteria, particularly those producing carbapenemases, requires the growing use of last-resort antibiotics, such as polymyxins (Grundmann et al., 2017).On the other hand, polymyxins have been continuously used in Europe in livestock for prophylactic, therapeutic, and, until 2006, growth promotion purposes (Regulation (EC) No 1831/2003). The frequent application of antibiotics in food-producing animals is associated with selection of resistant zoonotic strains with the risk of transmission, directly from animal to human, or indirectly via the food chain, and eventually causing difficult-to-treat illnesses in humans (Marshall and Levy, 2011). Nowadays, the One Health approach aims to address the urgent problem of AMR by reducing the use of antimicrobials in food-producing animals, since human health and animal health are interconnected (Min et al., 2013).

Specifically, the family of Enterobacteriaceae is among the most significant public health problems worldwide due to the high resistance to antibiotics. In early 2017, WHO published a pathogen priority list, which included carbapenem-resistant Enterobacteriaceae as "critical" antibiotic-resistant bacteria that represent an enormous threat to public health (De Freitas, 2013). Members of the Enterobacteriaceae account for about 80% of Gram-negative isolates with a variety of diseases in humans including UTIs, pneumonia, diarrhea, meningitis, sepsis, endotoxic shock, and others. *Escherichia, Klebsiella, Salmonella, Shigella, Enterobacter, Yersinia, Citrobacter* and *Proteus* are genera, which frequently affect humans. Even the

intestinal commensals, *Escherichia* and *Salmonella* spp. have also the potential to become pathogens causing infections such as diarrhea and colibacillosis, among others.

The SARS-CoV-2 pandemic has exacerbated the existing global crisis of AMR due to secondary infections by MDR bacteria along with the increased use of disinfectants. Altogether, is anticipated to lead to novel resistances in the coming years (García-Meniño et al., 2021a; Gonzalez-Zorn, 2021; Mahoney et al., 2021).

1.2.1. Main antibiotic families and resistance mechanisms

With the discovery of antibiotics as an effective therapeutic tool for infections in 1929, a new horizon was established in clinical medicine since fatal infections were now manageable. From the moment of their discovery, were used both as a therapeutic tool and as a production tool in the livestock sector. A high number of natural antibiotic families were discovered and soon after, synthetic and semi-synthetic derivative modifications were developed (Van Hoek et al., 2011).

Nevertheless, as a response to it the bacteria population started developing numerous and varied mechanisms of resistance against them to ensure their survival. Some of the more studied ones are:

- Intracellular reduction of antibiotic presence due to permeability changes in the bacterial cell wall or active efflux of the antibiotic from the interior of microbial cell.
- The enzymatic modification or degradation of the antibiotic, that makes it lose their antibiotic properties.
- The acquisition of alternative metabolic pathways to the ones inhibited by the antibiotic.
- The modification of the antibiotic targets of the cell.
- The overproduction of the target enzyme.

The acquisition of these resistance responses is mainly due to horizontal transfer of MGE but there is also the possibility of mutation events that can be transmitted vertically, nevertheless they have a low frequency of appearance ($1/10^7$ to $1/10^9$) (Van Hoek et al., 2011; Schwarz et al., 2017).

The antibiotic resistance mechanisms previously mentioned have become one of the most important health problems of society and a global priority recognized by the WHO and the European Union (WHO, 2017). Some of the antibiotic families discovered years ago are now a days still working as first line of defense against infections and are used daily in clinical medicine. Some of the most used are the following:

Aminoglycosides: First discovered in 1940s, this groups was led by streptomycin, first recovered from *Streptomyces griseus* (Schatz and Waksman, 1944). Later neomycin and kanamycin were found and recovered from other spp. of *Streptomycies*, and in the 1960s, gentamicin was recovered from the actinomycete *Micromonospora purpurea*. The first semisynthetic antibiotic, amikacin, was synthesized in the 1970s from kanamycin (Begg and Barclay, 1995). The action mechanism of this antibiotic family is the inhibition of protein synthesis and / or the alteration of the integrity of cell membranes. It has a wide spectrum of

action among Gram negative and positive bacterial species and often acts in synergy with other antibiotics, giving them greater antibacterial capacity (Vakulenko and Mobashery, 2003).

There are several types of resistance mechanisms known acting against this family of antibiotics, as for example, active expulsion, decreased permeability, alteration of ribosomes and inactivation of antibiotics by modifying enzymes. The main mechanisms of resistance are the target modification and the enzymatic inactivation. The modification of the target site can be achieved by methylation of residues of the site A of the 16S RNA, resulting in high level or resistance to amikacin, tobramycin, gentamicin and netilcmicin.16S RNA methylases include ArmA, RmtA/B/C/D/E/F/G/H and NmpA, being *armA* the first coding gene for methylases found in a plasmid. The enzymatic inactivation of aminoglycosides is achieved by the modification of the molecules do they are unable to get to the target point. The three enzymes known to this day are classified into acetyltransferases (AAC(3)-II/IV and AAC(6)Ib being the most frequently found in *E. coli*), nucleotidyltransferases (ANT(2") and ANT(3") the most commonly found in Gram negative) and phosphotransferases (APH(6)-Ia and APH(6)-Ib being the most common in *E. coli*) (Galimand et al., 2003; Poirel et al., 2018).

β-lactams: Penicillin was first antibiotic described, discovered in 1929 by scientist Alexander Flemming when he notice the presence of a substance with antimicrobial properties produced by the *Penicillium notatum* mold (Flemming, 1929) and also was the first member of the family of β-lactams . In the last 30 years, many new antibiotics of this family have been synthesized, most of them sharing a β-lactamase nucleus in their molecular structure. It includes penicillin, cephalosporins, carbapenems, monobactams, and β-lactamase inhibitors. They are grouped into first, second, third and fourth generation cephalosporins based on the spectrum of activity and the time of introduction of the agent.

First-generation cephalosporins are mainly effective against Gram positive, with minimal coverage against Gram negative, examples of this group are cefazolin or cephalothin.

Second-generation cephalosporins have less activity against Gram positive bacteria compared to first generation but a better antibiotic effect against Gram-negative bacilli. This group can be divided in two groups, the second generation (cefuroxime or cefprozil) and cephamycin subgroup (cefoxitin or cefmetazole).

In the third-generation group we can find cefotaxime, ceftazidime, ceftriaxone, cefixime and others as main representers of it. This generation has an extended spectrum against Gram negative bacteria, and importantly, against bacteria that are resistant to antibiotics from the first and second generation. When administrated intravenous can penetrate the blood-brain barrier and act against bacteria located in the spinal fluid, important in cases of meningitis.

Fourth-generation cephalosporin includes cefepime, a broad-spectrum antibiotic that can, as well as third-generation, penetrate the cerebral spinal fluid, but in addition, its composition allows them to penetrate the outer membrane of Gram negative more easily and is effective against β-lactamase-producing isolates (Bui and Preuss, 2021).

Carbapenems are also β-lactams that can easily diffuse into bacteria, which is why they are considered broad spectrum, examples of this subgroup are imipenem and ertapenem. In the case of monobactams, although they lack the central ring, they remain within this classification,

being the most used of them the aztreonam. Inhibitors of β-lactamases, such as clavulanic acid, contain the ring within their structure but exhibit a very low antimicrobial power, therefore they are used in combination with other β-lactams to increase their efficacy against β-lactamase producing bacteria (Van Hoek et al., 2011).

Its mechanism of action is based on the inhibition of cell wall synthesis, acting as a false structural component of it, the penicillin-binding proteins (PBP). As consequence, the wall is weakened, resulting in cytolysis or death due to poor regulation of osmotic pressure (Kotra and Mobashery, 1998; Bush, 2018).

The first enzyme described that had activity against penicillin was AmpC and it was found in *E. coli* in the 1940s, since then bacterial resistance to this antibiotic family has increased at a significant rate (Abrahan and Chain, 1940). There are diverse mechanisms of resistance against β-lactams, modification of the PBPs target, porin modifications that affect the permeability of the membrane or efflux pumps. Nevertheless, the most common mechanism of resistance is the production of β-lactamase enzymes such as ESBL or enzymes associated with AmpC-type plasmids or carbapenem hydrolyzing β-lactamases. These are versatile enzymes with a limited range of molecular structures that are found in a wide range of bacterial species due to their horizontal dissemination mainly associated with plasmid acquisition (Van Hoek et al., 2011; Bush, 2018).

There are currently two classification systems for these enzymes, one proposed by Ambler in 1980 where they are grouped into four classes based on their nucleotide and amino acid sequence (Ambler, 1980), and one proposed by Bush, Jacoby and Medeiros in 1995 and updated in 2010 based on structural and functional biochemical characteristics (Bush et al., 1995; Bush and Jacoby, 2010). The following table (Table 5) shows the correlation between both classifications.

Table 5. Classification schemes for bacterial β-lactamase. Adaptation on Bush & Jacoby. (Bush and Jacoby, 2010)

Bush-Jacoby (2010)	Ambler (1980)	Distinctive subtrate(s)	Inhibited by AC [a] o TZB [b]	Inhibited by EDTA [c]	Defining characteristic(s)	Representative enzyme(s)
1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycin	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β- lactams *	GC1, CMY-37
2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams *	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10

Bush-Jacoby (2010)	Ambler (1980)	Distinctive subtrate(s)	Inhibited by AC [a] o TZB [b]	Inhibited by EDTA [c]	Defining characteristic(s)	Representative enzyme(s)
2ber	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-ß-lactams * + Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	A	Carbenicillin (carboxypenicillin)	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime and cefpirome	RTG-4
2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-ß-lactams	OXA-11, OXA-15
2df	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Si	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-ß-lactams, cephamycin	KPC-2, IMI-1, SME-1
3a	B(B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
	B(B3)					L1, CAU-1, GOB-1, FEZ-1
3b	B(B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1

[a] Clavulanic acid; [b] Tazobactam; [c] Ethylenediaminetetraacetic acid * Cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam.

Now a days, resistance to β-lactams through the production of ESBL enzymes represents an important problem worldwide, with high-risk pandemic clones such as ST131, to which the CTX-M-15 enzyme is associated.

ESBL enzymes can hydrolyze monobactams, first, second, third and fourth generation cephalosporins but they are unable to inactivate cephamycins and carbapenems. These enzymes are inhibited by class A β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Gurrero and Sanchez, 2017) .

The main ESBL enzymes produced by *E. coli* are TEM, SHV and CTX-M. TEM-1, TEM-2 and SHV-1 are considered classic plasmidic β-lactamases from which new and with higher hydrolytic power enzymes are generated (Juan J et al., 2000; Cantón and Coque, 2006). The TEM family has more than 200 allelic variants described and some of them are characterized by an incremented hydrolysis of oxyimino β-lactams, meanwhile SHV family has been associated with 190 allelic variants which can be divided into three subgroups, subgroup 2b (can hydrolyze penicillin, first generation cephalosporins and is inhibited by clavulanic acid and tazobactam), subgroup 2br (with action against extended spectrum β-lactams and resistant to clavulanic acid) and subgroup 2be (can hydrolyze one or more oxyimino β-lactams) (Liakopoulos et al., 2016).

In the 90s, the antibiotic resistant enzymes scene was dominated by TEM and SHV enzymes worldwide but after the description of the CTX-M family this had been the one that arise as dominant, with the most rapid and dramatic expansion (Cantón and Coque, 2006; Cantón et al., 2012). Soon after its discovery was already observed not only in nosocomial environment but also in the community settings associated to MGEs. This expansion can be divided into three stages, first, the dissemination of diverse CTX-M enzymes in distant geographic areas until mid-90s, second, the rise of new CTX enzymes as CTX-M-3, CTX-M-9, CTX-M-14, and CTX-M-15 between 1994 and 2000 and the third event that took part from the 2000 when CTX-M-14, and CTX-M-15 become the most predominant ESBL worldwide mainly due to their association with the pandemic clonal group ST131 (Mora et al., 2010; Cantón et al., 2012; Poirel et al., 2018).

ESBL-producing *E. coli* have adaptative advantages against isolates that does not carry resistance genes, making certain STs more frequently found in both animals and humans, being examples of this ST10, ST23, ST38, ST88, ST131, ST167, ST410 and ST648. These clonal groups are found worldwide and located in varied ecosystems, which enables the spreading. of these resistances between niches, making the ESBL dissemination an important world health problem (Poirel et al., 2018).

Chloramphenicol: It was first described in 1947 produced by a *Strepotmyces venezuelae* (Ehrlich et al., 1947). This compound has a simple structure, which makes it very difficult to synthesize new compounds from it, since it easily loses its antibiotic characteristics. Chloramphenicol is a potent and specific inhibitor of protein synthesis through the affinity to peptidyltransferases of the 50S ribosomal subunit of 70S ribosomes. This antibiotic has a broad spectrum since it acts on Gram positive and negative bacteria, both aerobic and anaerobic (Schwarz et al., 2004).

The resistance to this antibiotic in *E. coli* is mediated by three main mechanisms, the most frequently encountered mechanism is the enzyme inactivation by acetylation of the antibiotic through the action of different types of acetyltransferases encoded by *cat* genes. Two types have been defined, the classic *catA* and the new or xenobiotic ones known as *catB*. Another resistance mechanism is the active efflux of nonfluorinated phenicols coded by the genes *cmlA* or by *floR* genes when dealing with fluorinated and nonfluorinated phenicols. The last major mechanism is the methylation of the target site by an rRNA methylase encoded by the multiresistance gene *cfr* (Schwarz et al., 2004; Poirel et al., 2018).

Quinolones and fluoroquinolones: In 1962, during the synthesis and purification of the chloroquine, used as an antimalarial agent, nalidixic acid was discovered and shown bactericidal capacities against Gram negative bacteria (Lesher et al., 1962). These capacities were increased with the addition of a fluorine atom, becoming known as fluoroquinolones, or second-generation quinolones (Wolfson and Hooper, 1989). In the 1980s, new fluoroquinolones such as ciprofloxacin, norfloxacin or ofloxacin were synthesized, in which the spectrum of action came to include Gram-positive ones. The main binding point for the quinolones are the DNA-gyrase and topoisomerase IV, essential enzymes for DNA replication. The DNA-gyrase is formed by 4 subunits, 2 GyrA and 2 GyrB, as well as topoisomerase IV with 2 A and 2 B subunits encoded by genes *parC* and *parE* respectively, and them are the targets for the point mutations that confer resistance to the bacterial organism (Van Hoek et al., 2011).

At first it was assumed that mechanisms of resistance towards these antibiotics were only encoded in the chromosome. The main mechanism of resistance arouse spontaneously due to point mutations that results in amino acid substitutions within the topoisomerase and gyrase subunits triggering the decreasing expression of outer membrane porins or overexpressing the multidrug efflux pumps (Hopkins et al., 2005). Mutations occur at specific points known as "quinolone resistance determining regions" in the two genes coding for the subunits of gyrase (*gyrA* and *gyrB*) and the two subunits of topoisomerase IV (*parC* and *parE*). Individual mutations in the *gyrA* gene can confer resistance to quinolones, but fluoroquinolone resistance requires mutations in both *gyrA* and *parC* (Poirel et al., 2018). An example of the global importance of these mutations is their presence in the pandemic clonal group ST131, where the H30R and H30Rx subclades are characterized by carrying these mutations (Stoesser et al., 2016).

In the 1990s, a quinolone resistance gene of plasmid origin was described for the first time, the *qnr* gene that encodes a protein that protects DNA gyrase and topoisomerase IV from inhibition by quinolones. A second type of plasmid resistance to quinolones are the *cr* variants of the *aac(6 ')-Ib, aac(60)-Ib-cr* gene responsible for low-level resistance to ciprofloxacin. And, finally, a third mechanism, the hydrophilic fluoroquinolone ejection pumps, mediated by the *qepA* plasmid gene (Van Hoek et al., 2011).

Sulfonamides: First synthesized in 1932, they have evolved to the present day, with sulfamethoxazole as their most widely used representative (Domagk, 1935). In 1968 its use was associated with trimethoprim, developing co-trimoxazole, widely used due to its synergistic action, the reduction of resistance and its costs. Sulfonamide has a structure analogous to the p-aminobenzoic acid, which is involved in the metabolic pathway of folic acid, therefore, its interference in this path causes problems in the growth of bacterial cells.

Resistance to this group of antibiotics arose shortly after the start of its clinical use. They were developed at the chromosome level by mutation of the *folP* gene that encodes the enzyme dihydropteroase synthase (DHPS) that participates in the metabolic pathway of folic acid synthesis. The first plasmid-type resistances appeared in the eighties, encoded in the *sul1* and *sul2* genes, although the *sul3* gene has now also been described. sul1 gene is spread worldwide because is a part of the 3'-conserved segment of Class 1 integrons, who are present in *E. coli* isolated from healthy and diseased food producing animals, companion animals and wildlife (Roberts, 2002; Poirel et al., 2018).

Trimethoprim: This antibiotic available since 1962 is considered the last new antibiotic introduced in the clinical therapeutic arsenal, since all those that have subsequently emerged have been variations of those already described (Roth et al., 1962). It is totally synthetic and belongs to the group of diaminopyrimidine compounds. It inhibits the enzyme dihydrofolate reductase (DHFR) by competitive binding on its target. What this enzyme does is to catalyze the NAHPH reductions dependent on dihydrofolate acid to the active coenzyme tetrahydrofolate, therefore, trimethoprim acts as an antifolate, a structural analog of folic acid, interfering in its metabolic pathway.

In the same way as sulfonamides, being synthetic antimicrobials, it was assumed that the appearance of natural enzymes that would degrade or modify them was unlikely. However, a low resistance could be seen through variations in the chromosomal *folA* gene that codes for the DHFR enzyme. High-level resistance was achieved through a bypass mechanism where a

plasmid DHFR replaces the chromosomal DHFR target of the antibiotic (Sköld, 2001). Plasmid DHFRs are grouped into two major groups, *dfrA* and *dfrB* based on their sizes and structures. *dfrA* genes have been identified in *E. coli* from dogs, cats, horses, pigs, cattle, chicken and giant pandas in contrast with *dfrB* genes that have rarely been detected in animals (Poirel et al., 2018).

Tetracyclines: The first tetracycline was characterized in 1948, chlortetracycline, produced by *Streptomyces aureofaciens* (Chopra, 1994). Since then, more natural tetracyclines have been found, such as oxytetracycline, and also semi-synthetic products, such as doxycycline, have begun to be obtained. This was the first family in which the term "broad-spectrum" was used, which together with the fact of their relative safety and low cost made them the second most used antibiotics after penicillin. Two different mechanisms of action have been described in this family. First, typical tetracyclines prevent bacterial growth by inhibiting protein synthesis by interacting with the ribosomes, and second, some tetracyclines with little affinity for ribosomes act on the bacterial membrane (Chopra, 1994; Van Hoek et al., 2011).

The first resistance did was not described until the 1950s but since these antibiotics were widely used in veterinary medicine, the selective pressure made appear large number of tetracycline resistances. The resistance mechanisms could be grouped in three categories: nine genes for energy-dependent ejection pumps (*tet(A, B, C, D, E, G, J, L, Y*)), two genes coding for ribosomal protection proteins (RPPs) (*tet(M, W*)) and one gene coding for an oxidoreductase that produces enzymatic inactivation (*tet(X*)) (Poirel et al., 2018).

Polymyxins: This family of antibiotics was described in the 1940s as fermentation products of the bacterium *Bacillus polymyxa*. It comprises five antimicrobial compounds (polymyxin A, B, C, D, and E). Due to their reduced renal toxicity compared to the other polymyxins, only polymyxin B and E (colistin) are used as last-resort defense against severe infections with carbapenem-resistant Enterobacteriaceae in clinical sector (Li, 2019). The polymyxins share a similar structure and are pentacationic polypeptides consisting of a cyclic heptapeptide linked to a linear tripeptide, whose N-terminus is acylated with a fatty acid moiety. Colistin is a secondary metabolite peptide produced by the soil bacterium *Paenibacillus polymyxa* (formerly named *Bacillus polymyxa*). The polymyxins have bactericidal activity against most members of the Enterobacteriaceae family including *E. coli*, *Klebsiella*, *Salmonella*, *Shigella*, and *Enterobacter*, as well as other clinically relevant Gram-negative pathogens such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. However, the polymyxins demonstrated no activity towards Gram-negative and Gram-positive cocci and Gram-positive bacilli. Besides, polymyxins lack activity against intrinsically resistant species, such as *Serratia* or *Proteus* spp. (Muyembe et al., 1973; Storm et al., 1977; Pogue et al., 2011).

Since its introduction in the 1950s, colistin has been used continuously in the veterinary medicine to treat and prevent animal infectious caused by Gram-negative bacteria. In human medicine, colistin was gradually abandoned in the early 1980s due to concerns about neurotoxicity and nephrotoxicity (Brown et al., 1970) Thereafter, colistin was re-introduced for systemic treatment due to MDR Gram-negative bacteria (Conway et al., 1997).

Initially, resistance mechanisms were caused by mutations in a small number of chromosomal encoded genes. Particularly in *E. coli*, it can be due to mutations in the two-component systems PmrAB and PhoPQ, or in the MgrB regulator. Thus, Quesada *et al.* (Quesada et al., 2015) detected two colistin-resistant *E. coli* recovered in 2011 and 2013 from

the stools of two pigs, which showed mutations in PmrB V161G and PmrA S39I, reporting the finding as a rare event. Subsequently, other authors have reported not only different mutations in the amino acid sequences of the MgrB, PhoP, PhoQ, and PmrB proteins, but also the co-occurrence of transmissible colistin resistance genes (García-Meniño et al., 2019).

Since the *mcr-1* (mobile colistin resistance gene 1) plasmid gene was first described (Liu et al., 2016), it has been identified in members of the Enterobacteriaceae family encoded in different plasmid types, including IncI2, IncX4, IncHI1, IncHI2, IncFI, IncFII, IncP, IncK (Sun et al., 2018). The encoding enzyme is responsible for the transfer of pEtN to lipid A thereby mediating colistin resistance. Further investigation showed that the mcr-1 gene was present in *E. coli* since 2011 and it has spread in isolates from livestock, raw meat products and even humans (El Garch et al., 2017), and WGS analysis showed that the mcr-1 gene was present in continents (Ling et al., 2020). In Europe, the mcr-1 gene seems to be present since 2004, when it was found in *E. coli* from diseased cattle (El Garch et al., 2017). To date, ten different *mcr-* genes (*mcr-2* (Xavier et al., 2016), *mcr-3* (Yin et al., 2017), *mcr-4* (Carattoli et al., 2017), *mcr-5* (Borowiak et al., 2017), *mcr-6* (AbuOun et al., 2018), *mcr-7* (Yang et al., 2018), *mcr-8* (Yang et al., 2018), *mcr-9* (Carroll et al., 2019), *mcr-10* (Wang et al., 2020)) have been characterized in a wide number of plasmid reservoirs. Several variants have been described for *mcr-1* to *mcr-9*, of which *mcr-1* and *mcr-3* genes comprise the largest groups.

1.2.2. Standardized definitions of MDR, XDR and PDR

In 2012 Magiorakos *et al.* (Magiorakos et al., 2012) together with a group of international experts and through the initiative of the ECDC and the Center for Disease Control and Prevention (CDC) established an internationally standardized terminology to describe the resistance profiles of different bacteria associated with human clinical infections and predisposed to multidrug-resistance.

Different classifications were considered for different bacteria, so the definitions vary according to the family, in the case of Enterobacteriaceae, multidrug-resistant strains or MDR are defined as those that are resistant to one or more agents of three or more categories. Strains known as extensively drug-resistant or XDR are those that are resistant to one or more agents of all except for two or fewer antimicrobial categories. And finally, pandrug-resistant strains or PDR are considered to be those resistant to agents of all categories (Table 6).

Table 6. Categories and antimicrobial agents used to define MRD, XDR and PDR in Enterobacteriaceae. Adapted from Magiorakos *et al.* (Magiorakos et al., 2012)

Antimicrobial category	Antimicrobial agent
Aminoglycosides	Gentamicin, Tobramycin, Amikacin, Netilmicin
Anti-MRSA Cephalosporins	Ceftaroline (approved only for *E. coli, Klebsiella pneumoniae, Klebsiella oxytoca*)
Antipseudomonal penicillins + ß-lactamase inhibitors	Ticarcillin-clavulanic acid, piperazine-tazobactam
Carbapenems	Ertapenem, imipenem, meropenem, doripenem
1st and 2nd generation cephalosporins, not extended spectrum	Cefazolin, cefuroxime
3rd and 4th generation cephalosporins, extended spectrum	Cefotaxime or ceftriaxone, ceftazidime, cefepime
Cephamycins	Cefoxitin, cefotetan

Antimicrobial category	Antimicrobial agent
Fluoroquinolones	Ciprofloxacin
Inhibitors of the folate pathway	Trimethoprim-sulfamethoxazole
Glycylglycines	Tigecycline
Monobactams	Aztreonam
Penicillins	Ampicillin
Penicillins + ß-lactamase inhibitors	Amoxicillin – clavulanic acid, ampicillin-sulbactam
Phenicols	Chloramphenicol
Phosphonic acids	Fosfomycin
Polymyxins	Colistin
Tetracyclines	Tetracycline, doxycycline, minocycline

1.3. THE ROLE OF FOOD IN MICROORGANISM'S TRANSMISSION

Microorganisms can access the human body through diverse ways, being one of them within contaminated food. This contact can develop a wide variety of situations in the host organism. Microorganisms can act as a beneficial health element with the stimulation of host antibodies, the release of chemicals to stimulate the health of the overall system, by the inhibition of pathogen development, as well as shapers of the diversity of the human microbiota. Some of them can cause minimal changes within the equilibrium of the host microbial community being able to balance itself again to the optimal situation, but these food-borne microorganisms can also act as a pathogenic element. When this happens and the consumption of food or water is associated with a pathogenic organism and a disease is caused, they are considered as food-borne infections (European Food Safety Authority, 2019). These infections are the most frequently reported pathology in the United States according to the CDC with an estimated annual number of food-borne illness of 9.4 million cases associated with 31 known food-borne pathogenic agents, usually concomitant with gastrointestinal symptoms (Scallan et al., 2011; CDC, 2018). However, these symptoms are not routinely reported unless they come from an outbreak situation or a programmed sampling. So, the actual number of cases is known to be much higher. The same situations happens when the data from the EU is analyzed, in this case only 13 zoonotic elements were considered and the final report only accounted for 350,000 confirmed human cases, nevertheless the real incidence is suspected to be higher (EFSA/ECDC, 2020).

For a food-borne disease event to occur, three situations have to overlap: presence of a contaminated food item, a susceptible host and a bacterial pathogen able to survive and multiply in the new environment niche. The pathogen usually is able to cause a food-borne disease by three different mechanisms, first, by the ingestion of a toxin produced by the bacteria and present on the food, second, by the production of the toxin within the gastrointestinal tract after the pathogen has been ingested, or third, by the invasion of the intestinal epithelial cells (Antunes et al., 2020).

To have full picture of the transmission of hazardous microorganisms from food to the human population, we need to take into account the three following related items. First, the bacterial pathogen itself, the food chain and lastly, the human host (Antunes et al., 2020). The bacterial pathogens need to be able to survive the stress situations suffered during the

transmission from reservoir to host and keep their capability to multiply in order to colonize the new environment. For this adaptation, the bacteria use their genetic plasticity to acquire genetic elements through horizontal transfer, genetic recombination, mutations or modifications of their metabolic pathways. Antibiotic resistance and / or virulence genes are some of the adaptations usually give the bacteria the best adaptations for its survival. Another related item is the food chain itself, from the production systems to the global distribution of the food. With the new global situation, the demand and manufacture of food has created the necessity of industrial scale production systems for animal production as well as for agriculture. This intensive livestock practices with high density animal densities makes it easy for the bacteria to disseminate and persist on the facilities, as well as to take as reservoir the production animals and spread themselves through the food chain, at slaughterhouses and during the distribution of the products. Also, the fact that products or animals can travel from countries with lower food safety standards or safety practices to countries with higher standards can make possible de dissemination of diverse microorganisms. This is the case of poultry products imported from Brazil, one of the most important exporters, that has been linked to the dissemination of epidemic clones of MDR *Salmonella enterica* into European countries (Campos et al., 2018). Moreover, is needed to take into account the paper of the final host. In the last decades the consumption habits of the populations have change drastically, ready-to-eat and / or ready-to-cook products, consumption of uncooked or undercooked food from animal origin and a more frequent habit of eating in food service establishments prone the population to be more expose to possible hazardous situations, as for example, inadequate time - temperature control of the products, cross contamination, insufficient cleaning or personal hygiene of the handler are some of the possibilities for an outbreak to develop. Also, is important to notice that the own characteristics of the host will make an important difference in how the infection develops. The elderly and immunocompromised, as well as the infants are more susceptible to the infections. Is frequent the assessment of cases of listeriosis among elderly population (Buyck et al., 2018), as well as STEC severe outbreaks among children younger than five years old.

Cross contamination can happen at any step of the food chain and affects the final microbiological quality of the product. EFSA defines cross contamination as the process by which microbes or substances are involuntarily transferred from one object to another, with harmful effects.

EFSA carries out food risk assessment programs which provide data on cross contamination for the quantitative assessment of microbiological risks. According to the European Union Zoonosis Report issued in 2018, it is estimated that 40.5% of the outbreaks of bacterial infections have occurred at the domestic level, with 15.6% being cases of outbreaks in which a contaminated food was the cause. In these reports there is clear evidence that cross contamination in these cases is one of the determining factors producing the outbreaks (EFSA, 2019). This cross contamination or bacterial transfer can occur at various points in the food preparation process, but frequently the starting point of it is contaminated meat as a source of microorganisms. The handling of the meat during its preparation usually has several critical points to consider, from its cut and the utensils used for it, such as boards or knives, to the hands of the person who is handling it. The main determinant of risk in these cases is the fact that the food that receives the cross contamination will be served uncooked and without heat treatments as is the case with salads. On the contrary, when the food will be consumed after a heat process, bacteria usually die. The recommendations issued by the EFSA include good hand hygiene, working with clean cutting boards and knifes, changing utensils when changing from one

product to another, as well as establishing a correct order of actions when working with food (EFSA/ECDC, 2020; Iulietto and Evers, 2020).

However, this cross contamination does not only occur at the domestic level. There are numerous studies on cross-contamination of microorganisms that originate in slaughterhouses, both in cattle (Mather et al., 2008), pigs (Botteldoorn et al., 2003) and poultry, chicken and turkey (Olsen et al., 2003; Nde et al., 2007). Most studies are biased towards the detection of zoonotic organisms described as classic food-borne pathogens, usually associated with digestive pathologies as well as ESBL-producing isolates. In fact, the most common causes of food-borne disease according to the EFSA and CDC are *Campylobacter* spp., *Salmonella* spp., *Yersinia* spp., *E. coli*, *Listeria* spp., *Clostridium perfringens*. In relation to viral agents, norovirus is the most frequently reported (EFSA/ECDC, 2020). Yet, this criterion does not include microorganisms that are currently describe as food-borne pathologies, more often associated with extraintestinal pathologies, such as ExPEC or *Klebsiella* spp. For this reason, some authors (Smith et al., 2007; Davis et al., 2015; Hartantyo et al., 2020; Riley, 2020; Hu et al., 2021) highlight in their reviews the importance of these new recognized food-borne pathogens and the need of conducting studies and include them in the actual monitoring systems in order to have a more deep knowledge about them.

Due to the importance of these diseases and their association with increasingly frequent outbreaks and antibiotic resistance spread, in recent decades it has become necessary to develop surveillance systems and make preventive decisions to try to reduce the risk of exposure for consumer. As mentioned before, factors as changes in the eating and consumption habits of the population, increase in international travel, changes in the production processes and food distribution, the adaptation of pathogens to new environments, the acquisition of virulence and resistance factors by microorganisms, the improvement of the detection methods, poor hygiene and vector control, inadequate health services or even a deficient information to the consumer (Schirone et al., 2019) make necessary a continuous surveillance system in order to assess the possible outbreaks as soon as possible and be able to trace its route of spread and stop it as soon as possible. Also, a comprehensive evaluation of antibiotic resistances in the food chain and in production animals is essential to understand the magnitude of the problem and be able to act accordingly to reduce its burden on humans. However, food surveillance is considered a sensitive issue, therefore, the information derived is, in some cases, incomplete or difficult to access or understand by society (Tacconelli et al., 2018). Some of the most recent data are summarized below.

In the EU in 2019 the number of reported cases of human campylobacteriosis was 220,682. From them, 58,074 were related with meat and meat products meanwhile 2,760 were related with milk and milk products. The number of cases has decreased 6.9% regarding the data reported in 2018. Using new approaches in methodology, such as the WGS, it is possible to trace the origin of the infection. This is the case of a *Campylobacter fetus* outbreak in the Netherlands due to the consumption of unpasteurized sheep milk processed into unripened cheese (Koppenaal et al., 2017). The presence of *Campylobacter* spp. at a farm level as well as in the transport and slaughtering houses for broilers is a matter of discussion. In 2018 a process hygiene criterion (Regulation (EU) 2017/1495) with a critical limit of < 1000 cfu/g neck skin has been implemented in the EU countries, but an intervention method is needed at slaughter level in order to have a better control of the situation(Rasschaert et al., 2020).

In 2019, *Salmonella* was the second most commonly reported gastrointestinal infection in humans and an important cause of food-borne outbreaks. Thus, 87,923 confirmed cases were reported in humans, which meant the same level of incidence as in 2018. In total, 926 outbreaks were reported, 17.9% of the total amount of food-borne outbreaks of 2019. The vehicles of the infections were mainly, eggs and egg products followed by bakery products and pig meat. Within the EU, the national control programs carried out in poultry found a significant increase of the prevalence of *Salmonella* in breeding flocks, laying hens and fattening turkey flocks over the last 4 to 6 years (EFSA/ECDC, 2020). As with campylobacteriosis, WGS was not only useful to determinate the profile of *Salmonella enteritidis* from an outbreak linked to eggs from Poland with international impact, but also to identify the source and the movement of the bacteria (Pijnacker et al., 2019).

Regarding other bacteria, *Listeria* spp. was reported from 2,621 cases in 2019, with the same level of incidence as in 2018. Usually, these infections are reported within the group of age over 64 years old, which presented a fatality rate of 17.6% (higher in comparison to previous years). From the 2,621 cases, 1,803 were considered as domestic cases, acquired at home. One of the biggest *Listeria* outbreaks of last years occurred in Spain in 2017, where 222 confirmed cases were reported, with three deaths, six miscarriages and one travel-related case in France. The infected food associated with the outbreak was ready-to-eat pulled pork meat. After it, studies to determinate the diversity as well as the virulence potential of isolates recovered from pigs were carried out in the same region, pointing out the presence of virulent strains among the samples recovered from farm pigs, highlighting the importance of the veterinary medicine regarding the food safety (Gómez-Laguna et al., 2020; EFSA, 2021).

In 2019, the report on STEC infections accounted for 8,313 cases of infection, these infections were the third most commonly stated to the European Food and Waterborne Diseases and Zoonoses Network (FWD-Net) after campylobacteriosis and salmonellosis being all of them associated with digestive symptoms. After six years of stable trend, in 2017 a large increase of the cases was observed probably due to the change in the detection methodology used, now with a higher presence of PCR replacing culture diagnostic methods. Ruminants are known to be the main natural reservoir of STEC and because of this, undercooked ground beef or other meats are found to be a significant risk factor for food-borne infections associated with STEC isolates. In 2019 these infections involved 273 human cases in 42 known outbreaks in 11 different countries of the EU, which accounted for 0.8% of all food and waterborne outbreaks and 5.6% of the reported domestic STEC cases at the EU level (Ecdc, 2021).

Antibiotic resistance is a major priority in food safety as well as a global public health issue for humans and animals referred by a high number of entities as the European Commission, WHO or the Food and Agriculture Organization of the United Nations. The European Union, in order to protect the consumer and the environment and with the wide assumption that the misuse of antibiotic drugs in the animal production played an important role in the spread of ESBL and resistant *E. coli* strains through the food chain to humans, on January 1st 2006 banned the use of antibiotics as prophylaxis tool or as growth promoter in veterinary medicine (Hindermann *et al.*, 2017; Mora *et al.*, 2010). And since then, the European Medicines Agency (EMA) has been following the veterinary antimicrobial consumption of the EU updating yearly the trends of the antimicrobial sales and use in animals. Also has been developing successive One Health action plans against AMR that have been successively reviewed until the last updated in 2020 (WHO, 2017).

In order to control the impact on public and animal health of the use of antibiotics allowed to be applied in production animals, the EMA in 2020 established a new categorization in the veterinary handling of these drugs to help clarify the guidelines for its use in order to prevent and control derived resistances (EMA, 2019, 2020). In the categorization process, defined criteria, based on evidence and experts' considerations, have been applied to provide a rationale for the ranking. The updated criteria on which the categorization is based are as follows:

- If the (sub)class or group is authorized for use as a veterinary medicine in the EU.
- The importance of the (sub)class or group to human medicine according to the WHO ranking and considering the EU situation.
- The knowledge of factors influencing the likelihood and possible consequences of AMR transfer from animals to humans, considering mechanisms where a single gene confers multiresistance (or resistance to several classes).
- The availability of alternative antibiotic (sub)classes in veterinary medicine with lower AMR risk to animal and public health.

Based on this criteria, four categories have been established, from A to D, with an associated keyword to facilitate its use (Table 7). They would be the following:

- Category A or "Avoid": includes antibiotics not authorized for use in veterinary medicine but authorized in human medicine in the EU. They can be used exceptionally in animals not intended for consumption whenever their treatment with an antibiotic of a lower category is not possible.
- Category B or "Restrict": These are substances that fall within the category of high priority for the WHO except for macrolides and those that are already included in category A. In these antibiotics, the risk to public health derived from its use in veterinary medicine must be mitigated by its control.
- Category C or "Caution": It is an intermediate category for those substances that there are in general alternatives in human medicine in the EU but there are few alternatives in veterinary medicine for certain indications.
- Category D or "Prudence": It is the category in which there is the least risk to public health. It is known that the use of these antibiotics does not have a negative impact on the development and spread of antibiotic resistance, due to co-selection.

Table 7. Summary of the Antimicrobial Advice Ad Hoc Expert Group (AMEG) categorization for antibiotics (EMA, 2019)

AMEG categories	Antimicrobial class, subclasses and substances	Example of antimicrobial
Category A ("Avoid")	Amdinopenicillins	Mecilinam, pivmecilinam
	Carbapenems	Meropenem, doripenem
	Other cephalosporins and penems, including combinations of 3rd-generation cephalosporins with β-lactamase inhibitors	Ceftobiprole, ceftaroline, ceftolozane-tazobactam, faropenem
	Glycopeptides	Vancomycin
	Glycylcyclines	Tigecycline
	Ketolides	Telithromycin

AMEG categories	Antimicrobial class, subclasses and substances	Example of antimicrobial
	Lipopeptides	Daptomycin
	Monobactams	Aztreonam
	Oxazolindinones	Linezolid
	Penicillin: carboxypenicillins and ureidopenicillins, including combinations with β-lactamase inhibitors	Piperacillin-tazobactam
	Phosphonic acid derivates	Fosfomycin
	Pseudomonic acids	Mupirocin
	Rifamycins (except rifaximin)	Rifampicin
	Riminofenazines	Clofazimine
	Streptogramins	Pristinamycin, virginiamycin
	Sulfones	Dapsone
	Drugs used solely to treat tuberculosis or other mycobacterial diseases	Isoniazid, ethambutol, pyrazinamide, ethionamide
	Substances newly authorized in human medicine following publication of the AMEG categorization.	To be determined
Category B ("Restrict")	Cephalosporins: 3rd- and 4th-generation, except combinations with β-lactamase inhibitors	Ceftiofur, cefovecin, cefquinome
	Polymyxins	Colistin, polymyxin B
	Quinolones: fluoroquinolones and other quinolones	Enrofloxacin, ciprofloxacin, ofloxacin, oxolinic acid
Category C ("Caution")	Aminoglycosides (except spectinomycin)	Etreptomycin, gentamicin
	Aminopenicillins in combination with β-lactamase inhibitors	Amoxicillin-clavulanic acid
	Amphenicols	Florfenicol, thiamphenicol
	Cephalosporins: 1st- and 2nd-generation, and cephamycins	Cefalexin, cefapirin
	Macrolides (not including ketolides)	Tylosin, tulathomycin
	Lincosamides	Clindamycin, lincomycin
	Pleuromutilins	Tiamulin, valnemulin
	Rifamycins: rifaximin only	Rifaximin
Category D ("Prudence")	Aminopenicillins, without β-lactamase inhibitors	Amoxicillin, ampicillin
	Cyclic polypeptides	Bacitracin
	Nitrofuran derivatives	Furazoldone
	Nitroimidazoles	Metronidazole
	Penicillins: Anti-staphylococcal penicillins (β-lactamase-resistant penicillins)	Cloxacillin
	Penicillins: Natural, narrow spectrum penicillins (β-lactamase-sensitive penicillins)	Benzylpenicillin, phenoxymethylpenicillin
	Aminoglycosides: spectinomycin only	Spectinomycin
	Steroid antibacterial	Fusidic acid
	Sulfonamides, dihydrofolate reductase inhibitors and combinations	Sulfadiazine, trimethoprim
	Tetracyclines	Oxytetracycline, doxycycline

The EU carried out a monitoring program for antibiotic resistant bacteria, both zoonotic and indicators of bacterial quality in for humans, animals and food in all the states members. The annual monitoring of resistance in animals and food set its target in 2017 on pigs and cattle less than one year old, as well as their carcasses and meat. Similarly, in 2018 the target was birds and their derived products, carcasses and meat. The data collected these years included information from *Salmonella* spp., *Campylobacter* spp. and *E. coli* as indicator, as well as data obtained directly from the monitoring of ESBL / AmpC / carbapenemases producing *E. coli* strains (EMA, 2019).

According to the Regulation (EC) No 2073/2005 since 2017 the monitoring of AMR *Salmonella* spp. at slaughter was mandatory in isolates recovered from carcass swabs of fattening pigs and calves under one year of age and in 2018 become mandatory as well for isolates recovered from carcass swabs of broilers and fattening turkeys. Also, data from human cases were reported. In 2018, high proportions of human *Salmonella* isolates were resistant to sulfonamides (30.5%), tetracyclines (28.8%) and ampicillin (25.9%) and 0.8% of the isolates were presumptive ESBL-producing, meanwhile AmpC resistance was less frequent, only identified in 0.2% of tested isolates. MDR was high in this species with overall in the EU with a 28.5%.

Regarding *Campylobacter jejuni*, AMR monitoring was mandatory in slaughter in 2018 for caecal samples of broilers and fattening turkeys, and voluntary for caecal samples of fattening pigs and calves under 1 year. In 2018 a very high to extremely high resistance levels to ciprofloxacin were reported in almost all EU countries, but on the other hand low proportions of *Campylobacter* isolates were resistant to gentamicin and amoxicillin-clavulanic acid, except in Luxembourg, Malta and Spain with between 20 and 27.3% of *C. coli* resistant to clavulanic acid-amoxicillin, this differences are probably associated with the differences in the use of antimicrobials (EMA, 2019).

Each year, 30 EU and European Economic Area (EEA) countries report antibiotic susceptibility testing (AST) results collected from medical microbiology laboratories to EARS-Net for eight bacterial species under surveillance (*E. coli, K. pneumoniae, P. aeruginosa, Acinetobacter species, S. pneumoniae, S. aureus, E. faecalis and E. faecium*). In 2019, more than half of the *E. coli* isolates reported to EARS-Net and more than a third of the *K. pneumoniae* isolates were resistant to at least one antimicrobial group under surveillance, and combined resistance to several antimicrobial groups was frequent. Resistance percentages were generally higher in *K. pneumoniae* than in *E. coli*. While carbapenem resistance remained rare in *E. coli*, several countries reported carbapenem resistance percentages above 10% in *K. pneumoniae*. Carbapenem resistance was also common in *P. aeruginosa* and *Acinetobacter* species, and at higher percentages than in *K. pneumoniae* (ECDC, 2020). Recent studies have suggested that *E. coli* and *Klebsiella pneumoniae*, both associated with extraintestinal infections as well as with the widespread of antibiotic resistances, may be considered food-borne pathogens. The epidemiologic observations of the key lineages of these organisms suggested a common point-source exposure, such as contaminated food. Since contaminated food is the main source for microorganisms causing gastrointestinal infections, is easy to assume that this should be as well the entrance of microorganisms that are able to cause extraintestinal diseases.

1.3.1. The role of food-borne *E. coli*

The role of *Escherichia coli* as a pathogen is difficult to adequate into just a single type of pathology, as well as a single type of transmission or end host. It presents a complexity and extension as a species that makes it necessary to group it accordingly to its characteristics and taking into account its form of transmission which is also heterogeneous. InPEC strains with their pathotypes, their own characteristics and differences with the commensal strains, already described previously, shows a clear and indisputable transmission from food to the host. In fact, the pathotypes of *E. coli* most commonly detected in food and water destinated to human consumption are STEC isolates, usually associated with diarrhea, HC, HUS and end-stage renal disease with the highest rate of confirmed cases observed in children from zero to four years old. Some countries have surveillance networks in order to detect possible outbreaks (Yun et al., 2021) and collect epidemiologic data of the isolates in order to support decision-making at national level. In EU, the ECDC publishes yearly an epidemiological report with data recovered from the different countries (EFSA, 2019). The characterization of the food STEC isolates is pivotal for the assessment of the risk for consumers posed by food, usually serotyping was an important part of this process nevertheless, a recent pathogenicity assessment (Koutsoumanis et al., 2020) affirms that this feature is not an indicator of pathogenicity, but they admit that has some importance as an epidemiological marker, useful to observe the circulation of the different STEC types in food and human cases. Regarding the major food-borne outbreaks caused by *E. coli* in the last two decades we find a wide diversity of sources and pathotypes (Yang et al., 2017). Below, the most recent events and pathotypes are cited.

Two different aEPEC serotypes, O157:H45 and O127a:K63 caused two different outbreaks in China and South Korea in 2010 and 2013, respectively. The Chinese outbreak affected 112 students, from 18 to 23 years old. They developed digestive symptoms after eating in the same dining room, nevertheless, in this case, the researchers were not able to identify the origin of the infection, but associated its high virulence to the presence of the *eae* intimin alongside with resistance to quinolone and extended spectrum cephalosporin, mediated by five mutations, two in *gyrA*, two in *parC*, one in *parE* and the resistance genes *aac(6')-Ib-cr* and $bla_{CTX\text{-}M\text{-}15}$ (Hao et al., 2012) In the Korean outbreak, the authors analyzed samples from stool, environment as well as preserved food items, to finally determinate the tuna bibimbap, a typical Korean soup breakfast, to be de vehicle for the aEPEC that infected 33 people (Park et al., 2014). In developed countries is uncommon to find food-borne outbreaks associated with EIEC, nevertheless, an outbreak with a total of 109 cases of EIEC O96:H19 happened in 2012 in Italy. It was associated with the consumption of vegetables and affected the canteen of the Milan Fire Brigade (Escher et al., 2014).

In Europe, in 2006 and 2013, two different outbreaks associated with EAEC isolates were registered, one in a food festival in England and another associated with cheese from unsterilized raw milk (Scavia et al., 2008). In the food festival, different serotypes were involved, including O131:H27, O104:H4, O20:H19 among the 592 registered cases. The EAEC O104:H4 strains found in this outbreak had a close phylogenetic relationship with the one from the large outbreak of HUS in Germany in 2011 (Dallman et al., 2014). The causing agent of the latter was a hybrid pathotype of STEC and EAEC, carrier of several virulence factors of the ExPEC group, and a wide range of antibiotic resistances. The outbreak accounted for 3,816 cases in humans, with 845 of them developing HUS and 36 dying from it and 2,971 cases of gastroenteritis with 18 deaths associated. The main traits of the isolate causing the situation

were: serotype O104:H4, virulence factors associated with STEC (*stx2a*) and with enteroaggregative pathotype, as well as the $bla_{CTX-M-15}$ gene (Mora et al., 2011b; Blanco, 2012). In May 31th of 2011, the Robert Koch Institute reported that the suspected origin of the outbreak were cucumbers from Spain, nevertheless in June 23rd fenugreek seeds imported from Egypt was determined as the real vehicle for the hybrid pathotype (EFSA, 2011).

However, the dominant pathotype / serotype in food-borne outbreaks is still by far the STEC/EHEC O157:H7, with cases reported all over the world associated mainly with meat and vegetables (Jay et al., 2007; Wendel et al., 2009; CDC, 2014, 2015, 2019a, 2020b; Watahiki et al., 2014). Comparing the incidence of O157 and non O157 STEC outbreaks, we can appreciate that those associated with non O157 are less frequently linked to meat, water or vegetables, but more prone to be linked to person-to-person infection. While outbreaks caused by O157 strains are more frequently produced by meat as well as contaminated water and vegetables watered with it (Doyle et al., 2008).

On the other hand, in ExPEC strains, the transmission through food, especially animal-derived products, has been proposed on multiple occasions but due to the lack of knowledge of the time period between the colonization of the intestinal tract and the development of the infection, it is difficult to detect the reservoirs through which these strains are transmitted (Manges and Johnson, 2012).

In a recent review, Riley (Riley, 2020), stated that the problem arises from not being able asses with full certainty the reservoir from where these strains came, since knowing it could give valuable information about the flow of movement that they follow to cause infection. The problem that arises with ExPEC stains is the dichotomy of its nature. On one hand, if the ExPEC strains are simply commensals that are able to cross the hematological barrier and cause disease, the reservoir should be the human digestive tract, but, on the other hand, if we consider that the ExPEC strains are pathogenic strains, their natural habitat must lie outside the human intestine, where they must reach and colonize to cause disease. So, if we consider the ExPEC strains as pathogens, then their transmission must come from external sources such as food or water (Riley, 2020). This whole process is also hampered by the fact that the colonization time of these strains until the moment of causing infection remains unknown (Manges and Johnson, 2012).

The hypothesis that food, particularly avian products, can act as a reservoir for extraintestinal pathogenic Enterobacteriaceae is based on scientific evidence obtained from different approaches (Mora et al., 2009b, 2013; Jakobsen et al., 2010; Davis et al., 2015; Hindermann et al., 2017; Mellata et al., 2018; Jørgensen et al., 2019; Riley, 2020). In fact, certain strains that cause avian pathology, APEC isolates, show a high genetic similarity to those that cause extraintestinal pathology in humans. The hypothesis emerged from several studies is that some human ExPEC strains might have evolved from APEC lineages (Manges and Johnson, 2012; Jørgensen et al., 2019). The evidence that suggests this hypothesis are, among others:

- The geographical and temporal grouping of ExPEC strains isolated from patients with extraintestinal infections, suggesting the appearance of an outbreak and / or a common source of exposure (Yamaji et al., 2018a).

- The global distribution of lineages of identical ExPEC strains, which would indicate the global spread of contamination carried through food (Liu et al., 2018).
- The detection of identical genotypes of ExPEC isolated from human infections, as well as from food products (Yamaji et al., 2018a).
- The disproportionate representation of pandemic ExPEC lineages among the hundreds of STs causing extraintestinal infections worldwide, indicating a high biological or fitness advantage within different reservoirs (Mora et al., 2018).
- The relatively recent occurrence of the ST69, ST131 and ST393 genotypes as ExPEC, suggesting the recent emergence of these genotypes into the human intestinal niche from external sources (Manges and Johnson, 2012).

Riley's review analyzes the most frequently reported STs identified in two or more regions within isolates of clinical (human) and animal origin. The following table summarizes the nine dominant lineages (Table 8).

Table 8. The nine prevalent STs of *E. coli* identified in human extraintestinal infections (Riley, 2020)

ST	Number of EnteroBase entries	Food and food-animal sources	Other sources
ST10	6432	Poultry, bovine, swine, dairy (raw-milk cheese), goat, sheep, fish	Dog, horse, rabbit, sea lion, camel, pigeon, gazelle
ST12	598	Swine, bovine, fish	Dog, cat, horse, mink, raccoon, rat
ST69	1529	Poultry, bovine, sheep, dairy (raw-milk cheese)	Dog, horse, dolphin, mink, bald eagle, seagull
ST73	1984	Poultry, swine, bovine	Cat, donkey, duck, horse, giraffe, orangutan, elephant, gorilla, rhesus monkey, ferret, mouse
ST95	1590	Poultry, bovine, lettuce	Dog, ostrich, swan, rat, gecko, poultry feed
ST117	925	Poultry, bovine, calf, swine	Dog, cat, mink, rabbit, rat, animal feed
ST127	525	Turkey, bovine, celery	Dog, gazelle, rat, horse
ST131	6574	Poultry, bovine, pork	Dog, cat, rook, horse, seagull, rodent
ST405	646	Bovine, whale	Dog, crow, marmoset

Yamaji *et al.* (Yamaji et al., 2018a) analyzed cases of UTIs and meat samples of different origins recovered in the same. The study shows an overlap between the STs of the *E. coli* recovered from patients with UTIs and those found, mainly, in poultry meat samples. Thus, 21% of the STs determined in isolates of human origin were also found in poultry meat (ST10, ST38, ST69, ST101, ST117, ST131, ST569 and ST1844 in chicken meat and ST10, ST69, ST80, ST88, ST117 and ST1844 in turkey meat). The authors concluded that poultry meat may be acting as a reservoir for ExPEC isolates responsible of UTIs.

But *E. coli* is not only important to be consider as a pathogen, as mentioned previously, it is considered a carrier and spreading agent of antibacterial resistances. According to the study carried out by the European Union between 2017 and 2018, where commensal *E. coli* from healthy production animals were analyzed at slaughterhouses, these could be acting as a reservoir for resistant strains that could potentially spread among animals, as well as from animals to humans through the food chain. The rates of resistance detected to ampicillin, sulfamethoxazole, trimethoprim and tetracyclines in the strains were classified in most of the state members, as high or very high for pigs and cattle in 2017, and for chicken and turkey in

2018. Furthermore, particularly in poultry, resistance to ciprofloxacin and nalidixic acid was also common and was given a level of very high or extremely high resistance, both for chickens and turkeys (EMA, 2019).

In the same study, when they compared the four different species studied regarding MDR, the higher levels were clearly found in chickens (49.4%) and turkeys (52.4%) compared to pigs (31.1%) and calves (28.4%) (EMA, 2019). A possible explanation for these large differences may corelate with the method of administration of the antibiotic treatments during the production stage, since in poultry farms, the whole group is treated through water or food, meanwhile in the case of pigs and cattle, the treatments are mainly individual. The importance of these data, as we said before, lies in the possibility that these antibiotic resistances spread worldwide to commensal strains and pathogenic strains, therefore complicating antibiotic treatments for common pathologies such as UTIs. An improvement of the situation is beginning to be appreciated thanks to the implementation of programs such as the National Plan against Antibiotic Resistance (PRAN) 2014-2018 and 2019-2021 and international measures like the legislation on the use of antibiotics, although it must be mentioned that each member country starts from different conditions and levels of resistance, which will determinate its evolution.

Due to the methodology applied, data obtained from the majority of studies regarding ExPEC isolates is generally biased towards the recovery of ESBL-producing isolates or strains causing clinical pathology in humans, missing all the possible pathways that ExPEC isolates may colonize the human gut (Singer, 2015; Liu et al., 2018). Currently, there are many studies oriented to the detection of AMR ExPEC, or specific clades of the clonal group ST131 associated with human pathology (Ghodousi et al., 2015; Johnson et al., 2017b; Park et al., 2019; Vounba et al., 2019; Nagaoka et al., 2020; Taati Moghadam et al., 2021), nevertheless, this trend is slowly changing since many authors point out the importance of analyzing the diversity of ExPEC isolates involved in the dissemination of virulence genes through the environment and the different reservoirs. The same approach based on surveys for STEC should be applied for ExPEC isolates to assess the risk exposure for the costumer (Smith et al., 2007; Johnson and Manges, 2015; Riley, 2020).

1.3.2. *Klebsiella* spp. as food-borne pathogen

The members of *Klebsiella* spp. have been commonly described as commensals located in a wide diversity of niches such as the gastrointestinal tract of humans. Before the 90s, when certain lineages associated with epidemic outbreaks emerged worldwide, UTIs caused by these bacteria normally affected only hospitalized patients or with previous pathologies. Unfortunately, the KPC resistance associated with the clonal group CC258 began to spread in the 2000s. Thus, this clonal group was reported in outbreaks of New York hospitals (Bradford et al., 2004) as well as elsewhere on the east coast of the United States, and in countries as far away as Israel (Schwaber et al., 2011).

In 1998, it was the first time that a case of sepsis attributed to *K. pneumoniae* and *E. coli* was associated with the ingestion of food, in this case a hamburger (Sabota et al., 1998). In 2008, in different hospitals in Barcelona, many cases of patients infected and intestinal colonization by *Klebsiella* spp. were registered in a period of nine months, and all the isolates showed the same PFGE profile. The researchers concluded that both infections and colonization were originated from a food-borne outbreak (Calbo et al., 2011), although the food product that

caused the outbreak was not identified. A more recent study carried out by Davis *et al.* (Davis et al., 2015) establishes more clearly the relationship between food and an outbreak of extraintestinal infections associated with *Klebsiella* spp. The authors performed WGS of 82 strains recovered from meat (chicken, turkey, and pig) and from clinical human cases, evidencing an overlap of STs between both sources (ST14, ST76, ST188 and ST111) and similar virulence patterns in a murine model.

The fact that retail meat contaminated with AMR *K. pneumoniae* is a potential vehicle for the transmission of resistance and / or virulent genes is supported by diverse studies (Davis et al., 2015; Davis and Price, 2016; Hu et al., 2021). As an example, it is important to highlight the report of one *K. pneumoniae* isolate recovered in February 2016 from unfrozen chicken from a local grocer in Japan. This isolate showed resistance to a wide range of antibiotics including carbapenems. In the study, the authors performed WGS of the isolate and detected the presence of six plasmids, two of which carried different antibiotic and heavy metal resistance genes, including *mcr-9* and bla_{VIM-1} in an IncHI2A plasmid. Furthermore, the bla_{NDM-1} gene was found in a large IncFII(K) plasmid alongside with other resistance genes. The isolate belonged to ST30, a ST previously reported in patients in the United States as well as in China (Khalifa et al., 2020). In another recent study conducted in Singapore, *K. pneumoniae* was recovered from 21% (147 of 698) of the raw and ready-to-eat retail food screened. In this study, the results showed that 10% of the isolates analyzed were MDR. 98% of isolates tested were resistant to ampicillin and 14% to tetracycline. Ciprofloxacin resistance was shown by 8% of the isolates, 7% were resistant to chloramphenicol, 6% to sulfamethoxazole-trimethoprim, 5% to amoxicillin-clavulanic acid, 2% to nalidixic acid, 1% to amikacin and 1% resistant to ceftriaxone. Although only 7% of the total isolates showed genetic virulence determinants, the finding of them could potentially be a public health hazard as they make resistance genes available for other bacteria present in the food chain (Hartantyo et al., 2020).

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2. OBJECTIVES

OBJETIVES

The motivation for this doctoral thesis was based on the findings reported in previous theses (Herrera, 2015; Viso, 2017; Meniño, 2019) and research project AGL2013-47852-R, which indicated that "*food, especially poultry products, may act as an ESBL-producing ExPEC reservoir for humans*", and that "*there has been a rapid dissemination of ESBL-producing strains associated with successful clonal groups such as the pandemic ST131 within different niches*".

The HYPOTHESIS of the present thesis was that poultry meat would act as a reservoir, and potentially transmitter, of pathogenic strains that might be implicated in human UTI. To demonstrate this hypothesis, the STRATEGY was:

a) To analyze retail poultry meat directly acquired at points of sale with the idea that the final product provides data on what is happening on the farm, at the slaughterhouse, and what goes into the consumer's kitchen.

b) To identify potential uropathogenic clonal groups of *E. coli* based on specific genetic markers.

c) To consider "high-risk" strain that with the capacity to develop a serious extraintestinal infection in humans, due to either its virulence potential and / or its antibiotic resistance.

The specific OBJECTIVES were:

1. To design an efficient protocol for the recovery of food-borne *E. coli* and other pathogenic and / or antimicrobial-resistant Enterobacteriaceae.

2. To acquire knowledge on the current situation regarding AMR in poultry farming, paying special attention to antimicrobial categories A and B of EMA.

3. To assess the consumer exposure, via poultry meat, to high-risk *E. coli* and other Enterobacteriaceae isolates with potential to develop severe infections by either bacterial virulence and / or antibiotic resistance traits.

4. To explore the food transmission route of specific *E. coli* clones of human and animal origin through comparative genetic and genomic analysis.

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3. MATERIAL AND METHODS

3.1. BACTERIAL COLLECTIONS

3.1.1. Control strains and conservation

Positive and negative control strains from the LREC-USC collection were used in all phenotypic and genotypic tests (Table 9). The Enterobacteriaceae strains characterized in this study, as well as all the reference control strains, were preserved on nutrient agar with 0.75% (w / v) agar and were stored at room temperature in Vacutainer ™ tubes. Under these conditions, the strains maintain their viability for at least five years. For the preparation of the preservation medium, a mixture of nutrient agar (11.5 g / l) (Applichem-Panreac) and nutrient broth (4 g / l) (Applichem-Panreac) was used.

Table 9. Reference control strains

Control strain	Genes
ExPEC	
FV14504	*iutA, iucD, tsh, neuC-K1*
Daec II	*afa/draBC*
pap	*papEF*
FV10041	*fimH, fimAvMT78, papEF, papG III, sfa/focDE, cnf1, hlyA, iroN, KpsM II, KpsM II-K2, neuC-K1, ibeA, malX, usp, uitA*
FV10042	*fimH, papEF, cnf1, hlyA, iroN, KpsM II, KpsM II-K2, kpsM II-K5, neuC-K1, malX, usp*
FV14043	*fimH, papEF, papG II, cdtB, sat, iroN, kpsM II, kpsM II-K2, kpsM II-K5, malX, usp*
FV10044	*iutA, cvaC, iss, traT, tsh*
FV10045	*afa/draBC, sat, iutA, traT*
FV14390 FV14391	*fimH, fimAvMT78*
FV14067	*ibeA*
FV12671	*sfa/focDE, cdtB*
FV17090	*afaFM*
FV17134	*kpsM III*
FV 17132	*hlyF, ompT, vat, yfcV, fyuA*
EPEC	
IH2859f	*eae, bfpA*
ETEC/VTEC	
FV15560	*estB, k88, hlyA*
FV 15556	*stx2/vt2, estA, estB ,f18, hlyA*
FV15568	*stx2/vt2, f18, hlyA*
FV15525	*estA, estB, p987*
FV15498	*eltA , estB, k88, hlyA*
FV15553	*estA, p987*
FV15567	*estA, k99, f41*

FV15528	*estA, k99, f41*
C143.4A	*Stx2e/ stx2e*
FV20299	*k88ab*
FV20373	*k88ac*
FV20298	*k88ad*
FV20372	*f18ab*
FV20374	*f18ac*
O157.34	*stx1/vt1, stx2/vt2*
EIEC	
EiEC26	*ipaH*
EAEC	
FV 167	*aatA*
Resistance	
FV9650	$bla_{CTX\text{-}M}$, $bla_{CTX\text{-}M\ group\ 9}$
FV19247	bla_{SHV}
FV17090	$bla_{CTX\text{-}M\ group\ 1}$, $bla_{CTX\text{-}M\text{-}15\ end\ 3'}$
FV14390	bla_{TEM}
FV17811	*LAT-1 a LAT-4, CMY-2 a CMY-7, BIL-1*
FV20151	*mcr-1*
FV 21136	*mcr-2*
FV 21074	*mcr-4*
FV 21078	*mcr-5*
FV 18691	*colEX*
Phylogenetic group	
FV10042	*chuA, yjaA, TSPE*
O157-1103	*arpA*, phylogroup E
O and H antigens	
FV17090	*rfbO25b*, $fliC_{H4}$
FV14067	*rfbO25b*
LREC-H1	$fliC_{H1}$
LREC-H2	$fliC_{H2}$
LREC-H4	$fliC_{H4}$
LREC-H7	$fliC_{H7}$
LREC-H8	$fliC_{H8}$
LREC-H9	$fliC_{H9}$
LREC-H10	$fliC_{H10}$
LREC-H11	$fliC_{H11}$
LREC-H18	$fliC_{H18}$
LREC-H21	$fliC_{H21}$
LREC-H25	$fliC_{H25}$
LREC-H28	$fliC_{H28}$

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3.1.2. Poultry meat Enterobacteriaceae collection

In total, 358 different Enterobacteriaceae isolates were recovered from 100 poultry meat samples (170 isolates recovered from 50 chicken samples and 188 isolates recovered from 50 turkey samples). Bacterial identification revealed that 323 out of 358 isolates were *E. coli*, 28 *K. pneumoniae*, six *Serratia fonticola* and one *Enterobacter cloacae*. This collection was fully characterized as detailed in section Material and Methods, Results and in Table 41.

3.1.3. EPEC O153 collection

Collections obtained during the period of 2005 to 2015 from different surveillance studies performed at LREC, in Lugo, Spain, aimed the detection of ESBL-producing *E. coli* within different sources of our region. These studies included samples from chicken, beef and pork meat, as well as poultry farm environment and wildlife.

On the other hand, human diarrheagenic *E. coli isolates*, mainly from the Hospital Universitario Lucus Augusti (HULA) of our city (Lugo, northwest Spain), were routinely screening in our laboratory for intestinal VF, and those positive, further analysed for extraintestinal traces and ESBL genes, as described below.

The number of isolates, origin and period of isolation of the collections are detailed in Table 10.

Table 10. Thirty-two isolates included in the study (in red) from our own collections.

Origin of isolation	Sampling period	No. ESBL aEPEC O153 isolates / total ESBL isolates [a]	No. NON-ESBL aEPEC O153 isolates[b]
Chicken meat study	2009-2010	**7** / 127	NA
Beef meat 1st study	2005-2009	**5** / DNA	**2**
Beef meat 2nd study	2011-2012	**1** / 5	NA
Pork meat study	2011-2012	**1** / 13	NA
Poultry farm environment	2010-2012	**1** / 96	NA
Wildlife study	2014-2015	**1** / 95	NA
Human diarrhoea	2006-2012	**5** / DNA	**9**
[a] Data not available (DNA); [b] Not analysed (NA)			

3.2. MEAT SAMPLING AND ENTEROBACTERIACEAE CHARACTERIZATION

3.2.1. Sampling, screening and Enterobacteriaceae recovery

Between September 2016 and September 2017, 100 poultry meat products were acquired in eight points of sale in the city of Lugo (northwest Spain), including six supermarket chains (Table 11). The meat products were transported in an isothermal container and processed within the next two hours after collection. Of the 100 meat samples, 50 were chicken breast (24 packaged in a modified atmosphere and 26 freshly cut by the butcher at the moment of sampling), and another 50 were turkey meat (25 in modified atmosphere and 25 freshly cut). The lab method designed was oriented to investigate i) the microbiological quality of poultry meat based on *E. coli* counts and ii) the prevalence of pathogenic and AMR food-borne *E. coli* and other Enterobacteriaceae. For the latter (AMR and potential pathogens), the method was subdivided in six protocols (I to VI) based on a combination of selective media and incubation temperatures, whose bacterial growth was eventually screened by PCR for specific genetic targets. Through the characterization of the recovered isolates, the adequacy of each protocol was finally evaluated.

Table 11. Sampling date, type and packaging system of the 100 meat products and points of sale

Sampling batch	Sampling date	Meat type (no. of samples)	Packaging system (C=chicken; T=turkey)	[a] Points of sale
1	05/09/2016	Chicken (10)	5 modified atmosphere 5 freshly cut	A, B, C, D, E
2	20/09/2016	Turkey (8)	4 modified atmosphere 4 freshly cut	B, C, E, F, G
3	19/10/2016	Chicken (2)	2 freshly cut	H
4	02/11/2016	Chicken (4) Turkey (4)	4 modified atmosphere (2 C + 2 T) 4 freshly cut (2 C + 2 T)	C, D, E
5	22/11/2016	Chicken (4) Turkey (4)	4 modified atmosphere (2 C + 2 T) 4 freshly cut (2 C + 2 T)	A, B, C, G
6	13/03/2017	Chicken (4) Turkey (4)	4 modified atmosphere (2 C + 2 T) 4 freshly cut (2 C + 2 T)	B, C, E, G
7	03/04/2017	Chicken (4) Turkey (4)	4 modified atmosphere (2 C + 2 T) 4 freshly cut (2 C + 2 T)	B, C D, E, G
8	24/04/2017	Chicken (4) Turkey (4)	4 modified atmosphere (2 C + 2 T) 4 freshly cut (2 C + 2 T)	A, B, D, E
9	22/05/2017	Chicken (4) Turkey (4)	4 modified atmosphere (2 C + 2 T) 4 freshly cut (2 C + 2 T)	B, C, E, G
10	08/06/2017	Chicken (4) Turkey (4)	4 modified atmosphere (2 C + 2 T) 4 freshly cut (2 C + 2 T)	B, D, E, G
11	27/06/2017	Chicken (4) Turkey (4)	4 modified atmosphere (2 C + 2 T) 4 freshly cut (2 C + 2 T)	B, C, E, G
12	04/09/2017	Chicken (6) Turkey (4)	5 modified atmosphere (3 C + 2 T) 5 freshly cut (3 C + 2 T)	C, D, E
13	18/09/2017	Turkey (6)	3 modified atmosphere 3 freshly cut	D, E, B, C

[a] Points of sale: A to F (supermarket chains); G, H (local retailers).

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Briefly, 25 g of each meat sample were aseptically cut and homogenized (2 min in a stomacher) with 225 ml of Buffer Peptone Water (BPW; ApplyChem Panreac). From the homogenate, 1 ml was plated into a 3M Petrifilm™ Select *E. coli,* which was examined after incubation 24 h/44 ºC for the *E. coli counts* following manufacturer's instructions. Then, the homogenized meat samples were incubated 6 h/37 ºC, from which 1 ml was inoculated in duplicate into 9 ml MacConkey Lactose broth (Oxoid) tubes, growth for 18-24 h at 37 ºC and 44 ºC, respectively. Finally, different selective agar media (protocols I to VI) were inoculated from the MacConkey Lactose broth tubes (Figure 2). The protocols I to IV were meant for the detection of potentially pathogenic *E. coli (*carriers of diarrheagenic or extraintestinal virulence traits): protocol I. MacConkey Lactose agar (ML) (Oxoid), 18-24 h/37 ºC; protocol II. MacConkey Sorbitol agar enriched with tellurite and cefixime (MSTC) (Oxoid), 18-24 h/37 ºC; protocol III. ML, 18-24 h/44 ºC; protocol IV. MSTC, 18-24 h/44 ºC. Additionally, ESBL-producing Enterobacteriaceae were screened by means of CHROMID® ESBL agar plates (bioMérieux) in protocol V, while the protocol VI screened carbapenemase-producing *E. coli in* CHROMID® CARBA SMART plates (bioMérieux).

As shown in Figure 2, the confluent growth of plates I to IV and the pooled colonies recovered from I to VI and plated on Tryptone Soy Agar (TSA) (Oxoid) were analysed by PCR for specific virulence factors (VF) associated with the InPEC pathotypes EPEC (*eae*, *bfpA*), STEC (*stx1*, *stx2, eae*) and EAEC (*aaiC*, *aggR*). Likewise, specific VF linked to the pathogenic potential of extraintestinal pathogenic *E. coli* (ExPEC status) and uropathogenic *E. coli* (UPEC status) were tested (Table 28; Table 29). The ExPEC status was assigned to isolates positive for ≥2 of these five markers (*papAH*, *sfa/focDE*, *afa/draBC*, *kpsM II* and *iutA*) (Johnson et al., 2003c), while the UPEC status was assigned to isolates positive for ≥ 3 of these four markers (*chuA*, *fyuA*, *vat* and *yfcV*) (Spurbeck et al., 2012). For those isolates exhibiting ExPEC and / or UPEC status, other extraintestinal VF were analyzed to complete their characterization (Table 28; Table 29). The O25b subtype (*rbfO25b*) associated with the clonal group ST131 was also screened by PCR (Clermont et al., 2008) and positive isolates were confirmed by multilocus sequence typing (MLST). PCR amplification of the β-D-glucuronidase-encoding gene (*uidA*) was routinely used to specifically identify *E. coli* (Gómez-Duarte et al., 2010) (Table 28; Table 29). Additionally, isolates suspected of being *E. coli* but *uidA* negative, as well as other Enterobacteriaceae, were identified by MALDI-TOF MS (Bruker Daltonik, Bremen, Germany) in duplicated; a reliable result (at the species level) was only considered if the score obtained was higher than 2. All the isolates recovered in this study were stored at room temperature in nutrient broth (Difco™) with 0.75% nutrient agar (Difco™) for further characterization.

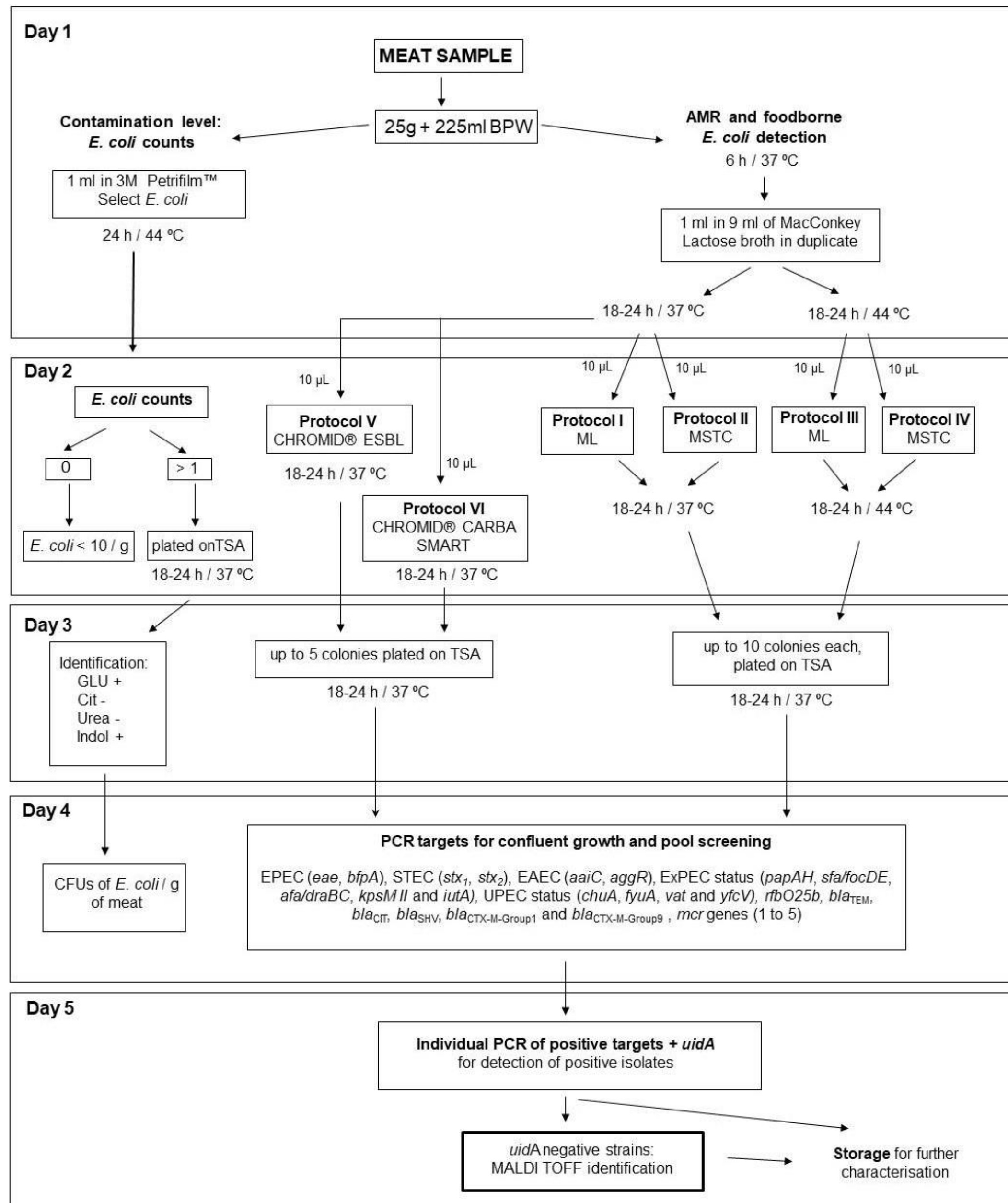
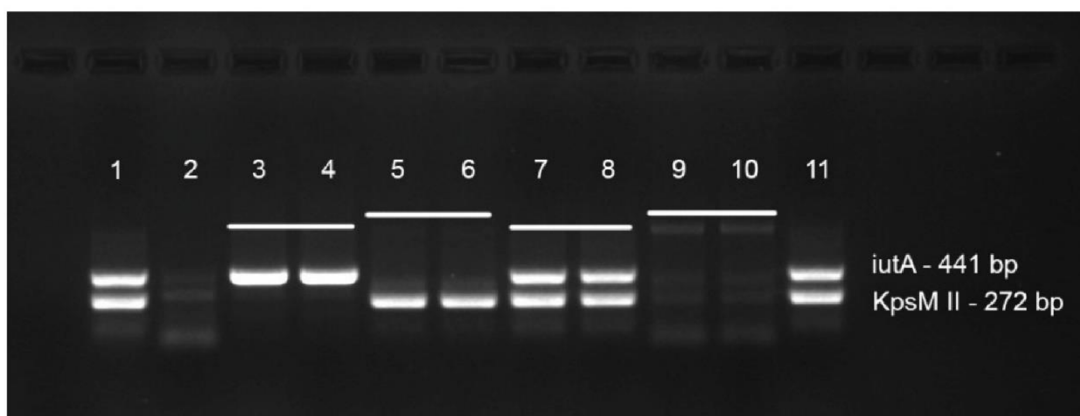


Figure 2. Lab workflow designed in this study to investigate the level of contamination, and the rates of AMR and food-borne pathogenic *E. coli*. *Note*: AMR: antimicrobial resistance; ML: MacConkey Lactose agar; MSTC: MacConkey Sorbitol agar enriched with tellurite and cefixime; TSA: tryptone soy agar; CFU: colony forming units; EPEC: enteropathogenic *E. coli;* STEC: Shiga toxin-producing *E. coli;* EAEC: enteroaggregative *E. coli;* ExPEC: extraintestinal pathogenic *E. coli*. From protocols V and VI, other Enterobacteriaceae were also investigated.

3.1.2.1.Rapid detection of *E. coli* conforming ExPEC status in meat samples

We designed a duplex PCR for a rapid and effective recovery of isolates with ExPEC status. Previous results indicated that >95% of the isolates present in meat and conforming ExPEC status were carriers of both *iutA* and *KpsM* II genes; furthermore, 100% of them were carriers of at least one of those genes (Herrera, 2015). Using these targets, the duplex PCR amplifies a

fragment of 272 bp with the kpsII f and r primers described elsewhere for *KpsM* II (Johnson and Stell, 2000), and 441 bp of *iutA*. For the latter, we designed the new primer "iutA-Al f" 5'GCCGGAGCTGTCTCCGGCGG 3´ within the locus tag "NRG857 30235" of *iutA* from the GenBank CP001856 genomic sequence, which was used with the previously aer-1152r primer described by Johnson *et al.* (Johnson et al., 1997) (Figure 3). For a 25 µl PCR reaction, the amplification mix includes 12,5 µl of NZYTaq 2x Green MasterMix (2,5 U), 0.6 µl of 20 µM of KpsM II primers, 1 µl of 20 µM of *iutA* primers, and 5 µl of sample DNA. The PCR conditions were validated with DNA pools of negative and positive isolates for one or both genes, as well as with individual colonies (Figure 3).



Target	Primers	Nucleotide sequence (5´- 3´)	Size (bp)	Reference
iutA	iutA-Al f	GCCGGAGCTGTCTCCGGCGG	441	This study
	aer-1152 r	CGTCGGGAACGGGTAGAATCG		Johnson *et al.* 1997
kpsM II	KpsII f	GCGCATTTGCTGATACTGTTG	272	Johnson & Stell 2000
	KpsII r	CATCCAGACGATAAGCATGAGCA		

Figure 3. Duplex PCR based on *iutA* (441 bp) and *KpsM* II (272 bp) targets and designed for the ExPEC screening on confluent growth, pooled and individual isolates. *Note*: Lines 1 and 11, positive control (+ +); line 2, negative control (- -); lines 3 and 4, *iutA* carriers (+ -); lines 5 and 6, *KpsM II* carriers (- +); lines 7 and 8, *iutA* and *KpsM II* carriers (+ +) and lines 9 and 10, negative carriers (- -). PCR products were loaded on a 1.5% agarose gels with nzytech GreenSafe as stain. After electrophoresis, images were captured in an ultraviolet BioRad GelDoc. The thermal cycle included 35 cycles of amplification (denaturation 94°C, 1 min; annealing 60°C, 1 min; extension 72°C, 1.30 min).

3.2.2. Phylogroups and clonotypes of *E. coli*

The phylogenetic relatedness of the *E. coli* population recovered from the poultry meat was determined by means of the phylogroup, sequence type (ST), clonotype (CH) and serotype assignment as described elsewhere (Díaz-Jiménez et al., 2020b). In brief, the Clermont method (Clermont et al., 2013, 2019) recognizes eight phylogroups (A, B1, B2, C, D, E, F, G) belonging to *E. coli sensu stricto* and also discriminates those belonging to *Escherichia* cryptic clades. The MLST was performed following Achtman's scheme based on seven genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) (Wirth et al., 2006) (Table 28; Table 29). The CHs were established based on the internal 469-nucleotide (nt) and 489-nt sequence of the *fumC* (allele obtained from MLST) and *fimH* genes, respectively (Weissman et al., 2012) (Table 28; Table

29). The isolates confirmed as ST131 were characterized for their virotypes according to the scheme defined by Dahbi *et al.* (Dahbi et al., 2014), based on the presence or absence of certain extraintestinal VF (*afa/draBC*, *afa* operon FM955459, *iroN*, *sat*, *ibeA*, *papG II*, *papG III*, *cnf1*, *hlyA*, *cdtB*, *kpsM II-K1*, *-K2 and -K5*) (Table 28; Table 29). The collection was also investigated by PCR for specific *bla* genes using the TEM, CIT, SHV, CTX-M-1 and CTX-M-9 group-specific primers, and further sequencing, as well as for the *mcr* genes (1 to 5) as previously described (Díaz-Jiménez et al., 2020b) (Table 28; Table 29).

3.2.3. O and H typing of *E. coli*

O:H antigens of *E. coli* were determined following the method described by Guinée *et al.* (Guinée *et al.*, 1981b) with O1 to O185 and H1 to H56 antisera, respectively. Isolates that did not react with any O antisera were classified as non-typeable (ONT), and non-motile isolates (HNM) were further analyzed by PCR for their flagellar genes (Mora et al., 2018) (Table 28).

3.2.4. Antimicrobial susceptibility and genetic characterization of β-lactamase and *mcr* genes of Enterobacteriaceae isolates

Antimicrobial susceptibility testing was conducted by disc diffusion assay and / or by the Microscan system (Beckman Coulter, CA, USA). The antibiotics tested included ampicillin (AMP), amoxicillin-clavulanic acid (AMC), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), aztreonam (ATM), imipenem (IMP), gentamicin (GEN), tobramycin (TOB), amikacin (AMK), fosfomycin (FOF), doxycycline (DOX), chloramphenicol (CHL), nitrofurantoin (NIT), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP), nalidixic acid (NAL) and tigecycline (TGC). Furthermore, MICs for colistin (CST) were manually obtained by broth microdilution for those suspected colonies. All results were interpreted according to the CLSI guidelines (The Clinical and Laboratory Standards Institute, 2020). Multidrug-resistant (MDR) isolates were defined according to Magiorakos *et al.* criteria, as those showing acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012). The collection was also investigated by PCR for screening of specific *bla* genes using the TEM, CIT, SHV, CTX-M-1 and CTX-M-9 group-specific primers, and further sequencing (Mora et al., 2013), as well as for the *mcr* genes (1 to 5) (Table 28).

3.2.5. Screening of ST131 and virotypes

The O25b subtype associated with the clonal group ST131 was screened by PCR (Clermont et al., 2008) within the *E. coli* collection (Table 28; Table 29). The isolates confirmed as O25b:H4-B2-ST31 were characterized for their virotypes according to the scheme defined by Dahbi *et al.* (Dahbi et al., 2014), based on the presence or absence of certain extraintestinal VF (*afa/draBC*, *afa* operon FM955459, *iroN, sat, ibeA, papG II, papG III, cnf1, hlyA, cdtB, kpsM II-K1, -K2* and *-K5*). The assignment to the corresponding virotype is also detailed in Table 3 of the introduction section.

3.2.6. *K. pneumoniae* characterization

Those *K. pneumoniae* recovered from CHROMID® plates, and identified by MALDI-TOF MS, were further characterized for their STs following the Institute Pasteur MLST scheme (Diancourt et al., 2005) (Table 28).

3.2.7. Pulsed field gel electrophoresis (PGFE)

XbaI-PFGE profiles were performed following the PulseNet protocol (https://www.cdc.gov/pulsenet/participants/international/index.html), and imported into BioNumerics (Applied Maths, St-Martens-Latern Belgium) to obtain a dendrogram with the UPGMA algorithm based on the Dice similarity coefficient and applying 1% of tolerance in the band position.

3.2.8. WGS of *mcr*-positive isolates

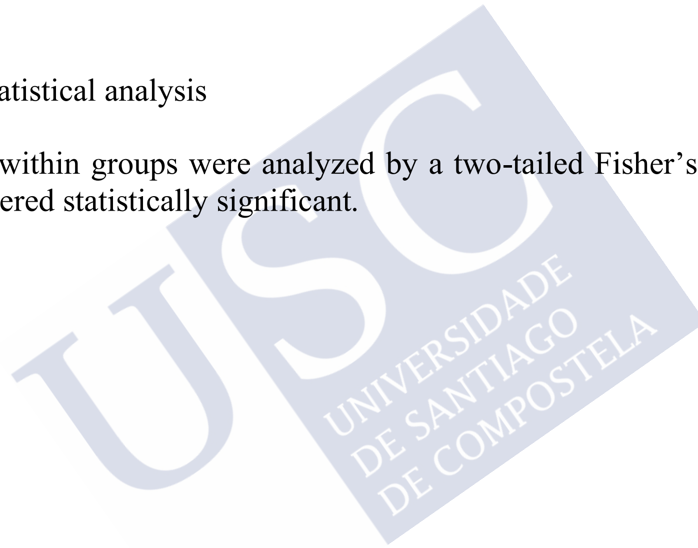
Two isolates positive by PCR for the presence of *mcr* genes were further characterized by WGS. Genomic libraries were constructed using the Nextera XT DNA library preparation kit (Illumina) following the manufacturer's instructions and sequenced on an Illumina HiSeq X with 150 bp x 2 read length. Quality control checks on the obtained raw sequence data was performed using FastQC version 0.11.3. Genome assembly was performed *de novo* using SPAdes 3.11 (Bankevich et al., 2012) and were *in silico* analyzed using the bioinformatics tools of the Center for Genomic Epidemiology (CGE) (https://cge.cbs.dtu.dk/services/) for the presence of antibiotic resistance (ResFinder V2.1.), virulence genes (VirulenceFinder v1.5.), plasmid replicon types (PlasmidFinder 1.3./PMLST 1.4.), and identification of clonotypes (CHTyper 1.0), sequence types (MLST 2.0) and serotypes (SerotypeFinder 2.0). All the CGE predictions were called applying a select threshold for identification and a minimum length of 95% and 80%, respectively. Phylogroups were predicted using the ClermonTyping tool at the iame-research center web (http://clermontyping.iame-research.center/).

3.2.9. WGS of EPEC O153 isolates

DNA from 17 isolates was extracted with the QIAamp 96 DNA Qiacube HT kit (Qiagen, Hilden, Germany) and libraries were prepared using the Nextera XT kit (Illumina). Pooled libraries were denatured following the Illumina protocol and 600 µl (approx. 20 pM) were loaded onto a MisSeq V2 -500 cycle cartridge (Illumina) and sequenced on a MiSeq to produces fastq files. Raw reads were uploaded and automatically assembled in EnteroBase using SPAdes Genome Assembler v 3.5. with a contig threshold of minimum 200 nucleotides. Subsequently, the *de novo* assembled contigs were MLST (7 gene Achtman ST scheme, whole genome MLST, core genome MLST and ribosomal MLST) and serotyped *in silico* using EnteroBase typing tools (Alikhan et al., 2018). The raw reads were also analyzed using the CGE databases: SerotypeFinder, MLSTtyper, CHtyper, PlasmidFinder, ResFinder, and VirulenceFinder (Camacho et al., 2009; Larsen et al., 2012; Zankari et al., 2012; Carattoli et al., 2014; Joensen et al., 2015). For genomic relatedness comparison, we used different approaches based on the cgMLST of EnteroBase. Thus, a MSTree was inferred using the MSTree V2 algorithm and the

asymmetric distance matrix based on the cgMLST scheme from EnteroBase. This cgMLST scheme consists of 2,513 genes present in over 98% of 3,457 genomes, which represented most of the diversity in EnteroBase https://enterobase.readthedocs.io/en/latest/pipelines/escherichia-statistics.html. We also investigated the HierCC designations for our collection and other related genomes of EnteroBase within each cluster group (Zhou et al., 2017; Alikhan et al., 2018). The SNP tree was also built in EnteroBase, where all assemblies were aligned against LREC-113 using Last (Hamada et al., 2011), and SNPs from these alignments were filtered to remove regions with low base qualities or ambiguous alignment. Specifically, any sites with low base qualities ($Q < 10$) or sites which could not be aligned unambiguously (ambiguity of alignment ≥ 0.1, as reported by Last) were excluded. Additionally sites were removed if disperse repetitive regions were aligned with ≥ 95% identities and longer than ≥ 100 bps according to nucleotide BLAST; or they were part of tandem repeats that were identified by TRF (Benson, 1999); or within CRISPR regions, which were identified by PILER-CR (Edgar, 2007). After removing repetitive regions, all core SNPs were then called in the core genomic regions that were conserved in ≥ 90% of the genomes.

3.2.10. Statistical analysis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Differences within groups were analyzed by a two-tailed Fisher's exact test. P values < 0.05 were considered statistically significant.

4. RESULTS

4.1. STUDY 1: CHICKEN AND TURKEY MEAT: CONSUMER EXPOSURE TO MULTIDRUG-RESISTANT Enterobacteriaceae INCLUDING MCR-CARRIERS, UROPATHOGENIC *E. coli* AND HIGH-RISK LINEAGES SUCH AS ST131

We aimed to design a protocol applicable in the routine of food microbiological laboratories to evaluate consumer exposure to antibiotic-resistant and / or potentially pathogenic Enterobacteriaceae. Thus, we assessed a reduced protocol based on the recovery of one representative *E. coli* isolate per sample and the recovery of ESBL/Carbapenemase-producing Enterobacteriaceae in chromogenic media.

4.1.1. Representative *E. coli* isolate per sample recovered from Lactose MacConkey agar (ML)

Of the 100 meat samples analyzed, 84% were positive for the presence of *E. coli* growth in ML (86% of the chicken and 82% of the turkey samples). From those positive, 84 representative *E. coli* (one colony per sample) were collected and characterized for their phylogroups, STs, clonotypes, serotypes, bla_{ESBL} genes, resistance profiles, ExPEC/UPEC status and VF associated with EPEC, STEC and EAEC (Table 12). As result, most isolates belonged to phylogroups B1 (25 isolates; 29.8%) and A (24; 28.6%); however, the other five phylogroups of *E. coli sensu stricto* were also present, without differences regarding the type of meat (chicken or turkey): C (15; 17.9%), F (8; 9.5%), E (6; 7.1%), B2 (5; 6%) and D (1; 1.2%) (Figure 15).

The analysis by MLST showed 41 different STs (Figure 4), including three new (STnew1, STnew2, and ST117-like with one single nucleotide of difference in *fumC*45). Despite this diversity, eight STs accounted for 52.4% of the isolates: ST10-A (eight isolates); ST23-C, ST58-B1 (seven isolates each); ST162-B1 (six isolates); ST410-C (five isolates); ST38-E, ST1485-F (four isolates each) and ST744-A (three isolates) (Table 30). Clonotyping also showed high heterogeneity, with 23 different *fimH* alleles (seven isolates were negative for the amplification of the 489-nt internal sequence) and 39 *fumC-fimH* combinations, but 51.2% of the isolates showed any of the following seven clonotypes: CH11-54 (13 isolates of the CC10-A); CH4-35 (seven ST23-C); CH4-32 (six CC155-B1), CH65-32 (four ST162-B1 and two ST3580-B1 isolates), CH26-65 (four ST38-E), CH231-58 (four ST1485-F) and CH4-24 (three ST410-C) (Table 30). Regarding the serotyping, only three O:H combinations were detected in more than two isolates belonging to the clonal groups O78:HNM-C-ST23 (three isolates), O83:H42-F-ST1485 (three isolates), ONT:H7-B1-ST3580 and ONT:H7-A- ST5826 (two and one isolate, respectively) (Table 12).

Table 12. Characterization of the 84 representative *E. coli* recovered from the 100 chicken and turkey meat samples

[1]Isolate code	[2]ExPEC status	[3]UPEC status	[4]Serotype	[5]PG	[6]ST	[7]Clonotype	[8]MDR	[9]Phenotypic resistance	[10]ESBL and *mcr*
Ch-15-R	-	-	O84:HNM	A	10	11-54	+	AMP, GEN, DOX, CIP, NAL	
Ch-17-R	-	-	O64:HNT	A	10	11-54	+	AMP, CAZ, ATM, DOX, CHL, CIP, NAL	SHV-12
Ch-18-R	-	-	O132:HNM	A	10	11-54	-	-	
Ch-25-R	-	-	O140:HNM	A	10	11-54	-	DOX	
Ch-29-R	-	-	O113:H4	A	10	11-24	-	GEN, TOB, CIP, NAL	

[1]Isolate code	[2]ExPEC status	[3]UPEC status	[4]Serotype	[5]PG	[6]ST	[7]Clonotype	[8]MDR	[9]Phenotypic resistance	[10]ESBL and *mcr*
Ch-36-R*	-	-	O153:H10	A	10	11-54	+	AMP, DOX, CHL, CIP, NAL	
T-2-R	-	-	O16:HNM	A	10	11-54	-	AMP, SXT	
T-16-R	-	-	O18:H25	A	10	11-54	+	AMP, CHL, CIP, NAL	
T-32-R	-	-	O6:H10	A	43	11-54	-	AMP	
T-24-R	-	-	O20:HNM	A	48	11-neg	-	AMP, SXT	
T-44-R	-	-	O176:H11	A	48	11-neg	-	AMP, NAL	
T-35-R	-	-	O7:H4	A	93	11-41	+	AMP, DOX, CHL, SXT, CIP, NAL	
T-29-R	-	-	O101:HNM	A	744	11-54	+	AMP, DOX, CHL, CIP, NAL	
T-31-R	-	-	O101:HNM	A	744	11-54	+	AMP, DOX, CHL, SXT, CIP, NAL	
T-39-R	-	-	O101:H9	A	744	11-54	+	AMP, DOX, CHL, SXT, CIP, NAL	
Ch-40-R*	-	-	O145:H40	A	752	11-24	-	AMP, NAL	
T-17-R	-	-	O162/O89:H37	A	853	11-54	+	AMP, CST, SXT	*mcr-1*
Ch-4-R	-	-	O8:H10	A	2705	11-23	+	AMP, DOX, CHL	
Ch-26-R	-	-	O101:H9	A	5507	11-54	+	AMP, AMC, DOX, NAL	
Ch-31-R	-	-	ONT:H7	A	5826	4-60	-	AMP	
Ch-3-R	-	-	O40:HNM	A	7199	7-neg	-	AMP	
T-45-R	-	-	O33-HNM	A	7315	11-398	+	AMP, GEN, TOB, CHL, CIP, NAL	
Ch-28	-	-	O88:HNT	A	new1	153-39	+	AMP, AMC, DOX, CIP, NAL	
T-12-R	-	-	ONT:HNM	A	new2	11-neg	+	AMP, CHL, SXT, CIP, NAL	
T-6-R	-	-	ONT:H4	B1	58	4-32	-	SXT	
T-18-R	-	-	O9:HNM	B1	58	4-32	+	AMP, GEN, CIP	
T-25-R	-	-	O8:HNM	B1	58	4-32	-	AMP, CIP	
T-26-R	-	-	O9:H12	B1	58	4-27	-	AMP	
T-27-R	-	-	O48:H30	B1	58	4-neg	+	AMP, CHL, SXT	
T-34-R	-	-	O8:HNT	B1	58	4-32	+	AMP, SXT, CIP, NAL	
T-36-R	-	-	O8:H25	B1	58	4-32	+	AMP, DOX, SXT, CIP, NAL	
Ch-21-R	-	-	O88:H8	B1	101	41-86	-	-	
Ch-49-R	-	-	O103:H21	B1	101	41-86	-	AMP	
T-13-R	-	-	O29:H9	B1	155	4-32	-	AMP, CIP, NAL	
T-43-R	-	-	O64HNM	B1	155	4-neg	+	AMP, AMC, CAZ, ATM	SHV-12
Ch-8-R	-	-	O109:HNT	B1	162	65-32	+	AMP, DOX, SXT, CIP, NAL	
Ch-14-R	-	-	O88:H10	B1	162	65-32	-	CIP, NAL	
Ch-32-R	-	-	O8:H19	B1	162	65-27	+	AMP, GEN, TOB, NAL	
Ch-33-R	-	-	O8:H19	B1	162	65-38	-	AMP, NAL	
T-5-R	-	-	O9:HNM	B1	162	65-32	+	AMP, DOX, CIP, NAL	
T-11-R	-	-	O9:H19	B1	162	65-32	-	SXT, CIP, NAL	
Ch-27-R	-	-	O19:HNT	B1	212	29-38	+	AMP, AMC, SXT, CIP, NAL	

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[1]Isolate code	[2]ExPEC status	[3]UPEC status	[4]Serotype	[5]PG	[6]ST	[7]Clonotype	[8]MDR	[9]Phenotypic resistance	[10]ESBL and *mcr*
T-21-R	-	-	O149:H45	B1	297	65-38	-	AMP	
T-7-R	-	-	O9:H53	B1	345	4-31	-	AMP, CIP, NAL	
T-9-R	-	-	O29:H10	B1	1720	270-54	+	AMP, DOX, SXT	
Ch-43-R	-	-	O7:HNT	B1	1730	69-32	-	AMP, CHL	
T-3-R	-	-	ONT:H2	B1	2599	6-32	+	AMP, SXT, CIP, NAL	
T-8-R	-	-	ONT:H7	B1	3580	65-32	-	AMP, GEN	
T-38-R	-	-	ONT:H7	B1	3580	65-32	-	-	
T-22-R	+	+	O1:H7	B2	95	38-30	-	AMP, NAL	
Ch-2-R	+	+	O25:H4	B2	131	40-22	+	GEN, DOX, NAL	
Ch-13-R	+	+	O25:H4	B2	131	40-22	+	GEN, DOX, NAL	
Ch-19-R	+	+	O120:H4	B2	428	40-neg	-	AMP, SXT	
T-14-R	+	+	O120:H4	B2	428	40-22	-	AMP, SXT	
Ch-1-R	-	-	O78:H9	C	23	4-35	-	DOX, NAL	
Ch-5-R	-	-	O78:HNM	C	23	4-35	-	NAL	
Ch-7-R	-	-	O78:HNM	C	23	4-35	-	NAL	
Ch-20-R	-	-	O15:HNT	C	23	4-35	+	AMP, TOB, DOX, SXT, CIP, NAL	
Ch-37-R	-	-	O78:HNM	C	23	4-35	-	AMP, NAL	
Ch-45-R	-	-	O60:H9	C	23	4-35	-	AMP	
Ch-48-R	-	-	O8:H9	C	23	4-35	-	AMP, CHL	
T-4-R	-	-	O8:H4	C	88	4-39	+	AMP, GEN, SXT, CIP, NAL	
Ch-10-R	-	-	O159:H16	C	295	4-38	-	AMP, NAL	
Ch-42-R	-	-	O162:H42	C	295	4-38	-	AMP, NAL	
T-15-R	-	-	O86:H9	C	410	4-24	+	AMP, DOX, CIP, NAL	
T-19-R	-	-	O86:H9	C	410	4-24	-	AMP, CIP, NAL	
T-41-R	-	-	O20:HNT	C	410	4-24	-	AMP, CIP, NAL	
T-42-R	-	-	O60:HNT	C	410	4-53	-	AMP, DOX	
T-46-R	-	-	O19:HNT	C	410	4-45	-	AMP, CIP, NAL	
Ch-24-R	+	-	O73:HNT	D	4243	3-1002	-	GEN, NAL	
Ch-23-R	-	-	O123:H15	E	38	26-65	-	NAL	
Ch-46-R	-	-	O7:H15	E	38	26-65	-	AMP, GEN, TOB	
Ch-50-R	-	-	O99:H15	E	38	26-65	+	AMP, DOX, SXT, NAL	
T-33-R	-	-	O7:H15	E	38	26-65	-	AMP, DOX	
T-30-R	-	-	ONT:H25	E	57	31-27	-	-	
Ch-34	-	-	O45:HNM	E	371	31-142	-	GEN, NAL	
Ch-35-R	-	-	O143:H4	F	117	45-97	-	AMP	
Ch-47-R	-	+	O53:HNM	F	117-like	new - 97	+	AMP, GEN, DOX, NAL	
Ch-16-R	+	+	O11:H25	F	457	88-145	-	GEN, DOX	
Ch-6-R	+	+	O15:H42	F	1485	231-58	+	AMP, AMC, GEN, TOB, SXT, CIP, NAL	

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[1]Isolate code	[2]ExPEC status	[3]UPEC status	[4]Serotype	[5]PG	[6]ST	[7]Clonotype	[8]MDR	[9]Phenotypic resistance	[10]ESBL and *mcr*
Ch-9-R	+	+	O83:H42	F	1485	231-58	+	AMP, GEN, SXT, NAL	
Ch-38-R	+	+	O83:H42	F	1485	231-58	+	AMP, SXT, CIP, NAL	
T-20-R	+	+	O83:H42	F	1485	231-58	+	AMP, SXT, CIP, NAL	
T-40-R	-	+	O8:HNM	F	5340	271-58	-	AMP, SXT	

[1]Origin of isolation-sample number-type of isolate: Ch (chicken meat), T (turkey meat), R (representative *E. coli*). *Isolates Ch-36-R and Ch-40-R showed a hybrid pathotype aEPEC/ExPEC, being both carriers of *eae*-beta1 and extraintestinal pathogenic genes. [2]ExPEC status +: *E. coli* strains considered with higher capacity of developing extraintestinal pathologies when positive for two or more of five markers, including *papAH* and / or *papC*, *sfa/focDE*, *afa/draBC*, *kpsM II* and *iutA*; ExPEC -: strains negative for those markers (Johnson et al., 2003b). [3]UPEC status +: strains considered with higher capacity of developing UTI pathologies when positive for three or more of four markers, including *chuA*, *fyuA*, *vat* and *yfcV*; UPEC -: strains negative for those markers (Spurbeck et al., 2012). [4]O antigen: non-typeable isolates were designated as ONT; H antigen: HNM for non-motile isolates and HNT for those which did not react with any antisera. [5]Phylogroup (PG) was designated by PCR according to Clermont scheme (Clermont et al., 2013). [6]Sequence type (ST) was performed following the Achtman scheme (Wirth et al., 2006). [7]Clonotype based on the internal 469-nucleotide (nt) and 489-nt sequence of the *fumC* (allele obtained from MLST) and *fimH* genes, respectively (Weissman et al., 2012). Seven isolates were negative (neg) for the amplification of the 489-nt internal sequence. [8]Multidrug-resistance (MDR) +: resistant to at least 1 agent of ≥ 3 different antimicrobial categories as defined by Magiorakos (Magiorakos et al., 2012). [9]Phenotypic resistance interpreted according to the CLSI standard guidelines: ampicillin (AMP), amoxicillin-clavulanic acid (AMC), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), aztreonam (ATM), imipenem (IMP), gentamicin (GEN), tobramycin (TOB), amikacin (AMK), fosfomycin (FOF), doxycycline (DOX), chloramphenicol (CHL), nitrofurantoin (NIT), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP), nalidixic acid (NAL), tigecycline (TGC) and colistin (CST). [10]*bla*$_{ESBL}$ and *mcr* typing. The collection was also investigated by PCR for specific *mcr* (1 to 5) and *bla* genes using the TEM, CIT, SHV, CTX-M-1 and CTX-M-9 group-specific primers, followed by amplicon sequencing for those positive.

Of the 84 representative *E. coli* isolates, 36 (42.9%) showed resistance to at least one agent of ≥ three different antimicrobial categories and were defined as MDR (Magiorakos et al., 2012). Moreover, only the isolates from four samples showed susceptibility to the 19 antibiotics tested. The highest rates of resistance were to AMP (78.6%), NAL (60.7%), CIP (39.3%), SXT (31.0%), DOX (29.8%) and GEN (19.0%), being of note that the isolates recovered from turkey meat showed higher values compared to those of chicken for AMP (90.2% *vs* 67.4%; P = 0.016), CIP (53.7% *vs* 25.6%; P = 0.013) and SXT (43.9% *vs* 18.6%; P = 0.018) (Table 13). The screening and typing of *bla*$_{ESBL}$ and *mcr* genes determined that two ceftazidime-resistant isolates (one from chicken and one from turkey meat) were SHV-12, and one colistin-resistant *E. coli* recovered from turkey was *mcr-1* (Table 12).

Table 13. Phenotypic resistances of the 256 isolates recovered from the 100 meat samples

Antibiotics tested	REPRESENTATIVE *E. COLI*			ESBL-PRODUCING *E. COLI*			ESBL-PRODUCING ENTEROBACTERIACEAE			Antibiotics tested
	Chicken n=43 (%)	Turkey n=41 (%)	TOTAL N=84 (%)	Chicken n=64 (%)	Turkey n=73 (%)	TOTAL N=137 (%)	Chicken n=71 (%)	Turkey n=101 (%)	TOTAL N=172 (%)	
AMP	29 (67.4)	37 (90.2)	66 (78.6)	64 (100.0)	73 (100.0)	137 (100.0)	71 (100.0)	101 (100.0)	172 (100.0)	AMP
AMC	4 (9.3)	1 (2.4)	5 (6.0)	4 (6.3)	6 (8.2)	10 (7.3)	8 (11.3)	7 (6.9)	15 (8.7)	AMC
CAZ	1 (2.3)	1 (2.4)	2 (2.4)	36 (56.3)	51 (69.9)	87 (63.5)	37 (52.1)	58 (57.4)	95 (55.2)	CAZ
CTX	0	0	0	43 (67.2)	42 (57.5)	85 (62.0)	50 (70.4)	70 (69.3)	120 (69.8)	CTX
FOX	0	0	0	1 (1.6)	0	1 (0.7)	2 (2.8)	1 (1.0)	3 (1.7)	FOX
ATM	1 (2.3)	1 (2.4)	2 (2.4)	43 (67.2)	57 (78.1)	100 (73.0)	44 (62.0)	66 (65.3)	110 (63.9)	ATM
IPM	0	0	0	0	0	0	0	0	0	IPM

GEN	12 (27.9)	4 (9.8)	16 (19.0)	10 (15.6)	5 (6.8)	15 (10.9)	11 (15.5)	12 (11.9)	23 (13.4)	**GEN**
TOB	5 (11.6)	1 (2.4)	6 (7.1)	4 (6.3)	4 (5.5)	8 (5.8)	5 (7.0)	15 (14.8)	20 (11.6)	**TOB**
AMK	0	0	0	0	0	0	0	0	0	**AMK**
FOF	0	0	0	0	0	0	0	1 (1.0)	1 (0.6)	**FOF**
CST	0	1 (2.4)	1 (1.2)	0	1 (1.4)	1 (0.7)	6 (8.5)	13 (12.9)	19 (11.0)	**CST**
DOX	15 (34.9)	10 (24.4)	25 (29.8)	26 (40.6)	45 (61.6)	71 (51.8)	29 (40.8)	64 (63.4)	93 (54.1)	**DOX**
CHL	5 (11.6)	8 (19.5)	13 (15.5)	22 (34.4)	40 (54.8)	62 (45.3)	24 (33.8)	47 (46.5)	71 (41.3)	**CHL**
NIT	0	0	0	1 (1.6)	1 (1.4)	2 (1.5)	2 (2.8)	7 (6.9)	9 (5.2)	**NIT**
SXT	8 (18.6)	18 (43.9)	26 (31.0)	11 (17.2)	28 (38.4)	39 (28.5)	13 (18.3)	50 (49.5)	63 (36.6)	**SXT**
CIP	11 (25.6)	22 (53.7)	33 (39.3)	32 (50.0)	50 (68.5)	82 (59.8)	35 (49.3)	74 (73.3)	109 (63.4)	**CIP**
NAL	29 (67.4)	22 (53.7)	51 (60.7)	46 (71.9)	54 (74.0)	100 (73.0)	49 (69.0)	76 (75.2)	125 (72.7)	**NAL**
TGC	0	0	0	0	1 (1.4)	1 (0.7)	2 (2.8)	18 (17.8)	20 (11.6)	**TGC**
Multidrug-resistant	17 (39.5)	19 (46.3)	36 (42.9)	63 (98.4)	73 (100.0)	136 (99.3)	70 (98.6)	101 (100.0)	171 (99.4)	**Multidrug-resistant**

Antimicrobial susceptibility tested by disc difussion assay and interpreted according to the CLSI standard breakpoints (CLSI, 2019): ampicillin (AMP), amoxicillin-clavulanic acid (AMC), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), aztreonam (ATM), imipenem (IMP), gentamicin (GEN), tobramycin (TOB), amikacin (AMK), fosfomycin (FOF), colistin (CST), doxycycline (DOX), chloramphenicol (CHL), nitrofurantoin (NIT), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP), nalidixic acid (NAL) and tigecycline (TGC). Highlighted in blue the statistically significant values (P values < 0.05), and in red the highest rates of prevalence.

The screening of VF associated with diarrheagenic and extraintestinal pathotypes of *E. coli* determined that 11 out of the 84 representative *E. coli (*13.1%) satisfied the status ExPEC, and 12 isolates (14.3%) the status UPEC (the late comprised 10 of those conforming also the ExPEC status). The 12 isolates positive for the UPEC status belonged to the B2 and F phylogroups, and seven exhibited STs associated with high-risk clonal groups (ST131-B2, ST95-B2, CC648-F) (Table 12; Table 14). None of the 84 isolates was positive for the presence of the diarrheagenic genes *bfpA, stx$_1$*, *stx$_2$ aaiC*, or *aggR*. However, two *E. coli* isolates characterized as O153:H10-A-ST10 (CH11-54) and O145:H40-A-ST752 (CH11-24) exhibited a hybrid atypical EPEC/ExPEC pathotype, since both were carriers of the intimin-encoding gene *eae*-beta1 as well as of extraintestinal VF (Table 31).

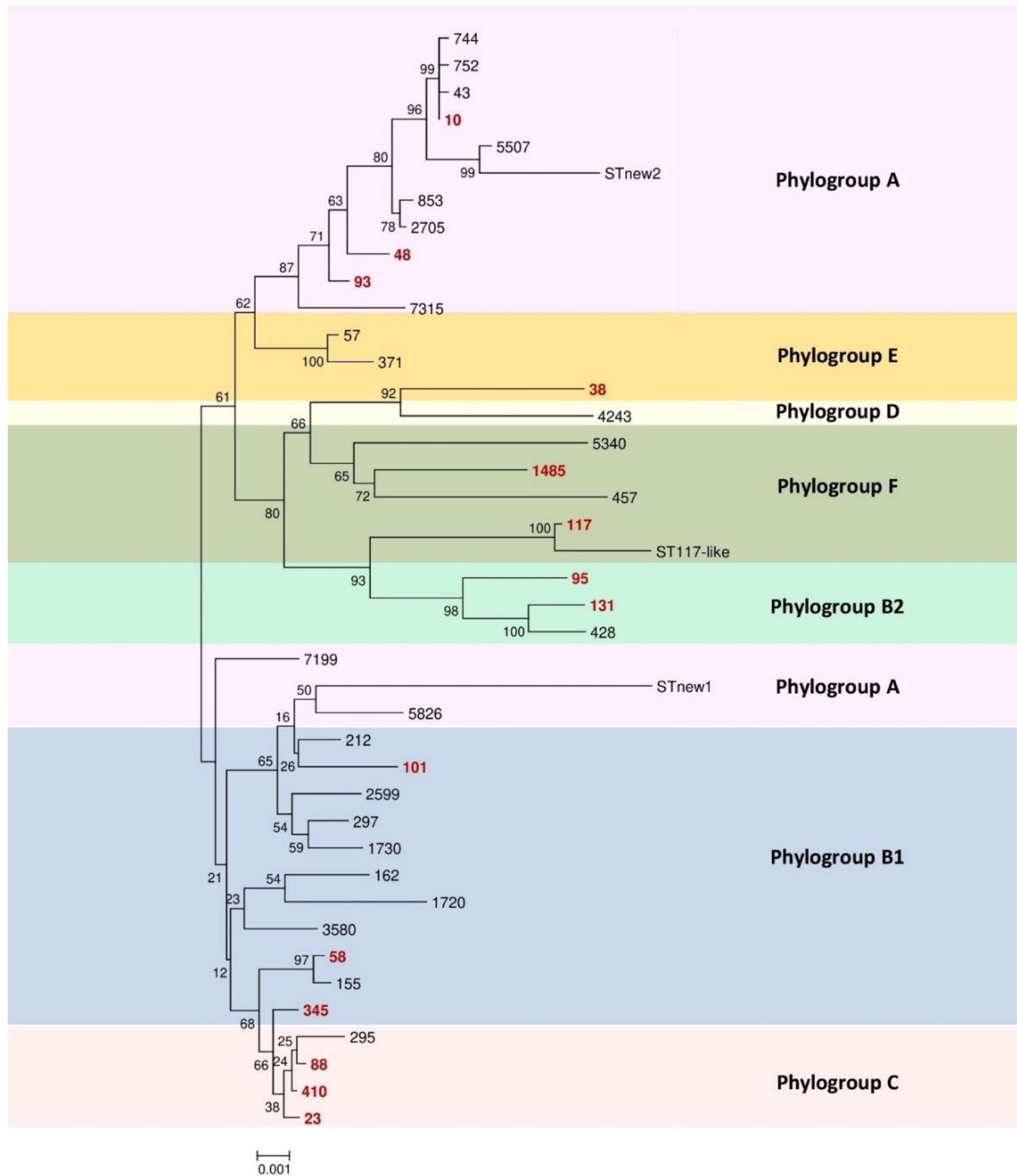

Figure 4. Representative *E. coli* collection. Phylogenetic tree based on concatenated sequences of the seven housekeeping genes from the MLST Achtman scheme by the Neighbor-Joining method using MEGA6. The optimal tree with the sum of branch length = 0.13442852 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 41 nucleotide sequences determined within the 84 representative *E. coli*. All positions containing gaps and missing data were eliminated. Highlighted in red those STs of *E. coli* associated with human extraintestinal and / or uropathogenic pathologies.

Table 14. Phenotypic and genotypic characterization of the 18 *E. coli* conforming UPEC status: 12 from the representative collection ("R" code) plus six recovered from CHROMID® ("ESBL" code)

[1]Isolate code	[2]Serotype	[3]PG	[4]ST	[5]CH	[6]ESBL	[7]Resistances	[8]Virulence-gene profile
T-24-ESBL	O50/O2:H6	B2	141	52-14	SHV-12	AMP, CAZ, ATM, DOX, CHL	*fimH14 hlyF iucD iutA iroN kpsM II-K1 cvaC traT ibeA malX usp tsh ompT iss chuA vat fyuA yfcV*
T-40-ESBL	O113:H5	B2	8611	24-26	SHV-12	AMP, CAZ, ATM, DOX, CHL, NAL	*fimH26 iroN traT malX usp ompT chuA vat fyuA yfcV*
T-48-ESBL	O115:HNM	B2	919	24-187	SHV-12	AMP, CAZ, CTX, ATM, SXT, NAL	*fimH187 iucD iutA iroN traT ibeA malX hlyF ompT iss chuA vat fyuA yfcV*
Ch-2-R	O25:H4	B2	131	40-22	-	GEN, DOX, NAL	*fimH22 iucD iutA iroN kpsM II-K1 traT ibeA malX usp tsh ompT iss chuA fyuA yfcV*
Ch-13-R	O25:H4	B2	131	40-22	-	GEN, DOX, NAL	*fimH22 hlyF iucD iutA iroN kpsM II-K1traT ibeA malX usp tsh ompT iss chuA fyuA yfcV*
Ch-19-R	O120:H4	B2	428	40-neg	-	AMP, SXT	*fimH fimAvMT78 hlyF iucD iutA iroN kpsM II-K1 cvaC traT ibeA malX usp ompT iss chuA vat fyuA yfcV*
T-14-R	O120:H4	B2	428	40-22	-	AMP, SXT	*fimH22 hlyF iucD iutA iroN kpsM II-K1 cvaC traT ibeA malX usp ompT iss chuA vat fyuA yfcV*
T-22-R	O1:H7	B2	95	38-30	-	AMP, NAL	*fimH30 hlyF papAH papaEF papC papG II cdtB iucD iutA iroN kpsM II-K1 cvaC traT malX usp ompT iss chuA vat fyuA yfcV*
Ch-1-ESBL	O24:H18	F	117	45-151	SHV-12	AMP, CAZ, ATM, DOX, CHL, CIP, NAL	*fimH151 cdtB hlyF iucD iutA traT malX ompT chuA vat fyuA*
Ch-43-ESBL	O57:HNM	F	117	45-97	SHV-12	AMP, CAZ, ATM, DOX, CHL	*fimH97 cdtB iucD iutA iroN traT malX tsh ompT iss chuA vat fyuA*
T-9-ESBL	O118:H4	F	117	45-97	SHV-12	AMP, CAZ, CTX, ATM, DOX, CHL	*fimH97 cdtB iucD iutA iroN traT malX tsh ompT iss chuA vat fyuA*
Ch-6-R	O15:H42	F	1485	231-58	-	AMP, AMC, GEN, TOB, SXT, CIP, NAL	*fimH58 hlyF iucD iutA iroN kpsM II-K5 cvaC traT malX tsh ompT iss chuA vat yfcV*
Ch-9-R	O83:H42	F	1485	231-58	-	AMP, GEN, SXT, NAL	*fimH58 hlyF iucD iutA iroN kpsM II-K5 cvaC traT malX tsh ompT iss chuA vat yfcV*
Ch-16-R	O11:H25	F	457	88-145	-	GEN, DOX	*fimH145 hlyF iucD iutA iroN kpsM II-K2 cvaC traT malX tsh ompT iss chuA vat fyuA yfcV*
Ch-38-R	O83:H42	F	1485	231-58	-	AMP, SXT, CIP, NAL	*fimH58 hlyF iucD iutA iroN kpsM II-K5 cvaC traT malX tsh ompT iss chuA vat yfcV*
Ch-47-R	O53:HNM	F	117-like	new - 97	-	AMP, GEN, DOX, NAL	*fimH97 hlyF iucD iutA iroN traT malX tsh ompT iss chuA vat fyuA*
T-20-R	O83:H42	F	1485	231-58	-	AMP, SXT, CIP, NAL	*fimH58 hlyF iucD iutA iroN kpsM II-K5 cvaC traT malX tsh ompT iss chuA vat yfcV*
T-40-R	O8:HNM	F	5340	271-58	-	AMP, SXT	*fimH58 hlyF iucD iutA iroN cvaC traT malX tsh iss chuA vat yfcV*

[1]Origin of isolation-sample number-type of isolate: Ch (chicken meat), T (turkey meat), R (representative *E. coli*), ESBL (ESBL-producing *E. coli*). [2]H antigen: HNM for non-motile isolates. [3]Phylogroup (PG) was designated by PCR according to Clermont scheme (Clermont *et al.*, 2013). [4]Sequence type (ST) was performed following the Achtman scheme (Wirth *et al.*, 2006). [5]Clonotype based on the internal 469-nucleotide (nt) and 489-nt sequence of the *fumC* (allele obtained from MLST) and *fimH* genes, respectively (Weissman *et al.*, 2012): neg when PCR was negative for the 489-nt internal sequence amplification. [6]bla_{ESBL} typing (Garcia-Meniño *et al.*, 2018). [7]Phenotypic resistance interpreted according to the CLSI guidelines (CLSI, 2019): ampicillin (AMP), ceftazidime (CAZ), cefotaxime (CTX), aztreonam (ATM), gentamicin (GEN), tobramycin (TOB), doxycycline (DOX), chloramphenicol (CHL), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP), nalidixic acid (NAL). [8]Specific extraintestinal VF (Johnson *et al.*, 2003; Spurbeck *et al.*, 2012).

4.1.2. ESBL-producing Enterobacteriaceae recovered from CHROMID® ESBL

While none carbapenemase-producing colony was recovered from the CHROMID®CARBA SMART medium, 82% of the 100 meat samples (40 of chicken and 42 of turkey) were positive in CHROMID® ESBL. From those 82 samples, 172 different ESBL-producing isolates (71 of chicken and 101 of turkey) were recovered and identified as *Escherichia coli* (137 isolates), *Klebsiella pneumoniae* (28 isolates), *Serratia fonticola* (six isolates) and *Enterobacter cloacae* (one isolate). Significantly, we found that 23 of the 50 turkey meat samples *versus* five of 50 chicken meat carried more than one ESBL-producing species (46% *vs* 10%; P = 0.00) (Figures 16a and 16b). All but one of the 172 ESBL-producing Enterobacteriaceae were MDR, showing the highest rates of resistance to AMP (100%), NAL (72.7%), CTX (69.8%), ATM (63.9%), CIP (63.4%), CAZ (55.2%), DOX (54.1%), CHL (41.3%) and SXT (36.6%). ESBL-producing isolates obtained from turkey were significantly more resistant to DOX (63.4% *vs* 40.8%; P = 0.005), SXT (49.5% *vs* 18.3%; P = 0.000), CIP (73.3% *vs* 49.3%; P = 0.002) and TGC (17.8% *vs* 2.8%; P = 0.003) (Table 13). Nineteen isolates showed resistance to colistin: 11 *K. pneumoniae*, two *E. coli* (MICs >4 mg/L) (Table 32), and the intrinsically resistant *S. fonticola* (six isolates).

4.1.2.1. ESBL-producing *E. coli* recovered from CHROMID® ESBL

E. coli represented 80% of the 172 ESBL-producing Enterobacteriaceae recovered by means of the CHROMID® ESBL (Figure 16b). The 137 ESBL-producing *E. coli (*64 isolates of chicken and 73 of turkey) from 76 meat samples (38 chicken and 38 turkey) showed a similar phylogroup distribution to that found within the representative *E. coli* collection obtained from ML. Thus, most isolates belonged to the phylogroups A (55 isolates; 40.1%) and B1 (40; 29.2%), but there was also presence of phylogroups E (18; 13.1%), F (10; 7.3%), C (4; 2.9%), B2 (3; 2.2%) and D (2; 1.4%). Interestingly, five isolates (5; 3.6%) belonged to Clade I (Figure 5). By MLST, the ESBL-producing *E. coli* showed high heterogeneity with 51different STs, including five new: STnew3 (related with the ST665-A), STnew4 (related with the ST350-E), STnew5 (related with the ST906-B1), STnew6 and STnew7 (Table 33). In fact, only seven STs (ST10-A, ST93-A, ST117-F, ST155-B1, ST354-F, ST602-B1 and ST770-Clade I) were found in ≥ three isolates. Sixteen of the 51 STs were also present within the representative *E. coli* collection (Figure 5). Accordingly, clonotyping also showed high diversity with 46 *fumC-fimH* different combinations, of which only six were determined in ≥ three isolates: CH4-32 (seven isolates of the CC155-B1), CH11-54 (ten CC10-A), CH19-86 (three ST602-B1), CH31-54 (three CC350-E), CH45-97 (six ST117-F) and CH116-552 (five ST770-Clade I) (Table 33). ESBL-typing determined that 68.6% of the 137 *E. coli* produced SHV (93 isolates SHV-12 and one SHV-2), 27% CTX-M (14 isolates CTX-M-1, nine CTX-M-32, six CTX-M-14, five CTX-M-15 and three CTX-M-9), and 4.4% showed type TEM-52. All but one chicken isolate were MDR, showing the highest rates of resistance to AMP (100%), NAL and ATM (73.0%), CAZ (63.5%), CTX (62.0%), CIP (59.8%), DOX (51.8%), CHL (45.3%). ESBL-producing *E. coli* from turkey were significantly more resistant to DOX (61.6% *vs* 40.6%; P = 0.023), CHL (54.8% *vs* 34.4%; P = 0.025); SXT (38.4% *vs* 17.2%; P = 0.008) and CIP (68.5% *vs* 50.0%; P = 0.036) (Table 13). Of note that one SHV-12 colistin-resistant *E. coli* recovered from turkey was positive for the *mcr-1* gene. The screening of VF determined that 18 out of the 137 ESBL-*E. coli* (13.1%) satisfied the status ExPEC, including isolates of phylogroups A, B1, B2, D, E, F and the five isolates of Clade I. Besides, six isolates (4.4%) conformed the UPEC status and

included risk clonal groups such as ST141-B2 and ST117-F (Table 12). Interestingly, two *E. coli* isolates from the ESBL-producing collection, recovered from different samples, also exhibited a hybrid atypical EPEC/ExPEC pathotype, being carriers of *eae*-beta1 and extraintestinal VF: O153:H10-A-ST10 (CH11-54), CTX-M-32, and O123/186:H34-A-ST752 (CH11-24), CTX-M-1 (Table 31).

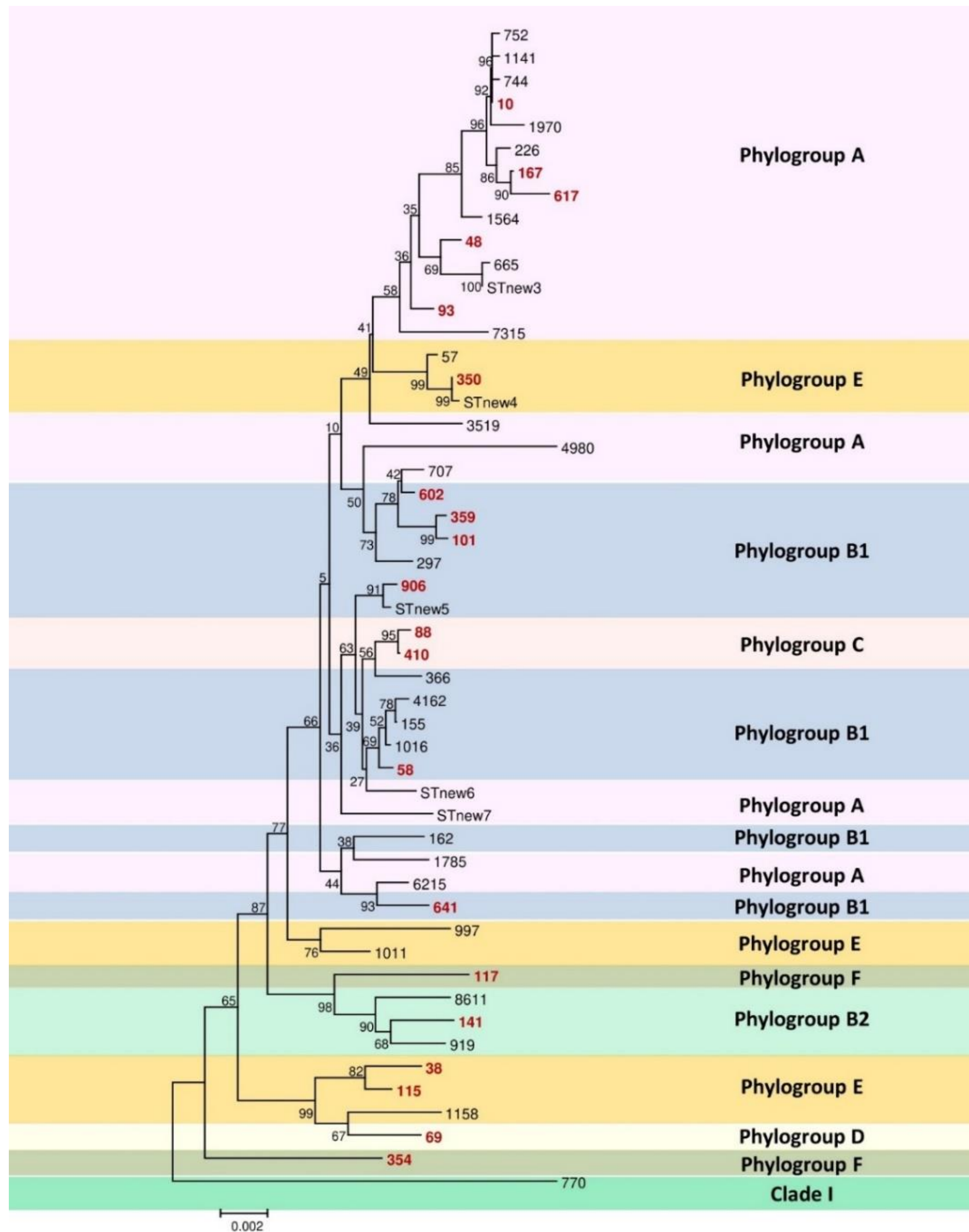

Figure 5. ESBL-producing *E. coli* collection. Phylogenetic tree based on concatenated sequences of the seven housekeeping genes from the MLST Achtman scheme by the Neighbor-Joining method using MEGA6. The optimal tree with the sum of branch length = 0.15093159 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 51 nucleotide sequences determined within the 137 ESBL-producing *E. coli* collection. All positions containing gaps and missing data were eliminated. There was a total of 3414 positions in the final dataset. Highlighted in red those STs of *E. coli* associated with human extraintestinal and / or uropathogenic pathologies.

4.1.2.2. ESBL-producing *K. pneumoniae* recovered from CHROMID® ESBL

Twenty-eight *K. pneumoniae* were recovered from 27 different samples, representing 16% of the ESBL-producing isolates (Figure 16b). Significantly, only two isolates were from chicken meat compared to 26 isolates from turkey. All isolates were MDR, with the highest rates of resistances to AMP and CTX (100%), CIP (89.3%), SXT and NAL (82.1%), DOX (67.9%), TGC (62.3%), TOB (42.9%), CST (39.3%), ATM (35.7%), CAZ (28.6%). The ESBL typing revealed that 13 isolates were CTX-M-15, and eight of those also positive for SHV-28. All isolates were negative by PCR for the presence of *mcr-1* to *mcr-5* genes, including eleven phenotypically resistant to colistin (Table 32). The 28 *K. pneumoniae* were further characterized by MLST and PFGE as shown in Figure 6. Eleven different STs were established, being the most prevalent: ST307 (seven isolates), ST147 (four isolates), the new assignation ST4028 (four isolates) and ST15 (three isolates). The 28 *K. pneumoniae* isolates exhibited 25 macrorestriction profiles which grouped in the *XbaI*-PFGE dendrogram according to their ST, with five clusters of similarity ≥85% (the two ST111 isolates, the four ST147, six of the seven ST307, two of the three ST15 and the four ST4028) (Figure 6).

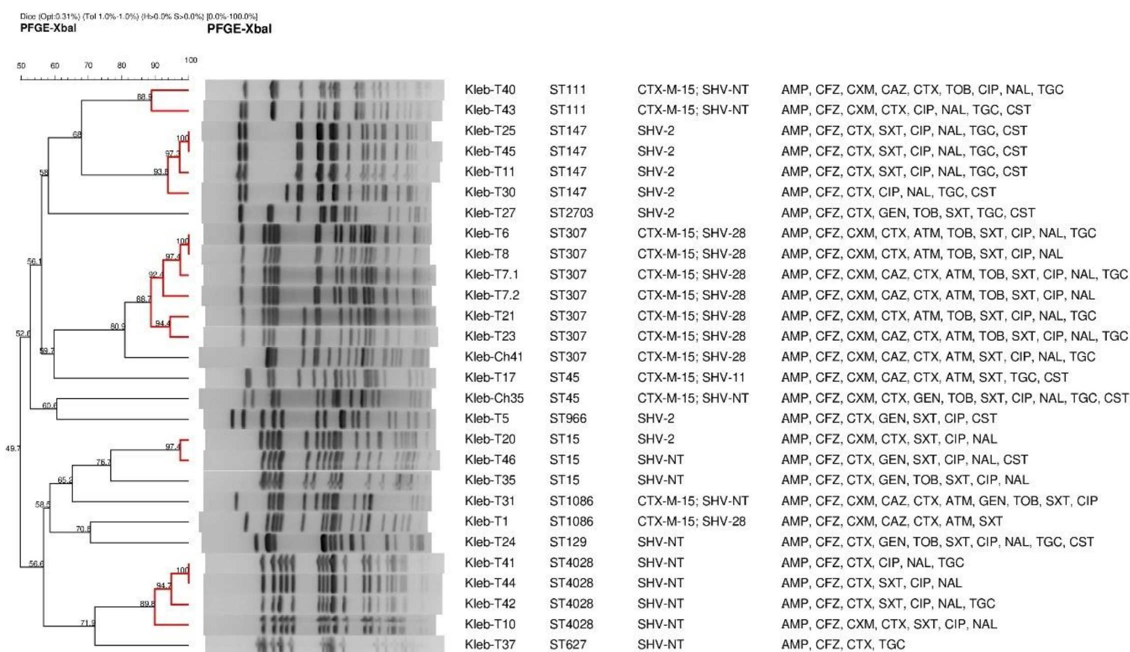

Figure 6. Dendrogram of the *Xba*I macrorestriction profiles of the 28 *Klebsiella pneumoniae* isolates. The dendrogram was obtained with the UPGMA algorithm based on the Dice similarity coefficient and applying 1% of tolerance in the band position using the BioNumerics software (Applied Maths, St-Martens-Latern Belgium). Association between isolation code, ST, ESBL type (NT: not typable) and resistance profile is indicated on the right. Clusters of ≥85% identity are highlighted in red.

4.1.3. WGS of *mcr-E. coli* isolates

Within the collection analyzed here (84 representative *E. coli* and 172 ESBL-producing Enterobacteriaceae), two colistin-resistant *E. coli* recovered from two meat turkey samples were *mcr*-carriers (2/50; 4% turkey and 0/50 chicken meat). They showed MIC values of >4 mg/L (ESBL-producing *E. coli)* and >32 mg/L (representative *E. coli)* (Table 32). Table 15 summarizes their phenotypic traits and *in silico* characterization

using CGE tools. The two isolates belonged to the clonal group CC10-A (CH11-54). The resistome analysis revealed that both genomes encoded mechanisms of antibiotic resistance for ≥ three different antimicrobial categories, and both were carriers of the *mcr-1.1* variant located in an IncX4 plasmid type. Furthermore, PlasmidFinder showed a high plasmid diversity based on the identified replicons, with five to seven different plasmid types per genome (Table 15). To highlight the fact that LREC-204 carried double-serine mutations in *gyrA* S83L and *parC* S80I, with additional substitutions in *gyrA* D87N and *parC* A56T, which corresponded to the fluoroquinolone resistance determined *in vitro* for its isolate.

4.1.4. Food-borne risk assessment

In order to assess the level of microbiological risk exposure for consumers, each meat sample was qualified between zero (lowest risk) to five (highest), on the basis of five parameters which were individually analyzed and considered as positive when happened: i) the recovery of more than one ESBL-producing Enterobacteriaceae species; ii) the identification of a high-risk clonal group of *E. coli*, according to recent studies, due to their association within human extraintestinal and uropathogenic pathologies (Yamaji et al., 2018b; Mamani et al., 2019; Manges et al., 2019); iii) the presence of *E. coli* conforming ExPEC status; iv) the presence of *E. coli* conforming UPEC status; v) the recovery of Enterobacteriaceae resistant to antimicrobials of categories A (“Avoid”) or B (“Restrict”) (EMA, 2019).

The results determined that the majority (97%) of meat samples were positive for the presence of Enterobacteriaceae. Besides, a comprehensive analysis based on the assessment of five parameters showed that 96% of the samples meant a consumer exposure to ≥ one risk, and 82% to ≥ two risks (Table 16). In detail, the result for each parameter was: i) 96 out of the 100 samples with positive recovery of isolates resistant to antimicrobials of the EMA categories A (“Avoid”) or B (“Restrict”), including 64 meat (31 chicken and 33 turkey) carriers of isolates resistant to monobactams (ATM), and one of those also to FOF (category A). ii) Sixty-two samples (31 chicken and 31 turkey) with presence of high-risk clonal groups of *E. coli* associated with human extraintestinal and / or uropathogenic pathologies (ST10, ST23, ST38, ST48, ST58, ST69, ST88, ST93, ST95, ST101, ST115, ST117, ST131, ST141, ST167, ST350, ST345, ST354, ST359, ST410, ST602, ST617, ST641, ST906, ST1485). iii) The ExPEC and iv) UPEC status, based on the presence of certain virulence markers associated with a higher capacity of developing extraintestinal or UTI pathologies, was determined in *E. coli* isolates recovered from 25 and 17 samples, respectively. v) More than one ESBL-producing bacterial species were recovered from 28 samples (23 turkey meat and five chicken; P = 0.000). Although it was not considered as an additional risk, it is of note that 37 samples carried more than one type of ESBL-producing *E. coli*.

4.1.5. Nucleotide sequence accession numbers

The nucleotide sequence of the *mcr*-positive isolates have been deposited in the NCBI sequence databases with accession codes SAMN12430141 (isolate T-1-V-e; genome LREC-204) and SAMN12430147 (isolate T-17-R; genome LREC-210) and these sequences are part of BioProject ID PRJNA558228.

Table 15. *In silico* characterization and phenotypic traits of the *mcr* positive isolates recovered from two turkey meat samples

Code[1]	O:H antigens[2]	Phylo Group[3]	ST[4]	CHType[5]	Acquired resistances (in black) and point mutations (in blue)[6]	Plasmid content Inc group (pMLST)[7]	*mcr* type / location[8]	Virulence genes[9]	Phenotypic resistances[10]	Virulence profile[11]
LREC-204 / T1-ESBL	O162/ O89:H9	A	744	11-54	***bla*$_{SHV-12}$**; *aadA1, aadA2, aadA5, aph(3")-Ib, aph(6)-Id; catA1, cmlA1; dfrA17; mdf(A); sul1, sul2, sul3; tet(A), tet(B);* ***mcr-1*** *gyrA S83L, gyrA D87N, parC A56T, parC S80I*	IncF (F18:A-:B20*) IncI1 (ST26) IncQ1 IncX1 IncX4 Col(MG828)-like ColpVC	*mcr-1.1* / IncX4	*mchF, iroN, iss, tsh, cba, cma*	AMP, CAZ, CTX, ATM, CST, DOX, CHL, SXT, CIP, NAL	*fimH54, fimAv*$_{MT78}$*, hlyF, iutA, iucD, iroN, cvaC, iss, traT, tsh*
LREC-210 / T17-R	O162/ O89:H37	A	853	11-54	*bla*$_{TEM-1B}$; *aadA1, aph(3")-Ib, aph(6)-Id; dfrA1; mdf(A); mph(B); sul1, sul2; tet(A);* ***mcr-1***	IncF (F18:A-:B1) IncQ1 IncX4 Col(MG828)-like Col156-like	*mcr-1.1* / IncX4	*iroN, cma, iss, celb*	AMP, CST, SXT	*fimH54, fimAv*$_{MT78}$*, hlyF, iutA, iucD, iroN, iss, traT, ompT*

[1]Genomic and isolate reference, respectively; [2]serotypes, [4]sequence types, [5]clonotypes, [6]acquired antimicrobial resistance genes and / or chromosomal mutations, [7]replicon/plasmid STs, and [9]virulence genes were determined using SerotypeFinder 2.0, MLST 2.0, CHtyper 1.0, ResFinder 3.1, PlasmidFinder 2.0, pMLST 2.0, and VirulenceFinder 2.0 online tools at the Center of Genomic Epidemiology, respectively; [3]phylogroups were predicted using the ClermonTyping tool at the Iame-research Center web (http://clermontyping.iame-research.center/) [6]Resistome. Acquired resistance genes: B-lactam: *bla*$_{TEM-1B}$, *bla*$_{SHV-12}$, aminoglycosides: *aadA, aph(3")-Ib, aph(6)-Id*; phenicols: *catA1, cmlA1*; macrolides: *mdf(A)*; sulphonamides: *sul1, sul2, sul3*; tetracycline: *tet(A), tet(B)*; trimethoprim: *dfrA1, dfrA17*; colistin: *mcr*. Point mutations: quinolones and fluoroquinolones: *gyrA* S83L: TCG-TTG, *gyrA* D87N: GAC-AAT, *parC* S80I: AGC-ATC, *parC* A56T: GCC-ACC. [7]Plasmid STs: "*" indicates the nearest ST allele (with less than 100% but >95% identity and 100% coverage). [8]*mcr* gene location determined by PlasmidFinder/ResFinder predictions.[9]Virulence genes: *cba*: colicin B, celb: endonuclease colicin E2, *cma*: colicin M, *iroN*: enterobactin siderophore receptor protein, *iss*: increased serum survival, *mchF*: ABC transporter protein MchF, *tsh*: temperature-sensitive hemagglutinin. [10]Phenotypic resistance interpreted according to the CLSI standard breakpoints (Clinical and Laboratory Standards Institute, 2019): ampicillin (AMP), ceftazidime (CAZ), cefotaxime (CTX), aztreonam (ATM), colistin (CST), doxycycline (DOX), chloramphenicol (CHL), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP), nalidixic acid (NAL). [11]Specific extraintestinal VF determined by PCR (Johnson *et al.*, 2003; Spurbeck *et al.*, 2012)(Spurbeck et al., 2012).

Table 16. Food-borne risk assessment of the 97 meat samples (48 chicken and 49 turkey) with positive bacterial isolation

[1]Type of sample	No. sample	[2]ESBL-producing spp.	[3]High-risk clonal groups of *E. coli*	[4]ExPEC	[5]UPEC	[6]Resistances to antimicrobials of categories A or B	[7]TOTAL risk
Ch	1	*E. coli, S. fonticola*	**ST23-C (CH4-35)**; ST117-F	0	1	MB-CF3rd-FQ-CST* (*S. fonticola*)	4
Ch	2	*E. coli*	**ST131-B2 (CH40-22)**	1	1	MB-CF3rd-Q	4
Ch	3	*E. coli*	-	0	0	MB-CF3rd-FQ	1
Ch	4	*E. coli*	ST117-F	0	0	CF3rd	2
Ch	5	*E. coli*	**ST23-C (CH4-35); ST69-D (CH35-27)**; ST115-E	1	0	MB-CF3rd-FQ	3
Ch	6	*E. coli*	CC648-F	1	1	CF3rd-FQ	4
Ch	7	*E. coli*	**ST23-C (CH4-35); ST93-A**	1	0	MB-CF3rd-FQ	3
Ch	8	*E. coli, S. fonticola*	-	0	0	MB-CF3rd-FQ-CST* (*S. fonticola*)	2
Ch	9	*0*	CC648-F	1	1	Q	4
Ch	10	*E. coli*	ST117-F; **CC10-A (*eae*-beta1)**	0	0	MB-CF3rd-Q	2*
Ch	13	*E. coli*	**ST131-B2 (CH40-22)**	1	1	MB-CF3rd-FQ	4
Ch	14	*0*	-	0	0	FQ	1
Ch	15	*0*	**ST10-A (CH11-54)**	0	0	FQ	2
Ch	16	*E. coli*	-	1	1	MB-CF3rd-Q	3
Ch	17	*E. coli*	**ST10-A (CH11-54)**	0	0	MB-CF3rd-FQ	2
Ch	18	*E. coli, S. fonticola*	**ST10-A (CH11-54)**	0	0	CF3rd-FQ-CST* (*S. fonticola*)	3
Ch	19	*E. coli*	-	1	1	MB-CF3rd-Q	3
Ch	20	*E. coli*	**ST23-C (CH4-35)**	0	0	MB-CF3rd-FQ	2
Ch	21	*E. coli*	ST101-B1	0	0	MB-CF3rd-FQ	2
Ch	22	*E. coli*	-	0	0	MB-CF3rd-FQ	1
Ch	23	*E. coli*	ST38-E	0	0	MB-CF3rd-FQ	2
Ch	24	*E. coli*	**ST10-A (CH11-54) *eae*-beta1**	1	0	MB-CF3rd-Q	3*
Ch	25	*E. coli*	**ST10-A (CH11-54)**	0	0	MB-CF3rd-FQ	2

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[1]Type of sample	No. sample	[2]ESBL-producing spp.	[3]High-risk clonal groups of *E. coli*	[4]ExPEC	[5]UPEC	[6]Resistances to antimicrobials of categories A or B	[7]TOTAL risk
Ch	26	*0*	-	0	0	Q	1
Ch	27	*E. coli*	**ST10-A (CH11-54)**	0	0	MB-FQ	2
Ch	28	*E. coli*	-	1	0	MB-CF3rd-FQ	2
Ch	29	*0*	**ST10-A (CH11-54)**	0	0	FQ	2
Ch	30	*S. fonticola*	-	0	0	CF3rd-CST* (*S. fonticola*)	1
Ch	31	*E. coli*	**ST93-A**	1	0	MB-CF3rd-Q	3
Ch	32	*E. coli*	-	0	0	Q	1
Ch	33	*E. coli*	-	1	0	MB-CF3rd-FQ	2
Ch	34	*E. coli*	-	1	0	MB-CF3rd-FQ	2
Ch	35	*E. coli, K. pneumoniae*	ST101-B1; ST117-F	0	0	MB-CF3rd-FQ-CST	3
Ch	36	*E. coli*	**ST10-A (CH11-54) *eae*-beta1; ST93-A**	1	0	FQ	3*
Ch	37	*0*	**ST23-C (CH4-35)**	0	0	Q	2
Ch	38	*0*	CC648-F	1	1	FQ	4
Ch	39	*E. coli*	-	0	0	MB	1
Ch	40	*E. coli*	**CCT10-A *eae*-beta1**	1	0	MB-CF3rd-FQ	3*
Ch	41	*E. coli, K. pneumoniae*	-	1	0	MB-CF3rd-FQ	3
Ch	42	*0*	-	0	0	Q	1
Ch	43	*E. coli*	ST117-F	0	1	MB-CF3rd	3
Ch	44	*E. coli*	-	1	0	CF3rd-FQ	2
Ch	45	*E. coli*	**ST23-C (CH4-35); ST410-C (CH4-24)**	0	0	MB-CF3rd-FQ	2
Ch	46	*E. coli*	ST38-E; **ST641-B1**	0	0	MB-CF3rd-Q	2
Ch	47	*E. coli*	-	0	1	CF3rd-FQ	2
Ch	48	*E. coli*	**ST23-C (CH4-35); ST10-A (CH11-54)**	0	0	MB-CF3rd-FQ	2
Ch	49	*E. coli*	ST101-B1; **ST617-A (CH11-neg)**	0	0	MB-CF3rd-FQ	2

[1]Type of sample	No. sample	[2]ESBL-producing spp.	[3]High-risk clonal groups of *E. coli*	[4]ExPEC	[5]UPEC	[6]Resistances to antimicrobials of categories A or B	[7]TOTAL risk
Ch	50	*S. fonticola*	ST38-E	0	0	CF3rd-Q-CST* (*S. fonticola*)	2
T	1	*E. coli, K. pneumoniae*	-	0	0	MB-CF3rd-FQ-CST (*mcr*-carrying *E. coli)*	2
T	2	*E. coli*	**ST10-A (CH11-54)**	0	0	MB-CF3rd-FQ	2
T	3	*E. coli*	-	0	0	MB-CF3rd-FQ	1
T	4	*E. coli*	**ST88-C (CH4-39)**; ST354-F	0	0	MB-CF3rd-FQ	2
T	5	*E. coli, K. pneumoniae*	-	0	0	CF3rd-FQ-CST	2
T	6	*E. coli, K. pneumoniae*	**ST58-B1 (CH4-32)**	0	0	MB-CF3rd-FQ	3
T	7	*E. coli, K. pneumoniae*	ST345-B1	0	0	MB-CF3rd-FQ	3
T	8	*E. coli, K. pneumoniae*	-	0	0	MB-CF3rd-FQ	2
T	9	*E. coli*	ST117-F	0	1	MB-CF3rd-FQ	3
T	10	*E. coli, K. pneumoniae*	-	0	0	CF3rd-FQ	2
T	11	*E. coli, K. pneumoniae*	-	0	0	MB-CF3rd-FQ-CST	2
T	12	*E. coli*	-	0	0	MB-FQ	1
T	13	*E. coli*	ST117-F	0	0	MB-CF3rd-FQ	2
T	14	*0*	-	1	1		2
T	15	*E. coli*	**ST410-C (CH4-24)**	0	0	MB-CF3rd-FQ	2
T	16	*0*	**ST10-A (CH11-54)**	0	0	FQ	2
T	17	*E. coli, K. pneumoniae*	-	0	0	MB-CF3rd-FQ-CST (*mcr*-carrying *E. coli)*	2
T	18	*E. coli*	**ST58-B1 (CH4-32)**	0	0	MB-CF3rd-FQ	2
T	19	*0*	**ST410-C (CH4-24)**	0	0	FQ	2
T	20	*E. coli, K. pneumoniae*	CC648-F	1	1	MB-CF3rd-FQ	5
T	21	*E. coli, K. pneumoniae*	-	0	0	MB-CF3rd-FQ	2
T	22	*0*	ST95-B2	1	1	Q	4

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[1]Type of sample	No. sample	[2]ESBL-producing spp.	[3]High-risk clonal groups of *E. coli*	[4]ExPEC	[5]UPEC	[6]Resistances to antimicrobials of categories A or B	[7]TOTAL risk
T	23	*K. pneumoniae*	-	0	0	MB-CF3rd-FQ	1
T	24	*E. coli, K. pneumoniae*	**ST48-A; ST141-B2 (CH52-14); ST354-F (CH88-58)**	1	1	MB-FOF-CF3rd-FQ-CST	5
T	25	*E. coli, K. pneumoniae*	**ST58-B1 (CH4-32)**; ST350-E	0	0	MB-CF3rd-FQ-CST	3
T	26	*E. coli*	**ST58-B1 (CH4-27); ST93-A**	1	0	Q	3
T	27	*E. coli, K. pneumoniae*	**ST58-B1 (CH4-32)**; ST350-E	0	0	MB-CF3rd-FQ-CST	3
T	28	*E. coli*	**ST10-A (CH11-54)**	0	0	MB-CF3rd-FQ	2
T	29	*0*	-	0	0	FQ	1
T	30	*K. pneumoniae*	-	0	0	CF3rd-FQ-CST	1
T	31	*E. coli, K. pneumoniae*	-	0	0	MB-CF3rd-FQ	2
T	32	*E. coli*	**ST10-A (CH11-54); ST617-A (CH11-neg)**	1	0	MB-CF3rd-FQ	3
T	33	*E. coli, E. cloacae**	**ST88-C (CH4-39)**; ST38-E	0	0	CF3rd-FQ	3
T	34	*0*	**ST58-B1 (CH4-32)**	0	0	FQ	2
T	35	*E. coli, K. pneumoniae*	**ST93-A**	0	0	MB-CF3rd-FQ	3
T	36	*0*	**ST58-B1 (CH4-32)**	0	0	FQ	2
T	37	*E. coli, K. pneumoniae*	**ST48-A**	0	0	CF3rd-FQ	3
T	38	*E. coli*	-	0	0	MB-CF3rd-Q	1
T	39	*E. coli, S. fonticola**	-	0	0	MB-CF3rd-FQ-CST* (*S. fonticola*)	2
T	40	*E. coli, K. pneumoniae*	**ST58-B1 (CH4-27)**; ST38-E	0	1	MB-CF3rd-FQ	4
T	41	*E. coli, K. pneumoniae*	**ST410-C (CH4-24)**	0	0	MB-CF3rd-FQ	3
T	42	*E. coli, K. pneumoniae*	**ST410-C; ST10-A (CH11-54); ST602-B1 (CH19-86)**	0	0	MB-CF3rd-FQ	3
T	43	*E. coli, K. pneumoniae*	**ST354-F (CH88-58)**	1	0	MB-CF3rd-FQ-CST	4
T	44	*E. coli, K. pneumoniae*	**ST48-A; ST359-B1 (CH41-35)**	0	0	MB-CF3rd-FQ	3
T	45	*K. pneumoniae*	-	0	0	CF3rd-FQ-CST	1

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[1]Type of sample	No. sample	[2]ESBL-producing spp.	[3]High-risk clonal groups of *E. coli*	[4]ExPEC	[5]UPEC	[6]Resistances to antimicrobials of categories A or B	[7]TOTAL risk
T	46	*K. pneumoniae*	ST410-C	0	0	CF3rd-FQ-CST	2
T	47	*E. coli*	ST167-A	0	0	CF3rd-FQ	2
T	48	*E. coli*	-	0	1	MB-CF3rd-FQ	2
T	50	*E. coli*	**ST602-B1 (CH19-86)**; ST906-B1	0	0	MB-CF3rd-FQ	2

[1]Type of sample: Ch (chicken meat), T (turkey meat). Highlighted in red those samples with recovery of more than one ESBL-producing type of *E. coli*. *Presumptively ESBL-producers since they were recovered from CHROMID® ESBL (the ESBL type could not be determined in those two isolates). [2]ESBL-producing bacteria recovered from CHROMID® ESBL, 0 = none recovered. [3]High-risk clonal groups of *E. coli* associated with human extraintestinal and / or uropathogenic pathologies according to recent studies (Yamaji et al., 2018b; Mamani et al., 2019; Manges et al., 2019); highlighted in bold those reported within our collection of clinical human isolates (Mamani et al., 2019). [4]ExPEC status +: *E. coli* strains considered with higher capacity of developing extraintestinal pathologies when positive for two or more of five markers, including *papAH* and / or *papC*, *sfa/focDE*, *afa/draBC*, *kpsM II* and *iutA*; ExPEC -: strains negative for those markers (Johnson *et al*. 2003). [5]UPEC status +: strains considered with higher capacity of developing UTI pathologies when positive for three or more of four markers, including *chuA*, *fyuA*, *vat* and *yfcV*; UPEC -: strains negative for those markers (Spurbeck et al., 2012). [6]Detection of isolates resistant to antimicrobials categorized as A or B (EMA/CVP/CHMP, 2019); FOF (fosfomycin); MB (monobactams); CF3rd (3rd-generation cephalosporins); FQ (fluoroquinolones); Q (quinolones); CST (colistin), *intrinsic resistance. [7]Meat samples were qualified between zero (lowest risk) to five (highest), being positive when happened: i) the recovery of more than one ESBL-producing bacterial species; ii) the presence of ExPEC and / or UPEC lineages of *E. coli*; iii) the presence of isolates conforming ExPEC status; iv) UPEC status; v) the recovery of resistant isolates to antimicrobials of categories A or B, *presence of an atypical EPEC/ExPEC hybrid pathotype.

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4.2. STUDY 2: MICROBIOLOGICAL RISK ASSESSMENT OF TURKEY AND CHICKEN MEAT FOR CONSUMER: SIGNIFICANT DIFFERENCES REGARDING MULTIDRUG RESISTANCE, MCR OR PRESENCE OF HYBRID AEPEC/EXPEC PATHOTYPES OF *E. coli*

Based on the same sampling as referred in 4.1. Study 1, we performed here a comprehensive analysis of all the pathogenic and AMR *E. coli* recovered within the VI protocols detailed in Figure 2 from 3.2.1. Sampling, screening and Enterobacteriaceae recovery.

Ninety-two of the 100 samples (46 of chicken and 46 of turkey) were positive for the presence and recover of *E. coli* isolates. From those, 323 isolates constituted the collection of study (163 *E. coli* from chicken and 160 from turkey). Per protocol, 137 (42.4%) *E. coli* were recovered from protocol V, 86 (26.6%) from protocol II, 79 (24.5%) from protocol I, 16 (5.0%) from protocol IV and 5 (1.5%) from protocol III. None carbepenemase-producing isolate was recovered in this study, which were specifically searched in the protocol VI. The screening of VF associated with InPEC pathotypes determined the presence of aEPEC (*eae*-positive, *bfp*-negative), but no STEC or EAEC. The screening of VF associated with the ExPEC and UPEC status, *rfbO25b, bla* and *mcr* were also positive as it is detailed below.

4.2.1. Microbiological quality of the poultry meat

As noted above, 92% of the meat samples were positive for *E. coli* isolation in 3M Petrifilm™ Select *E. coli*. While 27 samples showed < 10 cfu of *E. coli* per g, 43 showed ≥ 50 cfu/g with significant differences regarding meat origin (28 of 50 turkey *vs* 15 of 50 chicken; P = 0.015) (Table 17). Besides, five of the 100 samples obtained "not satisfactory" *E. coli counts* (> 500), if we take as reference the Commission Regulation (EC) No 2073/2005 for meat preparations at the end of the manufacturing process. In this Regulation, the limits to recommend improvements in selection and / or origin of raw materials are "m = 500 and M = 5000" cfu/g. Finally, similar levels of contamination were observed for the two packaging systems (modified atmosphere and freshly butchered) (P > 0.05) (Table 17).

Table 17. Association of *E. coli* counts with meat origin (chicken vs turkey) and packaging (modified atmosphere vs freshly butchered)

[1]CFU / g *E. coli*	Poultry meat n = 100	Chicken meat n = 50	Turkey meat n = 50	*P* two-tailed value	Modified atmosphere n = 49	Freshly butchered n = 51	*P* two-tailed value
< 10	27	16 (32%)	11 (22%)	0.367	10 (20.4%)	17 (33.3%)	0.179
oct-49	30	19 (38%)	11 (22%)	0.125	15 (30.6%)	15 (29.4%)	1.000
50-500	38	14 (28%)	24 (48%)	0.063	22 (44.9%)	16 (31.4%)	0.216
> 500	5	1 (2%)	4 (8%)	0.322	2 (4.1%)	3 (5.9%)	1.000
≥ 50	43	15 (30%)	28 (56%)	**0.015**	24 (49%)	19 (37.3%)	0.419

[1]CFU: colony forming units. Statistically significant differences (P < 0.05) are highlighted in bold.

4.2.2. Evaluation of protocols I to V

The method designed here was thought to detect *E. coli* potentially pathogenic for humans (diarrheagenic, extraintestinal and MDR), using different media, temperatures of incubation and specific genetic targets (named as protocols I to VI within the method). As summarized in Table 18 and Table 34, we evaluated the adequacy of this method through the characterization of 323 *E. coli* and the assessment of six virulence traits. We found that the protocols I and II (ML and MSTC incubated at 37 ºC, respectively) were the most effective for the recovery of isolates satisfying the ExPEC and UPEC status. In detail, of the 150 isolates from 78 different meat samples that satisfied the ExPEC status, 118 (78.7%) from 71 samples were recovered in plates of protocols I and II. Likewise, of the 83 isolates positive for UPEC status from 53 individual samples, 69 (83.1%) recovered in 47 samples come from plates of protocols I and II. The protocol V (CHROMID® ESBL agar plates 37 ºC) was key for the recovery of ESBL or pAmpC-producing *E. coli.* In fact, of 155 ESBL/pAmpC-producing *E. coli* isolated from 78 samples, 137 (88.4%) isolates and 76 samples were detected in protocol V. Although most *mcr* isolates were from protocols I and II, with 10 of 13 (76.9%) *mcr E. coli recovered* in six of the seven positive meat samples, they were also isolated in plates of protocols IV and V (two and one isolate, respectively). Of the 323 isolates analyzed here, 253 *E. coli* recovered from 88 meat samples were MDR according to Magiorakos *et al.* (Magiorakos et al., 2012). MDR isolates were mostly those 137 (54.1%) ESBL/pAmpC-producing *E. coli* recovered from 76 meat samples (protocol V), and 100 *E. coli from* protocols I and II. Finally, the screening by PCR of the *rbfO25b* associated with the clonal group ST131 allowed the detection of 13 isolates; 12 (92.3%) from nine samples were recovered in plates of protocols I and II (Table 18, Table 34) (Figure 7).

Table 18. Association of virulence traits with protocols and meat origin for the *E. coli* collection (N = 323)

Virulence trait	Protocol I ML 37 °C	Protocol II MLST 37 °C	Protocol III ML 44 °C	Protocol IV MLST 44 °C	Protocol V CHROMID® 37 °C	Chicken origin	Turkey origin	[7] *P* two tail Chicken vs Turkey
[1] ExPEC status (%) N = 150	57 (38)	61 (40.7)	4 (2.7)	10 (6.6)	18 (12)	87 (58)	63 (42)	**0.014**
[2] UPEC status (%) N = 83	32 (38.6)	37 (44.6)	1 (1.2)	7 (8.4)	6 (7.2)	47 (56.6)	36 (43.4)	0.205
[3] ESBL/ pAmpC producer (%) N = 155	7 (4.5)	9 (5.8)	0	2 (1.3)	137 (88.4)	71 (45.8)	84 (54.2)	0.119
[4] *mcr-1* carrier (%) N = 13	4 (30.8)	6 (46.1)	0	2 (15.4)	1 (7.7)	1 (7.7)	12 (92.3)	**0.001**
[5] MDR (%) N = 253	48 (19.0)	52 (20.6)	3 (1.2)	13 (5.1)	137 (54.1)	118 (46.6)	135 (53.4)	**0.010**
[6] *rbfO25b* (%) N = 13	6 (46.1)	6 (46.1)	1 (7.7)	0	0	10 (76.9)	3 (23.1)	0.086
No. isolates per protocol and meat origin	79	86	5	16	137	163	160	-

[1]No. of isolates conforming ExPEC status (Johnson *et al.*, 2003). [2]No. of isolates conforming status UPEC (Spurbeck *et al.*, 2012). [3]No. of ESBL/pAmpC-producing *E. coli*. [4]No. of isolates carriers of the *mcr-1* gene. [5]No. of MDR isolates according to Magiorakos *et al.* (Magiorakos *et al.*, 2012) criteria. [6]No. of *rbfO25b*-positive isolates: O25b subtype associated with the clonal group ST131 screened by PCR (Clermont *et al.*, 2008). [7]Statistically significant differences ($P < 0.05$) highlighted in bold.

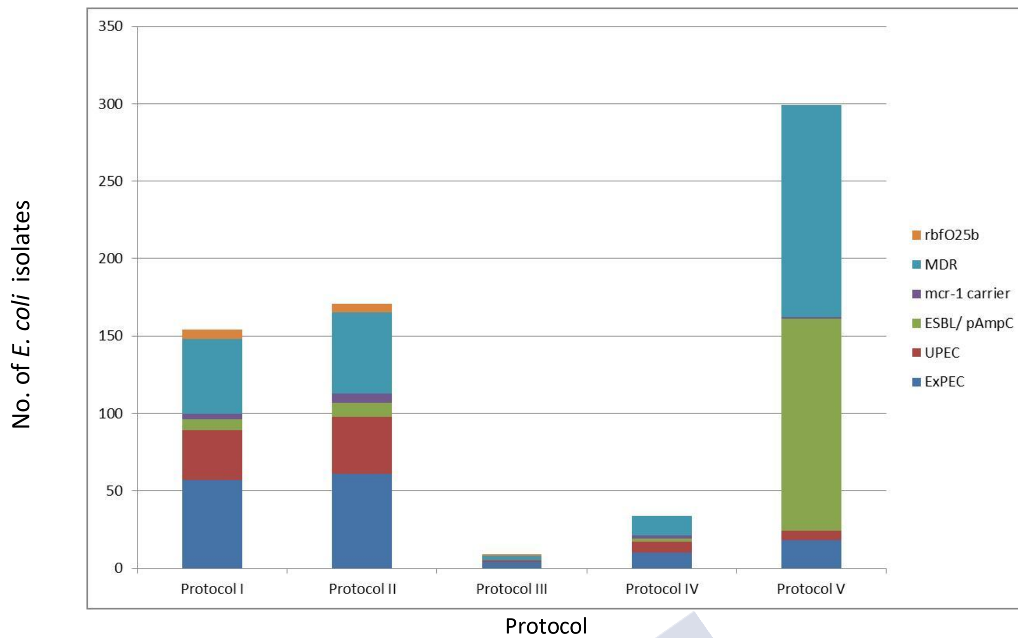

Figure 7. No. of *E. coli* isolates recovered per protocol in correlation with the six VF

Regarding meat origin, we found a significant higher prevalence of isolates with ExPEC status in chicken meat (58% *vs* 42% in turkey), while MDR and *mcr-1* isolates were more prevalent within *E. coli* of turkey origin (53.4% *vs* 46.6% in chicken and 92.3% *vs* 7.7% in chicken, respectively) ($P < 0.05$) (Table 18) (Figure 8).

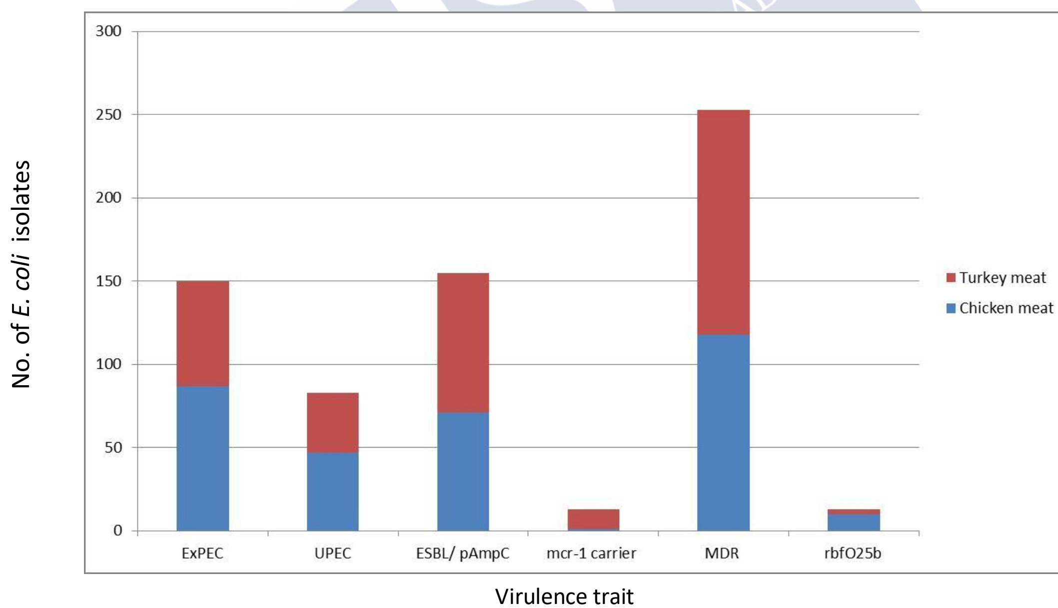

Figure 8. No. of *E. coli* isolates recovered per VF in correlation with the meat origin.

In summary, the microbiological method applied in this study showed high prevalence rates of ExPEC and UPEC status, ESBL/AmpC enzymes, *mcr-1* gene, MDR, or *rbfO25b* gene (positive isolates present in 78%, 53%, 78%, 7%, 88% and 10% of the meat samples, respectively). Importantly, the protocols I+II+V allowed the detection of around 85-90% of those positive samples (ML, MSTC and CHROMID® ESBL media incubated at 37 ºC), conforming an optimized workflow combination that would capture the greatest risk as analyzed here (Figure 9).

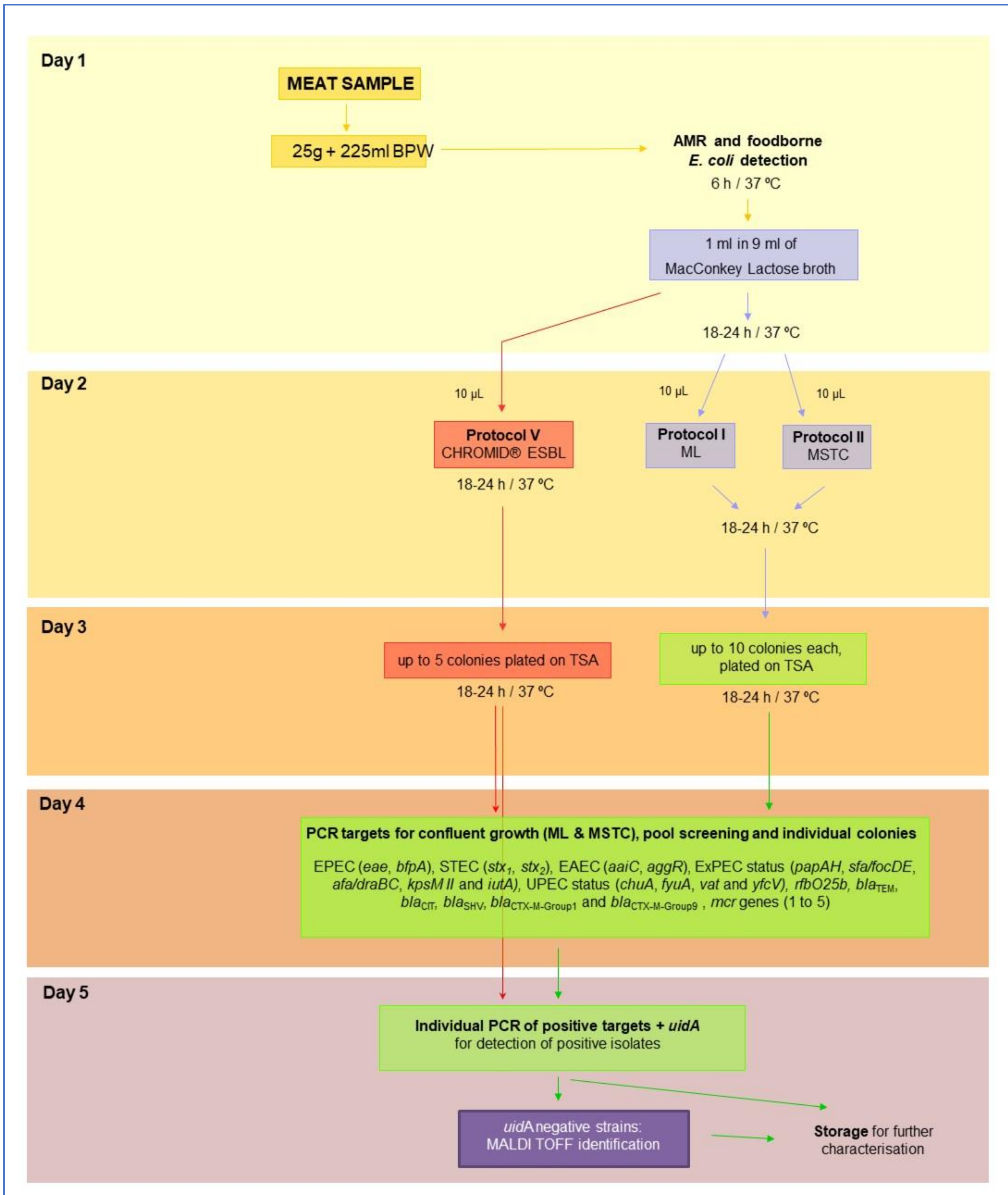

Figure 9. Proposal of optimized workflow to investigate the level of contamination, and the rates of AMR and food-borne pathogenic *E. coli*. AMR: antimicrobial resistance; ML: MacConkey Lactose agar; MSTC: MacConkey Sorbitol agar enriched with tellurite and cefixime; TSA: tryptone soy agar; CFU: colony forming units; EPEC: enteropathogenic *E. coli*; STEC: Shiga toxin-producing *E. coli*; EAEC: enteroaggregative *E. coli*; ExPEC: extraintestinal pathogenic *E. coli*.

4.2.3. *E. coli* characterization

The phylogenetic analysis revealed that the 323 *E. coli* isolates belonged to *Escherichia* clade I (8 isolates; 2,5%) and the eight phylogroups of *E. coli sensu stricto*: A (105 of 323; 32.5%), B2 (57; 17.6%), B1 (56; 17.3%), E (35; 10.8%), F (33; 10.2%), D (11; 3.4%), C (9; 2.8%), G (9; 2.8%). The isolates which exhibited ExPEC status were mainly of phylogroups B2, F, A and E (122 of 150; 81.3%), while the UPEC status appeared associated with phylogroups B2 and F (78 of 83; 94%). The ESBL producers, as well as MDR isolates, belonged mostly to phylogroups A, B1 and E, accounting for 127 of 155 (81.9%) and 179 of 253 (70.7%), respectively (Table 19) (Figure 10).

Table 19. Association of virulence traits with phylogroup distribution for the *E. coli* collection (N = 323)

Virulence trait	PG. A	PG. B1	PG. B2	PG. C	PG. D	PG. E	PG. F	PG. G	Clade I
[1]ExPEC status (%) N = 150	24 **(16)**	7 (4.7)	51 **(34)**	3 (2)	8 (5.3)	16 **(10.7)**	31 **(20.7)**	2 (1.3)	8 (5.3)
[2]UPEC status (%) N = 83	0	0	57 **(68.7)**	0	0	0	21 **(25.3)**	5 **(6)**	0
[3]ESBL/ pAmpC producer (%) N = 155	63 **(40.6)**	44 **(28.4)**	5 (3.2)	4 (2.6)	3 (1.9)	20 **(12.9)**	4 (2.6)	7 (4.5)	5 (3.2)
[4]*mcr-1* carrier (%) N = 13	6 **(46.2)**	4 **(30.8)**	2 **(15.4)**	0	1 (7.7)	0	0	0	0
[5]MDR (%) N = 253	91 **(36)**	55 **(21.7)**	20 (7.9)	8 (3.2)	6 (2.4)	33 **(13)**	25 (9.9)	9 (3.6)	6 (2.4)
[6]*rbfO25b* (%) N = 13	0	0	12 **(92.3)**	0	0	1 (7.7)	0	0	0
No. isolates per phylogroup (%) (N = 323)									
	105 (32.5)	56 (17.3)	57 (17.6)	9 (2.8)	11 (3.4)	35 (10.8)	33 (10.2)	9 (2.8)	8 (2.5)

[1]No. of isolates conforming ExPEC status (Johnson *et al.*, 2003). [2]No. of isolates conforming UPEC status (Spurbeck *et al.*, 2012). [3]No. of ESBL/pAmpC-producing *E. coli*. [4]No. of isolates carriers of the *mcr-1* gene. [5]No. of MDR isolates according to Magiorakos *et al.* (Magiorakos *et al.*, 2012) criteria. [6]No. of *rbfO25b*-positive isolates: O25b subtype associated with the clonal group ST131 screened by PCR (Clermont *et al.*, 2008). [7]In bold, the most prevalent associations.

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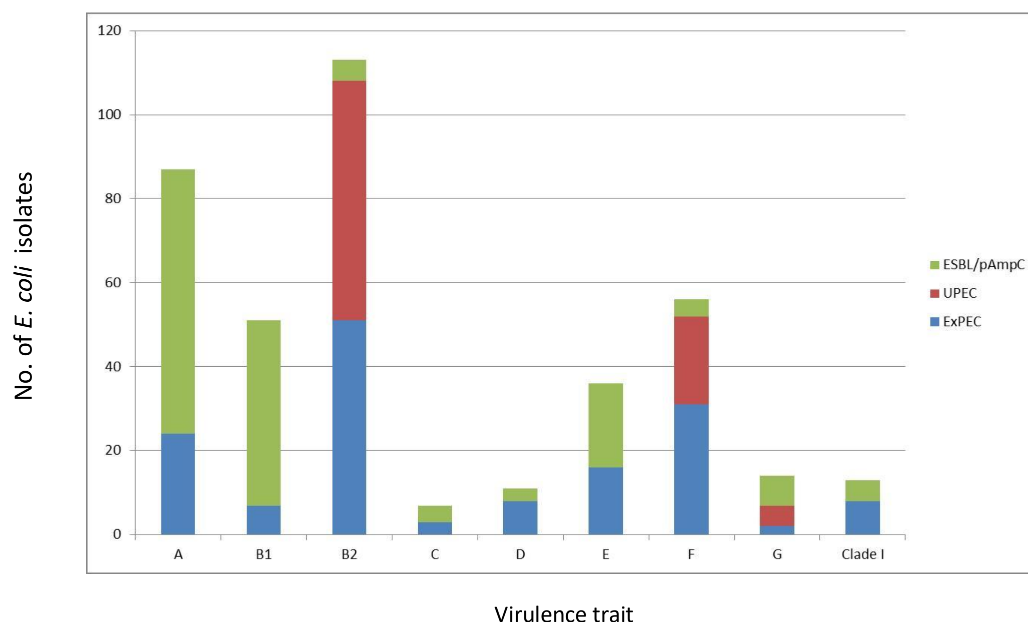
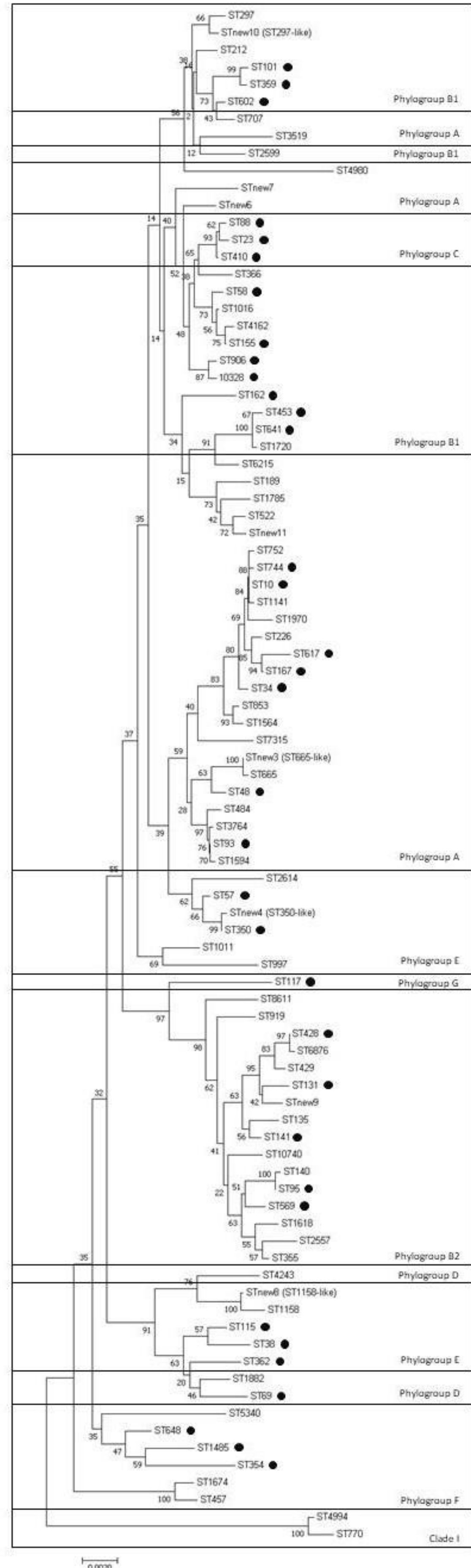


Figure 10. Phylogroup distribution within the isolates positive for the traits ESBL/pAmpC production, UPEC and ExPEC status

MLST was performed for 272 representative isolates. As a result, 89 different STs were determined, including eight new (Figure 11). However, 16 of those 89 STs detected in at least five isolates accounted for 153 of 272 isolates (56.2%): 10, 93, 95, 115,117, 131, 155, 355, 428, 648, 770, 752, 1158, 1485, 4243, 10740. And the most prevalent combination of STs and CHs revealed the following clonal groups: ST1485-F (CH231-58) (19 isolates); ST10-A (CH11-54) (12 isolates); ST93-A (CH11-neg) (11 isolates); ST752-A (CH11-24) (nine isolates); ST131-B2 (CH40-22) (eight isolates); ST117-G (CH45-97) (eight isolates); ST155-B1 (CH4-32) (seven isolates); ST355-B2 (24-154) (seven isolates); ST115-E (CH26-270) (seven isolates); ST770-clade I (CH116-552) (seven isolates); ST95-B2 (CH38-27) (six isolates); ST1158-E (CH3-47) (six isolates); ST648-F (CH4-58) (six isolates); ST10740-B2 (1544-9) (five isolates); and ST4243-D (3-1002) (five isolates) (Table 35). Besides, different clones could be distinguished within these prevalent clonal groups by serotype. In fact, serotyping showed high heterogeneity with 184 different O:H antigen combinations and only five serotypes determined for > 5 isolates in the characterized collection: O83:H42 (14 isolates); O25:H4 (12); O2:H5 (8); O2:HNM (7) and O5:H10 (6).

Figure 11. Representative *E. coli* collection. Phylogenetic tree based on concatenated sequences of the seven housekeeping genes from the MLST Achtman scheme by the Neighbor-Joining method. *Note*: The optimal tree with the sum of branch length = 0.21789331 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. The analysis involved 89 nucleotide sequences determined for 272 isolates. All positions containing gaps and missing data were eliminated. There were a total of 3423 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Marked with a black dot those STs of *E. coli* associated with human extraintestinal and / or uropathogenic pathologies.



An important finding in this study was the high prevalence of meat samples with isolation of hybrid pathotypes aEPEC/ExPEC (19% of samples and 22 isolates). Table 20 shows the characterization of the 22 aEPEC/ExPEC, which were all positive for the *eae*-beta1 intimin, belonged to phylogroup A and mostly to the CC10. Besides, four of the 22 were ESBL/pAmpC producers.

Table 20. Characterization of the 22 isolates exhibiting hybrid pathotype aEPEC/ExPEC recovered from 19 meat samples.

E. coli counts [1]cfu /g	[2]Sample code	Protocol	[3] Clones	*eae* type	Virulence profile	[4]Antibioresistance profile	ESBL /pAmpC typing
40	Ch2	IV	ONT:H40-A-ST752-CC10 (CH11-24)	B1	*fimH24 hlyF iucD iutA traT iss*	AMP, GEN, DOX, CIP, NAL	-
40	Ch3	II	O123:H34-A-ST752-CC10 (CH11-24)	B1	*fimH24 hlyF iucD iutA traT*	AMP, GEN, TOB*, DOX, SXT, CIP*, NAL	-
40	Ch4	II	O123:H34-A-ST752-CC10 (CH11-24)	B1	*fimH24 hlyF iucD iutA traT*	AMP, NIT*, CIP*, NAL	-
20	Ch5	II	O11:H40-A-ST752-CC10 (CH11-24)	B1	*fimH24 hlyF iucD iutA traT iss*	DOX, CIP*, NAL	-
20	Ch5	II	O80:H26-A-ST165-CC189 *	B1	*fimH fimAvMT78 traT fyuA*	AMP, GEN, TOB*, CIP, NAL	-
10	Ch6	I	O145:H40-A-ST752-CC10 (CH11-24)	B1	*fimH24 traT*	CIP*, NAL	-
100	Ch7	II	ONT:HNT-A-ST19-CC10 (CH11-122)	B1	*fimH122 hlyF iucD iroN cvaC traT tsh, ompT iss chuA yfcV*	AMP, CIP*, NAL	-
70	Ch8	II	O132:H37-ST10-CC10 (CH11-24)	B1	*fimH24 traT*	CIP*, NAL	-
40	Ch10	V	O123:H34-A-ST752-CC10 (CH11-24)	B1	*fimH24 hlyF iucD iutA traT*	AMP, CXM, CTX, FOX*, ATM*, CHL*, NIT*, CIP, NAL	CTX-M-1
10	Ch14	I	O153:H10-A-ST10-CC10 (CH11-54)	B1	*fimH54 fimAvMT78 traT fyuA*	-	-
<10	Ch16	II	O68:H51-A-ST10-CC10 (CH11-24)	B1	*fimH24 traT*	AMP, GEN, TOB*, NAL	-
20	Ch17	II	O153:HNM-A-ST10-CC10 (CH11-54)	B1	*fimH54 fimAvMT78 traT fyuA*	AMP, DOX, CHL	-
440	Ch18	IV	O153:H10-A-ST10-CC10 (CH11-54)	B1	*fimH54 fimAvMT78 traT fyuA*	AMP, GEN, DOX, CHL	-
40	Ch20	II	O11:HNT-A-ST752-CC10 (CH11-54)	B1	*fimH54 traT fyuA*	AMP, CIP*, NAL	-
40	Ch20	II	O123:H34-A-ST10-CC10 (CH11-54)	B1	*fimH54*	AMP*, CIP*, NAL	-

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E. coli counts [1]cfu /g	[2]Sample code	Protocol	[3] Clones	*eae* type	Virulence profile	[4]Antibioresistance profile	ESBL /pAmpC typing
40	Ch22	II	O154:H51-A-STnew11-CC10 (23-823)	ß1	*fimH823 fimAvMT78 traT usp*	AMP, DOX*, SXT, CIP, NAL	-
200	Ch24	V	O153:H10-A-ST10-CC10 (CH11-54)	ß1	*fimH54 fimAvMT78 traT fyuA*	AMP, CFZ, CXM, CAZ*, CTX, ATM, GEN, DOX, CHL	CTX-M-32
20	Ch36	I	O153:H10-A-ST10-CC10 (CH11-54)	ß1	*fimH54 papEF papC papG II traT fyuA*	AMP, DOX, CHL, CIP, NAL	-
<10	Ch40	I	O145:H40-A-ST752-CC10 (CH11-24)	ß1	*fimH24 traT*	AMP, NAL	-
40	T18	II	O57:HNT-A-ST165-CC189 *	ß1	*fimH fimAvMT78 hlyF iucD iroN cvaCtraT usp iss fyuA*	AMP, GEN, TOB, DOX, SXT, CIP, NAL	-
40	T47	II	O2:H40-A-ST10-CC10 (CH11-24)	ß1	*fimH24 hlyF traT*	AMP, AMC, CFZ, CXM, CAZ, CTX, FOX, SXT, CIP, NAL	CMY-2
40	T47	II	O2:H40-A-ST10-CC10 (CH11-24)	ß1	*fimH24 hlyF iucD iroN cvaC traT iss*	AMP, AMC, CFZ, CXM, CAZ, CTX, FOX, DOX, SXT, CIP, NAL	CMY-2

[1]cfu: colony forming units. [2]Ch (chicken meat), T (turkey meat). [3]Clone defined as combination of serotype-phylogroup-Sequence Type-Clonal Complex (Clonotype); *CH not determined. [4]Antimicrobial susceptibility tested by disc diffusion assay and interpreted according to the CLSI standard breakpoints (The Clinical and Laboratory Standards Institute, 2020): ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefuroxime (CXM), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), aztreonam (ATM), gentamicin (GEN), tobramycin (TOB), doxycycline (DOX), chloramphenicol (CHL), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP), nalidixic acid (NAL). Intermediate values are indicated with *.

The study of susceptibility showed that only 13 out of 323 isolates were susceptible to all the antibiotics tested. On the contrary, 258 (79.9%) were MDR, with acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012) (Table 21). The highest prevalence was against ampicillin (82.7%), nalidixic acid (74.0%), ciprofloxacin (71.8%), doxycycline (65.9%) and cefotaxime (46.7%). We found that turkey isolates exhibited significant higher rates of resistance against ampicillin, amoxicillin-clavulanic acid, ceftazidime, cefotaxime, aztreonam, doxycycline, chloramphenicol, sulfamethoxazole-trimethoprim, as well as in the number of MDR ($P < 0.05$). Chicken isolates only showed a significant difference in gentamicin resistance (Table 21). While the disc diffusion method for colistin resistance gave non-susceptible values only for six *mcr*-positive isolates out of the 323, the broth microdilution method, performed only for the *mcr*-bearing *E. coli,* showed CMI values of ≥ 2 µg/mL for 11 of 13 *mcr*-positive isolates.

Table 21. Study of susceptibility for the for the *E. coli* collection (N = 323)

[1]ATB	*E. coli* from chicken n = 163		*E. coli* from turkey n = 160		Total N = 323		Chicken *vs* turkey origin
	No. non-susceptible	%	No. non-susceptible	%	No. non-susceptible	%	[2] Two-tailed *P* value
AMP	118	72.4	149	**93.1**	267	82.7	**0.000**
AMC	26	16.0	47	**29.4**	73	22.6	**0.005**
CXM	54	33.1	58	36.3	112	34.7	0.561
CAZ	55	33.7	73	**45.6**	128	39.6	**0.031**
CTX	69	42.3	82	**51.3**	151	46.7	**0.119**
FOX	3	1.8	5	3.1	8	2.5	0.499
ATM	57	35.0	77	**48.1**	134	41.5	**0.018**
IPM	0	0.0	0	0.0	0	0.0	-
GEN	48	**29.4**	16	10.0	64	19.8	**0.000**
TOB	21	12.9	13	8.1	34	10.5	0.205
AMK	0	0.0	0	0.0	0	0.0	-
FOF	0	0.0	0	0.0	0	0.0	-
CST*	1	0.6	5	3.1	6	1.9	0.119
DOX	96	58.9	117	**73.1**	213	65.9	**0.010**
CHL	35	21.5	69	**43.1**	104	32.2	**0.000**
NIT	11	6.7	11	6.9	22	6.8	1
SXT	47	28.8	74	**46.3**	121	37.5	**0.001**
CIP	115	70.6	117	73.1	232	71.8	0.623
NAL	123	75.5	116	72.5	239	74.0	0.612
TGC	0	0.0	1	0.6	1	0.3	0.495
MDR	121	74.2	137	**85.6**	258	79.9	**0.012**

[1]Antimicrobial susceptibility tested by disc diffusion assay and interpreted according to the CLSI standard breakpoints (The Clinical and Laboratory Standards Institute, 2020), where number of isolates and prevalence include intermediate values: ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefuroxime (CXM), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), aztreonam (ATM), imipenem (IMP), gentamicin (GEN), tobramycin (TOB), amikacin (AMK), fosfomycin (FOF), colistin (CST), doxycycline (DOX), chloramphenicol (CHL), nitrofurantoin (NIT), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP), nalidixic acid (NAL), tigecycline (TGC) and multidrug-resistance (MDR) according to Magiorakos definition (Magiorakos *et al.*, 2012). *Resistance to colistin was also performed by broth microdilution for the 13 *mcr*-positive isolates, which gave MIC values of 4 µg/mL (11 isolates), 2 and 1 µg/mL (1 isolate each). [2] In bold, the statistically significant values ($P < 0.05$).

Seventy-eight meat samples (39 from chicken and 39 from turkey) were carriers of 153 different ESBL/pAmpC-producing isolates, of which 108 (70.6%) carried bla_{SHV}: bla_{SHV-12} (107 isolates) and bla_{SHV-2} (1 isolate). Besides, 39 (25.5%) were positive for bla_{CTX-M}: $bla_{CTX-M-1}$ (14), $bla_{CTX-M-14}$ (6), $bla_{CTX-M-15}$ (5), $bla_{CTX-M-32}$ (9), $bla_{CTX-M-9}$ (3) and two were not-typeable (NT) (2). In addition, six isolates (3.9%) from different meat samples were carriers of bla_{TEM-52}. We also recovered two bla_{CMY-2} isolates from one turkey sample (Table 24).

The *mcr* (1 to 5) screening resulted in 13 *mcr-1.1.* isolates recovered from seven samples. As shown in Table 22, the isolates belonged to different phylogroups (A, B1,

B2, D) and STs (10, 69, 101, 140, 155, 212, 522, 744, 853). To highlight three samples that carried more than one different *mcr*-positive clone. The MIC values for colistin were of 4 µg/mL (11 isolates), 2 and 1 µg/mL (1 isolate each).

Table 22. Characterization of the 13 *mcr-1* isolates recovered from seven meat samples.

E. coli counts [1]cfu /g	[2]Sample code	Protocol (code of isolate)	[3] Clones	ESBL /*mcr* typing	MIC value for colistin	[4] Resistance profile	Virulence profile
30	Ch9	II (a)	O18ac:H49-B1-ST212 (CH29-38)	*mcr1.1*	4 µg/mL	AMP, AMC*, GEN, TOB*, CST, DOX, CHL, NIT*, SXT, CIP, NAL	*fimH38 traT*
510	T1	IV (a)	O109:H51-B1-ST155-CC155 (CH4-32)	*mcr1.1*	4 µg/mL	AMP, AMC*, GEN, DOX, CHL, CIP, NAL	*fimH32 hlyF iroN traT iss*
	T1	V (b)	O162/O89:H9-A-ST744-CC10 (CH11-54)	SHV-12 / *mcr1.1*	4 µg/mL	AMP, CXM*, CAZ, CTX, ATM, CST, DOX, CHL, SXT, CIP, NAL	*fimH54 fimAvMT78 hlyF iucD iutA iroN cvaC traT tsh iss*
210	T13	II (a)	O105:H32-A-ST10-CC10 (CH11-23)	*mcr1.1*	4 µg/mL	AMP, AMC*, DOX, CHL, CIP*, NAL	*fimH23 hlyF iroN ompT iss*
	T13	II (b)	O15:H6-D-ST69-CC69 (CH35-27)	*mcr1.1*	4 µg/mL	AMP, DOX, CHL, NAL	*fimH27 hlyF iroN traT ompT iss chuA fyuA*
100	T16	II (a)	O21:HAA-B1-ST101-CC101 (CH41-86)	*mcr1.1*	4 µg/mL	AMP, DOX, CHL, SXT, CIP*	*fimH86 traT ompT*
	T16	IV (b)	O21:H21-B1-ST101-CC101 (CH41-86)	*mcr1.1*	4 µg/mL	AMP, CST, DOX, CHL, SXT, CIP*	*fimH86 traT ompT*
60	T17	I (a)	O162/O89:H37-A-ST853-CC10 (CH11-54)	*mcr1.1*	4 µg/mL	AMP, CST, DOX*, SXT	*fimH54 fimAvMT78 hlyF iucD iutA iroN traT ompT iss*
	T17	I (b)	O101:HNM-A-ST853-CC10 (CH11-54)	*mcr1.1*	4 µg/mL	AMP, CST, DOX*, SXT	*fimH54 fimAvMT78 hlyF iucD iutA iroN traT ompT iss*
	T17	I (c)	O101:HNM-A-ST853-CC10 (CH11-54)	*mcr1.1*	4 µg/mL	AMP, CST, DOX*, SXT	*fimH54 fimAvMT78 hlyF iucD iutA iroN traT ompT iss*
150	T24	II (a)	O50/O2:H5-B2-ST140-CC95 (CH38-15)	*mcr1.1*	4 µg/mL	AMP, GEN, TOB*, CHL, CIP*	*fimH15 fimAvMT78 hlyF iucD iutA kpsM II-K1 traT ibeA malX usp ompT chuA vat fyuA yfcV*
	T24	II (b)	O50/O2:H5-B2-ST140-CC95 (CH38-15)	*mcr1.1*	2 µg/mL	AMP, GEN, TOB, CHL, CIP*	*fimH15 fimAvMT78 hlyF iucD iutA kpsM II-K1 traT ibeA malX usp ompT chuA vat fyuA yfcV*
350	T31	I (a)	O148:H30-A-ST522-CC522 (CH23-neg)	*mcr1.1*	1 µg/mL	AMP, AMC*, CFZ, DOX, NIT, CIP*, NAL*	*hlyF iroN traT*

[1]CFU: colony forming units. [2]Ch (chicken meat), T (turkey meat).[3]Clone defined as combination of serotype-phylogroup-Sequence Type-Clonal Complex (Clonotype); “neg” when PCR was negative for the 489-nt internal sequence amplification of the *fimH* gene (Weissman *et al.*, 2012). [4]Antimicrobial susceptibility tested by disc diffusion assay and interpreted according to the CLSI standard breakpoints (The Clinical and Laboratory Standards Institute, 2020): ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefuroxime (CXM), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), aztreonam (ATM), gentamicin (GEN), tobramycin (TOB), colistin (CST), doxycycline (DOX), chloramphenicol (CHL), nitrofurantoin (NIT), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP), nalidixic acid (NAL). Intermediate values are indicated with *.

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The screening of *rbfO25b* gave as a result 13 positive isolates from 10 meat samples. MLST typing confirmed 12 as ST131 from nine meat samples. The remaining one belonged to ST1011 and phylogroup E. The virulence profile of the ST131 isolates conformed virotype D (*ibeA* carriers) with subtypes: D4 (10 isolates; *ibeA*, *kpsM II-K1* positive), D1 (1 isolate; *ibeA*, *cdtB*, *kpsM II-K5*) and D-not typeable (1 isolate). The 13 ST131 exhibited two clonotypes: CH40-22 (7 isolates) and CH40-neg (5 isolates) (Table 23).

Table 23. Characterization of the 13 *rbfO25b* isolates recovered from 10 meat samples.

E. coli counts [1]CFU /g	[2]Sample code	Protocol code of isolate	[3]Clones	[4]Virotype ST131	ExPEC/ UPEC status	[5]Resistance profile	Virulence profile
30	Ch2	I (a)	O25b:H4-B2-ST131-CC131 (CH40-neg)	D4	+ / +	GEN, DOX, CIP*, NAL	*fimH hlyF iucD iutA iroN kpsM II-K1 traT ibeA malX usp tsh ompT iss chuA fyuA yfcV*
	Ch2	I (b)	O25b:H4-B2-ST131-CC131 (CH40-neg)	D4	+ / +	GEN, DOX*, CIP*, NAL	*fimH hlyF iucD iutA iroN kpsM II-K1 traT ibeA malX usp tsh ompT iss chuA fyuA yfcV*
	Ch2	I (c)	O25b:H4-B2-ST131-CC131 (CH40-neg)	D4	+ / +	CIP*, NAL	*fimH hlyF iucD iutA iroN kpsM II-K1 traT ibeA malX usp tsh ompT iss chuA fyuA yfcV*
70	Ch8	II	O25b:H4-B2-ST131-CC131 (CH40-neg)	D4	+ / +	NAL*	*fimH hlyF iucD iutA iroN kpsM II-K1 cvaC traT ibeA malX usp tsh ompT iss chuA vat fyuA yfcV*
50	Ch13	I	O25b:H4-B2-ST131-CC131 (CH40-22)	D4	+ / +	GEN*, DOX, CIP*, NAL	*fimH22 hlyF iucD iutA iroN kpsM II-K1 traT ibeA malX usp tsh ompT iss chuA fyuA yfcV*
	Ch13	II	O25b:H4-B2-ST131-CC131 (CH40-22)	D4	+ / +	GEN, DOX, CIP*, NAL	*fimH22 hlyF iucD iutA iroN kpsM II-K1 traT ibeA malX usp tsh ompT iss chuA vat fyuA yfcV*
10	Ch27	III	O25b:H45-E-ST1011 (CH4-31)	-	- / -	AMP, DOX*, SXT, CIP,	*fimH31 hlyF iroN traT tsh ompT iss chuA fyuA*
<10	Ch29	II	O25b:H4-B2-ST131-CC131 (CH40-22)	D4	+ / +	CIP*, NAL	*fimH22 hlyF iucD iutA iroN kpsM II-K1 traT ibeA malX tsh ompT iss chuA vat fyuA yfcV*
<10	Ch39	I	O25b:H4-B2-ST131-CC131 (CH40-22)	D4	+ / +	CIP*, NAL	*fimH22 hlyF iucD iutA iroN kpsM II-K1 cvaC traT ibeA malX usp tsh ompT iss chuA vat fyuA yfcV*
80	Ch50	II	O25b:H4-B2-ST131-CC131 (CH40-22)	D4	+ / +	GEN, DOX*, NAL	*fimH22 hlyF iucD iutA iroN kpsM II-K1 traT ibeA malX usp tsh ompT iss chuA vat fyuA yfcV*
20	T26	I	O25b:H4-B2-ST131-CC131 (CH40-22)	D-nt	+ / +	-	*fimH22 hlyF iucD iutA iroN kpsM II-K5 cvaC traT ibeA malX usp tsh ompT iss chuA vat fyuA yfcV*
<10	T44	II	O25b:H4-B2-ST131-CC131 (CH40-22)	D1	- / +	AMP, AMC*, DOX*,	*fimH22 cdtB kpsM II-K5 traT ibeA malX usp ompT chuA fyuA yfcV*
1130	T46	II	O25b:H4-B2-ST131-CC131 (CH40-neg)	D4	+ / +	AMP, DOX*, NAL	*fimH22 hlyF iucD iutA iroN kpsM II-K1 cvaC traT ibeA malX usp tsh ompT iss chuA fyuA yfcV*

[1]CFU: colony forming units. [2]Ch (chicken meat), T (turkey meat).[3]Clone defined as combination of serotype-phylogroup-Sequence Type-Clonal Complex (Clonotype); "neg" when PCR was negative for the 489-nt internal sequence amplification of the *fimH* gene (Weissman *et al.*, 2012). [4]Virotypes according to Dahbi *et al.* (2014). [5]Antimicrobial susceptibility tested by disc diffusion assay and interpreted according to the CLSI standard breakpoints (The Clinical and Laboratory Standards Institute, 2020): ampicillin (AMP), amoxicillin-clavulanic acid (AMC), gentamicin (GEN), doxycycline (DOX), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP), nalidixic acid (NAL). Intermediate values are indicated with *.

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4.2.4. Risk assessment

In order to evaluate the microbiological risk exposure for consumers, we performed an assessment based on the food-risk definition described by Díaz-Jiménez *et al.* (Díaz-Jiménez et al., 2020a). In the present study, each meat sample was qualified between zero (lowest) to six (highest) in association with the following microbiological parameters, considered as summative risks when happened: i) *E. coli* counts > 500 cfu/g of poultry meat. We took as reference the Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. This Regulation establishes that for meat preparations at the end of the manufacturing process and using *E. coli* as an indicator of recent fecal contamination, the limits considered are "m = 500 and M = 5000" cfu/g to recommend improvements in production hygiene and improvements in selection and / or origin of raw materials. ii) The recovery of *E. coli* resistant to antimicrobials of categories A ("Avoid") or B ("Restrict") (EMA, 2019). iii) The recovery of ≥ 2 different isolates of *E. coli* positive for typically plasmid borne ESBL, AmpC (pAmpC) or *mcr* resistance genes. iv) The identification of high-risk lineages of *E. coli* associated with human extraintestinal pathologies (Yamaji et al., 2018b; Mamani et al., 2019; Manges et al., 2019; Flament-Simon et al., 2020a, 2020b). v) The isolation of *E. coli* conforming ExPEC status (Johnson et al., 2003c). vi) The isolation of *E. coli* conforming UPEC status (Spurbeck et al., 2012).

The Table 24 summarizes the risk assessment of the 100 poultry meat samples analyzed in this study. We considered the summative presence of events, based on the six microbiological parameters described in section 2.3. The results determined that the majority (92%) of meat samples were positive for any of those parameters, with 61% positive for ≥ 4 risks and 84% for ≥ 3 risks.

Per parameter, there was evidence of non-susceptible *E. coli against* monobactams, 3rd-generation cephalosporins and / or fluoroquinolones in 71% of the meat samples. Besides, 47% of the samples showed presence of ≥2 different isolates of *E. coli positive* for ESBL, pAmpC or *mcr* genes. *E. coli isolates* belonging to STs/CCs identified as global ExPEC high-risk lineages were present in 86% of the samples and, what is more important, 73% showed carriage of the same clones as those determined within clinic human isolates of our Health Area. Besides, the isolates from 78% of the samples exhibited ExPEC status, and 53% were carriers of isolates positive for UPEC status. Finally, five samples showed "not satisfactory" *E. coli counts* (> 500 cfu/g).

Table 24. Food-borne risk assessment of the 100 meat poultry samples based on six parameters.

[1] Type of sample	No. sample	[2] ESBL/pAmpC/*mcr* types (No. of isolates)	[3] High-risk lineages of *E. coli*	[4]ExPEC	[5] UPEC	[6] Resistances to antimicrobials of categories A or B	[7] *E. coli* count	[8] TOTAL risk	[9] HP
Ch	1	SHV-12 (1 isolate)	**ST648-F (CH4-58)**; ST117-G; ST162-B1	1	1	MB-CF3rd-FQ	10	4	
Ch	2	CTX-M-32 (1 isolate)	**O25b:H4-B2-ST131 (CH40-neg); ST115-E (CH26-270)**; CC10-A (*eae*-beta1)	1	1	MB-CF3rd-FQ	30	4	*
Ch	3	SHV-12 (1 isolate)	CC10-A (*eae*-beta1)	1	1	MB-CF3rd-FQ	40	4	*
Ch	4	CTX-M-1 (1 isolate)	**ST117-G (CH45-97); ST428-B2 (CH40-22)**; CC10-A (*eae*-beta1)	1	1	CF3rd-FQ	410	4	*
Ch	5	SHV-12, CTX-M-NT, CTX-M-9 **(1 isolate each)**	**O2:H9-E-ST115 (CH26-270); ST69-D (CH35-27); ST1485-F (CH231-58);** CC10-A (*eae*-beta1)	1	1	MB-CF3rd-FQ	20	5	*
Ch	6	TEM-52	**ST1485-F (CH231-58)**; CC10-A (*eae*-beta1)	1	1	CF3rd-FQ	10	4	*
Ch	7	SHV-12, CTX-M-32, CTX-M-1 **(1 isolate each)**	**ST23-C (CH4-35)**; ST93-A; ST10-A (*eae*-beta1)	1	1	MB-CF3rd-FQ	100	5	*
Ch	8	SHV-12 **(2 isolates)**	**O25b:H4-B2-ST131 (CH40-neg)**; CC10-A (*eae*-beta1)	1	1	MB-CF3rd-FQ	70	5	*
Ch	9	CTX-M-NT, *mcr1.1* (**1 isolate each**)	**ST1485-F (CH231-58)**; ST48-A	1	1	CF3rd-FQ-CST*	30	5	
Ch	10	CTX-M-1 (**2 isolates**)	**ST117-G (CH45-97)**; CC10-A (*eae*-beta1)	1	1	MB-CF3rd-FQ	40	5	*
Ch	11	-	-	0	0	-	<10	0	
Ch	12	-	-	0	0	-	<10	0	
Ch	13	SHV-12 (**2 isolates**)	**O25b:H4-B2-ST131 (CH40-22); ST1485-F (CH231-58)**	1	1	MB-CF3rd-FQ	50	5	
Ch	14	-	**O153:H10-A-ST10 (CH11-54) (*eae*-beta1)**	1	1	-	10	3	*
Ch	15	-	**ST93-A (CH11-41)**	1	0	FQ	<10	3	

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[1] Type of sample	No. sample	[2] ESBL/pAmpC/*mcr* types (No. of isolates)	[3] High-risk lineages of *E. coli*	[4]ExPEC	[5] UPEC	[6] Resistances to antimicrobials of categories A or B	[7] *E. coli* count	[8] TOTAL risk	[9] HP
Ch	16	SHV-12, CTX-M-1 (**2 isolates**)	ST10-A (*eae*-beta1)	1	1	MB-CF3rd-Q	20	5	*
Ch	17	SHV-12 (**2 isolates**)	**O153:HNM-A-ST10 (CH11-54) (*eae*-beta1)**; ST428-B2	1	1	MB-CF3rd-FQ	20	5	*
Ch	18	SHV-12, CTX-M-1 (**1 isolate each**)	**O153:H10-A-ST10 (CH11-54) (*eae*-beta1)**	1	1	MB-CF3rd-FQ	440	5	*
Ch	19	CTX-M-1, TEM-52 (**1 isolate each**)	ST428-B2	1	1	MB-CF3rd-FQ	510	6	
Ch	20	SHV-12 (1 isolate)	ST10-A (*eae*-beta1)	1	1	MB-CF3rd-FQ	40	4	*
Ch	21	SHV-12, TEM-52 (1 isolate each)	**ST95-B2 (CH38-27)**	1	1	MB-CF3rd-FQ	10	5	
Ch	22	SHV-12 (**2 isolates**)	**ST93-A (CH11-41)**	1	0	MB-CF3rd-FQ	40	4	*
Ch	23	SHV-12 (1 isolate)	-	0	0	MB-CF3rd-FQ	310	1	
Ch	24	CTX-M-32 (1 isolate)	**ST117-G (CH45-97); O153:H10-A-ST10 (CH11-54) (*eae*-beta1)**	1	1	MB-CF3rd-FQ	200	4	*
Ch	25	SHV-12, CTX-M-32, TEM-52 (**1, 2, and 1 isolates respectively)**	**ST10-A (CH11-54)**	0	0	MB-CF3rd-FQ	50	3	
Ch	26	-	**ST93-A (CH11-neg)**	1	0	FQ	<10	3	
Ch	27	SHV-12 (1 isolate)	**ST10-A (CH11-54)**	1	0	MB-CF3rd-FQ	10	3	
Ch	28	SHV-12 (**2 isolates**)	ST155-B1	1	0	MB-CF3rd-FQ	<10	4	
Ch	29	-	**O25b:H4-B2-ST131 (CH40-22)**	1	1	FQ	<10	4	
Ch	30	-	-	0	0	-	<10	0	
Ch	31	SHV-12 (1 isolate)	**ST93-A (CH11-neg)**	1	1	MB-CF3rd-FQ	10	4	

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[1] Type of sample	No. sample	[2] ESBL/pAmpC/*mcr* types (No. of isolates)	[3] High-risk lineages of *E. coli*	[4]ExPEC	[5] UPEC	[6] Resistances to antimicrobials of categories A or B	[7] *E. coli* count	[8] TOTAL risk	[9] HP
Ch	32	SHV-12 (1 isolate)	-	0	0	MB-CF3rd	<10	1	
Ch	33	SHV-12 (3 isolates)	ST162-B1	1	0	MB-CF3rd-FQ	20	3	
Ch	34	CTX-M-9 (1 isolate)	**ST93-A (CH11-neg)**	1	0	MB-CF3rd-FQ	80	3	
Ch	35	SHV-12, TEM-52, CTX-M-1 **(1, 1, and 2 isolates respectively)**	**ST117-G (CH45-97); ST101-B1 (CH41-86)**	1	0	MB-CF3rd-FQ	50	4	
Ch	36	SHV-12 (1 isolate)	**O153:H10-A-ST10 (CH11-54) (*eae*-beta1); ST93-A (CH11-41)**	1	0	MB-CF3rd-FQ	20	3	*
Ch	37	-	**ST1485-F (CH231-58)**	1	1	FQ	<10	4	
Ch	38	-	**ST1485-F (CH231-58)**	1	1	FQ	<10	4	
Ch	39	SHV-12 (1 isolate)	**O25b:H4-B2-ST131 (CH40-22)**	1	1	MB-CF3rd-FQ	<10	4	
Ch	40	SHV-12 (1 isolate)	CC10-A (*eae*-beta1)	1	1	MB-CF3rd-FQ	<10	4	*
Ch	41	SHV-12 **(3 isolates)**	**ST69-D (CH35-27)**; ST155-B1	1	0	MB-CF3rd-FQ	<10	4	
Ch	42	-	-	0	0	-	<10	0	
Ch	43	SHV-12 **(3 isolates)**	**ST117-G (CH45-97); ST1485-F (CH231-58)**; ST57-E	1	1	MB-CF3rd-FQ	30	5	
Ch	44	CTX-M-9 (1 isolate)	**ST95-B2 (CH38-27)**	1	1	CF3rd-FQ	120	4	
Ch	45	SHV-12, CTX-M-14 (**1 isolate each**)	**O20:H9-C-ST410 (CH4-24); ST648-F (CH4-58)**	1	1	MB-CF3rd-FQ	480	5	
Ch	46	SHV-12 (1 isolate)	ST641-B1	0	0	MB-CF3rd-FQ	<10	2	
Ch	47	CTX-M-32 (**2 isolates**)	**ST93-A (CH11-neg)**	1	0	MB-CF3rd-FQ	<10	4	

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[1] Type of sample	No. sample	[2] ESBL/pAmpC/*mcr* types (No. of isolates)	[3] High-risk lineages of *E. coli*	[4]ExPEC	[5] UPEC	[6] Resistances to antimicrobials of categories A or B	[7] *E. coli* count	[8] TOTAL risk	[9] HP
Ch	48	SHV-12 **(2 isolates)**	**ST10-A (CH11-54);** ST48-A; ST744-A	1	0	MB-CF3rd-FQ	120	4	
Ch	49	SHV-12, CTX-M-15 **(2 and 1 isolate, respectively)**	**ST617-A (CH11-neg);** ST155-B1	1	0	MB-CF3rd-FQ	30	4	
Ch	50	-	**O25b:H4-B2-ST131 (CH40-22)**	1	1	FQ	80	4	
T	1	SHV-12 and *mcr1.1* (1); SHV-12 (2); *mcr1.1* (1)	**ST744-A (CH11-54);** ST155-B1	1	1	MB-CF3rd-FQ-CST*	510	6	
T	2	SHV-12 (1 isolate)	**O46:H31-B2-ST569 (CH38-5); ST10-A (CH11-54)**	1	1	MB-CF3rd-FQ	90	4	
T	3	CTX-M-1, CTX-M-15 **(1 isolate each)**	-	0	0	MB-CF3rd-FQ	100	2	
T	4	SHV-12 **(3 isolates)**	ST354-F	1	1	MB-CF3rd-FQ	40	5	
T	5	SHV-2, SHV-12 **(1 isolate each)**	**O51:H52-A-ST93 (CH11-neg)**	1	0	MB-CF3rd-FQ	70	4	
T	6	SHV-12 **(2 isolates)**	**ST1485-F (CH231-58)**	1	1	MB-CF3rd-FQ	180	5	
T	7	CTX-M-1, CTX-M-15 **(1 isolate each)**	ST48-A	0	0	MB-CF3rd-FQ	<10	3	
T	8	SHV-12, CTX-M-15 **(1 isolate each)**	**ST453-B1 (CH6-31)**	1	0	MB-CF3rd-FQ	20	4	
T	9	SHV-12 **(5 isolates)**	**ST117-G (CH45-97)**	1	1	MB-CF3rd-FQ	250	5	
T	10	CTX-M-32 (1 isolate)	**O51:H52-A-ST93 (CH11-neg)**	1	0	MB-CF3rd-FQ	300	3	
T	11	SHV-12 (1 isolate)	**ST115-E (CH26-270);** ST162-B1	1	0	MB-CF3rd-FQ	200	3	
T	12	SHV-12 (1 isolate)	-	0	0	MB-CF3rd-FQ	440	1	
T	13	SHV-12, CTX-M-1, *mcr1.1* **(1, 1, and 2 isolates, respectively)**	**ST10-A (CH11-23); ST69-D (CH35-27); ST117-G (CH45-97)**	1	1	MB-CF3rd-FQ-CST*	210	5	

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[1] Type of sample	No. sample	[2] ESBL/pAmpC/*mcr* types (No. of isolates)	[3] High-risk lineages of *E. coli*	[4]ExPEC	[5] UPEC	[6] Resistances to antimicrobials of categories A or B	[7] *E. coli* count	[8] TOTAL risk	[9] HP
T	14	-	**O8:H4-C-ST88 (CH4-39); ST428-B2 (CH40-22)**	1	1	FQ	150	4	
T	15	CTX-M-15 (1 isolate)	**ST117-G (CH45-97)**	1	1	MB-CF3rd-FQ	70	4	
T	16	*mcr1.1* (2 isolates)	**ST93-A (CH11-41); ST101-B1 (CH41-86); ST1485-F (CH231-58)**	1	1	FQ-CST*	100	4	
T	17	SHV-12, TEM-52, mcr1.1 (**1, 1 and 3 isolates, respectively)**	**O153:H34-F-ST354 (CH88-58)**	1	1	MB-CF3rd-FQ-CST*	60	5	
T	18	SHV-12 (1 isolate)	**ST648-F (CH4-58);** ST93-A	1	0	MB-CF3rd-FQ	40	3	*
T	19	-	-	0	0	-	<10	0	
T	20	SHV-12, CTX- 14 **(2 and 1 isolates, respectively)**	**ST1485-F (CH231-58)**	1	1	MB-CF3rd-FQ	60	5	
T	21	SHV-12 (1 isolate)	**ST1485-F (CH231-58)**	1	1	MB-CF3rd-FQ	130	4	
T	22	-	**O8:H4-C-ST88 (CH4-39);** ST95-B2	1	1	FQ	80	4	
T	23	-	-	0	0	-	<10	0	
T	24	SHV-12, *mcr1.1* (**5 and 2 isolates, respectively)**	**ONT:H9-A-ST744 (CH11-54); O153:H34-F-ST354 (CH88-58); ST141-B2 (CH52-14);** ST57-E; ST34-A	1	1	MB-CF3rd-FQ	150	5	
T	25	SHV-12 (1 isolate)	ST350-E	0	0	MB-CF3rd-FQ	420	2	
T	26	SHV-12 **(2 isolates)**	**O25b:H4-B2-ST131 (CH40-22); ST93-A (CH11-neg); ST648-F (CH4-58)**	1	1	MB-CF3rd-FQ	20	5	
T	27	SHV-12 **(2 isolates)**	ST350-E; ST155-B1	0	0	MB-CF3rd-FQ	220	3	
T	28	SHV-12 (1 isolate)	**ST10-A (CH11-54); ST648-F (CH4-58)**	1	1	MB-CF3rd-FQ	70	4	
T	29	-	-	0	0	-	<10	0	

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[1] Type of sample	No. sample	[2] ESBL/pAmpC/*mcr* types (No. of isolates)	[3] High-risk lineages of *E. coli*	[4]ExPEC	[5] UPEC	[6] Resistances to antimicrobials of categories A or B	[7] *E. coli* count	[8] TOTAL risk	[9] HP
T	30	-	**ST115-E (CH26-270)**	1	0	FQ	<10	3	
T	31	SHV-12, *mcr1.1* **(1 isolate each)**	-	0	0	MB-CF3rd-FQ	350	2	
T	32	SHV-12, CTX-M-14 **(2 and 1 isolates, respectively)**	**ST10-A (CH11-54); ST617-A (CH11-neg)**	1	0	MB-CF3rd-FQ	100	4	
T	33	CTX-M-14 **(2 isolates)**	**O8:H4-C-ST88 (CH4-39)**; ST1141-A	1	1	MB-CF3rd-FQ	30	5	
T	34	-	**ST453-B1 (CH6-31)**	1	0	FQ	<10	3	
T	35	SHV-12 (1 isolate)	-	0	0	MB-CF3rd-FQ	<10	1	
T	36	-	**O7:H6-E-ST362 (CH100-96)**	1	0	FQ	<10	3	
T	37	CTX-M-1 (1 isolate)	ST48-A	1	0	MB-CF3rd-FQ	20	3	
T	38	SHV-12 **(3 isolates)**	**ST155-B1 (CH4-neg)**	0	1	MB-CF3rd-FQ	30	4	
T	39	SHV-12 **(2 isolates)**	**O51:H52-A-ST93 (CH11-neg); ST10328-B1**	1	0	MB-CF3rd-FQ	210	4	
T	40	SHV-12, CTX-M-14 **(3 and 1 isolates, respectively)**	**O8:HNM-B1-ST58 (CH4-27); ST38-E (CH26-65)**	1	1	MB-CF3rd-FQ	2320	6	
T	41	SHV-12 (1 isolate)	**ST1485-F (CH231-58); ST95-B2 (CH38-30)**	1	1	MB-CF3rd-FQ	<10	4	
T	42	SHV-12 **(2 isolates)**	**ST10-A (CH11-54); ST602-B1 (CH19-86)**	0	0	MB-CF3rd-FQ	680	4	
T	43	SHV-12 **(5 isolates)**	**ST95-B2 (CH38-27); ST155-B1 (CH4-neg); ST354-F (CH88-58)**; ST34-A	1	1	MB-CF3rd-FQ	<10	5	
T	44	SHV-12 (1 isolate)	**O25b:H4-B2-ST131 (CH40-22); ST1485-F (CH231-58); ST359-B1 (CH41-35)**	1	1	MB-CF3rd-FQ	<10	4	

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[1] Type of sample	No. sample	[2] ESBL/pAmpC/*mcr* types (No. of isolates)	[3] High-risk lineages of *E. coli*	[4]ExPEC	[5] UPEC	[6] Resistances to antimicrobials of categories A or B	[7] *E. coli* count	[8] TOTAL risk	[9] HP
T	45	-	-	0	0	-	<10	0	
T	46	SHV-12 (1 isolate)	**O25b:H4-ST131-B2 (CH40-22);** ST57-E	1	1	MB-CF3rd-FQ	1130	5	
T	47	CTX-M-1, CMY-2 **(1 and 2 isolates, respectively)**	**O101:HNM-A-ST167 (CH11-negative);** ST10-A (*eae*-beta1)	0	0	MB-CF3rd-FQ	40	3	*
T	48	SHV-12 **(4 isolates)**	**ST95-B2 (CH38-27); ST155-B1 (CH4-neg)**	1	1	MB-CF3rd-FQ	20	5	
T	49	-	**ST1485-F (CH231-58)**	1	0	FQ	20	3	
T	50	SHV-12, CTX-M-32 **(2 and 1 isolates, respectively)**	**ST115-E (CH26-270); ST1485-F (CH231-58); ST602-B1 (CH19-86);** ST906-B1	1	0	MB-CF3rd-FQ	110	4	

[1]Type of sample: Ch (chicken meat), T (turkey meat). [2]ESBL/pAmpC/mcr types determined by PCR and sequencing. Indicated in bold, the recovery of 2 different ESBL/pAmpC - producing or mcr-bearing isolates. [3]High-risk lineages of *E. coli* associated with human extraintestinal and / or uropathogenic pathologies according to recent studies (Yamaji et al., 2018b; Manges et al., 2019). In bold, those clonal groups (phylogroup, ST and CH) found within our own collections of clinical human isolates (Mamani et al., 2019; Flament-Simon et al., 2020b, 2020c). [4]ExPEC status = 1: *E. coli* strains considered with higher capacity of developing extraintestinal pathologies when positive for two or more of five markers, including papAH and / or papC, sfa/focDE, afa/draBC, kpsM II and iutA; ExPEC status = 0: strains negative for those markers (Johnson *et al.*, 2003). [5]UPEC status = 1: strains considered with higher capacity of developing UTI pathologies when positive for three or more of four markers, including chuA, fyuA, vat and yfcV; UPEC status 0: strains negative for those markers (Spurbeck *et al.*, 2012). [6]Detection of isolates resistant to antimicrobials categorized as A or B (European Medicines Agency, 2020); MB (monobactams); CF3rd (3rd-generation cephalo- sporins); FQ (fluoroquinolones); Q (quinolones); CST (colistin) performed by broth microdilution, * MIC values 4 µg/mL. [7]Count of CFU (colony forming units) per g. [8]Meat samples were qualified between zero (lowest) to six (highest) in association with the following microbiological parameters, considered as summative risks when happened: i) *E. coli* counts >500 cfu/g of poultry meat. ii) The recovery of *E. coli* resistant to antimicrobials of categories A (“Avoid”) or B (“Restrict”). iii) The recovery of ≥2 different ESBL/pAmpC - producing or mcr-bearing isolates. iv) The identification of high-risk clonal groups of *E. coli* associated with human extra- intestinal pathologies. v) The isolation of *E. coli* conforming ExPEC status. vi) The isolation of *E. coli* conforming UPEC status. [9]HP: the recovery of hybrid pathotypes aEPEC/ExPEC is indicated with asterisk (*).

4.3. STUDY 3: GENOMIC CHARACTERIZATION OF ESBL-PRODUCING *ESCHERICHIA COLI* ISOLATES BELONGING TO A HYBRID AEPEC/EXPEC PATHOTYPE O153:H10-A-ST10 *EAE*-BETA1 OCCURRED IN HUMAN DIARRHEAGENIC ISOLATES, MEAT, POULTRY AND WILDLIFE

From previous food and clinical surveys, we were conscious of the presence of *eae*-positive ESBL-producing isolates belonging to serogroup O153. Its recovery from the 100 meat samples (Table 20) reinforces the hypothesis of the potential food transmission. This was the motivation to explore the genetic and genomic relatedness between human and animal/meta isolates.

Thirty-two *eae*-positive *E. coli* (21 ESBL and 11 non-ESBL) belonging to the serotype O153:H10 constituted the collection of study. As detailed in Table 10 (3.1.3. EPEC O153 collection of Material and Methods), they were detected within different surveys in the period 2005 to 2015: 14 from human stools, eight from beef meat, seven from chicken meat, and one each of pork meat, wildlife (fox feces) and poultry farm environment.

4.3.1. Conventional typing

Table 25 summarizes the main traits determined by conventional typing for the 32 isolates. All were positive for the intimin *eae*-beta1, but negative for *bfpA* gene, conforming the aEPEC pathotype. Other virulence genes defining STEC, EIEC, EAEC or ETEC pathotypes were not detected; however, the $fimAv_{MT78}$ gene, which is a virulence *locus* that codify a *fimA* variant MT78 of type 1 fimbriae (Marc and Dho-Moulin, 1996) was present in all isolates. Besides, the *traT* gene that codifies an outer membrane protein implicated in serum survival (Johnson and Stell, 2000) was also present in 17 of the isolates (Table 25). By means of the serotype, phylogroup, ST and clonotyping, the isolates were assigned to the clonal group O153:H10-A-ST10 (CH11-54).

The highest rates of AMR were to: ampicillin (75%; 24/32), cefuroxime (68.7%; 22/32), cefotaxime (65.6%, 21/32), ceftazidime (65.6%, 21/32), cefepime (59.4%, 19/32) and gentamicin (59.4%, 19/32). The ESBL-typing determined that 19 isolates were CTX-M-32 and two SHV-12 (Table 25).

Table 25. Phenotypic and genotypic characterization of 32 aEPEC O153:H10-A-ST10 (CH11-54) isolates

Sample origin	Code[1]	Year	Geographic origin	Virulence gene profile	Resistance profile[2]	bla_{ESBL} type
Pork meat	*LREC-122	2011	Lugo	*fimH54, $fimAv_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Chicken meat	*LREC-115	2009	Lugo	*fimH54, $fimAv_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Chicken meat	FV 19517	2009	Lugo	*fimH54, $fimAv_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Chicken meat	*LREC-118	2009	Lugo	*fimH54, $fimAv_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Chicken meat	*LREC-110	2010	Lugo	*fimH54, $fimAv_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Chicken meat	FV 14703	2010	Lugo	*fimH54, $fimAv_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB, FOF	CTX-M-32

Sample origin	Code[1]	Year	Geographic origin	Virulence gene profile	Resistance profile[2]	*bla*$_{ESBL}$ type
Chicken meat	LREC-126	2010	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Chicken meat	*LREC-123	2010	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Beef meat	*LREC-119	2007	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Beef meat	*LREC-117	2007	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Beef meat	4-3a	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ	SHV-12
Beef meat	85-5a	2008	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, GEN	-
Beef meat	*LREC-125	2008	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, FEP	CTX-M-32
Beef meat	*LREC-114	2008	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Beef meat	65-6a	2009	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	-	-
Beef meat	*LREC-120	2011	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ, FEP	SHV-12
Wildlife (Fox)	*LREC-111	2015	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Poultry farm	*LREC-127	2010	Pontevedra	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN	CTX-M-32
Human	*LREC-116	2006	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Human	*LREC-113	2007	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Human	*LREC-121	2007	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Human	*LREC-124	2007	Lugo	*fimH54, fimAv$_{MT78}$, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, GEN, TOB	CTX-M-32
Human	31952. 07	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	-	-
Human	32651. 07	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	NAL, CIP	-
Human	32884. 07	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CAZ, AMC, SXT	-
Human	34535. 07	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	NAL, CIP	-
Human	39044. 07	2007	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	-	-
Human	21011. 08	2008	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	-	-
Human	38506. 08	2008	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	CIP	-
Human	40237. 08	2008	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	NAL, CIP	-
Human	*LREC-112	2011	Santiago de Compostela	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, CXM, CTX, CAZ, FEP, NAL	CTX-M-32
Human	55515.12	2012	Lugo	*fimH54, fimAv$_{MT78}$, traT, eae-beta1*	AMP, GEN	-

[1]Strains further analyzed by WGS are those marked with (*); [2]ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefuroxime (CXM), ceftazidime (CAZ), cefotaxime (CTX), cefepime (FEP), cefoxitin (FOX), gentamicin (GEN), tobramycin (TOB), fosfomycin (FOF), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP) and nalidixic acid (NAL).

The PFGE comparison of the *XbaI*-macrorrestriction profiles of the ESBL-producing aEPEC isolates revealed high similarity. Thus, all but one clustered with an identity >85% in the dendrogram shown in Figure 12. It is of note that three human clinical isolates, recovered in different years, clustered each with a fox (95.2% of similarity) and with two beef meat isolates (100% and 97.6% of similarity, respectively).

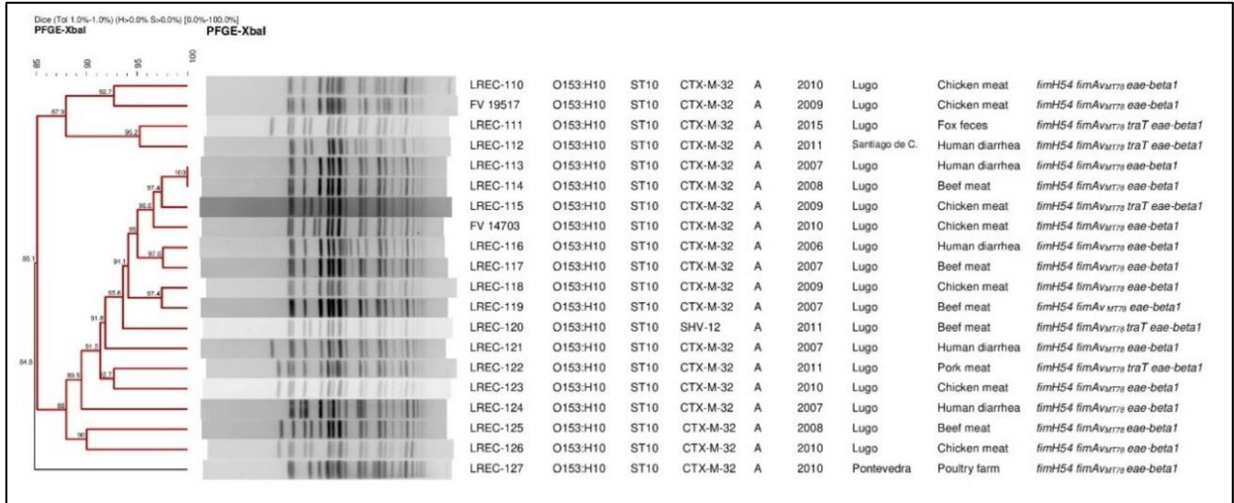

Figure 12. PFGE of *XbaI*-digested DNA from 20 ESBL-producing aEPEC isolates of the clonal group O153:H10-A-ST10 (one autodigested). On the right of the dendrogram: Isolate designation, O:H serotype, ST, ESBL type, phylogroup, year of isolation, geographic origin, source and virulence-gene profile

4.3.2. Whole genome sequencing (WGS)

Based on the high similarity shown by PFGE and to further investigate the virulence profile, resistome, plasmid content and relatedness, 17 representative aEPEC/ExPEC isolates of different origins were WG sequenced. The *de novo* assembled contigs were then typed *in silico* using the EnteroBase tools (Table 36), as well as the Center for Genomic Epidemiology (CGE) databases (Table 26).

SerotypeFinder and EnteroBase predictions corroborated O and H antigens, except for LREC-120 and LREC-121, for which O153 was solved by serotyping. MLST (CGE and EnteroBase), CHtyper and ClermonTyping also confirmed conventional data for ST (10), CH (11-54) and phylogroup (A) (Table 26, Table 36). Additionally, the wgST, cgST, and rST of the genomes were determined using the schemes of EnteroBase based on 25,002; 2,513 and 53 loci, respectively (Table 36). WgMLST and cgMLST are powerful schemes with extreme and high resolution, respectively, which determined different STs for each of the 17 genomes analyzed, while rST (medium resolution) established the same ST (2021) for all genome but for LREC-127 (58738) (Table 36).

VirulenceFinder corroborated the hybrid pathotype nature of the isolates, predicting in all genomes the *eae* gene (intimin) together with other components encoded in the LEE pathogenicity island, as well as the increased serum survival gene *iss* recognized for its role in ExPEC virulence (Johnson et al., 2008). Besides, the *astA* gene, which encodes the heat-stable enterotoxin 1, was also present in all 17 isolates (Table 26).

ResFinder identified the genes associated to resistances observed *in vitro* (acquired resistances for β-lactams, aminoglycosides, and point mutations for quinolones). Only, the *bla*$_{CTX-M-32}$ was not predicted *in silico* for LREC-112 and LREC-119, but by conventional sequencing. Furthermore, ResFinder determined other acquired resistances which had not been tested *in vitro*, such as to phenicols and macrolides in all genomes, and to tetracyclines in 16 out of the 17 genomes (Table 26).

Based on replicon identification, PlasmidFinder revealed a homogenous profile of four/five plasmid types. Thus, the concomitant presence of IncF (F2:A-:B-), IncI1 (ST unknown) and IncX1, together with non-conjugative Col156-like plasmids, was detected in 15 of 17 genomes. Four of those 15 genomes were also carriers of Col (MG828)-like plasmids (Table 26).

In the asymmetric distance matrix on the cgMLST scheme from EnteroBase, based on the presence/absence of 2,513 genes, the 17 genomes showed <20 differences (range 5-19) in relation to the human diarrheagenic isolate LREC-113 (Table 27, Figure 13). We also looked into the static Hierarchical Clustering (HierCC) designations in EnteroBase. The 17 genomes were assigned into the same HierCC HC50 (37600), which means all strains in this cluster have links no more than 50 alleles apart. Besides, using HC20, three human genomes (LREC-113, LREC-116, LREC-124) and two beef meat (LREC-119, LREC-125) clustered together (37606) with links no more than 20 alleles apart (Table 37). A dendrogram based on the SNPs of the core genomic regions present in 90% of the compared genomes and using LREC-113 as reference, was also built in EnteroBase, downloaded and modified with FigTree v1.4.3 (Figure 14). Within 1,068 variant sites, the number of SNPs was <62 for 13 of the 17 genomes (Table 37).

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Table 26. *In silico* characterization of 17 *E. coli* genomes from the study collection using CGE databases and ClermonTyping (in red, results obtained only by conventional typing)

Code	Serotype[1]	PG[2]	CHType[3]	ST[4]	Plasmid content Inc group (pMLST)[5]	Acquired resistances (black) and point mutations (blue)[6]	Virulence genes[7]
LREC-110	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (ST unknown); IncX1; Col156	*$bla_{CTX-M-32}$, bla_{TEM-1A}; aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP, tir*
LREC-111	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (ST unknown); IncX1; Col156; Col (MG828)	*$bla_{CTX-M-32}$, bla_{TEM-1A}; aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tccP, tir*
LREC-112	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncX1; Col156; Col (MG828)	*$bla_{CTX-M-32}$; aadA1; catA1; mdf(A); tet(A); gyrA* S83L	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP*
LREC-113	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (ST unknown); IncX1; Col156	*$bla_{CTX-M-32}$, bla_{TEM-1A}; aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, tir*
LREC-114	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (ST unknown); IncX1; Col156; Col (MG828)	*$bla_{CTX-M-32}$, bla_{TEM-1A}; aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tir*
LREC-115	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (ST unknown); IncX1; Col156	*$bla_{CTX-M-32}$, bla_{TEM-1A}; aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP, tir*
LREC-116	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (ST unknown); IncX1; Col156	*$bla_{CTX-M-32}$, bla_{TEM-1A}; aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, tccP, tir*
LREC-117	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (ST unknown); IncX1; Col156	*$bla_{CTX-M-32}$; aadA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, tccP, tir*
LREC-118	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (ST unknown); IncX1; Col156; Col(MG828)	*$bla_{CTX-M-32}$, bla_{TEM-1A}; aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP, tir*
LREC-119	O153:H10	A	11-54	10	Col156	*$bla_{CTX-M-32}$, aadA1; catA1; mdf(A)*	*astA, eae, espA, espB, gad, iss, nleA, tccP, tir*

Code	Serotype[1]	PG[2]	CHType[3]	ST[4]	Plasmid content Inc group (pMLST)[5]	Acquired resistances (black) and point mutations (blue)[6]	Virulence genes[7]
LREC-120	O153:H10	A	11-54	10	IncI1 (ST22-CC2); IncQ1; IncX1; Col156; Col (MG828)	*bla*$_{SHV-12}$; *aadA1, aadA2; catA1, cmlA1; mdf(A); sul3; tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tccP, tir*
LREC-121	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (STunknown); IncX1; Col156	*bla*$_{CTX-M-32}$, *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tccP, tir*
LREC-122	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (STunknown); IncX1; Col156; Col (MG828)	*bla*$_{CTX-M-32}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tccP, tir*
LREC-123	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (STunknown); IncX1; Col156; Col (MG828)	*bla*$_{CTX-M-32}$, *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tccP, tir*
LREC-124	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (STunknown); IncX1; IncY; Col156	*bla*$_{CTX-M-32}$, *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, tccP, tir*
LREC-125	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (STunknown); IncX1; Col156	*bla*$_{CTX-M-32}$; *aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP, tir*
LREC-127	O153:H10	A	11-54	10	IncF (F2:A-:B-); IncI1 (STunknown); IncX1; Col156; Col (MG828)	*bla*$_{CTX-M-32}$, *bla*$_{TEM-1A}$; *aac(3)-IIa, aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, espF, gad, iss, mchF, nleA, tccP, tir*

[1]Serotypes, [3]clonotypes, [4]sequence types, [5]replicon/plasmid STs, [6]acquired antimicrobial resistance genes and / or chromosomal mutations, [7]virulence genes were determined using SerotypeFinder 2.0, CHtyper 1.0, MLST 2.0, PlasmidFinder 2.0, pMLST 2.0, ResFinder 3.1 and VirulenceFinder 2.0 online tools at the CGE, respectively. While [2]PG; phylogroups were predicted using the ClermonTyping tool at the Iame-research Center web. [1]Serotypes: underlined and in red those (LREC-121, LREC-120) that were not predicted (ONT) by SerotypeFinder but assigned as O153 by conventional typing.[6]Resistome: Acquired resistance genes: β-lactam: *bla*$_{TEM-1A}$, *bla*$_{CTX-M-32}$, *bla*$_{SHV-12}$; aminoglycosides: *aac(3)-IIa, aadA1, aadA2;* phenicols: *catA1, cmlA1;* macrolides: *mdf(A)*; sulphonamides: *sul3*; tetracycline: *tet(A)*. Point mutations (marked in blue): quinolones and fluoroquinolones: *gyrA* S83L: TCG-TTG. Underlined and in red those bla$_{CTX-M-32}$ genes (LREC-112, LREC-119) that were not predicted by ResFinder but determined in conventional typing [8]Virulence genes: *astA*: EAST-1, *eae*: intimin, *espA*: type III secretions system, *espB*: secreted protein B, *espF*: type III secretions system, *gad*: glutamate descarboxylase, *iss*: increased serum survival, *mchF*: ABC transporter protein MchF, *nleA*: non LEE encoded effector A, *tccP*: Tir cytoskeleton coupling protein, *tir*: translocated intimin receptor protein. bp: base pairs; CHType: clonotype (*fumC-fimH*); ST: sequence type according to Achtman scheme; pMLST: plasmid sequence type.

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Table 27. Asymmetric distance matrix based on the cgMLST scheme from EnteroBase in which D (a, b) equals all sites that are present in (b) and different from (a)

Genome code / cgMLST		LREC-110	LREC-111	LREC-127	LREC-112	LREC-113	LREC-120	LREC-117	LREC-121	LREC-119	LREC-116	LREC-115	LREC-114	LREC-123	LREC-122	LREC-124	LREC-125	LREC-118
		37600	**37601**	**37602**	**37605**	**37606**	**37607**	**37609**	**37610**	**37611**	**37612**	**37613**	**37614**	**37615**	**37616**	**37617**	**37618**	**38299**
LREC-110	**37600**	**0**	14	17	19	13	12	18	19	12	15	27	8	15	15	18	16	14
LREC-111	**37601**	14	**0**	21	23	16	20	22	23	16	19	30	11	19	18	22	20	17
LREC-127	**37602**	17	21	**0**	24	9	22	14	16	9	11	24	15	13	13	15	13	13
LREC-112	**37605**	19	23	24	**0**	19	25	24	25	18	21	33	17	22	22	24	23	21
LREC-113	**37606**	13	16	9	19	**0**	18	9	11	5	6	18	10	9	8	11	9	8
LREC-120	**37607**	12	20	22	25	18	**0**	23	23	18	20	33	14	20	20	24	22	19
LREC-117	**37609**	18	22	14	24	9	23	**0**	17	8	12	22	15	14	13	14	12	13
LREC-121	**37610**	19	23	16	25	11	23	17	**0**	10	13	25	15	16	16	16	14	14
LREC-119	**37611**	12	16	9	18	5	18	8	10	**0**	6	17	10	9	9	7	6	8
LREC-116	**37612**	15	19	11	21	6	20	12	13	6	**0**	22	12	11	11	13	11	10
LREC-115	**37613**	27	30	24	33	18	33	22	25	17	22	**0**	22	24	23	23	15	20
LREC-114	**37614**	8	11	15	17	10	14	15	15	10	12	22	**0**	13	12	15	14	11
LREC-123	**37615**	15	19	13	22	9	20	14	16	9	11	24	13	**0**	7	15	13	8
LREC-122	**37616**	15	18	13	22	8	20	13	16	9	11	23	12	7	**0**	15	13	9
LREC-124	**37617**	18	22	15	24	11	24	14	16	7	13	23	15	15	15	**0**	12	14
LREC-125	**37618**	16	20	13	23	9	22	12	14	6	11	15	14	13	13	12	**0**	12
LREC-118	**38299**	14	17	13	21	8	19	13	14	8	10	20	11	8	9	14	12	**0**

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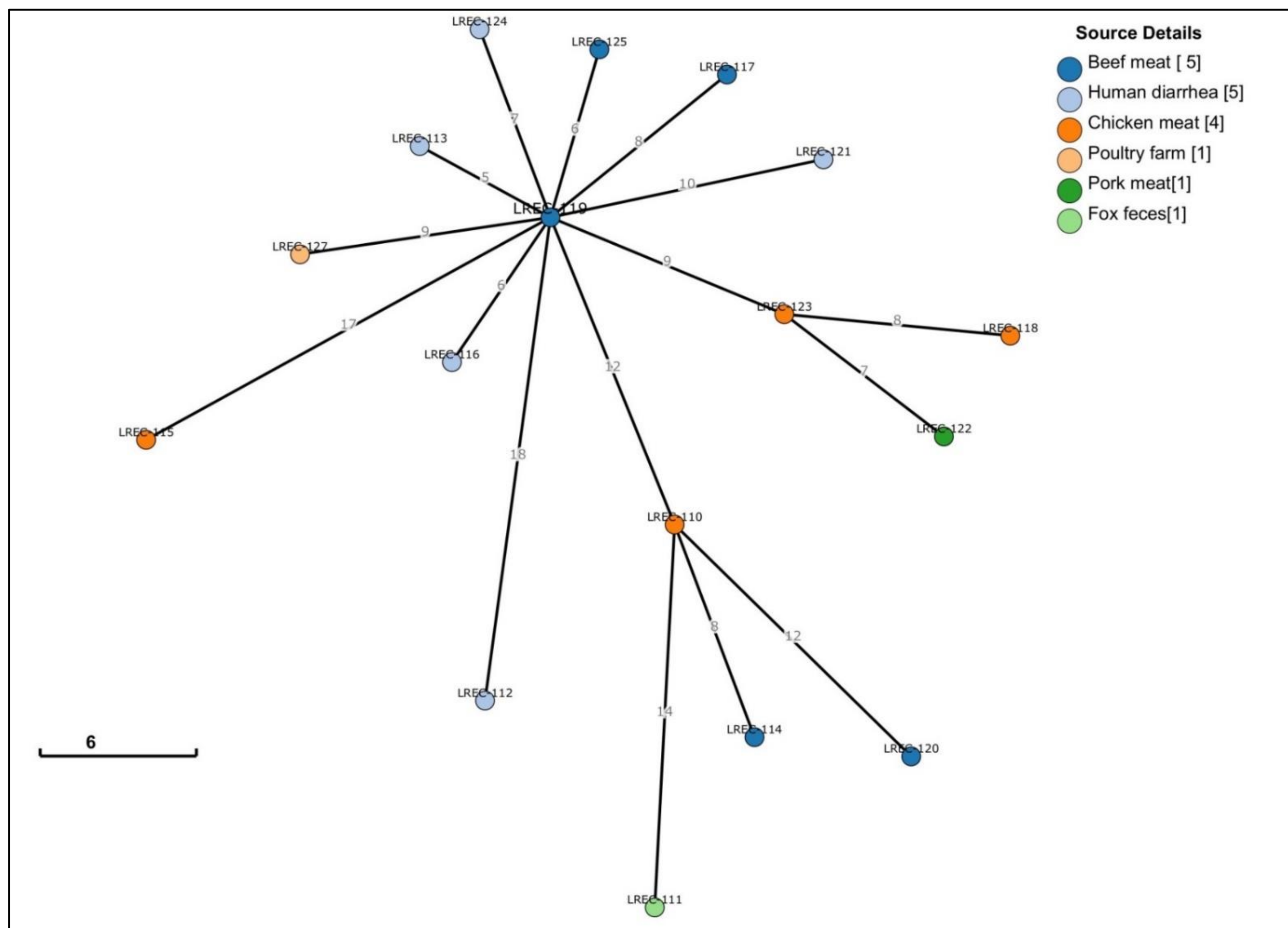

Figure 13. GrapeTree inferred using the MSTree V2 algorithm based on the cgMLST V1 + HierCC V1 scheme from EnteroBase

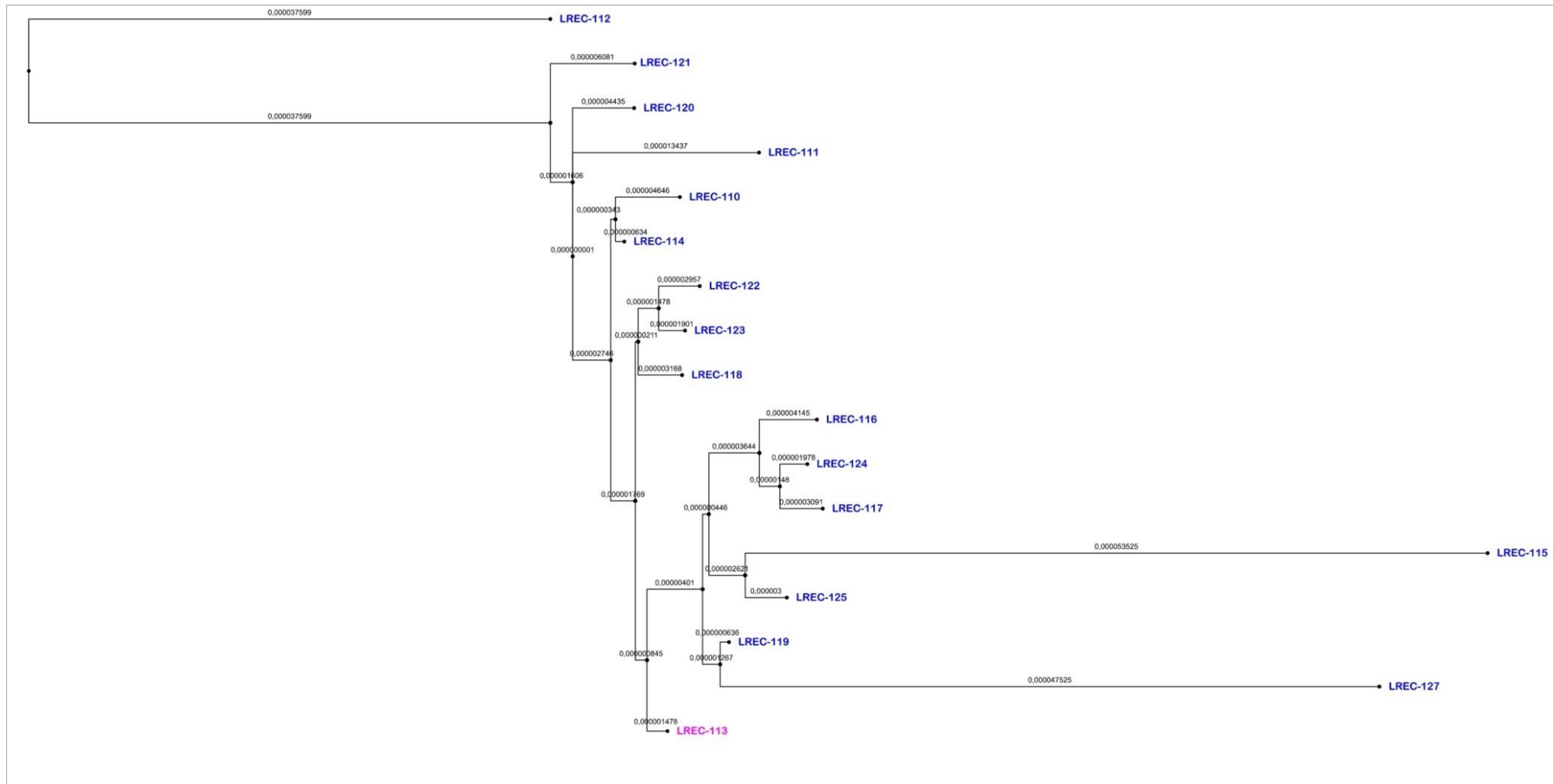

Figure 14. Dendrogram based on the SNPs of the core genomic regions present in 90% of the compared genomes and using LREC-113 as reference, built in EnteroBase and modified with FigTree v1.4.3

5. DISCUSSION

5.1. CONSUMER EXPOSURE TO HIGH RISK ENTEROBACTERIACEAE FROM CHICKEN AND TURKEY MEAT

Common human extraintestinal diseases, namely, UTIs or blood stream infections, may be caused by bacteria not traditionally defined as food-borne pathogens. Currently, there is not a surveillance system of ExPEC genotypes, or other Enterobacteriaceae causing extraintestinal infections, to elucidate their real role (Riley, 2020). For first time, and based on a comprehensive characterization of 256 isolates, this study evaluates the consumer exposure via poultry meat to Enterobacteriaceae with capacity to develop, not only intestinal, but also severe extraintestinal infections by either bacterial virulence and / or antibiotic resistance traits. For this purpose, we aimed to develop a suitable protocol potentially applicable in the routine of food microbiological laboratories. This protocol comprises a meat sample enrichment, followed by the characterization of one representative *E. coli* colony grown on Lactose MacConkey agar (LMA), and those species suspected of being ESBL/Carbapenemase-producing Enterobacteriaceae grown on CHROMID® ESBL or CHROMID®CARBA SMART. The combination of these selective media effectively provided complementary information on the presence and prevalence of specific high-risk clonal groups of *E. coli,* as well as other ESBL-producing Enterobacteriaceae.

A high-risk clone, such as the ST131 of *E. coli* , is that defined as globally distributed, associated with multiple AMR determinants, able to colonize and persist in hosts for more than six months, capable of effective transmission between hosts, enhanced pathogenicity and fitness, and able to cause severe and / or recurrent infections (Mathers et al., 2015). It is within the group of ExPEC where the successful risk clones of *E. coli* emerge. According to a recent meta-analysis, 20 major ExPEC STs accounted for 85% of the studies included, being considered global extraintestinal pathogenic lineages (Manges et al., 2019). In this study, 13 of those top 20 ExPEC lineages were detected in 50% of our meat samples (ST10, ST23, ST38, ST58, ST69, ST88, ST95, ST117, ST131, ST167, ST354, ST410, ST617). Seven of the 13 STs were determined within both the representative 84 *E. coli* and the 137 ESBL-*E. coli;* however, certain isolates could be recovered only via LMA (those belonging to ST23, ST95 and the pandemic ST131) while others (ST69, ST167, ST354, and ST617) of the ESBL-producing isolates, were detected mostly via CHROMID® ESBL.

The increasing evidence that retail food may serve as a source of *E. coli* implicated in UTIs was recently analyzed by Yamaji *et al.* (Yamaji et al., 2018a) through the characterization of 233 *E. coli* isolates from human urine samples and 177 *E. coli* from retail meat (poultry, pork and beef) collected in the same geographic region. Within their collection, 21% of *E. coli isolates* from suspected cases of UTIs belonged to STs found in poultry, stating that poultry may serve as possible reservoir of UPEC. In our study, 40% of the meat poultry samples carried *E. coli* belonging to STs (ST10, ST38, ST69, ST88, ST95, ST101, ST117, ST131, ST141, ST354, ST906) identified by Yamaji *et al.* (2018) within the UTI human cases, corresponding to 20.8% of our 221 *E. coli* isolates. Significantly, we found a higher prevalence of turkey isolates belonging to STs associated to UPEC in comparison with those of chicken origin (32 of 114; 28.1% *vs* 16 of 107; 14.9%) (P = 0.022).

We further investigated, within the 221 *E. coli* , the presence of four genes (*yfcV*, *vat*, *fyuA*, and *chuA*) that predicts whether isolates can colonize the bladder more efficiently

than *E. coli* isolates without these genes (Spurbeck et al., 2012). We found that 18 *E. coli* from 17 different meat samples conformed the UPEC status (Table 14). The 18 isolates belonged to B2 and F phylogroups, exhibited a high number of extraintestinal VF and included reported ST/CC linked to UTIs, such as ST95, ST117, ST131, ST141 or CC648. Importantly, the isolates of the following seven clones carried the four genes *yfcV*, *vat*, *fyuA*, and *chuA*: O1:H7-B2-ST95 (CH38-30); O50/O2:H6-B2-ST141 (CH52-14); O115:HNM-B2-ST187 (CH24-187); O120:H4-B2-ST428 (CH40-22); O120:H4-B2-ST428 (CH40-neg); O11:H25-F-ST457 (CH88-145); O113:H5-B2-ST8611 (CH24-26).

E. coli ST131 has clearly become the major cause of MDR UTIs worldwide within healthcare and community settings. WGS-analysis of the population structure of *E. coli* ST131 identified three genetically distinct Clades (A, B, C), and numerous subclades from the dominant fluoroquinolone-resistant Clade C (Johnson et al., 2010; Price et al., 2013; Stoesser et al., 2016); Clade C carries a type 1 fimbrial adhesin gene *H30* variant (*fimH*30; clonotype CH40-30), and compensatory mutations at regulatory regions which seems to confer adaptive advantages for the fitness cost of AMR, plasmid acquisition and maintenance, differently from the fluoroquinolone-susceptible Clades A (*fimH*41; clonotype CH40-41) and B (*fimH*22; clonotype CH40-22) (Stoesser et al., 2016; Decano and Downing, 2019). While ST131-*H*30 is the most prevalent, Clades A and B are also important agents of community and hospital-acquired UTIs (Mora et al., 2014; de Toro et al., 2017b; Liu et al., 2018). ST131 isolates can be further classified into 12 virotypes (A to F), regarding the presence/absence of certain virulence genes, which show different host distribution, prevalence, and *in vivo* virulence in the mouse model (Blanco et al., 2013; Dahbi et al., 2014; Mora et al., 2014). In this study, ST131-*H*22 (CH40-22) was determined in two *E. coli* isolates recovered from two chicken samples in the LMA medium. Both ST131 isolates conformed virotype D4 (carriers of *ibe*A gene and K1 variant of group II capsule) and showed MDR to aminoglycosides, tetracyclines and quinolones. In previous studies, we proved that the ST131 poultry linage typically conforms virotype D4 (Cortés et al., 2010; Mora et al., 2010; Solà-Ginés et al., 2015). Importantly, we also found virotype D4 within clinical human ST131, with a prevalence of 3.8% among 157 isolates (unpublished data), and some of them showing a high genetic similarity compared to avian isolates (Mora et al., 2010). Recently, we also proved by WGS, that porcine (meat and animal origin) and clinical human ST131-H22 isolates of new subclades B6 and B7, were strongly related (average distance of 20 and 15 SNP/Mb, respectively) (Flament-Simon et al., 2020c). Liu *et al.* (2018), combining detection of poultry associated ColV plasmids with high-resolution phylogenetics, quantified the proportion of human infections (from urine and blood cultures). From their results, the authors stated that sub lineage ST131-H22 has become established in poultry populations around the world and that meat may serve as a vehicle for human exposure and infection. According to the authors, ST131-*H22* would be just one of many *E. coli* lineages that may be transmitted from food animals to humans.

We also studied within meat isolates the presence of diarrheagenic *E. coli.* While none of the 221 *E. coli* was positive for the specific VF associated with the verotoxigenic (*stx_1*, *stx_2*) or enteroaggregative (*aaiC*, *aggR*) pathotypes, four CC10-A isolates obtained from four different meat samples carried the *eae*-beta1 intimin gene, together with extraintestinal pathogenic genes, and conforming an atypical EPEC/ExPEC hybrid pathotype: two isolates O153:H10-A-ST10 (CH11-54); one O145:H40-A-ST752 (CH11-24) and one O123/186:H34-A-ST752 (CH11-24). In our geographical region (NW Spain), we have been periodically detecting a hybrid MDR aEPEC/ExPEC of clonal

group O153:H10-A-ST10 (CH11-54) recovered from different sources (food-producing animals; chicken, beef and pork meat; wildlife and human clinical samples). Importantly, we proved genomic evidence of the close relatedness of the isolates that may be playing a successful role in spreading ESBLs (CTX-M-32) in our region within different hosts, including wildlife. Besides, it would be potentially implicated in human diarrhea via food (meat) transmission (Díaz-Jiménez et al., 2020b). Since 2011, when a novel STEC/EAEC *E. coli* O104:H4 emerged in Germany and neighboring countries (Mora et al., 2011b), other hybrid virulent *E. coli* have been reported. The most outstanding is the recently emerged STEC/ExPEC O80:H2 hybrid reported to cause HUS and bacteremia (Mariani-Kurkdjian et al., 2014), but there are also STEC/UPEC hybrids which have been identified from hospitalized patients (Toval et al., 2014), or some STEC/ETEC strains associated with diarrheal disease and HUS in humans (Nyholm et al., 2015). Given the public health importance of hybrid pathotypes, it seems necessary the surveillance of potentially emerging types.

According to the diversity of STs found within the 221 *E. coli* isolates of our study, and despite more than 50% of the 84 representative *E. coli as* well as the ESBL-producing *E. coli* belonged to the phylogroups A + B1, the other five (B2, C, D, E, F) of *E. coli sensu stricto* were represented in the collection. The most anciently diverged phylogroups B2, F and D comprises the majority of ExPEC isolates, whereas the intestinal pathologies are linked to the most recently diverged phylogroups (E, C, B1 and A) (Clermont et al., 2019). Interestingly, five isolates from different samples belonged to *Escherichia* clade I, which is also considered a phylogroup of *E. coli* based on the extent of recombination detected between strains belonging to clade I and *E. coli* (Clermont et al., 2013). The five isolates, recovered from CHROMID® ESBL, belonged to the clonal group ST770 (CH116-552), conformed the ExPEC status, were CTX-M-9 (two) or SHV-12 (three isolates) and MDR (the five FQ-resistant).

Besides the virulence traits associated to intestinal and extraintestinal *E. coli* pathotypes, we investigated here the consumers´ exposure to antibiotic-resistant bacteria. Based on the complementary analysis of Enterobacteriaceae recovered from the two selective media, we found that 90% of the meat samples were carriers of MDR isolates. Specifically, 96% samples carried resistant isolates to antimicrobials of categories A or B, including 18% of the meat samples with colistin-resistant isolates, 64% with resistance to monobactams, and one of those also to FOF (category A). Resistance prevalence was significantly higher among turkey isolates (in both representative *E. coli and* ESBL-producing Enterobacteriaceae) for SXT and CIP. In a study conducted in USA on poultry meat, the authors found higher resistance prevalence among *E. coli* isolates from conventionally-raised turkey for most of the antibiotics tested compared to chicken meat (Davis et al., 2018). We also found in our study that turkey meat was significantly more contaminated with other ESBL-producing species than chicken. The differences found for turkey meat can be probably associated with a longer exposition to antibiotics due to the much longer fattening period.

On the other hand, the marked variation of prevalence and type of antibiotic resistances reported by the countries would be linked to the current and past usage of antibiotics in the respective animal species. The European Union summary report on AMR in indicator *E. coli* (EFSA, 2021) shows comparable results to ours from the 84 representative *E. coli* , however, this *E. coli* collection alone would not reflect the real figures of MDR occurrence in the poultry samples.

The ESBL types determined in our study within the *E. coli* isolates are mostly the same as those reported in other studies for poultry meat (Egea et al., 2012; Kaesbohrer et al., 2019; Nüesch-Inderbinen et al., 2019), but with an outstanding prevalence of SHV (SHV-12, mainly) (71.5% of the 172 ESBL-producing Enterobacteriaceae and 68.6% of the 137 ESBL-producing *E. coli*). In the south of Spain, Egea *et al.* also found this predominance, but with a decrease in favor of CTX-M ESBLs in comparison with a previous study (Doi et al., 2010; Egea et al., 2012). Interestingly, our studies on poultry, suggest an increase of the SHV isolates. Thus, of the 84 avian ESBL-producing *E. coli* recovered from faecal avian samples in 52 farms located in the same geographical area (2010-2012), 70.2% were of CTX-M type and 29.8% of SHV (García et al., 2018). Likewise, 62.8% and 37.2% of 98 ESBL-producing *E. coli* from chicken meat sampled in our city (2010-2011) were CTX-M and SHV, respectively (Herrera, 2015).

We also investigated here the colistin resistance linked to *mcr* genes within the meat isolates. Since the *mcr-1* plasmid gene was first described (Liu et al., 2016), different authors corroborate that large conjugative plasmids of types IncHI2, IncX4 and IncI2 would be the maximum responsible for the dissemination of the *mcr-1* gene among *E. coli* isolates from different sources and geographical locations (Hasman et al., 2015; Doumith et al., 2016; Dominguez et al., 2019). We report in this study two CC10-A (CH11-54) carriers of the *mcr-1.1* variant located in an IncX4 plasmid type. Based on the different replicons identified by PlasmidFinder, it is of note the high plasmid diversity found within these isolates. In a recent study, we investigated the characteristics of colistin-resistant *E. coli* clones successfully spread in swine in Spain. We found high variability in the location of *mcr-1.1* genes, although they were located mainly on plasmids of the IncHI2 and IncX4 types (six and four of the 12 *mcr-1.1* plasmid-located genes, respectively); however, *mcr-1.1* also appeared integrated in the chromosome of four genomes (García-Meniño et al., 2019).

We also recovered 28 ESBL-producing *K. pneumoniae* from 27 meat samples (mainly from turkey). *K. pneumoniae* is a major cause of nosocomial infections worldwide, capable to persist in a wide range of reservoirs including health care settings, retail meat, livestock and wastewater (Holt et al., 2015; Ludden et al., 2020). A recent study explored the genetic relatedness of *K. pneumoniae* isolated from the same and different reservoirs within a defined geographic region of England. The authors found few STs shared between the different sources, and the WGS-based analysis showed no evidence for livestock as a source of *K. pneumoniae* infecting humans (Ludden et al., 2020). In our collection, at least eight of the 11 STs identified were previously reported within human clinic isolates: ST15, ST45, ST111, ST147, ST307, ST627, ST966 and ST1086 (Hu et al., 2013; uz Zaman et al., 2014; Holt et al., 2015; Moradigaravand et al., 2017; Esposito et al., 2018). Since *K. pneumoniae* is an opportunistic pathogen, the main concern here would be the high rates of resistance to CTX, CIP, SXT, DOX and TGC (more than 60% of isolates), together with the high prevalence of $bla_{\text{CTX-M-15}}$ (13 isolates from 12 meat samples). In contrast, CTX-M-15 producing *E. coli* was recovered only from five samples, and two of them with co-occurrence of *K. pneumoniae* SHV-28, CTX-M-15 isolates.

Our results show that poultry meat microbiota is a source of genetically diverse Enterobacteriaceae*,* resistant to relevant antimicrobials and potentially pathogenic for humans, including hybrid pathotypes of *E. coli,* high-risk clonal groups of *E. coli* associated with human extraintestinal and / or uropathogenic pathologies, as well as *K.*

pneumoniae clonal groups of clinical interest. Given this scenario, antibiotic pressure reduction in poultry as well as surveillance of bacterial evolution is a public health priority. It would be highly recommended the implementation of a systematic AMR and ExPEC monitoring of food at retail as a follow-up tool "from the farm to the table" under the One Health strategy.

5.2. LABORATORY WORKFLOW FOR THE COMPREHENSIVE ANALYSIS IN FOOD OF AMR AND PATHOGENIC *E. COLI*, INCLUDING EXPEC ISOLATES

We aimed to develop a standardized protocol to assess exposure risk via food to drug-resistance genes and *E. coli* strains potentially pathogenic to humans. To the best of our knowledge, this would be the first study that reports a comprehensive typing of the *E. coli* isolates per food sample, which, on the other side, helped us to show the relevance of our proposal. In previous studies, we had observed the genetic similarity between isolates of certain ExPEC clonal groups recovered from poultry and human pathologies (Mora et al., 2009b, 2010, 2013). We had also demonstrated close genomic relatedness between isolates of a hybrid MDR aEPEC/ExPEC O153:H10-A-ST10 (CH11-54) from different sources, including avian farm, chicken meat and human diarrheagenic samples (Díaz-Jiménez et al., 2020b). We had found, in another study, a short distance of less than 55 SNPs on the core genome comparison between a human and an avian isolates of ST131 subclade B3 (Flament-Simon et al., 2020c). Other authors also investigated the genomic overlap between APEC and human ExPEC of the specific ST95, and found that certain ExPEC clones may indeed have the potential to cause infection in both poultry and humans (Jørgensen et al., 2019). For those evidences, and in agreement with Riley (Riley, 2020), we claim the need of looking at ExPEC genotypes to elucidate their role as extraintestinal food-borne pathogen.

The selective media, genetic targets and virulence traits of the protocol proposed here are based on results from previous studies. In relation to InPEC targets, we included clinically important *E. coli* for humans, and potentially prevalent in poultry meat. Thus, the analysis of all InPEC pathotypes in 200 poultry samples showed that none of the 200 meat samples was positive for EIEC or ETEC (Herrera, 2015). Nor were these pathotypes relevant within the diarrheagenic stools of patients of our Health Area (Mora et al., 2011b). Also, in the study of Herrera (2015), we had isolated ESBL-producing *E. coli* in 45.5% of the samples by means of ML and MLST. Subsequently, we proved that the CHROMID® ESBL medium is essential for the rapid and accurate recovery of ESBL-producing isolates (Díaz-Jiménez et al., 2020a). We performed here the selective characterization of ESBL-producing *E. coli as* indicator of drug-resistance gene exposure via food, due to being by far the most prevalent species isolated in CHROMID® ESBL (77%). Taken into account the presence of other ESBL-producers, the global rate of positive samples would be 82% (Díaz-Jiménez et al., 2020a). To assess exposure risk to ExPEC, we used the virulence traits which are statistically associated with the pathogenic potential of causing extraintestinal infections, conforming the ExPEC status (Johnson et al., 2003c); and then, those specifically linked to uropathogenic isolates, conforming the UPEC status (Spurbeck et al., 2012). The duplex PCR based on *iutA* and *KpsM II* genes on ML and MLST was essential for the accurate screening of the isolates with ExPEC status, as well as for the recovery of those with UPEC status since most of the latter also satisfies the ExPEC status (but not the other way around). As a result, we found worrying prevalence rates of positivity for the ExPEC and the UPEC status (78% and 53%, respectively). There are few comparable data available. Two studies on AMR and ExPEC

in retail foods performed in Minneapolis (1999-2000 and 2001-2003), found a prevalence of 35.7% and 46% of ExPEC contamination in poultry meat, respectively (Johnson et al., 2005a, 2005b). The media used here, ML and MSTC, inoculated with the MacConkey Lactose broth (growth for 18-24 h at 37 ºC), together with the specific PCR on confluents and pools of colonies, probably explains the significant differences with the US findings. In those, the virulence traits associated to ExPEC status were investigated on a selection of colonies obtained from a non-specific protocol (Johnson et al., 2005a, 2005b).

The finding here of aEPEC/ExPEC O153:H10-A-ST10 (CH11-54) *eae*-beta1 and similar hybrids in 19% of the meat sampled, reinforces the role of poultry meat in their maintenance and transmission. The prevalence and implication of hybrid pathotypes of *E. coli* in food and infections are probably underestimated since there is no systematic search of them. Recently, we described the hybrid MDR aEPEC/ExPEC of the clonal group O153:H10-A-ST10 (CH11-54) found within different surveillance studies (2005-2015), and the close genomic relatedness between isolates of human and animal origin belonging to it. This hybrid has been circulating in our region within different hosts, including wildlife, and seems implicated in human diarrhea via meat transmission and in the spreading of ESBL genes. Furthermore, we found genomic evidence of a related hybrid in at least one other country (Díaz-Jiménez et al., 2020b). Curiously, Flament-Simon *et al.* (Flament-Simon et al., 2020b) detected a hybrid EAEC/ ExPEC isolate O153:HNT-A-ST10 (CH11-54) among 96 *E. coli* implicated in UTIs and other extraintestinal human infections in the Hospital of Beaujon (Clichy, Paris) in 2016. Lindstedt *et al.* (Lindstedt et al., 2018) reported that a high frequency (> 93%) of routinely submitted faecal *E. coli* isolates from Norwegian hospitals (2012-2013), previously characterized as DEC, harbored ExPEC virulence factors. In view of our and other author´s findings, we believe that hybrid *E. coli* isolates should be monitored as a pre-warning of altered virulence capabilities.

In addition to O153:H10-A-ST10 (CH11-54), other human-associated clonal groups characterized in our own Health Area (Flament-Simon et al., 2020a, 2020b) were determined in 73% of our meat samples (as detailed in Table 24). What is more, around 25% of the meat samples showed co-occurrence of two or more different human associated ExPEC clones. To highlight, the concomitant presence in four meat samples of isolates belonging to the pandemic clonal group O25b:H4-B2-ST131 (subclones CH40-22 and CH40-neg), together with others such as ST648-F (CH4-58); or a turkey meat sample (T40) with the co-occurrence of the human-associated ExPEC clones ONT:H9-A-ST744 (CH11-54), O153:H34-F-ST354 (CH88-58), ST141-B2 (CH52-14), together with *mcr-1.1*-positive ST140-B2 isolates.

Within the 323 isolates analyzed in this study, we found representatives of the eight phylogroups of *E. coli* and of the *Escherichia* clade I, being the phylogroup A the most prevalent (32.5%), followed by phylogroups B1 and B2 (around 17.5% each) and phylogroups E and F (around 10.5% each). This would be a close picture of the *E. coli* population present in poultry farming and meat products, based on the comprehensive method performed here. Previous data showed that if we only take a representative *E. coli* recovered from ML into consideration, the phylogroups A and B1 would account for around 30% each, and B2 for 6%; while considering only ESBL-producing *E. coli* , the figures would be 40.1%, 29.2% and 2.2%, respectively (Díaz-Jiménez et al., 2020a). Similar distribution to the latter was observed within 84 ESBL-producing *E. coli*

recovered from 52 avian farms in our region (39.3% A, 33.3% B1, 3.5% B2) (García et al., 2018).

It is outstanding here, the high prevalence of meat samples with carriage of *E. coli exhibiting* the UPEC status (53%). The 83 isolates recovered from positive samples belonged to phylogroups B2, F and G (68.7%, 25.3% and 6%, respectively). Within the 22 STs established for the 83 meat isolates, we found some of the most prevalent in UPEC human collections, such as ST95-B2, ST131-B2 and ST141-B2 (Flament-Simon et al., 2020b). In concordance with the referenced study, we observed that the 22 isolates belonging to STs 95, 131 or 141 of our study conformed to the UPEC status. The relevant presence of isolates belonging to phylogroups F and G within poultry meat was mostly due to the clones ST648-F (CH4-58), ST1485-F (CH231-58) and ST117-G (CH45-97), which were also in the human clinic collection, but especially within the ESBL-producing *E. coli* (Flament-Simon et al., 2020a). Isolates belonging to the phylogroup F seems to be of particular significance as they have been reported as extraintestinal pathogens of companion animals, food-producing animals and humans. Further, specific F lineages such as CC648 or CC354 are resistant to fluoroquinolones (FQ) and / or extended-spectrum cephalosporins, and are increasingly associated with extraintestinal pathologies (Vangchhia et al., 2016; Johnson et al., 2017a; Abreu-Salinas et al., 2020). On the other hand, the phylogroup G has been recently defined as a group intermediate between the F and B2 phylogroups. CC117 is its most prevalent G lineage, whose isolates commonly possess many traits associated with extraintestinal virulence and exhibit multidrug resistance. Epidemiologic data suggest that CC117 is a poultry-associated lineage that appears also established in humans and cause extraintestinal diseases (Clermont et al., 2019). In the present study, we recovered nine ST117 isolates, all of them MDR, seven were ESBL producers (three CTX-M-1 and four SHV-12) and five were positive for the UPEC status.

We also recovered in this study eight isolates belonging to *Escherichia* clade I (ST770, 7 isolates; ST4994, 1 isolate). The eight isolates exhibited the ExPEC status and five were ESBL producers (2 CTX-M-9 and 3 SHV-12). Although ST770 *Escherichia* clade I is infrequently reported, it has been associated with *bla*$_{\text{CTX-M-1}}$ carriage in poultry in the Netherlands and Switzerland (Dierikx et al., 2013; Vogt et al., 2014). It has been also associated with pAmpC production, specifically CMY-2, isolated from rooks wintering in Czechia and from broilers in Sweden (Börjesson et al., 2013; Jamborova et al., 2015). Recently, we recovered *bla*$_{\text{CTX-M-14}}$-carrying ST770 isolates from five healthy dogs of our region (Abreu-Salinas et al., 2020). But importantly, ST770 isolates have been also found implicated in UTI cases: in a dog in Argentina by an *mcr*-1 and *bla*$_{\text{CTX-M-2}}$ isolate, and in a patient in Spain (Valverde et al., 2009; Rumi et al., 2019).

Globally, we found significant differences regarding meat origin. Thus, turkey meat showed worse microbiological quality (56% of turkey samples with *E. coli* counts > 50 cfu/g *vs* 30% of chicken), higher rates of multidrug resistance and higher rates of *mcr*-carriage. These differences are probably associated with a longer fattening period and so, with a longer exposition to antibiotics. There are also different reports suggesting that poultry production systems alternative to the conventional broiler production are associated with reduced frequency of antibiotic-resistant *E. coli* among the commensal gut microbiota, posing a lower risk to the environment and the consumer (Davis et al., 2018; Pesciaroli et al., 2020). Davis *et al.* (2018) found that the resistance prevalence

varied by meat type and was higher among *E. coli* isolates from turkey for most antibiotics tested compared to chicken meat.

The finding that more than 80% of the poultry meat samples posed ≥ 3 risks including resistance genes, virulence traits, and human-associated pathogenic clones of *E. coli* means that consumers are highly exposed to those threats. To which extend poultry participates in the human microbiota composition and extraintestinal pathologies such as MDR UTIs needs deep elucidation. But first it is necessary the implementation of a systematic AMR surveillance in food, together with the monitoring of ExPEC and DEC, which would enable effective food safety interventions under both "farm to fork strategy" and One Health perspective. Based on our observations, we propose an optimized workflow combination. The microbiological method (pre-enrichment, enrichment in ML broth, and inoculation onto ML/MSTC/CHROMID® ESBL), followed by the screening of six virulence/AMR traits, and including a duplex PCR for the screening of ExPEC, would estimate the greatest risk for consumers.

5.3. Genetic and genomic relatedness of the hybrid aEPEC/ExPEC pathotype O153:H10-A-ST10 *eae*-beta1

The recovery, over the time, of *eae*-positive isolates of serotype O153:H10 from different sources and its association with ESBL enzymes triggered this investigation. From independent studies on ESBLs, we found that O153 aEPEC represented 5.5% of the ESBL-producing *E. coli* recovered from chicken meat (2009-2010), 7.7% of pork meat (2011-2012), 5.5% 20% of beef meat (2011-2012), 1% of poultry farm environment (2010-2012) and 1% of wildlife feces (2014-2015) in our region (Díaz-Jiménez et al., 2017). Besides, we had detected 23 (0,24%) O153 aEPEC as the only pathogen within 9,523 stools of epidemiologically unrelated patients (2006-2012), in the routine testing of human diarrheagenic samples. From those 23, 14 (0.15%) were O153:H10 *eae*-beta1 fim_{AvMT78}, and five of them *bla*CTX-M-32 producers (Table 38, Table 39, Figure 12). By conventional typing, all animal and human isolates were assigned to the clonal group O153:H10-A-ST10 (CH11-54), conforming a hybrid aEPEC/ExPEC pathotype. The symptomatology reported in humans was mainly mild diarrhoea, but there were also some cases of acute/haemorrhagic gastroenteritis (Table 39). Epidemiological studies have indicated that aEPEC are emerging enteropathogens, implicated in human diarrhoea, with higher prevalence than tEPEC in both developed and developing countries (Hu and Torres, 2015). aEPEC are present in both healthy and diseased animals and humans (Blanco et al., 2006; Alonso et al., 2017; Mora et al., 2018), are phylogenetically heterogeneous and carry virulence factors of other diarrheagenic *E. coli* more often than tEPEC strains (Hernandes et al., 2009; Hu and Torres, 2015; Xu et al., 2017). However, the main feature of the EPEC diarrheagenic group is the ability to induce A/E lesions on intestinal epithelium encoded in the chromosomal pathogenicity island (LEE). Within more than 30 intimin types and subtypes based on the polymorphism of *eae*, the subtype determined here (β-1) is first or second in prevalence within different studies on isolates from humans with diarrhoea in Spain (Blanco et al., 2006), Australia (Robins-Browne et al., 2004), Brasil (Abe et al., 2009; Vieira et al., 2016), Peru (Contreras et al., 2010) or China (Xu et al., 2016).

It is of note that we have detected this clonal group in subsequent and current studies on meat sampled in supermarkets of our city. In fact, we recovered aEPEC/ExPEC from

15 out of 100 poultry meat samples (2016-2017); from those, five were carriers of isolates belonging to the clonal group O153:H10-A-ST10, being one CTX-M-32 carrier (unpublished data). Recently, Zhang *et al.* (Zhang et al., 2018) reported a 2.75% prevalence of aEPEC in retail foods at markets in the People's Republic of China, being the β-1 intimin and the ST10 the second intimin and ST most prevalent within their isolates. According to the authors, the presence of virulent and MDR aEPEC in retail foods poses a potential threat to consumers.

Since the occurrence of the major outbreak of HUS in Europe caused in 2011 by an EAEC/STEC O104:H4, other hybrid pathotypes have been recognized, and new are expected, either by novel assemblies of *E. coli* virulence determinants or through acquisition of new virulence genes from other bacterial species (Robins-Browne et al., 2016). In Norway, Lindstedt *et al.* (Lindstedt et al., 2018), expressed their concern regarding the detection of *E. coli* from human faecal content with a combination of intestinal and ExPEC virulence genes (InPEC/ExPEC) in a high frequency (64.3%). Several other studies have also identified STEC- and ETEC-associated virulence genes coexisting in *E. coli* isolates from humans, animals or environmental origin (Nyholm et al., 2015; Michelacci et al., 2018). But probably one of the most outstanding is the EPEC/STEC O80:H2-ST301, emerged in France over the last few years and diffused within Europe, associated with invasive infections, which combines intestinal VFs (*stx2d*, *eae*-xi and *ehxA* genes) and extraintestinal genes characteristic of the plasmid pS88 (Cointe et al., 2018, 2020). To highlight in this O80 clone, the location of MDR and pS88 genes in the same plasmid; and in addition to this plasmid, another two (a carrier of *ehxA* gene and a cryptic one) were described within the isolates (Cointe et al., 2018, 2020). The clonal group described here also poses the threat of being MDR and characteristically associated with ESBL type CTX-M-32. CTX-M-32 enzyme is derived from CTX-M-1 by a single amino acid replacement, being probably an ancestor among CTX-M-1 and CTX-M-15 (Cartelle et al., 2004). The $bla_{\text{CTX-M-32}}$ gene was first described in 2004 in an *Escherichia coli* isolate in our Health Area (A Coruña, northwest Spain) (Cartelle et al., 2004). Furthermore, it was described in three human isolates O25b:H4-ST131 *ibeA*-positive of our region, as early as in 2008 (Mora et al., 2010). Of the 2,427 *E. coli* bloodstream isolates recovered in the hospital of our city (HULA) in the period 2000-2011, 96 were positive for ESBL production, from which 4.2% were CTX-M-32 and 4.2% SHV-12 (Mamani et al., 2019). The same prevalence was observed in this hospital in 2015 (unpublished data).

The *in silico* analysis of 17 representative genomes O153:H10-A-ST10 corroborated the main traits determined by conventional typing. In a recent study, we had proved the good correlation and usefulness of SerotypeFinder or EnteroBase predictions (Mora et al., 2018; García-Meniño et al., 2019). Here, only the serotype of two genomes could not be predicted *in silico*, probably due to the limitation of the assembly based on Illumina short reads (Wick et al., 2017). MLST, CHTyper from CGE and EnteroBase also confirmed conventional results. Like in the previous study, we found that VirulenceFinder properly identifies *E. coli* pathotypes (hybrid in this case), although based on different traits for the ExPEC pathotype. Thus, this clonal group O153:H10-A-ST10 typically carries the *locus* that codify a *fimA* variant MT78 of type 1 fimbriae (Marc and Dho-Moulin, 1996) and the *traT* gene for an outer membrane protein implicated in serum survival (Johnson and Stell, 2000). Both VFs are not included in the VirulenceFinder scheme, and so they were not predicted. On the contrary, CGE tool identified in all genomes the increased serum survival gene *iss*, recognized for its role in

ExPEC virulence (Johnson et al., 2008), which was not determined by PCR. This is because CGE database predicts 14 variants of the *iss* gene (Joensen et al., 2014), including the one described in *E. coli* IAI1 (CU928160), and harbored by the O153:H10-A-ST10 genomes. Our specific PCR detects the plasmid-borne *iss* allele (designated type 1), which is highly prevalent among APEC and NMEC isolates but not among UPEC isolates (Johnson et al., 2008). The phenotypic AMR determined *in vitro* correlated with the results based on ResFinder databases, with the exception of $bla_{\text{CTX-M-32}}$ not predicted in two genomes but solved by conventional sequencing. Based on this and previous studies (de Toro et al., 2017a; García-Meniño et al., 2019), we consider both conventional and genomic-based analysis complementary for a better understanding and characterization of emerging isolates.

An interesting trait of our isolates was the concomitant presence of IncF (F2:A-:B-), IncI1 (ST unknown) and IncX1, together with non-conjugative Col156-like plasmids. Although carriage of plasmids means a fitness cost on the hosts (San Millan and MacLean, 2017), different studies support the hypothesis that interference between conjugative plasmids may reduce fitness costs by decreasing the efficiency of transfer. However, the mechanisms of such inhibitory systems need further investigation (Dionisio et al., 2019). On the other hand, small plasmids was shown to increase its stability in cells containing big plasmids (San Millan and MacLean, 2017).

Another objective in this study was to know if this was a restricted genetic lineage. For this purpose, we searched related genomes uploaded in EnteroBase based on the HierCC Cluster ID. As a result, we found a hybrid aEPEC/ExPEC pathotype A-ST10 *eae*-beta1 within its database associated to five human, one avian, and one unknown isolates (Table 37). Of note, the two human isolates (Code Name: 853984 and 866428) from United Kingdom, which clustered with the 17 Spanish genomes in the HC100 HierCC group (37600) (Table 37, Figure 17). The *in silico* analysis of these two genomes showed they belonged to the clonal group O153:H10-A-ST10 CH11-54 *eae*-beta1, were MDR carried similar virulence traits (conforming hybrid aEPEC/ExPEC pathotype), and plasmid combination: IncF (F2:A-:B-), IncX1, Col156-like (Table 40). To highlight that six of the seven genomes were carriers of IncF (F2:A-:B-) and Col156-like plasmids (Table 40). As above suggested, further investigation on the interplay between these plasmids and other MGEs affecting their transmission and persistence, as well as their role in the maintenance and acquisition of resistance genes is necessary.

In summary, our results demonstrate that a hybrid MDR aEPEC/ExPEC belonging to the clonal group O153:H10-A-ST10 (et CH11-54) *eae*-beta1 is circulating in our region within different hosts, including wildlife. It seems implicated in human diarrhoea via food (meat) transmission, and in the spreading of ESBL genes (mainly of CTX-M-32 type). The concomitant presence of IncF (F2:A-:B-), IncI1 (STunknown) and IncX1, together with non-conjugative Col156-like plasmids might be implicated in the successful persistence of this hybrid pathotype. We found genomic evidence of a related hybrid aEPEC/ExPEC in at least one other country.

5.4. FINAL REMARKS

COVID-19 has been threatening the world for almost two years now. Fortunately, the care of many researchers has allowed the development of precise combat weapons in the form of vaccines in record time. But this pandemic will leave us many absences, and many consequences, such as those derived from the temporary eclipse of the greatest health challenge: the AMR. So far 3.4 million lives have been lost due to COVID-19, but the death figures derived from AMR could be higher unless urgent action is not taken (Mora, 2021). Our group early warned on the impact of secondary infections by MDR bacteria in patients infected by COVID-19 (García-Meniño et al., 2021a), in agreement with the reports of increasing use of antibiotics in hospitals (Gonzalez-Zorn, 2021). We hope that the work showed in this thesis has also contributed to raising awareness about this global priority.

Animals, like people, may carry resistant bacteria in their guts. These bacteria can get in food, contaminating meat or other animal products when animals are slaughtered and processed for food, or through animal waste contaminating soil, water, or fertilizer in contact with fruits and vegetables. People are exposed to resistant bacteria when handling or consuming contaminated vegetables and animal-derived food. The danger is not only because food-borne infections caused by MDR bacteria have more serious health consequences than infections caused by sensitive bacteria; the danger is also derived from these bacteria getting in contact with the host's normal microbiota, and sharing resistance genes ((CDC, 2020) last access: 08/06/2021)

Traditionally, only InPEC have been accepted as food-borne pathogens (Kaper et al., 2004; Kai et al., 2010). Recently, Riley (Riley, 2020) indicated that a surveillance system of ExPEC genotypes causing extraintestinal infections, which does not currently exist, could provide traceback investigations to elucidate their role as new extraintestinal food-borne pathogens. Apart from InPEC and ExPEC, new hybrid pathotypes have been increasingly reported since it happened the major outbreak of HUS in Europe in 2011 by an EAEC/STEC O104:H4 (Mora et al., 2011a; Cointe et al., 2020). In fact, a comprehensive assessment of AMR in animals and the food chain is essential to reduce the burden of antimicrobial resistance in humans. However, food surveillance is considered commercially sensitive and so, the information derived from it is generally incomplete (Tacconelli et al., 2018).

On this base, we analyzed 100 retail poultry meat directly acquired at points of sale with the idea that the final product provides data on what is happening throughout the entire food chain "from farm to fork". Besides, we considered as "risk" strain that with the capacity to develop a serious extraintestinal infection in humans, either due to its virulence potential and / or due to its antibiotic resistance.

As a result, we have designed a lab workflow for a comprehensive microbiological risk assessment, including a PCR for the screening of ExPEC (Díaz-Jiménez et al., 2021). For the first time, we evaluated consumer exposure via poultry meat to Enterobacteriaceae with capacity to develop severe extraintestinal infections by either bacterial virulence and / or antibiotic resistance traits, and we showed the high level of consumer exposure to MDR bacteria *via* poultry meat. Our findings indicate that poultry meat is a rich source of *E. coli* (phylogroups A to G) and *Escherichia* clade I, including (Díaz-Jiménez et al., 2020a, 2021):

- *E. coli* CC10-A (CH11–54) isolates carrying *mcr*-1.1-bearing IncX4 plasmids in meat.

- Presence of high-risk lineages of *E. coli,* including the pandemic ST131-*H22*, in more than 70% of the meat samples.

- Clinical relevant ESBL-producing *K. pneumoniae* recovered from 27% of meat samples

- Detection of a hybrid pathotype aEPEC / ExPEC CC10-A (*eae*-beta1).

The latter confirms that the clonal group O153:H10-A-ST10 (CH11-54) is circulating in our region within different hosts, including poultry. It seems implicated in human diarrhea via meat transmission, and in the spreading of ESBL genes (mainly of CTX-M-32 type). The core genome investigation based on the cgMLST scheme from EnteroBase proved close relatedness between isolates of human and animal origin. We also found genomic evidence of a related hybrid aEPEC/ExPEC in at least one other country (Díaz-Jiménez et al., 2020b).

As a general conclusion, and given this scenario, antibiotic pressure reduction in poultry farming as well as surveillance of bacterial evolution is a public health priority. It would be highly recommended the implementation of a systematic AMR and ExPEC monitoring of food at retail as a follow-up tool “from farm to fork” under the One Health strategy.

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6. CONCLUSIONS

1. Our results determined that poultry meat microbiota is a source of genetically diverse Enterobacteriaceae, resistant to relevant antimicrobials (categories A and B of EMA) and potentially pathogenic for humans, including hybrid pathotypes of *E. coli*, high-risk clonal groups of *E. coli* associated with human extraintestinal and / or uropathogenic pathologies, as well as *K. pneumoniae* clonal groups of clinical interest.

2. Our results would indicate that the industrial production system for turkey meat seems to exert greater selection pressure of antibiotic resistant strains compared to chicken, which is reflected in significant higher rates of *mcr*-positive *E. coli* and MDR isolates, including ESBL-producing *K. pneumoniae*, in turkey meat.

3. The protocols I and II, based on MacConkey Lactose and MacConkey Sorbitol with telurite and cefixime agar incubated at 37 ºC, are the most effective for the recovery of isolates satisfying the ExPEC and UPEC status, as well as the *rbfO25b*-positive isolates associated with the clonal group STl31.

4. The protocol V (CHROMID® ESBL agar plates 37 ºC) is key for the recovery of ESBL or pAmpC-producing Enterobacteriaceae.

5. The duplex PCR based on *iutA* and *KpsM II* genes on MacConkey Lactose and MacConkey Sorbitol with telurite and cefixime agar is essential for the accurate screening of the isolates conforming ExPEC status, as well as for the recovery of those with UPEC status.

6. The microbiological method proposed here (pre-enrichment, enrichment in ML broth, and inoculation onto MacConkey Lactose broth, and inoculation onto MacConkey Lactose agar / MacConkey Sorbitol with telurite and cefixime agar / CHROMID® ESBL), followed by the screening of six virulence/AMR traits (ExPEC status, UPEC status, ESBL/pAmpC producer, *mcr-1* carrier, MDR, rfbO25b), would help to elucidate the role of ExPEC as new extraintestinal food-borne pathogens.

7. Our results prove that a hybrid MDR aEPEC/ExPEC belonging to the clonal group O153:H10-A-ST10 (CH11-54) *eae*-beta1 is circulating in our region within different hosts, including wildlife. It seems implicated in human diarrhea *via* food (meat) transmission, and in the spreading of ESBL genes (mainly of CTX-M-32 type). The concomitant presence of IncF (F2:A-:B-), IncI1 and IncX1, together with non-conjugative Col156-like plasmids might be implicated in the successful persistence of this hybrid pathotype.

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7. ANNEX

7.1. General primers compilation

Table 28. Primers used for the detection and / or sequencing of *E. coli* and *Klebsiella pneumoniae*

Target	Primers	Nucleotide sequence (5´- 3´)	Size (bp)	Reference
bla$_{CTX-M}$	CTX-C3	ATGTGCAGCACCAGTAAAGTGATG	542	(Mora et al., 2013)
	CTX-C4	ACCGCGATATCGTTGGTGGTGCC		
bla$_{CTX-M}$ group1	M13U	GGTTAAAAAATCACTGCGTC	863	(Saladin et al., 2002)
	M13L	TTGGTGACGATTTTAGCCGC		
bla$_{CTX-M}$ group9	[a]CTX-M9-F	GTGACAAAGAGAGTGCAACGG	856	(Simarro et al., 2000)
	[a]CTX-M9-R	ATGATTCTCGCCGCTGAAGCC		
bla$_{CTX-M}$ group9	[b]CTX-M9-14-14B-24F	GAATACTGATGTAACACGGA	998	(García-Meniño et al., 2018)
	[b]CTX-M9-R	AGCTGAAGATGTATATCAAG		
bla$_{CTX-M}$ group9	[b]CTX-M9-14-14B-24F	GAATACTGATGTAACACGGA	989	(García-Meniño et al., 2018)
	[b]CTX-M14-24-R	CTGCGTTGTCGGGAAGATACG		
bla$_{CTX-M}$ group9	[b]CTX-M9-14B-F	CCTATACCCGAGGCGCGACAG	1059	(García-Meniño et al., 2018)
	[b]CTX-M9-R	AGCTGAAGATGTATATCAAG		
bla$_{CTX-M}$ group9	[b]CTX-M14-24-F	CTAAATTCTTCGTGAAATAGTG	1049	(García-Meniño et al., 2018)
	[b]CTX-M14-24-R	CTGCGTTGTCGGGAAGATACG		
bla$_{SHV}$	SHV-F2	TTGTCGCTTCTTTACTCGCC	879	(Mora et al., 2013)
	SHV-R2	CCCGGCGATTTGCTGATTTCGC		
bla$_{SHV}$	[b]SHV-1	GGGTTATTCTTATTTGTCGC	930	(Rasheed et al., 1997)
	[b]SHV-2	TTAGCGTTGCCAGTGCTC		
bla$_{TEM}$	[a]TEM-1-F	ATGAGTATTCAACATTTCCG	868	(Rasheed et al., 1997)
	[a]TEM-1-R	CTGACAGTTACCAATGCTTA		
LAT-1 a LAT-4, CMY-2 a CMY-7, BIL-1	CITMF	TGGCCAGAACTGACAGGCAAA	462	(Pérez-Pérez and Hanson, 2002)
	CITMR	TTTCTCCTGAACGTGGCTGGC		
CMY-2	[b]CMY-2F	AACACACTGATTGCGTCTGAC	1226	(Pérez-Pérez and Hanson, 2002)
	[b]CMY-2R	CTGGGCCTCATCGTCAGTTA		
mcr-1	CLR5-F	CGGTCAGTCCGTTTGTTC	309	(Liu et al., 2016)
	CLR5-R	CTTGGTCGGTCTGTAGGG		
mcr-2	mcr-2 IF	TGTTGCTTGTGCCGATTGGA	567	(Xavier et al., 2016)
	mcr-2 IR	AGATGGTATTGTTGGTTGCTG		
mcr-3	MCR3-F	TTG GCACTGTATTTTGCATTT	542	(Yin et al., 2017)
	MCR3-R	TTAACGAAATTGGCTGGAACA		
mcr-4	mcr-4 FW	ATTGGGATAGTCGCCTTTTT	487	(Carattoli et al., 2017)
	mcr-4 RV	TTACAGCCAGAATCATTATCA		
mcr-5	MCR5_FW	ATGCGGTTGTCTGCATTTATC	1644	(Borowiak et al., 2017)
	MCR5_RV	TCATTGTGGTTGTCCTTTTCTG		

Target	Primers	Nucleotide sequence (5´- 3´)	Size (bp)	Reference
mcr-1	[b]mcrS1-F	GGGATTGCGCAATGATTGC	548	(García-Meniño et al., 2018)
	[b]mcrS1-R	CACCCAAACCAATGATACG		
Target	**Primers**	**Nucleotide sequence (5´- 3´)**	**Size (bp)**	**Reference**
Quadruplex phylogroup method of Clermont *et al.*, 2013[c]				
chuA	chuA.1b	ATGGTACCGGACGAACCAAC	288	(Clermont et al., 2013)
	chuA.2	TGCCGCCAGTACCAAAGACA		(Clermont et al., 2000)
yjaA	yjaA.1b	CAAACGTGAAGTGTCAGGAG	211	(Clermont et al., 2013)
	yjaA.2b	AATGCGTTCCTCAACCTGTG		
TspE4C2	TspE4C2.1b	CACTATTCGTAAGGTCATCC	152	(Clermont et al., 2013)
	TspE4C2.2b	AGTTTATCGCTGCGGGTCGC		
arpA	AceK.f	AACGCTATTCGCCAGCTTGC	400	(Clermont et al., 2013)
	ArpA1.r	TCTCCCCATACCGTACGCTA		
trpAgpC	trpAgpC.1	AGTTTTATGCCCAGTGCGAG	219	(Lescat et al., 2013)
	trpAgpC.2	TCTGCGCCGGTCACGCCC		
arpA (E)	ArpAgpE.f	GATTCCATCTTGTCAAAATATGCC	301	(Lescat et al., 2013)
	ArpAgpE.r	GAAAAGAAAAAGAATTCCCAAGAG		
trpA	trpBA.f	CGGCGATAAAGACATCTTCAC	489	(Clermont et al., 2008)
	trpBA.r	GCAACGCGGCCTGGCGGAAG		
Target	**Primers**	**Nucleotide sequence (5´- 3´)**	**Size (bp)**	**Reference**
$fliC_{H1}$	H1-F2	TATCCGGTCAGACCCAGTTC	828	(García-Meniño et al., 2018)
	H1-R2	TTGCGGATGTATCACCGTTA		
$fliC_{H2}$	H2-F	AACGACGGCGAAACAATTAC	828	(Alonso et al., 2017)
	H2-R	AGAACGCAACGAGTCAACCT		
$fliC_{H4}$	H4-F	GCAGCGTATTCGTGAACTGA	713	(Mora et al., 2011b)
	H4-R	GCTGGATAATCTGCGCTTTC		
$fliC_{H7}$	H7-F	GCGCTGTCGAGTTCTATCGAGC	625	(Gannon et al., 1997)
	H7-R	CAACGGTGACTTTATCGCCATTCC		
$fliC_{H8}$	H8-F	TAACAGCGCAAAAGACGATG	393	(Mora et al., 2012)
	H8-R	CCGAGAGTTTTCGCATCAAT		(García-Meniño et al., 2018)
$fliC_{H9}$	H28-F	ACGAAATCAAATCCCGTCTG	649	(Mora et al., 2012)
	H9-R	GCGGTATCGTTACCTGCATT		(García-Meniño et al., 2018)
$fliC_{H10}$	H10-F	AGCAAGTGGCAGTAGGTGCT	624	(Alonso et al., 2017)
	H10-R	GCTGGATAATCTGCGCTTTC		
$fliC_{H11}$	H11-F	ACTGTTAACGTAGATAGC	248	(Durso et al., 2005)
	H11-R	TCAATTTCTGCAGAATATAC		
$fliC_{H18}$	H18-F1	TTCTGACCTGGACTCCATCC	827	(Mora et al., 2018)
	H18-R1	CGTTAGCAAACGTTGAAGCA		
$fliC_{H21}$	H21-F	GGCGATTGCTAACCGTTTTA	549-556	

Target	Primers	Nucleotide sequence (5´- 3´)	Size (bp)	Reference
	H21-R3	CGTAAGTGAACCATCCGCAG		(Mora et al., 2012)
fliC$_{H25}$	H25-F	ATGAAATTGACCGCGTATCC	212	(Alonso et al., 2017)
	H25-R	TTGCGGGATAGATGTGATAGC		
fliC$_{H28}$	H28-F	ACGAAATCAAATCCCGTCTG	856	(Mora et al., 2012)
	H28-R	GCCGATTGAAGAGACTCAGC		
rfbO25b	rfb. 1bis.f	ATACCGACGACGCCGATCTG	300	(Clermont et al., 2008)
	rfbO25b.r	TGCTATTCATTATGCGCAGC		
Primers used for amplification and sequencing in the clonotyping method[d]				
fimH	fimH-F	CACTCAGGGAACCATTCAGGCA	locus size 469	(Weissman et al., 2012)
	fimH-R	CTTATTGATAAACAAAAGTCAC		
Target	**Primers**	**Nucleotide sequence (5´- 3´)**	**Locus size (bp)**	**Reference**
Achtman seven-locus scheme for *E. coli*				
adk	adkF	ATTCTGCTTGGCGCTCCGGG	536	(Wirth et al., 2006)
	adkR	CCGTCAACTTTCGCGTATTT		
fumC	fumCF	TCACAGGTCGCCAGCGCTTC	469	
	fumCR	GTACGCAGCGAAAAAGATTC		
gyrB	gyrBF	TCGGCGACACGGATGACGGC	460	
	gyrBR	ATCAGGCCTTCACGCGCATC		
icd	icdF	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	518	
	icdR	GGACGCAGCAGGATCTGTT		
mdh	mdhF	ATGAAAGTCGCAGTCCTCGGCGCTGCTGGCGG	452	
	mdhR	TTAACGAACTCCTGCCCCAGAGCGATATCTTTCTT		
purA	purAF	CGCGCTGATGAAAGAGATGA	478	
	purAR	CATACGGTAAGCCACGCAGA		
recA	recAR1	AGCGTGAAGGTAAAACCTGTG	510	
	recAF1	ACCTTTGTAGCTGTACCACG		
Institute Pasteur MLST for *Klebsiella pneumoniae*				
rpoB	Vic3	GGCGAAATGGCWGAGAACCA	501	((Diancourt et al., 2005)
	Vic2	GAGTCTTCGAAGTTGTAACC		
gapA	gapA173	TGAAATATGACTCCACTCACGG	450	
	gapA181	CTTCAGAAGCGGCTTTGATGGCTT		
mdh	mdh130	CCCAACTCGCTTCAGGTTCAG	477	
	mdh867	CCGTTTTTCCCCAGCAGCAG		
pgi	pgi1F	GAGAAAAACCTGCCTGTACTGCTGGC	432	
	pgi1R	CGCGCCACGCTTTATAGCGGTTAAT		
phoE	phoE604.1	ACCTACCGCAACACCGACTTCTTCGG	420	
	phoE604.2	TGATCAGAACTGGTAGGTGAT		
infB	infB1F	CTCGCTGCTGGACTATATTCG	318	
	infB1R	CGCTTTCAGCTCAAGAACTTC		
tonB	tonB1F	CTTTATACCTCGGTACATCAGGTT	414	

Target	Primers	Nucleotide sequence (5´- 3´)	Size (bp)	Reference
	tonB2R	ATTCGCCGGCTGRGCRGAGAG		
For the seven targets	[a]Primer oF	GTTTTCCCAGTCACGACGTTGTA		
	[e]Primer oR	TTGTGAGCGGATAACAATTTC		

[a]Primers used for amplification and sequencing. [b]Primers used for sequencing. [c]Phylogroup assignment method of Clermont (2013). With this method, eight phylogroups are recognized based on the presence/absence of the four genetic targets *arpA*, *chuA*, *yjaA* and TspE4.C2: seven (A, B1, B2, C, D, E, F) belonging to *E. coli sensu stricto*, and the remaining one to *Escherichia* cryptic clade I. [d]Allele assignments for fimH were determined using the fimtyper database available at the Center for Genomic Epidemiology (CGE) website https://bit.ly/35jD3Qx, and the combination of *fumC* and *fimH* allele designations determined the CH "type". [e]Universal primers used for sequencing. The STs were assigned through the EnteroBase website for *E. coli* (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search), and the Institute Pasteur website (https://bigsdb.pasteur.fr/klebsiella/) for *K. pneumoniae*. Novel STs were submitted to curator to determine new designations.

Table 29. Targets and primers associated with diarrheagenic and extraintestinal pathotypes of *E. coli*.

Pathotype	Target	Primers	Nucleotide sequence (5´- 3´)	Size (bp)	Reference
STEC	*stx₁*	VT1-F	TCGCTGAATGTCATTCGCTCTGC	539	(Mora et al., 2011b)
		VT1-R	TCAGCAGTCATTACATAAGAAC		
	stx₂	VT2-F1	TTTCTTCGGTATCCTATTCCC	358	
		VT2-F2	TGTCTTCAGCATCTTATGCAG		
		VT2-R	CTGCTGTCCGTTGTCATGGAA		
STEC EPEC	*eae*	EAE-V3F	CATTGATCAGGATTTTTCTGGT	510	
		EAE-MBR	TCCAGAATAATATTGTTATTACG		
STEC EPEC	*eae*	[c]EAE-R11	TCTTCGGAGGGTTTTTTATT	1125	(Alonso et al., 2017)
		[c]EAE-FBN	CAGGTCGTCGTGTCTGCTAAAAC		
STEC EPEC	*eae*	[c]EAE-R12	CCAGACGAATATATACATATTC	1181	
		[c]EAE-FBN	CAGGTCGTCGTGTCTGCTAAAAC		
tEPEC	*bfpA*	BFP-NF1	ATGGTTTCTAAAATCATGAATAAG	262	(Bennett, 2003)
		BFP-NR1	ATTATTCCGGAATTGCAGATGTGT		(García-Meniño et al., 2018)
EAEC	*aaiC*	aaiC-F	TGGTGACTACTTTGATGGACATTGT	313	(Boisen et al., 2012)
		aaiC-F	GACACTCTCTTCTGGGGTAAACGA		
	aggR	aggR-F	GCAATCAGATTAARCAGCGATACA	426	
		aggR-R	CATTCTTGATTGCATAAGGATCTGG		
ExPEC	*fimH*	FimH-f	TGCAGAACGGATAAGCCGTGG	508	(Johnson and Stell, 2000)
		FimH-r	GCAGTCACCTGCCCTCCGGTA		
	fimAv$_{MT78}$	fimA201	TCTGGCTGATACTACACC	266	(Marc and Dho-Moulin, 1996)
		fimA215	ACTTTAGGATGAGTACTG		
	papC	Forward	GTGGCAGTATGAGTAATGACCGTTA	205	(Johnson and Manges, 2015)
		Reverse	ATATCCTTTCTGCAGGGATGCAATA		
	[a]*papAH*	papA-F	ATGGCAGTGGTGTCTTTTGGTG	720	(Johnson and Stell, 2000)
		papA-R	CGTCCCACCATACGTGCTCTTC		
	papEF	PapEF-F	GCAACAGCAACGCTGGTTGCATCAT	336	

Pathotype	Target	Primers	Nucleotide sequence (5´- 3´)	Size (bp)	Reference
		PapEF-R	AGAGAGAGCCACTCTTATACGGACA		(Yamamoto et al., 1995)
	papG I	pap-I F	TTAGCTGGATGGCACAATG	335	(Mora et al., 2013)
		pap-I R	TTGTCCATGTATCCCATTCAT		
	papG II	pap-II F	GGGCATTGCTACGGTAACCTG	545	
		pap-II R	CGCTATTAATAGACAGATCACC		
	papG III	pap-III F	CGGCAACTTTAAGCTATGTG	720	
		pap-III R	TGTACCATCTCATCGTTGTCTC		
	[a]*sfa/focDE*	sfa1	CTCCGGAGAACTGGGTGCATCTTAC	410	(Le Bouguenec et al., 1992)
		sfa2	CGGAGGAGTAATTACAAACCTGGCA		
	[a]*afa/draBC*	afa1	GCTGGGCAGCAAACTGATAACTCTC	750	
		afa2	CATCAAGCTGTTTGTTCGTCCGCCG		
	afaFM955459	AFA-O25F	GAGTCACGGCAGTCGCGGCGG	207	(Blanco et al., 2009)
		AFA-O25R	TTCACCGGCGCACAGCCATCTCC		
	cnf1	cnf1-f2	CAGGAGGTACTTAGCAGCGT	468	(Mora et al., 2013)
		cnf1-rc	TAATTTTGGGTTTGTATC		
	cdtB	cdt-s1	GAAAGTAAATGGAATATAAATGTCCG	466	(Toth et al., 2003)
		cdt-as1	AAATCACCAAGAATCATCCAGTTA		
		cdt-s2	GAAAATAAATGGAACACACATGTCCG		
		cdt-as2	AAATCTCCTGCAATCATCCAGTTA		
	sat	SatF	GCAGCTACCGCAATAGGAGGT	937	(Johnson et al., 2003a)
		SatR	CATTCAGAGTACCGGGGCCTA		
	hlyA	hly F	AACAAGGATAAGCACTGTTCTGGCT	1177	(Yamamoto et al., 1995)
		hly R	ACCATATAAGCGGTCATTCCCGTCA		
	hlyF	hlyF f	TCGTTTAGGGTGCTTACCTTCAAC	444	(Morales et al., 2004)
		hlyF r	TTTGGCGGTTTAGGCATTCC		
	iucD	Aer F	TACCGGATTGTCATATGCAGACCGT	602	(Yamamoto et al., 1995)
		Aer R	AATATCTTCCTCCAGTCCGGAGAAG		
	estA	STa-A	ATTTTTATTTCTGTATTGTCTTT	176	(Penteado et al., 2002)
		STa-B	GGATTACAACACAGTTCACAGCAGT		
	estB	Stb-F	ATCGCATTTCTTCTTGCATC	175	(Blanco et al., 1997)
		Stb-R	GGGCGCCAAAGCATGCTCC		
	eltA	LT-A-1	GGCGACAGATTATACCGTGC	696	(Schultsz et al., 1994)
		LT-A-2	CCGAATTCTGTTATATATGTC		
	ipaH	EI1	GCTGGAAAAACTCAGTGCCT	424	(Tornieporth et al., 1995)
		EI2	CCAGTCCGTAAATTCATTCT		
	aatA	pCVD432/start	CTGGCGAAAGACTGTATCAT	630	(Schmidt et al., 1995)
		pCVD432/stop	CAATGTATAGAAATCCGCTGTT		
	iroN	Ironec-F	AAGTCAAAGCAGGGGTTGCCCG	665	(Johnson et al., 2000)
		Ironec-R	GACGCCGACATTAAGACGCAG		

Pathotype	Target	Primers	Nucleotide sequence (5´- 3´)	Size (bp)	Reference
	[a]*iutA*	aer-851F	GGCTGGACATCATGGGAACTGG	301	(Johnson et al., 1997)
		aer-1152R	CGTCGGGAACGGGTAGAATCG		
	[a]*kpsM II*	KpsII f	GCGCATTTGCTGATACTGTTG	272	(Johnson and Stell, 2000)
		KpsII r	CATCCAGACGATAAGCATGAGCA		
	kpsM II-K2	KpsII f	GCGCATTTGCTGATACTGTTG	570	(Johnson and O'Bryan, 2004)
		KpsII-K2r	AGGTAGTTCAGACTCACACCT		
	kpsM II-K5	K5-f	CAGTATCAGCAATCGTTCTGTA	159	(Johnson and Stell, 2000)
		KpsII r	CATCCAGACGATAAGCATGAGCA		
	neuC (K1)	neu1	AGGTGAAAAGCCTGGTAGTGTG	676	(Moulin-Schouleur et al., 2006)
		neu2	GGTGGTACATCCCGGGATGTC		
	kpsM III	KpsIII f	TCCTCTTGCTACTATTCCCCCT	392	(Johnson and Stell, 2000)
		KpsIII r	AGGCGTATCCATCCCTCCTAAC		
	cvaC	ColV-CF	CACACACAAACGGGAGCTGTT	680	(Johnson and Stell, 2000)
		ColV-CR	CTTCCCGCAGCATAGTTCCAT		
	traT	TraT f	GGTGTGGTGCGATGAGCACAG	290	(Johnson and Stell, 2000)
		TraT r	CACGGTTCAGCCATCCCTGAG		
	ibeA	ibe10 f	AGGCAGGTGTGCGCCGCGTAC	170	(Johnson and Stell, 2000)
		ibe10 r	TGGTGCTCCGGCAAACCATGC		
	malX	MALX-F	GCATGAGCAGTGCGATACATCGC	828	(Mora et al., 2013)
		MALX-R	AGGGCTGGGAAGTGGTTTAGCC		
	usp	usp-F	ACATTCACGGCAAGCCTCAG	440	(Bauer et al., 2002)
		usp-R	AGCGAGTTCCTGGTGAAAGC		
	ompT	ompT-F	ATCTAGCCGAAGAAGGAGGC	559	(Johnson et al., 2015)
		ompT-R	CCCGGGTCATAGTGTTCATC		
	tsh	tsh03	GGTGGTGCACTGGAGTGG	640	(Dozois et al., 2000)
		tsh15	AGTCCAGCGTGATAGTGG		
	[b]*vat*	vat-F	TCAGGACACGTTCAGGCATTCAGT	1100	(Spurbeck et al., 2012)
		vat-R	GGCCAGAACATTTGCTCCCTTGTT		
	[b]*fyuA*	fyuA-F	GTAAACAATCTTCCCGCTCGGCAT	850	(Spurbeck et al., 2012)
		fyuA-R	TGACGATTAACGAACCGGAAGGGA		
	[b]*yfcV*	yfcV-F	ACATGGAGACCACGTTCACC	292	(Spurbeck et al., 2012)
		yfcV-R	GTAATCTGGAATGTGGTCAGG		
	[b]*chuA*	ChuA-F	CTGAAACCATGACCGTTACG	652	(Spurbeck et al., 2012)
		ChuA-R	TTGTAGTAACGCACTAAACC		
	uidA	uidA-F	GCGTCTGTTGACTGGCAGGTGGTGG	503	(Gómez-Duarte et al., 2010)
		uidA-R	GTTGCCCGCTTCGAAACCAATGCCT		

[a]Virulence factors (VF) screened to assess the extraintestinal pathogenic *E. coli* status (ExPEC status). [b]VF screened to assess the uropathogenic *E. coli* status (UPEC status). [c] Primers used for the *eae* typing (sequencing). Those isolates exhibiting ExPEC and /or UPEC status, were further characterized for other extraintestinal VF: $fimAv_{MT78}$, *papEF*, *papC*, *papG I*, *papG II* and *papG III*, *cnf1*, *cdtB*, *sat*, *hlyA*, *hlyF*, *iucD*, *iroN*, *kpsM II* (establishing neuC-K1, K2 and K5 variants), *kpsM III*, *cvaC*, *iss*, *traT*, *ibeA*, *malX*, *usp*, *tsh* and *ompT*.

7.2. Study 1: Chicken and turkey meat: consumer exposure to multidrug-resistant Enterobacteriaceae including *mcr*-carriers, uropathogenic *E. coli* and high-risk lineages such as ST131

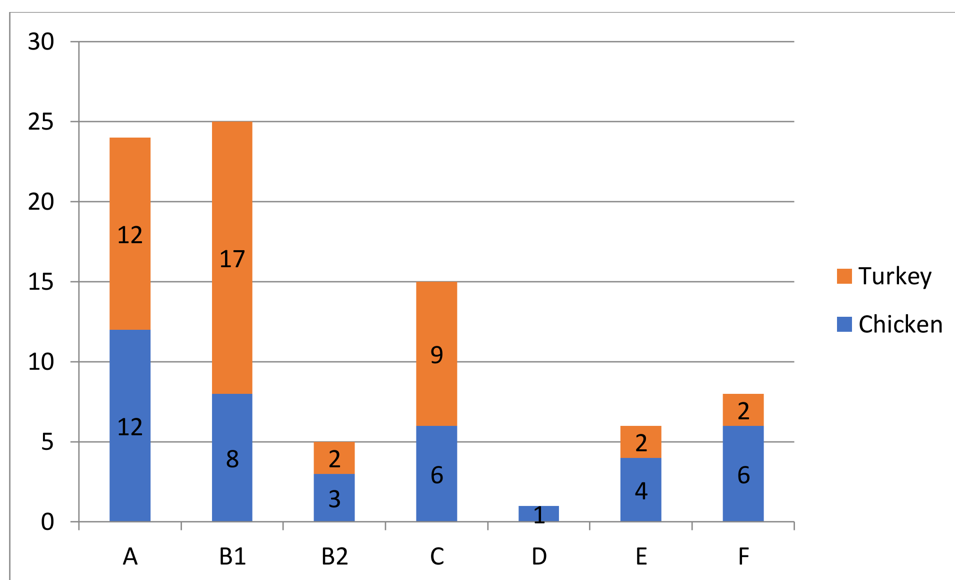

Figure 15. Graph of phylogroup distribution within the 84 representative *E. coli* obtained from ML. The value on the y-axis and bars indicates number of isolates.

Table 30. Clonotypes and ST combinations within the 84 representative *E. coli* isolates

adK	*fumC*	*gyrA*	*icD*	*mdh*	*purA*	*recA*	[1]CC	[1]ST	[2]Clonotype	[3]No. isolates and origin (C=chicken; T=turkey)
10	11	4	8	8	8	2	10	10	11-24	1C
10	11	4	8	8	8	2	10	**10**	**11-54**	**7 (5C+2T)**
24	11	4	8	8	8	2	10	43	11-54	1T
6	11	4	8	8	8	2	10	48	11-neg	2T
10	11	135	8	8	8	2	10	**744**	**11-54**	**3T**
10	11	4	8	8	8	49	10	752	11-24	1C
10	11	4	8	20	8	2	10	853	11-54	1T
10	11	4	10	7	8	2	10	2705	11-23	1C
10	11	4	560	8	8	2	10	5507	11-54	1C
10	11	4	8	8	13	new	10	STnew2	11-neg	1T
6	4	12	1	20	13	7	23	**23**	**4-35**	**7C**
6	4	12	1	20	12	7	23	88	4-39	1T
6	4	12	1	9	2	7	23	295	4-38	2C
6	4	12	1	20	18	7	23	**410**	**4-24**	**3T**
6	4	12	1	20	18	7	23	410	4-45	1T
6	4	12	1	20	18	7	23	410	4-53	1T
4	26	2	25	5	5	19	38	**38**	**26-65**	**4 (3C+1T)**
9	270	33	131	24	8	7	86	1720	270-54	1T
37	38	19	37	17	11	26	95	95	38-30	1T

adK	*fumC*	*gyrA*	*icD*	*mdh*	*purA*	*recA*	[1]CC	[1]ST	[2]Clonotype	[3]No. isolates and origin (C=chicken; T=turkey)
43	41	15	18	11	7	6	101	101	41-86	2C
53	40	47	13	36	28	29	131	131	40-22	2C
6	4	4	16	24	8	14	155	58	4-27	1T
6	4	4	16	24	8	14	155	**58**	**4-32**	**5T**
6	4	14	16	24	8	14	155	155	4-32	1T
6	4	14	16	24	8	14	155	155	4-neg	1T
6	4	4	16	24	8	14	155	58	4-neg	1T
6	11	4	10	7	8	6	168	93	11-41	1T
6	31	5	28	1	1	2	350	57	31-27	1T
6	31	83	28	1	1	67	350	371	31-142	1C
9	65	5	1	9	13	6	469	162	65-27	1C
9	65	5	1	9	13	6	469	162	65-38	1C
9	65	5	1	9	13	6	469	**162**	**65-32**	**4 (2C+2T)**
6	65	344	1	11	13	6	None	3580	65-32	2T
92	231	87	96	70	58	2	648	**1485**	**231-58**	**4 (3C+1T)**
new	153	188	83	7	8	6	new	STnew1	153-39	1C
20	45	41	43	5	32	2	None	117	45-97	1C
20	new	41	43	5	32	2	None	ST117-like	New-97	1C
6	29	4	18	11	8	6	None	212	29-38	1C
6	65	32	26	9	8	2	None	297	65-38	1T
6	69	158	18	9	8	7	None	1730	69-32	2C
443	271	24	198	7	214	359	None	5340	271-58	1T
80	4	57	18	55	8	6	None	5826	4-60	1C
136	11	4	1	9	18	7	None	7315	11-398	1T
6	4	14	1	20	62	7	None	345	4-31	1T
96	40	13	100	23	28	66	None	428	40-22	1T
96	40	13	100	23	28	66	None	428	40-neg	1C
101	88	97	108	26	79	2	None	457	88-145	1C
267	6	5	26	9	13	98	None	2599	6-32	1T
79	3	206	451	5	16	182	None	4243	3-1002	1C
6	7	5	1	618	8	2	None	7199	7-neg	1C

[1] Clonal complexes (CC) and Sequence types (ST) according to the Achtman scheme (Wirth et al., 2006). [7] Clonotype based on the internal 469-nucleotide (nt) and 489-nt sequence of the *fumC* (allele obtained from MLST) and *fimH* genes, respectively (Weissman et al., 2012). Seven isolates were negative (neg) for the amplification of the 489-nt internal sequence.

Table 31. *E. coli* isolates showing atypical EPEC (aEPEC)/ExPEC pathotypes

[1]Isolate code	Clonal group	ESBL	Phenotypic resistance	Virulence gene profile
Ch-10-ESBL	O123/186:H34-A-ST752 (CH11-24)	CTX-M-1	AMP, CTX, NAL	*fimH24 hlyF iucD iutA traT eae-beta1*
Ch-24-ESBL	O153:H10-A-ST10 (CH11-54)	CTX-M-32	AMP, CTX, ATM, GEN, DOX, CHL	*fimH54* $fimAv_{MT78}$ *traT eae-beta1*
Ch-36-R	O153:H10-A-ST10 (CH11-54)	-	AMP, DOX, CHL, CIP, NAL	*fimH54* $fimAv_{MT78}$ *papGII traT eae-beta1*
Ch-40-R	O145:H40-A-ST752 (CH11-24)	-	AMP, NAL	*fimH24 traT eae-beta1*
[1] Origin of isolation-sample number-type of isolate: Ch (chicken meat), T (turkey meat), R (representative *E. coli*), ESBL (ESBL-producing *E. coli*).				

Table 32. MIC values for colistin-resistant *K. pneumoniae* and *E. coli* isolates

[1]Isolate code	Identification	MIC value mg/L
Ch-35-ESBL	*Klebsiella pneumoniae*	>4
T-5-ESBL	*Klebsiella pneumoniae*	>4
T-11-ESBL	*Klebsiella pneumoniae*	>16
T-17-ESBL	*Klebsiella pneumoniae*	>128
T-24-ESBL	*Klebsiella pneumoniae*	>8
T-25-ESBL	*Klebsiella pneumoniae*	>128
T-27-ESBL	*Klebsiella pneumoniae*	>8
T-30-ESBL	*Klebsiella pneumoniae*	>16
T-43-ESBL	*Klebsiella pneumoniae*	>8
T-45-ESBL	*Klebsiella pneumoniae*	>32
T-46-ESBL	*Klebsiella pneumoniae*	>4
T-1-ESBL	*Escherichia coli*	>4
T-17-R	*Escherichia coli*	>32
[1] Origin of isolation-sample number-type of isolate: Ch (chicken meat), T (turkey meat), R (representative *E. coli*), ESBL (ESBL-producing isolates).		

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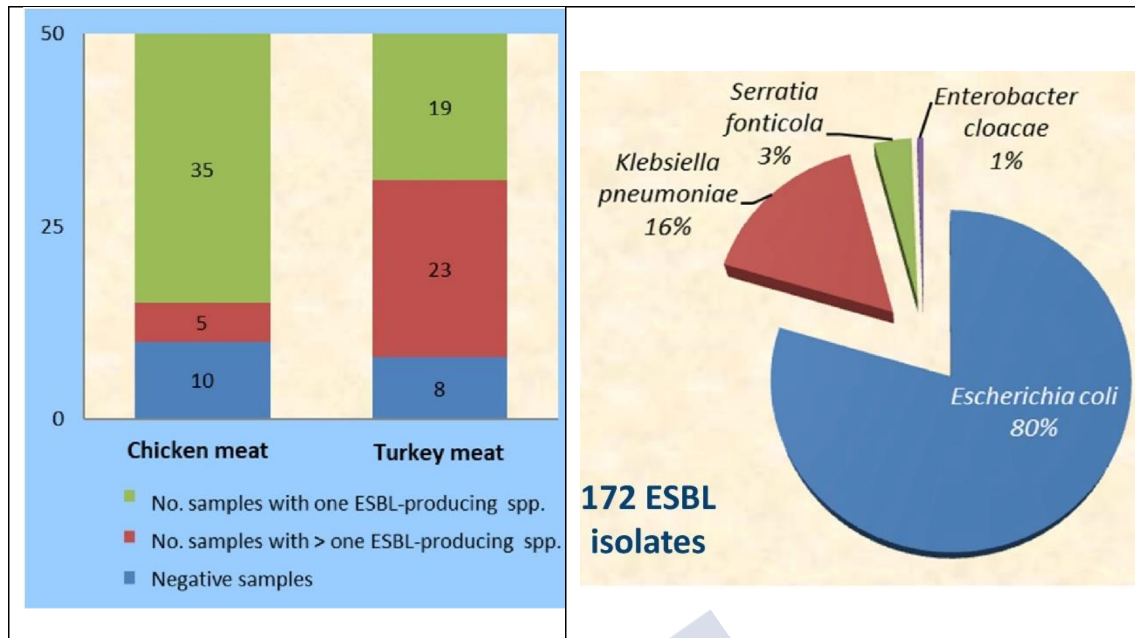

Figure 16. a) Presence of ESBL-producing Enterobacteriaceae within the 100 meat samples analyzed. The value on the y-axis and bars indicates number of samples; **b)** Species identification of the 172 ESBL-producing isolates recovered from 82 positive meat samples.

Table 33. Clonotypes and ST combinations of the 137 ESBL-producing *E. coli*.

adK	*fumC*	*gyrB*	*icD*	*mdh*	*purA*	*recA*	[1]ST	[1]CC	[2]CH
10	11	183	8	8	8	2	1141	10	11-32
6	11	4	8	8	8	2	48	10	11-41
10	11	4	8	8	8	2	10	10	11-54
10	11	135	8	8	8	2	744	10	11-54
10	11	57	8	8	8	185	1970	10	11-54
10	11	4	8	8	8	49	752	10	11-24
10	11	4	8	8	13	2	167	10	11-neg
10	11	4	8	8	13	73	617	10	11-neg
6	4	12	1	20	12	7	88	23	4-39
6	4	12	1	20	18	7	410	23	4-24
18	3	17	6	5	5	4	1158	31	3-47
4	26	39	25	5	31	19	115	38	26-270
4	26	2	25	5	5	19	38	38	26-65
21	35	27	6	5	5	4	69	69	35-27
9	6	33	131	24	8	7	641	86	6-25
43	41	15	90	11	8	6	359	101	41-35
43	41	15	18	11	7	6	101	101	41-86
6	4	14	16	7	8	14	1016	155	4-32
6	4	14	16	24	2	14	4162	155	4-38
6	4	4	16	24	8	14	58	155	4-27
6	4	14	16	24	8	14	155	155	4-32

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adK	*fumC*	*gyrB*	*icD*	*mdh*	*purA*	*recA*	[1]ST	[1]CC	[2]CH
6	4	14	16	24	8	14	155	155	4-121
6	4	14	16	24	8	14	155	155	4-neg
6	11	4	10	7	8	6	93	168	11-41
6	11	4	10	7	8	6	93	168	11-58
6	11	4	10	7	8	6	93	168	11-neg
10	27	5	8	8	7	2	226	226	27-41
6	31	5	28	1	1	2	57	350	31-27
6	31	5	28	1	1	2	57	350	31-31
6	31	83	28	1	1	2	350	350	31-54
6	31	83	28	1	1	new	STnew4	350	31-54
85	88	78	29	59	58	62	354	354	88-58
85	88	78	29	59	58	62	354	354	88-neg
6	19	33	26	11	8	6	602	446	19-86
9	65	5	1	9	13	6	162	469	65-32
6	new	4	16	7	13	2	STnew6	None	new-new
6	7	57	1	new	8	2	STnew7	None	7-54
6	4	15	1	22	8	7	366	None	4-30
122	11	125	12	96	8	2	665	None	11-30
122	11	125	12	8	8	2	STnew3	None	11-30
6	4	159	44	112	1	17	1011	None	4-31
6	8	32	159	9	23	7	3519	None	8-31
9	7	1	8	24	8	7	6215	None	7-34
6	8	32	159	9	23	7	3519	None	8-39
6	4	3	16	11	8	6	906	None	4-61
6	4	14	16	11	8	6	STnew5	None	4-32
136	11	4	1	9	18	7	7315	None	11-398
52	116	55	101	113	40	38	770	None	116-552
410	153	118	83	7	8	6	4980	None	153-39
10	168	4	8	12	35	2	1785	None	168-54
83	23	155	170	133	1	2	997	None	23-31
38	24	84	13	17	30	34	919	None	24-187
88	24	19	36	17	11	91	8611	None	24-26
10	252	5	8	7	8	2	1564	None	252-neg
6	41	33	18	9	8	6	707	None	41-60
20	45	41	43	5	32	2	117	None	45-151
20	45	41	43	5	32	2	117	None	45-97
13	52	10	14	17	25	17	141	None	52-14
6	65	32	26	9	8	2	297	None	65-38

[1]Clonal complexes (CC) and Sequence types (ST) according to the Achtman scheme (Wirth et al., 2006).
[2]Clonotype based on the internal 469-nucleotide (nt) and 489-nt sequence of the *fumC* (allele obtained from MLST) and *fimH* genes, respectively (Weissman et al., 2012). Seven isolates were negative (neg) for the amplification of the 489-nt internal sequence.

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7.3. STUDY 2: MICROBIOLOGICAL RISK ASSESSMENT OF TURKEY AND CHICKEN MEAT FOR CONSUMER: SIGNIFICANT DIFFERENCES REGARDING MULTIDRUG RESISTANCE, *MCR* OR PRESENCE OF HYBRID AEPEC/EXPEC PATHOTYPES OF E. COLI

Table 34. No. of positive isolates and positive samples regarding virulence traits and protocols

Virulence traits	**Protocol I + II** (ML + MSTC 37 ºC) No. positive /total: [a] isolates (%); [b] samples (%)	**Protocol V** (CHROMID® 37 ºC) No. positive /total: [a] isolates (%); [b] samples (%)
[1]**ExPEC status** N = 150 from 78 meat samples	[a] 118/150 (78.7); [b] 71/78 (91)	
[2]**UPEC status** N = 83 from 53 meat samples	[a] 69/83 (83.1); [b] 47/53 (88.7)	
[3]**ESBL/AmpC producer** N = 155 from 78 meat samples		[a] 137/155 (88.4); [b] 76/78 (97.4)
[4]***mcr-1* carrier** N = 13 from 7 meat samples	[a] 10/13 (76.9); [b] 6/7 (85.7)	
[5]**MDR** N = 253 from 88 meat samples	[a] 100/253 (39.5); [b] 59/88 (67)	[a] 137/253 (54.1); [b] 76/88 (87.5)
[6]***rbfO25b*** N = 13 from 10 meat samples	[a] 12/13 (92.3); [b] 9/10 (90)	

This table shows only the results for the protocol(s) of election in relation to each virulence trait.[1] No. of isolates conforming ExPEC status (Johnson et al., 2003c). [2] No. of isolates conforming status UPEC (Spurbeck et al., 2012). [3] No. of extended-spectrum β-lactamase (ESBL) or AmpC-β-lactamase (pAmpC)-producing *E. coli*. [4] No. of isolates carriers of the mcr-1 gene. [5] No. of MDR isolates according to Magiorakos *et al.* criteria (Magiorakos et al., 2012). [6] No. of rbfO25b-positive isolates: O25b subtype associated with the clonal group ST131 screened by PCR (Clermont et al., 2008).

Table 35. STs and clonotypes of 272 *E. coli* isolates

adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	[1]CC	[2]ST	[3]Clonotype	No. isolates and origin (C=chicken; T=turkey)
10	11	4	8	8	8	2	10	10	11-23	1T
10	11	4	8	8	8	2	10	10	11-24	3 (1C + 2T)
10	11	4	8	8	8	2	10	**10**	**11-54**	**12 (8C + 4T)**
10	11	4	8	8	8	2	10	10	11-122	1C
10	11	4	8	8	8	2	10	10	11-neg	2C
10	11	4	1	8	8	2	10	34	11-neg	2T
6	11	4	8	8	8	2	10	48	11-23	1C
6	11	4	8	8	8	2	10	48	11-41	2 (1C + 1T)
6	11	4	8	8	8	2	10	48	11-400	1T
10	11	4	8	8	13	2	10	167	11-neg	1T
10	11	4	8	8	13	73	10	617	11-neg	2 (1C + 1T)
10	11	135	8	8	8	2	10	744	11-54	2T

adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	[1]CC	[2]ST	[3]Clonotype	No. isolates and origin (C=chicken; T=turkey)
10	11	135	8	8	8	2	10	744	11-58	1C
10	11	4	8	8	8	49	10	**752**	**11-24**	**9C**
10	11	4	8	20	8	2	10	853	11-54	3T
10	11	183	8	8	8	2	10	1141	11-32	1T
10	11	57	8	8	8	185	10	1970	11-54	1C
6	4	12	1	20	13	7	23	23	4-35	1C
6	4	12	1	20	12	7	23	**88**	**4-39**	**4T**
6	4	12	1	20	18	7	23	410	4-24	1C
18	3	17	6	5	5	4	31	**1158**	**3-47**	**6C**
18	3	32	6	5	5	4	31	STnew8 (ST1158-like)	3-47	1C
4	26	2	25	5	5	19	38	38	26-65	1T
4	26	39	25	5	31	19	38	**115**	**26-270**	**7 (4C + 3T)**
21	35	27	6	5	5	4	69	**69**	**35-27**	**4 (2C + 2T)**
36	24	10	13	17	10	25	73	**355**	**24-154**	**7 (3C + 4T)**
36	24	9	13	17	11	159	73	1618	24-9	2C
99	6	33	33	24	8	7	86	453	6-31	2T
9	6	33	131	24	8	7	86	641	6-25	2C
9	270	33	131	24	8	7	86	1720	270-54	1T
37	38	19	37	17	11	26	95	**95**	**38-27**	**6 (4C + 2T)**
37	38	19	37	17	11	26	95	95	38-30	2T
55	38	19	37	17	11	26	95	140	38-15	3T
43	41	15	18	11	7	6	101	101	41-86	3 (1C + 2T)
43	41	15	90	11	8	6	101	359	41-35	1T
6	4	14	16	11	8	6	115	10328	4-32	1C
53	40	47	13	36	28	29	131	**131**	**40-22**	**8 (5C + 3T)**
53	40	47	13	36	28	29	131	**131**	**40-neg**	**4C**
6	4	4	16	24	8	14	155	58	4-27	1T
6	4	14	16	24	8	14	155	**155**	**4-32**	**7 (3C + 4T)**
6	4	14	16	24	8	14	155	155	4-121	1C
6	4	14	16	24	8	14	155	155	4-neg	3T
6	4	14	16	7	8	14	155	1016	4-32	1C
6	4	14	16	24	2	14	155	4162	4-38	1C
10	27	5	10	12	8	49	165	189	27-neg	2 (1C + 1T)
6	11	4	10	7	8	6	168	**93**	**11-41**	**4 (3C + 1T)**
6	11	4	10	7	8	6	168	93	11-47	1T
6	11	4	10	7	8	6	168	93	11-58	1C
6	11	4	10	7	8	6	168	**93**	**11-neg**	**11 (6C + 5T)**
6	11	4	10	7	84	6	168	484	11-neg	1T
6	11	4	234	7	8	6	168	1594	11-31	1C

adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	[1]CC	[2]ST	[3]Clonotype	No. isolates and origin (C=chicken; T=turkey)
6	482	4	10	7	8	6	168	3764	482-41	1T
10	27	5	8	8	7	2	226	226	27-41	2C
6	31	5	28	1	1	2	350	57	31-27	2 (1C + 1T)
6	31	5	28	1	1	2	350	57	31-31	1T
6	31	83	28	1	1	2	350	350	31-54	2T
6	31	83	28	1	1	new	350	STnew4 (ST350-like)	31-54	1T
85	88	78	29	59	58	62	354	354	88-58	3T
85	88	78	29	59	58	62	354	354	88-neg	1T
6	19	33	26	11	8	6	446	602	19-86	3T
9	65	5	1	9	13	6	469	**162**	**65-32**	**4 (2C + 2T)**
10	23	109	8	8	8	2	522	522	23-neg	1T
92	4	87	96	70	58	2	648	**648**	**4-58**	**6 (2C + 4T)**
92	231	87	96	70	58	2	648	**1485**	**231-58**	**19 (10C + 9T)**
6	new	4	16	7	13	2	None	STnew6	new-1319	2C
13	new	19	13	23	28	109	None	STnew9	new-664	1T
6	7	57	1	new	8	2	None	STnew7	7-54	1T
20	45	41	43	5	32	2	None	**117**	**45-97**	**8 (5C + 3T)**
20	45	41	43	5	32	2	None	117	45-151	1C
13	39	50	13	16	37	25	None	135	39-2	2C
13	52	10	14	17	25	17	None	141	52-14	1T
6	29	4	18	11	8	6	None	212	29-38	1C
6	65	32	26	9	8	2	None	297	65-38	1C
6	65	32	26	5	8	2	None	STnew10 (ST297-like)	65-276	1T
62	100	17	31	5	5	4	None	362	100-96	1T
6	4	15	1	22	8	7	None	366	4-30	2T
96	40	13	100	23	28	66	None	428	40-22	2 (1C + 1T)
96	40	13	100	23	28	66	None	**428**	**40-neg**	**4C**
96	40	93	13	23	28	66	None	429	40-20	2C
101	88	97	108	26	79	2	None	457	88-145	1T
13	38	84	13	17	64	34	None	569	38-5	1T
122	11	125	12	96	8	2	None	665	11-30	1C
122	11	125	12	8	8	2	None	STnew3 (ST665-like)	11-30	1C
6	41	33	18	9	8	6	None	707	41-60	1C
52	116	55	101	113	40	38	None	**770**	**116-552**	**7 (5C + 2T)**
6	4	3	16	11	8	6	None	906	4-61	1T
38	24	84	13	17	30	34	None	919	24-187	2T
83	23	155	170	133	1	2	None	997	23-31	1T
6	4	159	44	112	1	17	None	1011	4-31	2 (1C + 1T)

adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	[1]CC	[2]ST	[3]Clonotype	No. isolates and origin (C=chicken; T=turkey)
10	252	5	8	7	8	2	None	1564	252-neg	1T
101	88	97	108	7	13	2	None	1674	88-138	1T
10	168	4	8	12	35	2	None	1785	168-54	1T
18	22	67	31	5	5	4	None	1882	22-123	1T
36	43	19	13	16	10	25	None	2557	43-225	2C
267	6	5	26	9	13	98	None	2599	6-32	1T
31	276	83	140	1	187	19	None	2614	276-108	1C
6	8	32	159	9	23	7	None	3519	8-31	1C
6	8	32	159	9	23	7	None	3519	8-39	1C
79	3	206	451	5	16	182	None	**4243**	**3-1002**	**5 (4C + 1T)**
410	153	118	83	7	8	6	None	4980	153-39	2C
52	116	55	101	113	31	38	None	4994	116-270	1C
443	271	24	198	7	214	359	None	5340	271-58	1T
9	7	1	8	24	8	7	None	6215	7-34	1T
96	925	13	100	23	28	66	None	6876	925-neg	1T
136	11	4	1	9	18	7	None	7315	11-398	1T
88	24	19	36	17	11	91	None	8611	24-26	2T
76	1544	19	89	17	1	10	None	**10740**	**1544-9**	**5 (4C + 1T)**
101	88	97	108	26	79	2	None	457	88-145	1C
10	23	4	8	571	1	2	None	STnew11	23-823	1C

[1] Clonal complexes (CC) and [2] Sequence types (ST) according to the Achtman scheme (Wirth *et al.*, 2006): STnew was assigned to allelic combinations not found in EnteroBase, or to those including a new allele within the 7 gene; ST-like indicates one nucleotide of difference with the original ST. [2] Clonotype (CH) based on the internal 469-nucleotide (nt) and 489-nt sequence of the *fumC* (allele obtained from MLST) and *fimH* genes, respectively (Weissman *et al.*, 2012); neg: negative result for the amplification of the 489-nt internal sequence.

7.4. STUDY 3: GENOMIC CHARACTERIZATION OF ESBL-PRODUCING ESCHERICHIA COLI ISOLATES BELONGING TO A HYBRID AEPEC/EXPEC PATHOTYPE O153:H10-A-ST10 EAE-BETA1 OCCURRED IN HUMAN DIARRHEAGENIC ISOLATES, MEAT, POULTRY AND WILDLIFE

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Table 36. Assembly data from EnteroBase of the 17 O153:H10-A-ST10 genomes sequenced using Illumina NextSeq technology

Code	Assembly barcode	Coverage	N50	Length	Contig No. (>=200 bp)	ST_ 7 gene	ST Complex	wgMLST 25,002 loci	cgMLST 2,513 loci	rST 53 loci	O antigen prediction	H antigen prediction
LREC-110	ESC_KA7423AA_AS	361	147271	5152970	178	10	ST10 Cplx	38372	37600	2021	O153	H10
LREC-111	ESC_KA7425AA_AS	370	126323	5239837	221	10	ST10 Cplx	38373	37601	2021	O153	H10
LREC-112	ESC_KA7429AA_AS	124	109355	5084929	342	10	ST10 Cplx	38377	37605	2021	O153	H10
LREC-113	ESC_KA7430AA_AS	92	93205	5172711	213	10	ST10 Cplx	38378	37606	2021	O153	H10
LREC-114	ESC_KA7438AA_AS	163	126323	5201046	213	10	ST10 Cplx	38386	37614	2021	O153	H10
LREC-115	ESC_KA7437AA_AS	141	126291	5232022	228	10	ST10 Cplx	38385	37613	2021	O153	H10
LREC-116	ESC_KA7436AA_AS	118	124442	5187480	212	10	ST10 Cplx	38384	37612	2021	O153	H10
LREC-117	ESC_KA7433AA_AS	163	124771	5160744	169	10	ST10 Cplx	38381	37609	2021	O153	H10
LREC-118	ESC_KA7706AA_AS	39	69529	5166783	292	10	ST10 Cplx	39187	38299	2021	O153	H10
LREC-119	ESC_KA7435AA_AS	296	125664	4994631	189	10	ST10 Cplx	38383	37611	2021	O153	H10
LREC-120	ESC_KA7432AA_AS	150	102481	5263192	230	10	ST10 Cplx	38379	37607	2021	-	H10
LREC-121	ESC_KA7434AA_AS	71	73833	5134535	170	10	ST10 Cplx	38382	37610	2021	-	H10
LREC-122	ESC_KA7440AA_AS	168	124771	5209684	223	10	ST10 Cplx	38388	37616	2021	O153	H10
LREC-123	ESC_KA7439AA_AS	78	123102	5208501	253	10	ST10 Cplx	38387	37615	2021	O153	H10
LREC-124	ESC_KA7441AA_AS	201	119599	5258246	171	10	ST10 Cplx	38389	37617	2021	O153	H10
LREC-125	ESC_KA7442AA_AS	166	119599	5274856	272	10	ST10 Cplx	38390	37618	2021	O153	H10
LREC-127	ESC_KA7426AA_AS	208	126318	5253322	213	10	ST10 Cplx	38374	37602	58738	O153	H10

Raw reads were uploaded and automatically assembled in EnteroBase (https://enterobase.warwick.ac.uk/) using SPAdes Genome Assembler v3.5. with a threshold on contigs of minimum 200 nt. Subsequently, the *de novo* assembled contigs were MLST (7 gene ST, wgST, cgST and rST) and serotype predicted using EnteroBase typing tools

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Table 37. HierCC designations from EnteroBase for the 17 Spanish collection and other 7 related genomes within each cluster group. SNPs of the core genomic regions.

Name (EnteroBase)	Source Details [a]	Collection Year [a]	Country [a]	O Antigen	H Antigen	ST	Lineage	fimH allele	cgMLST	HC0	HC2	HC5	HC10	HC20	HC50	HC100	HC200	HC400	SNPs [b]
110084	DNA	DNA	DNA	O5	H27	10	A	54	8886	8886	8886	8886	8886	8886	8886	8886	8886	8224	
166357	Human; Homo sapiens	2015	United Kingdom	O40	H10	10	A	54	21500	21500	21500	21500	21500	21500	21500	21500	8224	8224	
208917	Human; Homo sapiens	2016	United Kingdom	O40	H10	10	A	54	21361	21361	21361	21361	21361	21361	21361	21361	8839	8224	
853984	Homo sapiens; human	2019	United Kingdom	O153	H10	10	A	54	124093	124093	124093	124093	124093	124093	124093	37600	8224	8224	
866428	Homo sapiens; human	2019	United Kingdom	O153	H10	10	A	54	129194	129194	129194	129194	129194	129194	124093	37600	8224	8224	
AM_LREC-110	Chicken meat	2010	Spain	O153	H10	10	A	54	37600	37600	37600	37600	37600	37600	37600	37600	8224	8224	37
AM_LREC-111	Fox faeces	2015	Spain	O153	H10	10	A	54	37601	37601	37601	37601	37601	37601	37600	37600	8224	8224	61
AM_LREC-112	Human clinical faeces	2011	Spain	O153	H10	10	A	54	37605	37605	37605	37605	37605	37605	37600	37600	8224	8224	361
AM_LREC-113	Human clinical faeces	2007	Spain	O153	H10	10	A	54	**37606**	**37606**	**37606**	**37606**	**37606**	**37606**	37600	37600	8224	8224	0
AM_LREC-114	Beef meat	2008	Spain	O153	H10	10	A	54	37614	37614	37614	37614	37614	37600	37600	37600	8224	8224	20
AM_LREC-115	Chicken meat	2009	Spain	O153	H10	10	A	54	37613	37613	37613	37613	37613	37613	37600	37600	8224	8224	101
AM_LREC-116	Human clinical faeces	2006	Spain	O153	H10	10	A	54	37612	37612	37612	37612	37612	**37606**	37600	37600	8224	8224	22
AM_LREC-117	Beef meat	2007	Spain	O153	H10	10	A	54	37609	37609	37609	37609	37609	37609	37600	37600	8224	8224	36
AM_LREC-118	Chicken breast	2009	Spain	O153	H10	10	A	54	38299	38299	38299	38299	38299	37615	37600	37600	8224	8224	24
AM_LREC-119	Beef meat	2007	Spain	O153	H10	10	A	54	37611	37611	37611	37611	37611	**37606**	37600	37600	8224	8224	15
AM_LREC-120	Beef meat	2011	Spain	-	H10	10	A	54	37607	37607	37607	37607	37607	37607	37600	37600	8224	8224	537
AM_LREC-121	Human clinical faeces	2007	Spain	-	H10	10	A	54	37610	37610	37610	37610	37610	37610	37600	37600	8224	8224	51

AM_LREC-122	Pork meat	2011	Spain	O153	H10	10	A	54	37616	37616	37616	37616	37616	37615	37600	37600	8224	8224	28
AM_LREC-123	Chicken meat	2010	Spain	O153	H10	10	A	54	37615	37615	37615	37615	37615	37615	37600	37600	8224	8224	25
AM_LREC-124	Human clinical faeces	2007	Spain	O153	H10	10	A	54	37617	37617	37617	37617	37617	**37606**	37600	37600	8224	8224	31
AM_LREC-125	Beef meat	2008	Spain	O153	H10	10	A	54	37618	37618	37618	37618	37618	**37606**	37600	37600	8224	8224	21
AM_LREC-127	Poultry farm environment	2010	Spain	O153	H10	10	A	54	37602	37602	37602	37602	37602	37602	37600	37600	8224	8224	54
E89	Broiler; Liver	2015	Denmark	uncertain	H10	7003	A	54	36964	36964	36964	36964	36964	36964	36964	36964	8224	8224	
Escherichia coli 2312	DNA	DNA	DNA	O40	H10	10	A	54	8224	8224	8224	8224	8224	8224	8224	8224	8224	8224	

[a] Data not available (DNA); [b] Not analysed (NA); [b] SNPs of the core genomic regions present in 90% of the 17 compared genomes of our collection and using LREC-113 as reference

Table 38. Number of human stool samples analyzed and positive for aEPEC O153

Year	Nº stool samples	No. of positive samples (%) for aEPEC O153	No. of positive samples (%) for O153:H10 *eae*-beta1 *fim*$_{AVMT78}$
2006	1,842	4 (0.22)	1 (0.05)
2007	2,095	11 (0.52)	8 (0.4)
2008	1,001	5 (0.50)	3 (0.3)
2009	550	0 (0)	0 (0)
2010	514	0 (0)	0 (0)
2011	1,207	2 (0.50)	1 (0.08)
2012	2314	1 (0.04)	1 (0.04)
Total	9,523	23 (0.14)	14 (0.15)

Table 39. Twenty-three aEPEC O153 human isolates recovered in the period 2006-2012

Isolate code	Year of isolation	Symptomatology	O153 Serogroup	H10 Antigen	*eae* gene	*eae* ß-1 intimin	*fimH*$_{AVMT78}$ gene
22250.06	2006	Diarrhea	+	-	+	+	-
37979. 06	2006	Diarrhea	+	+	+	-	-
41824. 06	2006	Diarrhea	+	-	+	-	-
45990. 06 (LREC 116)*	2006	Diarrhea	+	+	+	+	+
57646. 06	2007	Diarrhea	+	-	+	-	+
18396.07 (LREC 124)*	2007	Diarrhea	+	+	+	+	+
19979. 07 (LREC 113)*	2007	Diarrhea	+	+	+	+	+
30981. 07 (LREC 121)*	2007	Diarrhea	+	+	+	+	+
31952. 07	2007	Diarrhea	+	+	+	+	+
32182. 07	2007	Diarrhea	+	-	+	-	-
32651. 07	2007	Hemorrhagic gastroenteritis	+	+	+	+	+
32884. 07	2007	Diarrhea	+	+	+	+	+
34535. 07	2007	Acute gastroenteritis	+	+	+	+	+
39044. 07	2007	Acute gastroenteritis	+	+	+	+	+
65905/07	2007	Hemorrhagic colitis	+	-	+	-	+
110431.08	2008	Hemorrhagic colitis	+	-	+	+	-
2477.08	2008	Diarrhea	+	-	+	-	-
21011. 08	2008	Diarrhea	+	+	+	+	+
38506. 08	2008	Diarrhea	+	+	+	+	+
40237. 08	2008	Diarrhea	+	+	+	+	+

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Isolate code	Year of isolation	Symptomatology	O153 Serogroup	H10 Antigen	*eae* gene	*eae* β-1 intimin	*fimH*$_{AVMT78}$ gene
48633.11	2011	Diarrhea	+	-	+	-	-
9727.011 (LREC 112)*	2011	Hemorrhagic colitis	+	+	+	+	+
55515.12	2012	Diarrhea	+	+	+	+	+
(*) code of those strains which were WG sequenced							

Table 40. *in silico* characterization of seven *E. coli* related genomes from EnteroBase using CGE databases

Name	Serotype	Phylo group	CHType	ST	Plasmid content Inc group (pMLST)	Acquired resistances	Virulence genes
866428	O153:H10	A	11-54	10	**IncF (F2:A-:B-)** IncX1 Col156	*aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tir*
853984	O153:H10	A	11-54	10	**IncF (F2:A-:B-)** IncX1 Col156	*aadA1; catA1; mdf(A); tet(A)*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tir*
166357	O40:H10	A	11-54	10	**IncF (F2:A-:B-)** IncHI2 (ST4) IncQ Col156	*bla_{TEM-1B}; aph(3´´)-Ib, aph(3´)-Ia, aph(6)-Ib; catA1; mdf(A); florR; tet(A); sul2; dfrA8*	*astA, eae, espA, espB, gad, iss, mchF, nleA, nleC, tir*
E. coli 2312	O40:H10	A	11-54	10	**IncF (F2:A-:B-)** IncI1 (STunknown) Col156 Col (MG828)	*aac(3)-IV, aph(3´´)-Ib, aph(3´)-Ia, aph(4)-Ia, aph(6)-Ib; mdf(A); tet(A); sul2*	*astA, eae, espA, espB, gad, ireA, iss, mchF, nleA, nleC, tir*
E89	ND:H10	A	11-54	10	**IncF (F2:A-:B-)** Col156	*aadA1; mdf(A);*	*astA, eae, espA, espB, gad, iss, mchF, nleA, tir*
208917	O40:H10	A	11-54	10	**IncF (F2:A-:B-)** Col156	*mdf(A)*	*astA, eae, espA, gad, ireA, mchF, nleA, nleC, tir*
110084	O5:H27	A	11-54	10	IncF (F-:A-:B-) pO111	*aph(6)-Ib; mdf(A); sul2; dfrA8*	*astA, celB, eae, espA, gad, iss, mchF, nleA, tir*

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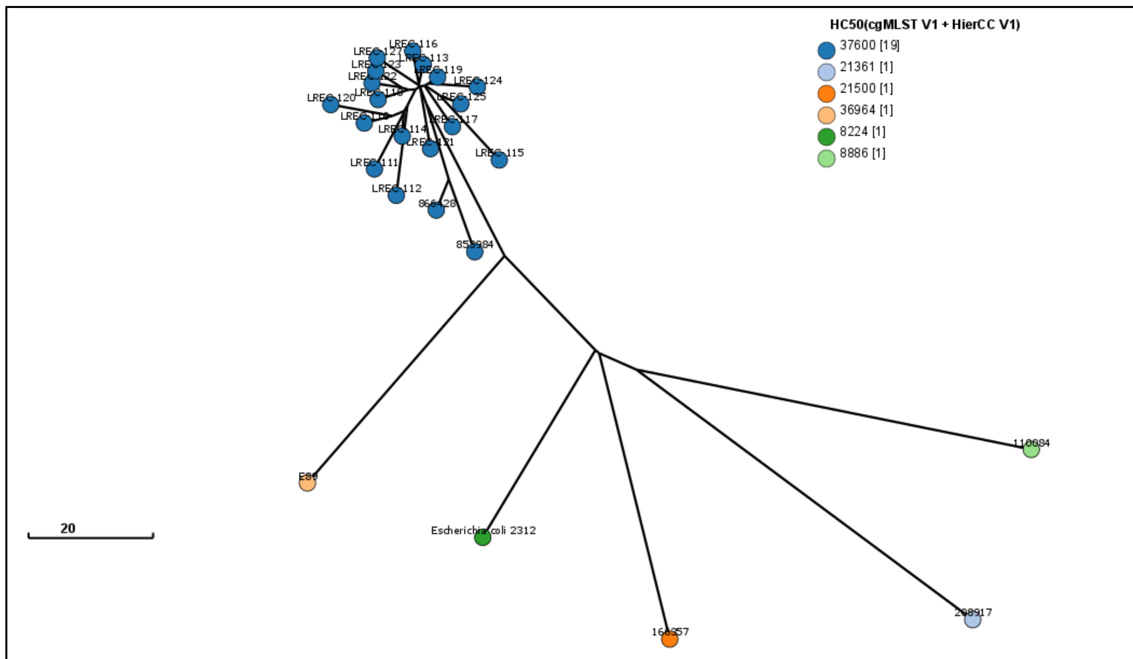
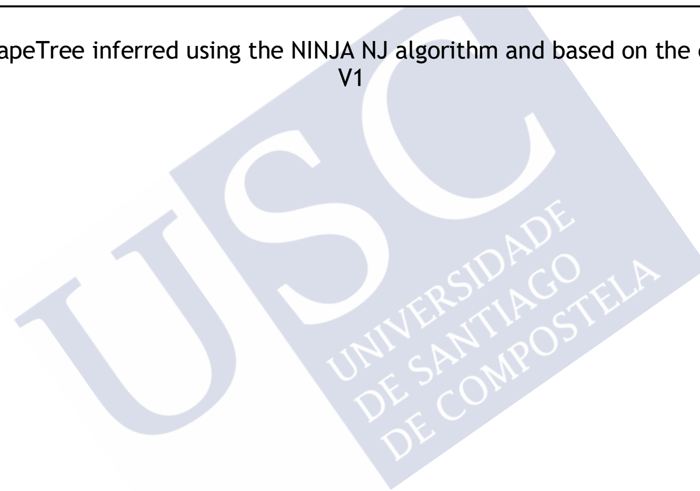


Figure 17. GrapeTree inferred using the NINJA NJ algorithm and based on the cgMLST V1 + HierCC V1



7.5. CHARACTERIZATION OF THE *E. COLI* COLLECTION RECOVERED FROM THE 100 MEAT SAMPLES

Table 41: Characterization of the 391 *E. coli* isolates recovered from 100 meat samples

CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
10	Ch1.I.R	**C**	O78:H9	23	23	35	4-35	*iutA*	-	0	NR	0	0	DOX, NAL
	Ch1.I.a	**F**	O2:H42	648	648	58	4-58	*iutA, KpsM II - K5*	*chuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, iutA, hlyF, ompT, iss*	0	0	NAL
	Ch1.I.b	**B1**	O78:H9	162	469	32	65-32	*papAH, iutA*	*fyuA*	0	*fimH, papC, papAH, papEF, papGII, iucD, iroN, cvaC, traT, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, DOX, CHL, SXT, CIP, NAL
	Ch1.V.a	**G**	O24:H18	117	None	151	45-151	*iutA*	*chuA, vat, fyuA*	0	*fimH, cdtB, iucD, traT, malX, iutA, hlyF, ompT*	**SHV-12** - TEM	0	AMP, CFZ, CAZ, ATM, DOX, CHL, NAL
30	Ch2.I.R	**B2**	O25:H4	131	131	22	40-22	*iutA, KpsM II - K1*	*chuA, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	GEN, DOX, NAL
	Ch2.I.c	**B2**	O25:H4	131	131	neg	40-neg	*iutA, KpsM II - K1*	*chuA, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	GEN, DOX, NAL
	Ch2.I.e	**B2**	O25:H4	131	131	neg	40-neg	*iutA, KpsM II - K1*	*chuA, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	GEN, NAL
	Ch2.I.g	**E**	O2:HNM	115	38	270	26-270	*iutA, KpsM II*	*chuA, fuyA*	0	NR	TEM	0	AMP, DOX, SXT, CIP, NAL
	Ch2.I.h	**E**	O2:HNM	115	38	270	26-270	*iutA, KpsM II*	*chuA, fuyA*	0	NR	TEM	0	AMP, DOX, SXT, CIP, NAL
	Ch2.I.i	**B2**	O25:H4	131	131	neg	40-neg	*iutA, KpsM II - K1*	*chuA, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	TEM	0	NAL
	Ch2.IV.e	**A**	ONT:H40	752 *	10	24	11-24	*iutA*	-	**1***	*fimH, iucD, traT, iutA, hlyF, iss*	TEM	0	AMP, GEN, DOX, NAL
	Ch2.V.a	**A**	O2:H40	NR	NR	NR	NR	-	-	0	NR	**CTX-M-32** - TEM	0	AMP, CFZ, CXM, CTX, ATM, GEN
40	Ch3.I.R	**A**	O40:HNM	7199	None	neg	7-neg	-	-	0	NR	TEM	0	AMP
	Ch3.II.g	**A**	O123:H34	752 *	10	24	11-24	*iutA*	-	**1**	*fimH, iucD, traT, iutA, hlyF*	TEM	0	AMP, GEN, DOX, SXT, NAL
	Ch3.III.f	**Clade I**	O1:H45	4994	None	270	116-270	*iutA, KpsM II - K5*	*chuA*	0	*fimH, iucD, KspM II, KpsM II - K2, KspM II - K5, malX, iutA, ompT*	0	0	NAL
	Ch3.III.h	**B2**	O2:H5	10740	None	9	1544-9	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	TEM	0	-
	Ch3.V.a	**B1**	O103:HAA	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, DOX, NAL

CFU/g[1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH[6]	Clonotype[6]	ExPEC[7]	UPEC[8]	*eae*[9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
410	Ch4.I.R	**A**	O8:H10	2705	10	23	11-23	*iutA*	-	0	NR	TEM	0	AMP, DOX, CHL
	Ch4.II.h	**B2**	O117:H4	428	None	22	40-22	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAv, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, DOX, CHL, SXT, CIP, NAL
	Ch4.II.i	**A**	O123:H34	752 *	10	24	11-24	*iutA*	-	**1**	*fimH, iucD, traT, iutA, hlyF*	TEM	0	AMP, NAL
	Ch4.II.j	**E**	O73:H34	1158	31	47	3-47	*iutA, KpsM II*	*chuA*	0	*KpsM II, iuta*	TEM	0	AMP, DOX, SXT, CIP, NAL
	Ch4.V.a	**G**	O111:H4	117	None	97	45-97	*iutA*	*chuA, vat*	0	*fimH, iucD, iroN, cvaC, traT, malX, iutA, hlyF, ompT, iss*	**CTX-M-1**	0	AMP, CFZ, CXM, CTX, DOX
20	Ch5.I.R	**C**	O78:HNM	23	23	35	4-35	*iutA*	-	0	NR	0	0	NAL
	Ch5.II.a	**E**	O2:H9	115	38	270	26-270	*iutA, KpsM II*	*chuA, fuyA*	0	NR	**CTX-M-NT**	0	AMP, CFZ, CXM, CTX, SXT, CIP, NAL
	Ch5.II.c	**A**	O11:H40	752 *	10	NR	NR	*iutA*	-	**1***	*fimH, iucD, traT, iutA, hlyF, iss*	0	0	DOX, NAL
	Ch5.II.f	**A**	O80:H26	189	165	NR	NR	-	*fyuA*	**1***	*fimH, fimAv, traT*	TEM	0	AMP, GEN, NAL
	Ch5.II.g	**F**	O21:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, GEN, SXT, CIP, NAL
	Ch5.V.a	**E**	O2:H9	115	38	270	26-270	*iutA, KpsM II - K2*	*chuA, fuyA*	0	*fimH, iucD, KpsM II, KpsM II - K2, traT, iutA, hlyF, ompT*	**CTX-M-9**	0	AMP, CFZ, CXM, CTX, SXT, CIP, NAL
	Ch5.V.c	**D**	ONT:H18	69	69	27	35-27	*iutA, KpsM II - K1*	*chuA, fuyA*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, traT, iutA, hlyF, ompT, iss*	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM
10	Ch6.I.R	**F**	O15:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, AMC, GEN, TOB, SXT, CIP, NAL
	Ch6.I.a	**F**	O15:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, GEN, TOB, DOX, SXT, CIP, NAL
	Ch6.I.f	**A**	O145:H40	752	10	24	11-24	-	-	**1***	*fimH, traT*	0	0	NAL
	Ch6.V.a	**E**	O140:H34	1158	31	47	3-47	*iutA, KpsM II - K5*	*chuA*	0	*fimH, iucD, KpsM II, KpsM II - K2, KpsM II - K5, traT, iutA, hlyF, ompT*	**TEM-52**	0	AMP, CFZ, CXM, CAZ, CTX, CIP, NAL
100	Ch7.I.R	**C**	O78:HNM	23	23	35	4-35	*iutA*	*yfcV*	0	NR	0	0	NAL
	Ch7.II.a	**D**	O73:H45	4243	None	1002	3-1002	*iutA, KpsM II*	*chuA*	0	NR	**SHV-12**	0	AMP, CFZ, ATM, GEN, NAL
	Ch7.II.b	**A**	ONT:HNT	10	10	122	11-122	-	*fyuA*	**1***	*fimH, iucD, iroN, cvaC, traT, tsh, hlyF, ompT, iss*	TEM	0	AMP, NAL

CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	Ch7.II.g	**B2**	O175:H5	2557	None	225	43-225	*KpsM II - K5, sfa/foc*	*chuA, vat, fyuA, yfcV*	0	*fimH, sfa/foc, iroN, KpsM II, KpsM II - K2, KpsM II - K5, ibeA, malX, usp, ompT*	0	0	-
	Ch7.II.i	**B2**	O175:H6	2557	None	225	43-225	*iutA, KpsM II - K5, sfa/foc*	*chuA, vat, fyuA, yfcV*	0	*fimH, sfa/foc, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	NAL
	Ch7.II.j	**C**	O78:H45	23	23	35	4-35	*iutA*	*chuA, fuyA*	0	NR	0	0	GEN, NAL
	Ch7.V.a	**A**	O2:H40	NR	NR	NR	NR	-	-	0	NR	**CTX-M-32** - TEM	0	AMP, CFZ, CXM, CAZ, CTX, ATM, GEN, NAL
	Ch7.V.b	**A**	O5:H10	93	168	58	11-58	*iutA, KpsM II - K2*	-	0	*iucD, iroN, KpsM II, KpsM II- K2, cvaC, traT, iuta, tsh, hlyF, iss*	**CTX-M-1**	0	AMP, CFZ, CXM, CTX, DOX, NAL
70	Ch8.I.R	**B1**	O109:HNT	162	469	32	65-32	*iutA*	*fyuA*	0	NR			AMP, DOX, SXT, CIP, NAL
	Ch8.II.a	**A**	O132:H37	752	10	24	11-24	-	-	**1***	*fimH, traT*	0	0	NAL
	Ch8.II.c	**B2**	O25:H4	131	131	neg	40-neg	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	TEM	0	-
	Ch8.V.a	**E**	O19:HNT	NR	NR	NR	NR	*iutA*	*chuA*	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, CTX, ATM
	Ch8.V.b	**E**	ONT:H10	NR	NR	NR	NR	*iutA*	*chuA, fuyA*	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, CTX, ATM
30	Ch9.I.R	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, GEN, SXT, NAL
	Ch9.I.e	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, GEN, DOX, SXT, NAL
	Ch9.II.a	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, GEN, SXT, NAL
	Ch9.II.i	**B1**	O18ac:H49	212	None	38	29-38	-	-	0	*fimH, traT*	TEM	**mcr1.1**	AMP, GEN, CST, DOX, CHL, SXT, CIP, NAL
	Ch9.IV.a	**A**	O20:H11	48	10	41	11-41	-	-	0	NR	**CTX-M-NT** - TEM	0	AMP, CFZ, CXM, CTX, DOX, SXT, NAL
40	Ch10.I.R	**C**	O159:H16	295	23	38	4-38	-	*yfcV*	0	NR	TEM	0	AMP, NAL
	Ch10.I.g	**B2**	O18:H12	1618	73	9	24-9	*KpsM II - K5, sfa/foc*	*chuA, vat, fyuA, yfcV*	0	*fimH, sfa/foc, cdtB, iroN, KpsM II, KpsM II - K2, KpsM II - K5, traT, malX, usp, hlyF, ompT*	0	0	NR
	Ch10.IV.c'	**E**	O19:HNM	2614	None	108	276-108	-	*chuA*	0	NR	TEM	0	AMP, DOX, CIP, NAL

CFU/g[1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH[6]	Clonotype[6]	ExPEC[7]	UPEC[8]	*eae*[9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	Ch10.V.a	**A**	O123/186: H34	752	10	24	11-24	*iutA*	-	**1**	*fimH, iucD, traT, iutA, hlyF*	**CTX-M-1**	0	AMP, CFZ, CXM, CTX, NAL
	Ch10.V.b	**G**	O111:H4	117	None	97	45-97	*iutA*	*chuA, vat*	0	*fimH, iucD, iroN, cvaC, traT, malX, iutA, hlyF, ompT, iss*	**CTX-M-1**	0	AMP, CFZ, CXM, CTX, ATM, DOX, SXT, NAL
50	Ch13.I.R	**B2**	O25:H4	131	131	22	40-22	*iutA, KpsM II - K1*	*chuA, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	GEN, DOX, NAL
	Ch13.II.b	**B2**	O25:H4	131	131	22	40-22	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	DOX, NAL
	Ch13.II.h	**Clade I**	O15:H16	770	None	552	116-552	*iutA, KpsM II - K5*	*chuA, fuyA*	0	*fimH, iucD, KspM II, KpsM II - K2, KspM II - K5, traT, malX, iutA, hlyF, ompT*	0	0	-
	Ch13.IV.a	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, GEN, DOX, SXT, CIP, NAL
	Ch13.V.a	**B1**	O8:H19	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, NAL
	Ch13.V.b	**A**	O88:H7	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CIP, NAL
10	Ch14.I.R	**B1**	O88:H10	162	469	32	65-32	*iutA*	-	0	NR	0	0	CIP, NAL
	Ch14.I.g	**B2**	O2:H5	10740	None	9	1544-9	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	0	0	-
	Ch14.I.j	**A**	O153:H10	10	10	54	11-54	-	*fyuA*	**1***	*fimH, fimAv, traT*	0	0	-
<10	Ch15.I.R	**A**	O84:HNM	10	10	54	11-54	-	-	0	NR	TEM	0	AMP, GEN, DOX, CIP, NAL
	Ch15.I.b	**A**	O86:H5	93	168	41	11-41	*iutA, KpsM II*	-	0	NR	TEM	0	AMP, AMC, GEN, DOX, CIP, NAL
20	Ch16.I.R	**F**	O11:H25	457	None	145	88-145	*iutA, KpsM II - K2*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	0	0	GEN, DOX
	Ch16.II.a	**A**	O68:H51	10	10	24	11-24	-	-	**1***	*fimH, traT*	TEM	0	AMP, GEN, NAL
	Ch16.V.a	**E**	O128:H25	NR	NR	NR	NR	*iutA*	*chuA*	0	NR	**CTX-M-1** - TEM	0	AMP, CFZ, CXM, CTX, GEN
	Ch16.V.c	**B1**	O8:H51	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, DOX, CHL, NAL
20	Ch17.I.R	**A**	O64:HNT	10	10	54	11-54	-	-	0	NR	**SHV-12**	0	AMP, CAZ, ATM, DOX, CHL, NAL

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CFU/g[1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH[6]	Clonotype[6]	ExPEC[7]	UPEC[8]	*eae*[9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	Ch17.II.b	**B2**	O120:H4	428	None	neg	40-neg	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAv, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	TEM	0	AMP, SXT
	Ch17.II.b	**A**	O153:HNM	10	10	NR	NR	-	*fyuA*	**1***	*fimH, fimAv, traT*	TEM	0	AMP, DOX, CHL
	Ch17.II.j2	**B2**	O2:H1	135	None	2	39-2	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAv, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, ibeA, malX, usp, iuta, tsh, hlyF, ompT, iss*	0	0	DOX, NAL
	Ch17.V.a	**A**	O154:H28	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, DOX, CHL, CIP, NAL
440	Ch18.I.R	**A**	O132:HNM	10	10	54	11-54	-	-	0	NR			-
	Ch18.IV.b	**B2**	O2:H5	10740	None	9	1544-9	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	0	0	-
	Ch18.IV.d	**A**	O153:H10	10	10	54	11-54	-	*fyuA*	**1***	*fimH, fimAv, traT*	TEM	0	AMP, GEN, DOX, CHL
	Ch18.IV.g	**B2**	O2:HNM	10740	None	9	1544-9	*iutA, KpsM II - K5, afa/dra*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAv, afa/dra, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	0	0	-
	Ch18.V.a	**E**	O140:H25	NR	NR	NR	NR	-	*chuA*	0	NR	**CTX-M-1**	0	AMP, CFZ, CXM, CTX, NAL
	Ch18.V.b	**A**	O154:H28	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CFZ, DOX, CHL, CIP, NAL
510	Ch19.I.R	**B2**	O120:H4	428	None	neg	40-neg	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAv, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	TEM	0	AMP, SXT
	Ch19.I.a	**B2**	O120:H4	428	None	neg	40-neg	*iutA, KpsM II - K1, afa/dra*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAV, afa/dra, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	0	0	AMP, SXT
	Ch19.II.c1	**B2**	O120:H4	428	None	neg	40-neg	*iutA, KpsM II - K1, afa/dra*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAV, afa/dra, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iuta, hlyF, ompT, iss*	0	0	AMP, SXT
	Ch19.V.a	**A**	O88:H7	NR	NR	NR	NR	-	-	0	NR	**TEM-52**	0	AMP, CFZ, CXM, CTX, NAL
	Ch19.V.b	**E**	O140:H25	NR	NR	NR	NR	-	*chuA*	0	NR	**CTX-M-1**	0	AMP, CFZ, CXM, CTX, ATM, NAL
40	Ch20.I.R	**C**	O15:HNT	23	23	32	4-35	*iutA*	*fyuA*	0	NR	TEM	0	AMP, TOB, DOX, SXT, CIP, NAL

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CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	Ch20.I.j	**B2**	O2:H1	429	None	20	40-20	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	NAL
	Ch20.II.a	**A**	O11:HNT	752 *	10	NR	NR	-	*fyuA*	**1***	*fimH, traT*	TEM	0	AMP, NAL
	Ch20.II.c	**B2**	O2:H1	429	None	20	40-20	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	NAL
	Ch20.II.g	**A**	O123:H34	10	10	NR	NR	-	-	**1***	*fimH*	TEM	0	NAL
	Ch20.V.a	**E**	O19:H45	NR	NR	NR	NR	-	*chuA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, ATM, DOX, CHL
10	Ch21.I.R	**B1**	O88:H8	101	101	86	41-86	-	*vat*	0	NR	0	0	-
	Ch21.I.d	**B2**	O2:HNM	95	95	27	38-27	*papAH, iutA, KpsM II - K1*	*chuA, fyuA, yfcV*	0	*fimH, papC, papAH, papEF, papGII, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, malX, usp, iutA, hlyF, ompT, iss*	TEM	0	NAL
	Ch21.II.a	**B2**	O2:HNM	95	95	27	38-27	*papAH, iutA, KpsM II - K1*	*chuA, fyuA, yfcV*	0	*fimH, papC, papAH, papEF, papGII, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, malX, usp, iutA, hlyF, ompT, iss*	0	0	NAL
	Ch21.V.a	**A**	O88:H7	NR	NR	NR	NR	-	-	0	NR	**TEM-52**	0	AMP, CXM, CTX, NAL
	Ch21.V.e	**C**	O19:HNM	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CAZ, ATM, DOX, CHL, CIP, NAL
40	Ch22.II.l	**A**	ONT:H51	STnew11	None	823	23-823	-	-	**1**	*fimH, fimAv, traT, usp*	TEM	0	AMP, SXT, CIP, NAL
	Ch22.II.o	**A**	O7:HNM	93	168	41	11-41	*iutA, KpsM II*	*chuA*	0	NR	**SHV-12** - TEM	0	AMP, GEN, TOB, CHL, NAL
	Ch22.V.b	**E**	O154:H38	NR	NR	NR	NR	*iutA*	*chuA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, ATM, DOX, CIP, NAL
310	Ch23.I.R	**E**	O123:H15	38	38	65	26-65	-	*chuA, fyuA*	0	NR	0	0	NAL
	Ch23.V.a	**B1**	O8:H51	NR	NR	NR	NR	*iutA*	-	0	NR	**SHV-12**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, DOX, CIP, NAL
200	Ch24.I.R	**D**	O73:HNT	4243	None	1002	3-1002	*iutA, KpsM II - K5*	*chuA*	0	*fimH, iucD, KspM II, KpsM II - K2, KspM II - K5, traT, iutA, iss*	0	0	GEN, NAL
	Ch24.II.d	**A**	O88:H28	NR	NR	27	152-27	*iutA, KpsM II*	*chuA, vat*	0	NR	0	0	-
	Ch24.IV.h	**G**	O161:H4	117	None	97	45-97	*papAH, iutA*	*chuA, vat, fyuA*	0	*fimH, papC, papAH, papEF, papGII, iucD, iroN, traT, malX, iutA, hlyF, ompT, iss*	0	0	DOX, CHL, NAL

CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	Ch24.V.a	**A**	O153:H10	10	10	54	11-54	-	*fyuA*	**1***	*fimH, fimAv, traT*	**CTX-M-32** - TEM	0	AMP, CFZ, CXM, CTX, ATM, GEN, DOX, CHL
50	Ch25.I.R	**A**	O140:HNM	10	10	54	11-54	-	-	0	NR	0	0	DOX
	Ch25.V.a	**A**	O19:H17	665	None	30	11-30	-	-	0	NR	**CTX-M-32**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, CIP, NAL
	Ch25.V.b	**A**	O88:H7	4980	None	39	153-39	-	-	0	NR	**TEM-52**	0	AMP, CFZ, CXM, CTX, NAL
	Ch25.V.d	**A**	O19:H17	STnew3 (ST665-like)	None	30	11-30	*iutA*	-	0	NR	**CTX-M-32**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, CIP, NAL
	Ch25.V.e	**A**	O23:H32	10	10	54	11-54	*iutA*	-	0	*fimH, fimAv, iucD, iroN, traT, iutA, hlyF, iss*	**SHV-12**	0	AMP, CFZ, ATM, NAL
<10	Ch26.I.R	**A**	O101:H9	5507	10	54	11-54	*iutA*	-	0	NR	TEM	0	AMP, AMC, DOX, NAL
	Ch26.I.b	**A**	O5:H10	93	168	neg	11-neg	*iutA, KpsM II*	-	0	NR	0	0	GEN, TOB, CIP, NAL
	Ch26.I.h	**A**	O5:H10	93	168	neg	11-neg	*iutA, KpsM II*	-	0	NR	TEM	0	GEN, CIP, NAL
10	Ch27.I.R	**B1**	O19:HNT	212	None	38	29-38	-	-	0	NR	TEM	0	AMP, AMC, SXT, CIP, NAL
	Ch27.II.b	**E**	ONT:H34	1158	31	47	3-47	*iutA, KpsM II*	*chuA*	0	NR	TEM	0	AMP, GEN, DOX, CIP, NAL
	Ch27.II.e	**D**	O17:H45	4243	None	1002	3-1002	*iutA, KpsM II*	-	0	NR	0	0	GEN, NAL
	Ch27.III.b	**E**	O25:H45	1011	None	31	4-31	-	*chuA, fuyA*	0	*fimH, iroN, traT, tsh, hlyF, ompT, iss*	TEM	0	AMP, SXT
	Ch27.V.a	**A**	O132:H28	10	10	54	11-54	-	-	0	NR	**SHV-12**	0	AMP, CFZ, ATM, CHL, NAL
<10	Ch28.I.R	**A**	O88:HNT	STnew1	None	39	153-39	-	-	0	NR	TEM	0	AMP, AMC, DOX, NAL
	Ch28.V.a	**B1**	O86:H51	155	155	32	4-32	*iutA, KpsM II - K5*	-	0	*fimH, iucD, KpsM II, KpsM II - K2, KpsM II - K5, iutA, hlyF*	**SHV-12** - TEM	0	AMP, AMC, CFZ, CAZ, CTX, ATM, GEN, TOB, DOX, SXT, CIP, NAL
	Ch28.V.e	**B1**	O86:H51	1016	155	32	4-32	-	-	0	NR	**SHV-12** - TEM	0	AMP, AMC, CFZ, CAZ, ATM, GEN, TOB, DOX, SXT, CIP, NAL
<10	Ch29.I.R	**A**	O113:H4	10	10	54	11-54	-	-	0	NR	0	0	GEN, TOB, CIP, NAL
	Ch29.II.d	**B2**	O25:H4	131	131	22	40-22	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, iutA, tsh, hlyF, ompT, iss*	0	0	NAL

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CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
10	Ch31.I.R	**A**	ONT:H7	5826	None	60	4-60	-	-	0	NR	0	0	AMP
	Ch31.II.a	**B2**	O15:H5	355	73	154	24-154	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	GEN, DOX, NAL
	Ch31.II.j	**B2**	O2:H5	355	73	154	24-154	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	TEM	0	DOX, NAL
	Ch31.V.a	**A**	O18:H11/H21	93	168	neg	11-neg	*iutA, KpsM II - K5*	-	0	*iucD, KpsM II, KpsM II- K2, KpsM II- K5, iuta, ompT*	**SHV-12**	0	AMP, AMC, CFZ, CXM, CAZ, CTX, ATM, CHL, NAL
<10	Ch32.I.R	**B1**	O8:H19	162	469	27	65-27	*iutA*	*vat*	0	NR	**TEM**	0	AMP, GEN, TOB, NAL
	Ch32.V.a	**B1**	O45:H8	297	None	38	65-38	*iutA*	-	0	NR	**SHV-12**	0	AMP, CFZ
20	Ch33.I.R	**B1**	O8:H19	162	469	38	65-38	*iutA*	-	0	NR	**0**	0	AMP, NAL
	Ch33.V.a	**A**	O8:H8	STnew6	None	1319	new-1319	*papAH, iutA*	*fyuA*	0	*fimH, papC, papAH, papEF, iucD, iroN, cvaC, traT, iutA, hlyF, ompT, iss*	**SHV-12 - TEM**	0	AMP, CXM, CAZ, ATM, CHL, SXT
	Ch33.V.b	**A**	O8:H8	STnew6	None	1319	new-1319	*papAH, iutA*	*fyuA*	0	*fimH, papC, papAH, papEF, iucD, iroN, cvaC, traT, iutA, hlyF, ompT, iss*	**SHV-12 - TEM**	0	AMP, CAZ, ATM, CHL, SXT
	Ch33.V.c	**B1**	O8:H19	162	469	32	65-32	*iutA*	-	0	NR	**SHV-12**	0	AMP, CAZ, ATM, CHL, SXT, CIP, NAL
80	Ch34.I.R	**E**	O45:HNM	371	350	142	31-142	*iutA*	*chuA*	0	NR	0	0	GEN, NAL
	Ch34.I.a	**A**	O5:H10	93	168	neg	11-neg	*iutA, KpsM II*	*chuA*	0	NR	0	0	GEN, CIP, NAL
	Ch34.V.a	**Clade I**	O1:H45	770	None	552	116-552	*iutA, KpsM II - K5*	-	0	*fimH, iucD, KpsM II, KpsM II - K2, KpsM II - K5, traT, malX, iutA, ompT*	**CTX-M-9**	0	AMP, CFZ, CXM, CTX, ATM, GEN, CIP, NAL
50	Ch35.I.R	**G**	O143:H4	117	None	97	45-97	*iutA*	*chuA, fyuA*	0	*fimH, iucD, traT, malX, iutA,tsh, hlyF, ompT, iss*	0	0	AMP
	Ch35.I.e	**A**	O7:H4	1594	168	31	11-31	*iutA, KpsM II*	*chuA*	0	NR	0	0	-
	Ch35.V.a	**G**	O132:H4	117	None	97	45-97	*iutA*	*chuA, vat*	0	*fimH, iucD, iroN, cvaC, traT, malX, iutA, hlyF, ompT, iss*	**SHV-12**	0	AMP, CFZ, CAZ, ATM, CHL, NAL
	Ch35.V.b	**A**	O88:H7	4980	None	39	153-39	-	-	0	NR	**TEM-52**	0	AMP, CFZ, CXM, CTX, DOX, SXT, NAL
	Ch35.V.c	**B1**	O51:H21	101	101	86	41-86	*iutA*	-	0	NR	**CTX-M-1 - TEM**	0	AMP, AMC, CFZ, CXM, CTX, NAL
	Ch35.V.d	**A**	ONT:H23	707	None	60	41-60	*iutA*	-	0	NR	**CTX-M-1 - TEM**	0	AMP, CFZ, CXM, CTX, DOX, CIP, NAL

CFU/g[1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH[6]	Clonotype[6]	ExPEC[7]	UPEC[8]	*eae*[9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
20	Ch36.I.R	**A**	O153:H10	10	10	54	11-54	-	*fyuA*	**1***	*fimH, fimAV, papC, papEF, papGII, traT*	0	0	AMP, DOX, CHL, CIP, NAL
	Ch36.V.a	**A**	O11:NHM	93	168	41	11-41	*iutA, KpsM II - K5*	*chuA*	0	*fimH, fimAv, iucD, KpsM II, KpsM II - K2, KpsM II - K5, traT, iutA, hlyF*	**SHV-12**	0	AMP, CFZ, CHL, NIT, CIP, NAL
<10	Ch37.I.R	**C**	O78:HNM	23	23	35	4-35	*iutA*	*vat, fyuA*	0	NR	0	0	AMP, NAL
	Ch37.II.c	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, SXT, CIP, NAL
	Ch37.II.i	**B2**	O2:H5	355	73	154	24-154	*iutA, KpsM II - K1*	*chuA, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	0	0	-
<10	Ch38.I.R	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, SXT, CIP, NAL
	Ch38.II.j	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, SXT, CIP, NAL
<10	Ch39.I.h'	**B2**	O25:H4	131	131	22	40-22	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	NAL
	Ch39.V.a	**B1**	O8:H51	4162	155	38	4-38	*iutA*	*vat*	0	NR	**SHV-12**	0	AMP, CFZ, ATM
<10	Ch40.I.R	**A**	O145:H40	752	10	24	11-24	-	-	**1***	*fimH, traT*	TEM	0	AMP, NAL
	Ch40.I.g	**B2**	O19:HNT	1618	73	9	24-9	*iutA, KpsM II - K5, sfa/foc*	*chuA, vat, fyuA, yfcV*	0	*fimH, sfa/foc, cdtB, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, usp, iutA, hlyF, ompT, iss*	TEM	0	AMP, GEN, TOB
	Ch40.V.a	**Clade I**	O153:HNM	770	None	552	116-552	*iutA, KpsM II - K5*	*fyuA*	0	*fimH, iucD, KpsM II, KpsM II - K2, KpsM II - K5, traT, malX, iutA, hlyF, ompT*	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, CHL, NAL
<10	Ch41.II.g	**D**	O15:H6	69	69	27	35-27	*iutA, KpsM II*	*chuA, fuyA*	0	NR	TEM	0	AMP, NAL
	Ch41.V.a	**B1**	O8:H51	155	155	32	4-32	-	*chuA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, CHL, CIP, NAL
	Ch41.V.b	**Clade I**	O1:H45	770	None	552	116-552	*iutA, KpsM II - K5*	*chuA*	0	*fimH, iucD, KpsM II, KpsM II - K2, KpsM II - K5, traT, malX, iutA, ompT*	**SHV-12**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, CHL, CIP, NAL
	Ch41.V.c	**B1**	O8:H51	155	155	32	4-32	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, ATM, CHL, CIP, NAL
60	Ch42.I.R	**C**	O162:H42	295	23	38	4-38	-	*fyuA*	0	NR	TEM	0	AMP, NAL
30	Ch43.I.R	**B1**	O7:HNT	1730	None	32	69-32	-	-	0	NR	0	0	AMP, CHL

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CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	Ch43.II.b	**B2**	O2:HAA	135	None	2	39-2	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAv, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, traT, ibeA, malX, usp, iuta, tsh, hlyF, ompT, iss*	TEM	0	AMP
	Ch43.II.e	**E**	O73:H34	1158	31	47	3-47	*iutA, KpsM II*	*chuA*	0	NR	TEM	0	AMP, GEN, TOB, SXT, CIP, NAL
	Ch43.II.f	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	**SHV-12** - TEM	0	AMP, CFZ, CAZ, CTX, ATM, SXT
	Ch43.V.a	**E**	ONT:H25	57	350	27	31-27	*iutA*	*chuA*	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, ATM, CHL
	Ch43.V.b	**G**	O57:HNM	117	None	97	45-97	*iutA*	*chuA, vat, fyuA*	0	*fimH, cdtB, iucD, iroN, traT, malX, iutA, tsh, hlyF, ompT, iss*	**SHV-12**	0	AMP, CFZ, CAZ, ATM, DOX, CHL
480	Ch44.I.d	**B2**	O50/O2:H4	95	95	27	38-27	*papAH, iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, papC, papAH, papEF, papGII, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	CIP, NAL
	Ch44.II.g	**E**	O73:H34	1158	31	47	3-47	*iutA, KpsM II*	*chuA*	0	NR	TEM	0	AMP, DOX, SXT, CIP, NAL
	Ch44.II.h	**B2**	O15:HNM	95	95	27	38-27	*papAH, iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, papC, papAH, papEF, papGII, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	CIP, NAL
	Ch44.V.a	**Clade I**	O1:HNT	770	None	552	116-552	*iutA, KpsM II - K5*	*chuA*	0	*fimH, iucD, KpsM II, KpsM II - K2, KpsM II - K5, traT, malX, iutA*	**CTX-M-9**	0	AMP, CFZ, CXM, CTX, GEN, CIP, NAL
480	Ch45.I.R	**C**	O60:H9	23	23	35	4-35	*iutA*	-	0	NR	TEM	0	AMP
	Ch45.I.p	**F**	O174:H42	648	648	58	4-58	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP
	Ch45.I.t	**E**	O20:H34	STnew8 (ST1158-like)	31	47	3-47	*iutA, KpsM II*	*chuA*	0	NR	TEM	0	AMP, GEN, TOB, CIP, NAL
	Ch45.V.a	**A**	O18:NHM	226	226	41	27-41	-	*fyuA*	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, ATM, DOX
	Ch45.V.c	**C**	O20:H9	410	23	24	4-24	*iutA*	*fyuA*	0	NR	**CTX-M-14**	0	AMP, CFZ, CXM, CTX, GEN, TOB, DOX, CHL, SXT, CIP, NAL
<10	Ch46.I.R	**E**	O7:H15	38	38	65	26-65	-	*chuA*	0	NR	0	0	AMP, GEN, TOB
	Ch46.II.c	**B1**	O159:H11/ H21	641	86	25	6-25	-	*chuA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, SXT, NAL
	Ch46.V.a	**B1**	O159:H11/ H21	641	86	25	6-25	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, DOX, SXT, NAL

CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
<10	Ch47.I.R	**F**	O53:HNM	STnew (ST117-like)	None	97	New-97	*iutA*	*chuA, vat, fyuA*	0	*fimH, iucD, iroN, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, GEN, DOX, NAL
	Ch47.I.a	**A**	O5:H10	93	168	neg	11-neg	*iutA, KpsM II*	*chuA*	0	NR	0	0	GEN, CIP, NAL
	Ch47.I.o	**A**	O5:H10	93	168	neg	11-neg	*iutA, KpsM II*	-	0	NR	0	0	GEN, CIP, NAL
	Ch47.V.a	**A**	O39:H48	3519	None	39	8-39	-	-	0	NR	**CTX-M-32**	0	AMP, CFZ, CXM, CTX, DOX, CIP, NAL
	Ch47.V.b	**A**	O39:H48	3519	None	31	8-31	-	-	0	NR	**CTX-M-32**	0	AMP, CFZ, CXM, CTX, DOX, CIP, NAL
120	Ch48.I.R	**C**	O8:H9	23	23	35	4-35	*iutA*	-	0	NR	TEM	0	AMP, CHL
	Ch48.I.m	**A**	O162:H3	744	10	58	11-58	*iutA*	*fyuA*	0	NR	TEM	0	AMP, GEN, TOB, DOX, SXT, CIP, NAL
	Ch48.I.o	**A**	O21:H6	48	10	23	11-23	*iutA, KpsM II*	-	0	NR	TEM	0	AMP, CHL, SXT, CIP, NAL
	Ch48.V.a	**A**	O18:HNM	226	226	41	27-41	-	*fyuA*	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, ATM, DOX
	Ch48.V.b	**A**	O127:H21	10	10	54	11-54	-	*fyuA*	0	*fimH*	**SHV-12**	0	AMP, CFZ, CXM, CAZ, CTX, FOX, ATM, DOX, CHL, NAL
30	Ch49.I.R	**B1**	O103:H21	101	101	86	41-86	*iutA*	-	0	NR	0	0	AMP
	Ch49.I.c	**D**	O44:H45	4243	None	1002	3-1002	*iutA, KpsM II*	*chuA*	0	NR	0	0	NAL
	Ch49.V.a	**A**	O162:H9	617	10	neg	11-neg	*iutA*	*fyuA*	0	NR	**CTX-M-15**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, DOX, SXT, CIP, NAL
	Ch49.V.b	**A**	O88:HNM	1970	10	54	11-54	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM
	Ch49.V.d	**B1**	O5:H11	155	155	121	4-121	-	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, CTX, ATM, GEN, TOB
80	Ch50.I.R	**E**	O99:H15	38	38	65	26-65	-	*fyuA*	0	*fimH, traT, hlyF*	TEM	0	AMP, DOX, SXT, NAL
	Ch50.II.a	**B2**	O25:H4	131	131	22	40-22	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	GEN, NAL
	Ch50.II.d	**E**	ONT:HNM	1158	31	47	3-47	*iutA, KpsM II*	*chuA*	0	*KpsM II, iuta*	TEM	0	AMP, GEN, DOX, CIP, NAL

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CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
510	T1.II.h	**B2**	O2:H5	355	73	154	24-154	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	0	0	DOX, CHL, NAL
	T1.IV.d	**B2**	O2:H5	140	95	15	38-15	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAv, iucD, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iutA, hlyF, ompT*	TEM	0	AMP, DOX, SXT
	T1.IV.e	**B1**	O109:H51	155	155	32	4-32	-	-	0	*fimH, iroN, traT, hlyF, iss*	TEM	**mcr1.1**	AMP, GEN, DOX, CHL, CIP, NAL
	T1.V.e	**A**	O162/O89:H9	744	10	54	11-54	*iutA*	-	0	*fimH, fimAv, iucD, iroN, cvaC, traT, iutA, tsh, hlyF, iss*	SHV-12	**mcr1.1**	AMP, CFZ, CAZ, CTX, ATM, CST, DOX, CHL, SXT, CIP, NAL
	T1.V.d	**B1**	O8:HNT	NR	NR	NR	NR	-	-	0	NR	SHV-12 - TEM	0	AMP, CFZ, CAZ, ATM, DOX, CHL, CIP, NAL
	T1.V.a	**D**	OAA:H23	NR	NR	NR	NR	-	*chuA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, DOX, CHL, CIP, NAL
90	T2.I.R	**A**	O16:HNM	10	10	54	11-54	-	-	0	NR	0	0	AMP, SXT
	T2.II.b	**B1**	O2:H34	STnew10 (ST297-like)	None	276	65-276	*iutA, KpsM II*	*chuA, fuyA*	0	NR	TEM	0	AMP, DOX, SXT, CIP, NAL
	T2.II.d	**B2**	O46:H31	569	None	5	38-5	*KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, usp, hlyF, ompT, iss*	TEM	0	DOX, NAL
	T2.V.a	**B1**	O19:H21	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, ATM, DOX, SXT, CIP, NAL
100	T3.I.R	**B1**	ONT:H2	2599	None	32	6-32	*iutA*	-	0	NR	TEM	0	AMP, SXT, CIP, NAL
	T3.I.a	**B1**	ONT:H2	2599	None	32	6-32	*iutA*	-	0	NR	TEM	0	AMP, SXT, CIP, NAL
	T3.V.c	**A**	ONT:H9	NR	NR	NR	NR	*iutA*	*fyuA*	0	NR	**CTX-M-15**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, DOX, CHL, CIP, NAL
	T3.V.a	**B1**	ONT:HNM	NR	NR	NR	NR	-	-	0	NR	**CTX-M-1** - TEM	0	AMP, CFZ, CXM, CTX, ATM, DOX, CHL, SXT, NAL
40	T4.I.R	**C**	O8:H4	88	23	39	4-39	*iutA*	-	0	NR	TEM	0	AMP, GEN, SXT, CIP, NAL
	T4.I.e	**B2**	O2:HNM	355	73	154	24-154	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	**SHV-12** - TEM	0	AMP, CFZ, ATM, DOX, NAL
	T4.V.c	**B1**	O20:HNM	NR	NR	NR	NR	-	-	0	NR	SHV-12 - TEM	0	AMP, CFZ, CAZ, ATM, DOX, CHL, CIP, NAL

CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	T4.V.a	**F**	O153:HNM	354	354	neg	88-neg	*KpsM II - K2*	*chuA, yfcV*	0	*fimH, KpsM II, KpsM II - K2, traT, ibeA, malX, usp*	**SHV-12**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, CHL, CIP, NAL
70	T5.I.R	**B1**	O9:HNM	162	469	32	65-32	*iutA*	*fyuA*	0	NR	TEM	0	AMP, DOX, CIP, NAL
	T5.II.a	**A**	O51:H52	93	168	neg	11-neg	*iutA, KpsM II*	-	0	NR	TEM	0	AMP, SXT, CIP, NAL
	T5.V.a	**E**	O119:HNT	NR	NR	NR	NR	-	*chuA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, SXT, CIP, NAL
	T5.V.b	**E**	O40/O8:H45	1011	None	31	4-31	-	*chuA*	0	*fimH, iroN, traT, hlyF, iss*	**SHV-2** - TEM	0	AMP, AMC, CFZ, CXM, CTX, DOX, SXT, CIP, NAL
180	T6.I.R	**B1**	ONT:H4	58	155	32	4-32	*iutA*	*fyuA*	0	NR	0	0	SXT
	T6.II.b	**F**	O171:H4	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	0	0	AMP, SXT, CIP, NAL
	T6.V.b	**A**	ONT:H10	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, DOX, CHL, NAL
	T6.V.a	**B1**	O19:H21	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, ATM, DOX, SXT, CIP, NAL
<10	T7.I.R	**B1**	O9:H53	345	None	31	4-31	-	-	0	NR	0	0	AMP, CIP, NAL
	T7.I.i	**A**	O9:H11	48	10	400	11-400	-	*fyuA*	0	NR	0	0	-
	T7.V.a	**A**	ONT:H9	NR	NR	NR	NR	*iutA*	*fyuA*	0	NR	**CTX-M-15**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, DOX, CHL, CIP, NAL
	T7.V.b	**B1**	ONT:HNM	NR	NR	NR	NR	-	-	0	NR	**CTX-M-1** - TEM	0	AMP, CFZ, CXM, CTX, DOX, CHL, SXT, NAL
20	T8.I.R	**B1**	ONT:H7	3580	None	32	65-32	*iutA*	-	0	NR	TEM	0	AMP, GEN
	T8.I.d	**B1**	O38:HNM	453	86	31	6-31	*iutA, KpsM II*	*fyuA*	0	NR	TEM	0	AMP, CIP, NAL
	T8.V.a	**A**	O101:HNM	NR	NR	NR	NR	*iutA*	*fyuA*	0	NR	**CTX-M-15**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, DOX, CHL, CIP, NAL
	T8.V.b	**B1**	O19:H21	NR	NR	NR	NR	*iutA*	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, DOX, SXT, CIP, NAL

CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
250	T9.I.R	**B1**	O29:H10	1720	86	54	270-54	*sfa/foc*	-	0	NR	TEM	0	AMP, DOX, SXT
	T9.I.h	**B1**	O9:H25	NR	NR	NR	NR	*iutA*	*fyuA*	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, CTX, ATM, DOX, CHL, SXT
	T9.II.a	**B1**	O9:HNT	NR	NR	NR	NR	*iutA*	*chuA*	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, ATM, GEN, CHL, CIP, NAL
	T9.II.b	**B2**	O101:H4	6876	None	neg	925-neg	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAv, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	CHL
	T9.II.h	**B2**	O2:H5	355	73	154	24-154	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	TEM	0	AMP, NAL
	T9.V.a	**B1**	O51:HNM	NR	NR	NR	NR	*sfa/foc*	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, SXT, CIP, NAL
	T9.V.b	**B1**	O9:HNM	NR	NR	NR	NR	*iutA*	*fyuA*	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, CTX, ATM, DOX, CHL, SXT
	T9.V.e	**G**	O118:H4	117	None	97	45-97	*iutA*	*chuA, vat, fyuA*	0	*fimH, cdtB, iucD, iroN, traT, malX, iutA, tsh, hlyF, ompT, iss*	**SHV-12**	0	AMP, CFZ, CXM, CAZ, CTX, ATM, DOX, CHL
300	T10.I.e	**A**	O51:H52	93	168	neg	11-neg	*iutA, KpsM*	-	0	NR	TEM	0	AMP, NAL
	T10.V.a	**B1**	O83:H23	NR	NR	NR	NR	-	-	0	NR	**CTX-M-32** - TEM	0	AMP, CFZ, CXM, CTX, DOX, CIP, NAL
200	T11.I.R	**B1**	O9:H19	162	469	32	65-32	*iutA*	-	0	NR	0	0	SXT, CIP, NAL
	T11.I.b	**B1**	O9:H19	162	469	32	65-32	*iutA, KpsM II*	*fyuA*	0	NR	TEM	0	AMP, SXT, CIP, NAL
	T11.II.a	**E**	O68:HNM	115	38	270	26-270	*iutA, KpsM II*	*chuA, fuyA*	0	NR	TEM	0	AMP, CIP, NAL
	T11.II.c	**B1**	O9:H19	162	469	32	65-32	*iutA, KpsM II*	*chuA, fuyA*	0	NR	TEM	0	AMP, DOX, SXT, CIP, NAL
	T11.V.a	**C**	OAA:H28	NR	NR	NR	NR	-	-	0	NR	**SHV-12** - TEM	0	AMP, AMC, CFZ, CAZ, CTX, ATM, DOX, CIP, NAL
440	T12.I.R	**A**	ONT:HNM	STnew2	10	neg	11-neg	-	-	0	NR	TEM	0	AMP, CHL, SXT, CIP, NAL
	T12.I.j	**C**	O8:H4	NR	NR	NR	NR	*papAH*	*fyuA*	0	*fimH, papC, papAH, papEF, traT, ompT*	0	0	AMP, AMC, GEN, TOB, DOX, CHL, SXT, CIP, NAL
	T12.V.a	**A**	O16:HNM	NR	NR	NR	NR	-	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, DOX, SXT
	T12.V.d	**A**	O16:HNM	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CFZ, ATM, DOX, SXT

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CFU/g[1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH[6]	Clonotype[6]	ExPEC[7]	UPEC[8]	*eae*[9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
210	T13.I.R	**B1**	O29:H9	155	155	32	4-32	-	-	0	NR	TEM	0	AMP, CIP, NAL
	T13.I.e	**Clade I**	ONT:H1	770	None	552	116-552	*iutA, KpsM II - K5*	*chuA, fuyA*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, traT, malX, iutA, hlyF, ompT, iss*	TEM	0	AMP, CHL, NAL
	T13.II.a	**A**	O105:H32	10	10	23	11-23	-	-	0	*fimH, iroN, hlyF, ompT, iss*	TEM	**mcr1.1**	AMP, DOX, CHL, NAL
	T13.II.f.1	**D**	O15:HNT	69	69	27	35-27	-	*chuA, fuyA*	0	NR	TEM	0	AMP, CHL, NAL
	T13.II.f.2	**B2**	O15:H5	355	73	154	24-154	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	TEM	0	AMP, AMC, NAL
	T13.II.h	**D**	O15:H6	69	69	27	35-27	-	*chuA, fuyA*	0	*fimH, iroN, traT, hlyF, ompT, iss*	TEM	**mcr1.1**	AMP, DOX, CHL, NAL
	T13.V.a	**B1**	O37:H10	NR	NR	NR	NR	-	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, CTX, ATM, DOX, CHL, CIP, NAL
	T13.V.b	**G**	O143:HNT	117	None	97	45-97	*iutA*	*chuA, fuyA*	0	*fimH, iucD, traT, malX, iutA, tsh, hlyF, ompT, iss*	**CTX-M-1**	0	AMP, CFZ, CXM, CTX, ATM, DOX
150	T14.I.R	**B2**	O120:H4	428	None	22	40-22	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAv, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	TEM	0	AMP, SXT
	T14.I.b	**C**	O8:H4	88	23	39	4-39	*papAH, iutA*	*fyuA*	0	*fimH, papC, papAH, papEF, iucD, iroN, cvaC, traT, iutA, hlyF, ompT, iss*	0	0	AMP, AMC, GEN, TOB, DOX, CHL, SXT, CIP, NAL
	T14.I.f	**C**	O8:H4	88	23	39	4-39	*papAH, iutA*	*fyuA*	0	*fimH, fimAV, papC, papAH, papEF, cdtB, iucD, iroN, cvaC, traT, iutA, hlyF, ompT, iss*	0	0	AMP, AMC, GEN, TOB, DOX, CHL, SXT, CIP, NAL
70	T15.I.R	**C**	O86:H9	410	23	24	4-24	*iutA*	-	0	NR	TEM	0	AMP, DOX, CIP, NAL
	T15.IV.b	**G**	O33:H4	117	None	97	45-97	*papAH, iutA*	*chuA, vat, fyuA*	0	*fimH, papC, papAH, papEF, iucD, iroN, traT, mlaX, iutA, hlyF, ompT, iss*	TEM	0	AMP, DOX, SXT, CIP, NAL
	T15.IV.j	**E**	O111:H45	NR	NR	31	11-31	*iutA, KpsM II*	*chuA*	0	NR	TEM	0	AMP, SXT, CIP, NAL
	T15.V.a	**B1**	O20:HNM	NR	NR	NR	NR	*iutA*	-	0	NR	**CTX-M-15** - TEM	0	AMP, CFZ, CXM, CAZ, CTX, ATM, DOX, CIP, NAL
100	T16.I.R	**A**	O18:H25	10	10	54	11-54	*iutA*	-	0	*fimH, fimAv, iucD, iutA, iss*	TEM	0	AMP, CHL, CIP, NAL
	T16.I.a	**A**	O11:HNM	93	168	41	11-41	*iutA, KpsM II*	-	0	NR	TEM	0	AMP, DOX, NIT, SXT, CIP, NAL
	T16.II.m	**B1**	O21:HAA	101	101	86	41-86	-	-	0	*fimH, traT, ompT*	TEM	mcr	AMP, DOX, CHL, SXT

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CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	T16.IV.c	**B1**	O21:H21	101	101	86	41-86	-	-	0	*fimH, traT, ompT*	TEM	**mcr1.1**	AMP, CST, DOX, CHL, SXT
	T16.IV.f	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, DOX, SXT, CIP, NAL
60	T17.I.R	**A**	O162/O89:H37	853	10	54	11-54	*iutA*	-	0	*fimH, fimAv, iucD, iroN, traT, iutA, hlyF, ompT, iss*	TEM	**mcr1.1**	AMP, CST, SXT
	T17.I.a	**A**	O101:HNM	853	10	54	11-54	*iutA*	-	0	*fimH, fimAv, iucD, iroN, traT, iutA, hlyF, ompT, iss*	TEM	mcr	AMP, CST, SXT
	T17.I.b	**A**	O101:HNM	853	10	54	11-54	*iutA*	-	0	*fimH, fimAv, iucD, iroN, traT, iutA, hlyF, ompT, iss*	TEM	mcr	AMP, CST, SXT
	T17.I.h	**F**	O153:H34	354	354	58	88-58	*iutA, KpsM II - K1*	*chuA, vat, yfcV*	0	*fimH, papEF, iucD, iroN, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iuta, hlyF, iss*	TEM	0	AMP, DOX, SXT, CIP, NAL
	T17.II.a	**A**	O7:HNT	484	168	neg	11-neg	*iutA, KpsM II*	*chuA*	0	NR	TEM	0	AMP, SXT
	T17.V.a	**A**	O8:HAA	NR	NR	NR	NR	-	-	0	NR	**TEM-52**	0	AMP, CFZ, CAZ, CTX, ATM, DOX
	T17.V.b	**A**	O8:20	NR	NR	NR	NR	-	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, CTX, ATM, DOX
40	T18.I.R	**B1**	O9:HNM	58	155	32	4-32	-	*fyuA*	0	NR	TEM	0	AMP, GEN
	T18.II.a	**F**	O2:H42	648	648	58	4-58	*iutA, KpsM II - K5*	*chuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, hlyF, iss*	0	0	-
	T18.II.d	**A**	O57:HNT	189	165	NR	NR	-	*fyuA*	**1***	*fimH, fimAv, iucD, iroN, cvaC, traT, usp, hlyF, iss*	TEM	0	AMP, GEN, TOB, DOX, SXT, CIP, NAL
	T18.II.j	**A**	O21:H16	93	168	47	11-47	*iutA, KpsM II - K5*	-	0	*fimH, iucD, KspM II, KpsM II - K2, KspM II - K5, traT, iutA*	TEM	0	AMP, GEN, TOB, CIP, NAL
	T18.V.a	**A**	ONT:H9	NR	NR	NR	NR	*iutA*	*vat*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, ATM, DOX, CHL, CIP, NAL
90	T19.I.R	**C**	O86:H9	410	23	24	4-24	*iutA*	-	0	NR	TEM	0	AMP, CIP, NAL
60	T20.I.R	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, SXT, NAL
	T20.I.a	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, SXT, NAL
	T20.V.a	**A**	O101:H9	NR	NR	NR	NR	*iutA*	*vat*	0	NR	**CTX-M-14**	0	AMP, CFZ, CXM, CTX, DOX, CHL, CIP, NAL
	T20.V.c	**A**	O81:23	NR	NR	NR	NR	-	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, ATM, DOX, SXT, CIP, NAL

CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	T20.V.d	**A**	O172:23	NR	NR	NR	NR	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, CIP, NAL
130	T21.I.R	**B1**	O149:H45	297	None	38	65-38	*iutA*	*vat*	0	NR	0	0	AMP
	T21.II.h	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, GEN, TOB, CHL, SXT, CIP, NAL
	T21.V.a	**E**	O53:H51	997	None	31	23-31	-	*chuA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, ATM, GEN, TOB, CHL, SXT, CIP, NAL
80	T22.I.R	**B2**	O1:H7	95	95	30	38-30	*papAH, iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, papC, papAH, papEF, papGII, cdtB, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, malX, usp, iutA, hlyF, ompT, iss*	0	0	AMP, NAL
	T22.III.q	**C**	O8:H4	88	23	39	4-39	*papAH, iutA*	*fyuA*	0	*fimH, papC, papAH, papEF, iucD, iroN, cvaC, traT, iutA, hlyF, ompT, iss*	0	0	AMP, AMC, DOX, CHL, SXT, CIP, NAL
150	T24.I.R	**A**	O20:HNM	48	10	neg	11-neg	-	-	0	NR	**TEM**	0	AMP, SXT
	T24.II.b	**A**	ONT:H10	34	10	neg	11-neg	*iutA*	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, CHL, NAL
	T24.II.f	**B2**	O50/O2:H5	140	95	15	38-15	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAv, iucD, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iutA, hlyF, ompT*	TEM	**mcr1.1**	AMP, GEN, CHL
	T24.II.i	**B2**	O50/O2:H5	140	95	15	38-15	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAv, iucD, KpsM II, KpsM II - K2, neuC, traT, ibeA, malX, usp, iutA, hlyF, ompT*	TEM	**mcr1.1**	AMP, GEN, TOB, CHL
	T24.V.a	**E**	O83:HNM	57	350	31	31-31	*iutA*	*chuA, fuyA*	0	NR	**SHV-12 - TEM**	0	AMP, CFZ, CAZ, CTX, ATM, DOX, CHL, SXT
	T24.V.b	**B2**	O50/O2:H6	141	None	14	52-14	*iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	**SHV-12**	0	AMP, CFZ, CAZ, ATM, DOX, CHL
	T24.V.c	**F**	O153:H34	354	354	58	88-58	*KpsM II - K5, sfa/foc*	*chuA, yfcV*	0	*fimH, sfa/foc, KpsM II, KpsM II - K2, KpsM II - K5, traT, ibeA, malX, usp*	**SHV-12**	0	AMP, CFZ, ATM, CHL, CIP, NAL
	T24.V.d	**A**	ONT:H9	744	10	54	11-54	-	-	0	NR	**SHV-12 - TEM**	0	AMP, CFZ, CAZ, ATM, DOX, CHL, CIP, NAL
420	T25.I.R	**B1**	O8:HNM	58	155	32	4-32	*iutA*	*vat, fyuA*	0	NR	0	0	AMP
	T25.V.a	**E**	O119:HNT	350	350	54	31-54	*iutA*	*chuA, vat*	0	NR	**SHV-12 - TEM**	0	AMP, CFZ, CAZ, CTX, ATM, SXT, CIP, NAL
20	T26.I.R	**B1**	O9:H12	58	155	27	4-27	*iutA*	*vat, fyuA*	0	NR	0	0	AMP
	T26.I.a	**F**	O1:H42	648	648	58	4-58	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	0	0	NAL

CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	T26.I.g	**B2**	O25:H4	131	131	22	40-22	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	-
	T26.IV.a	**A**	O18:H11/2 1/47	93	168	neg	11-neg	*iutA, KpsM II*	-	0	NR	**SHV-12**	0	AMP, CHL, NAL
	T26.IV.h	**D**	ONT:H45	4243	None	1002	3-1002	*iutA, KpsM II*	*chuA*	0	NR	0	0	GEN, NAL
	T26.V.a	**A**	O18:H11/2 1/47	93	168	neg	11-neg	*iutA, KpsM II - K5*	-	0	*iucD, KpsM II, KpsM II- K2, KpsM II- K5, iuta, ompT*	**SHV-12**	0	AMP, CFZ, CHL, NAL
220	T27.I.R	**B1**	O48:H30	58	155	neg	4-neg	*iutA*	-	0	NR	**TEM**	0	AMP, CHL, SXT
	T27.V.a	**B1**	O86:H51	155	155	32	4-32	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, ATM, CHL
	T27.V.c	**E**	O126:HNT	350	350	54	31-54	*iutA*	*chuA, fyuA*	0	NR	**SHV-12 - TEM**	0	AMP, CFZ, CAZ, ATM, GEN, DOX, CIP, NAL
70	T28.II.c	**F**	O1:H42	648	648	58	4-58	*iutA, KpsM II - K5*	*chuA, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	0	0	NAL
	T28.II.g	**F**	O1:H42	648	648	58	4-58	*iutA, KpsM II - K5*	*chuA, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	0	0	NAL
	T28.V.a	**A**	O101:H9	10	10	54	11-54	-	*fyuA*	0	-	**SHV-12 - TEM**	0	AMP, AMC, CAZ, CTX, ATM, CHL, CIP, NAL
20	T29.I.R	**A**	O101:HNM	744	10	54	11-54	*iutA*	-	0	NR	TEM	0	AMP, DOX, CHL, CIP, NAL
<10	T30.I.R	**E**	ONT_H25	57	350	27	31-27	-	*chuA, fyuA*	0	NR	0	0	-
	T30.II.b	**E**	O25:HNM	115	38	270	26-270	*iutA, KpsM II*	*chuA, fuyA*	0	NR	TEM	0	AMP, CIP, NAL
350	T31.I.R	**A**	O101:HNM	744	10	54	11-54	*iutA*	*vat*	0	NR	TEM	**0**	AMP, DOX, CHL, SXT, CIP, NAL
	T31.I.b	**A**	O148:H30	522	522	neg	23-neg	-	-	0	*iroN, traT, hlyF*	TEM	**mcr1.1**	AMP, CFZ, DOX, NIT
	T31.V.a	**A**	O33:HNM	7315	None	398	11-398	-	-	0	NR	**SHV-12 - TEM**	0	AMP, CFZ, CAZ, ATM, CIP, NAL
100	T32.I.R	**A**	O6:H10	43	10	54	11-54	-	*fyuA*	0	NR	**0**	0	AMP
	T32.V.a	**Clade I**	O1:H45	770	None	552	116-552	*iutA, KpsM II - K5*	-	0	*fimH, iucD, KpsM II, KpsM II - K2, KpsM II - K5, traT, malX, iutA, ompT*	**SHV-12**	0	AMP, CFZ, CAZ, ATM, CHL, CIP, NAL
	T32.V.b	**A**	O101:HNM	617	10	neg	11-neg	-	-	0	NR	**CTX-M-14**	0	AMP, CFZ, CXM, CTX, DOX, CIP, NAL

CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	T32.V.e	**A**	O154:H28	10	10	54	11-54	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CHL, CIP, NAL
30	T33.I.R	**E**	O7:H15	38	38	65	26-65	*KpsM II*	*chuA*	0	NR	TEM	0	AMP, DOX
	T33.I.d	**F**	O11:H25	1674	None	138	88-138	*iutA, KpsM II - K5*	*chuA, yfcV*	0	*fimH, fimAv, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iuta, hlyF, iss*	TEM	0	AMP
	T33.I.f	**B2**	O1:H17	STnew9	None	664	new-664	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, traT, malX, iutA, hlyF, ompT, iss*	TEM	0	AMP
	T33.V.a	**A**	ONT:H4	1141	10	32	11-32	-	*fyuA*	0	NR	**CTX-M-14**	0	AMP, CFZ, CXM, CTX
	T33.V.b	**C**	O9:H17	88	23	39	4-39	-	-	0	*fimH, papEF, traT, ompT*	**CTX-M-14/OXA-1**	0	AMP, AMC, CFZ, CXM, CTX, GEN, TOB, DOX, CHL, SXT, CIP, NAL
<10	T34.I.R	**B1**	O8:HNT	58	155	32	4-32	*iutA*	*fyuA*	0	NR	TEM	0	AMP, SXT, CIP, NAL
	T34.II.i	**B1**	O23:H16	453	86	31	6-31	*iutA, KpsM II*	-	0	NR	TEM	0	AMP, CIP, NAL
<10	T35.I.R	**A**	O7:H4	93	168	41	11-41	*iutA*	-	0	NR	**TEM**	0	AMP, DOX, CHL, SXT, CIP, NAL
	T35.V.a	**B1**	ONT:H8	366	None	30	4-30	-	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, ATM, DOX, CHL
<10	T36.I.R	**B1**	O8:H25	58	155	32	4-32	-	*fyuA*	0	NR	0	0	AMP, DOX, SXT, CIP, NAL
	T36.I.e	**E**	O7:H6	362	None	96	100-96	*iutA, KpsM II*	*chuA*	0	NR	TEM	0	AMP, CHL, SXT, CIP, NAL
20	T37.I.c	**A**	O11:H52	3764	168	41	482-41	*iutA, KpsM II*	*chuA, fuyA*	0	NR	TEM	0	AMP
	T37.V.a	**A**	O15:H11	48	10	41	11-41	-	-	0	NR	**CTX-M-1**	0	AMP, CFZ, CXM, CTX, SXT, CIP, NAL
30	T38.I.R	**B1**	ONT:H7	3580	None	32	65-32	*iutA*	-	0	NR	0	0	-
	T38.I.g	**B2**	O113:HNT	8611	None	26	24-26	*sfa/foc*	*chuA, vat, fyuA, yfcV*	0	*fimH, sfa/foc, iroN, malX, usp, hlyF, ompT, iss*	TEM	0	AMP
	T38.V.a	**A**	O20:HNM	1564	None	neg	252-neg	*iutA*	*fyuA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM
	T38.V.b	**B1**	O64:HNM	155	155	neg	4-neg	-	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, ATM, DOX, CHL
	T38.V.c	**A**	O9:HNM	STnew7	None	54	7-54	-	*fyuA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, DOX, CHL, NAL
210	T39.I.R	**A**	O101:H9	744	10	54	11-54	*iutA*	*vat*	0	NR	TEM	0	AMP, DOX, CHL, SXT, CIP, NAL

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CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	T39.II.j	**A**	O51:H52	93	168	neg	11-neg	*iutA, KpsM II*	*vat*	0	NR	TEM	0	AMP, SXT, CIP, NAL
	T39.V.a	**E**	O20:HNT	STnew4 (ST350-like)	None	54	31-54	*iutA*	*chuA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, SXT, CIP, NAL
	T39.V.c	**B1**	O37:H21	10328	None	32	4-32	-	-	0	*fimH, traT*	**SHV-12** - TEM	0	AMP, AMC, CFZ, CAZ, CTX, ATM, CIP, NAL
2320	T40.I.R	**F**	O8:HNM	5340	None	58	271-58	*iutA*	*chuA, vat, yfcV*	0	*fimH, iucD, iroN, cvaC, traT, malX, iutA, tsh, hlyF, iss*	TEM	0	AMP, SXT
	T40.I.k	**F**	O11:H6	457	None	145	88-145	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	0	0	-
	T40.V.a	**E**	O102:H45	38	38	65	26-65	*KpsM II - K5*	*chuA, vat*	0	*fimH, iroN, KpsM II, KpsM II - K2, KpsM II - K5, ompT*	**SHV-12**	0	AMP, CFZ, CXM, CTX, ATM, DOX, NAL
	T40.V.b	**B2**	O113:H5	8611	None	26	24-26	-	*chuA, vat, fyuA, yfcV*	0	*fimH, iroN, traT, malX, usp, ompT*	**SHV-12**	0	AMP, CFZ, CAZ, ATM, DOX, CHL, NAL
	T40.V.c	**A**	O9:HNM	6215	None	34	7-34	-	*chuA*	0	NR	**CTX-M-14**	0	AMP, CFZ, CXM, CTX, CHL, CIP, NAL
	T40.V.e	**B1**	O8:HNM	58	155	27	4-27	*iutA*	*vat, fyuA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, ATM, DOX, CHL
<10	T41.I.R	**C**	O20:HNT	410	23	24	4-24	*iutA*	*fyuA*	0	NR	0	0	AMP, CIP, NAL
	T41.I.h	**F**	O102:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, SXT, CIP, NAL
	T41.II.a	**B2**	O1:H7	95	95	30	38-30	*papAH, iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, papC, papAH, papGII, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, malX, usp, iutA, hlyF, ompT, iss*	TEM	0	AMP
	T41.II.e	**B2**	O15:H5	10740	None	9	1544-9	*iutA, KpsM II - K5*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, ibeA, malX, usp, iutA, hlyF, ompT, iss*	0	0	-
	T41.V.a	**B1**	ONT:H8	366	None	30	4-30	-	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, ATM, CHL
680	T42.I.R	**C**	O60:HNT	410	23	53	4-53	-	*fyuA*	0	NR	**TEM**	0	AMP, DOX
	T42.V.a	**B1**	ONT:H21	602	446	86	19-86	*iutA*	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, ATM, GEN, TOB, DOX, SXT, CIP, NAL
	T42.V.b	**A**	O101:H9	10	10	54	11-54	-	*fyuA*	0	NR	**SHV-12** - TEM	0	AMP, AMC, CAZ, ATM, CIP, NAL
<10	T43.I.R	**B1**	O64HNM	155	155	Neg	4-Neg	*iutA*	-	0	NR	**SHV-12**	0	AMP, AMC, CFZ, CAZ, ATM

CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
	T43.I.a	**A**	ONT:HNM	34	10	neg	11-neg	*iutA*	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CXM, CAZ, CTX, ATM, DOX, NAL
	T43.I.g	**B2**	O15:HNM	95	95	27	38-27	*papAH, iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAV, papC, papAH, papEF, papGII, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	-
	T43.V.a	**B1**	O9:HNM	155	155	32	4-32	*iutA*	*fyuA*	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, ATM, CHL, SXT, NAL
	T43.V.b	**F**	O153:H36	354	354	58	88-58	*KpsM II - K2, sfa/foc*	*chuA, yfcV*	0	*fimH, sfa/foc, KpsM II, KpsM II - K2, traT, ibeA, malX, usp*	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, CHL, CIP, NAL
	T43.V.c	**A**	O20:HNM	1785	None	54	168-54	-	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, ATM, NIT
<10	T44.I.R	**A**	O176:H11	48	10	neg	11-neg	-	-	0	NR	TEM	0	AMP, NAL
	T44.II.a	**B2**	O25:H4	131	131	22	40-22	*KpsM II - K5*	*chuA, fyuA, yfcV*	0	*fimH, cdtB, KpsM II, KpsM II- K2, KpsM II - K5, traT, ibeA, malX, usp, ompT*	TEM	0	AMP
	T44.II.d	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, CHL, SXT, CIP, NAL
	T44.III.j	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, SXT, CIP, NAL
	T44.V.a	**B1**	O100:H25	359	101	35	41-35	*iutA*	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, ATM, CHL, SXT, CIP, NAL
10	T45.I.R	**A**	O33:HNM	7315	None	398	11-398	-	-	0	NR	**TEM**	0	AMP, GEN, TOB, CHL, CIP, NAL
	T46.I.R	**C**	O19:HNT	410	23	45	4-45	*iutA*	-	0	NR	**TEM**	0	AMP, CIP, NAL
1130	T46.I.g	**E**	O37:H25	57	350	27	31-27	*iutA*	*chuA, fuyA*	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, CHL, SXT, CIP, NAL
	T46.II.h	**B2**	O25:H4	131	131	22	40-22	*iutA, KpsM II - K1*	*chuA, fyuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, ibeA, malX, usp, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, NAL
	T47.II.a	**A**	O2:H40	10	10	24	11-24	-	-	**1***	*fimH, traT, hlyF*	**CMY-2**	0	AMP, AMC, CFZ, CXM, CAZ, CTX, FOX, SXT, NAL
40	T47.II.f	**A**	O2:H40	10	10	24	11-24	-	-	**1**	*fimH, iucD, iroN, cvaC, traT, hlyF, iss*	**CMY-2**	0	AMP, AMC, CFZ, CXM, CAZ, CTX, FOX, DOX, SXT, CIP, NAL
	T47.V.a	**A**	O101:HNM	167	10	neg	11-neg	-	*fyuA*	0	NR	**CTX-M-1**	0	AMP, CFZ, CXM, CTX, CHL, CIP, NA L

CFU/g [1]	Isolate[2]	PG[3]	Serotype[4]	ST[5]	CC[5]	fimH [6]	Clonotype [6]	ExPEC[7]	UPEC[8]	*eae* [9]	Virulence profile[10]	ESBL /other BL	mcr	Antibiotic profile[11]
20	T48.I.d	**B2**	O2:HNM	95	95	27	38-27	*papAH, iutA, KpsM II - K1*	*chuA, vat, fyuA, yfcV*	0	*fimH, fimAV, papC, papAH, papEF, papGII, iucD, iroN, KpsM II, KpsM II - K2, neuC, cvaC, traT, malX, usp, iutA, tsh, hlyF, ompT, iss*	0	0	-
	T48.I.e	**B2**	O115:HNM	919	None	187	24-187	*iutA*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, traT, ibeA, malX, iutA, hlyF, ompT, iss*	**SHV-12** - TEM	0	AMP, CFZ, CAZ, CTX, ATM, SXT, NAL
	T48.I.j	**D**	O23:H4	1882	None	123	22-123	*iutA, KpsM II*	*chuA*	0	NR	0	0	-
	T48.V.a	**B1**	O64:HNM	155	155	neg	4-neg	-	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CAZ, CTX, ATM, DOX, CHL
	T48.V.a	**B1**	ONT:HNM	155	155	32	4-32	-	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, CTX, ATM, NAL
	T48.V.c	**B2**	O115:HNM	919	None	187	24-187	*iutA*	*chuA, vat, fyuA, yfcV*	0	*fimH, iucD, iroN, traT, ibeA, malX, iutA, hlyF, ompT, iss*	**SHV-12** - TEM	0	AMP, CFZ, CAZ, CTX, ATM, SXT, NAL
20	T49.II.g	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, SXT, NAL
110	T50.II.b	**F**	O83:H42	1485	648	58	231-58	*iutA, KpsM II - K5*	*chuA, yfcV*	0	*fimH, iucD, iroN, KpsM II, KpsM II - K2, KpsM II - K5, cvaC, traT, malX, iutA, tsh, hlyF, ompT, iss*	TEM	0	AMP, DOX, SXT, NAL
	T50.II.d	**E**	O153:HNM	115	38	270	26-270	*iutA, KpsM II*	*chuA, fuyA*	0	NR	TEM	0	AMP, SXT, CIP, NAL
	T50.V.a	**B1**	O83:H23	906	None	61	4-61	-	-	0	NR	**CTX-M-32** - TEM	0	AMP, CFZ, CXM, CTX, DOX, CIP, NAL
	T50.V.b	**B1**	O19:H21	602	446	86	19-86	*iutA*	-	0	NR	**SHV-12** - TEM	0	AMP, CFZ, CXM, CAZ, CTX, ATM, GEN, TOB, DOX, SXT, CIP, NAL
	T50.V.e	**B1**	O19:H21	602	446	86	19-86	*iutA*	-	0	NR	**SHV-12**	0	AMP, CFZ, CAZ, ATM, DOX, SXT, CIP, NAL

[1]CFU: colony forming units; [2]Isolate: Origin of isolation-sample number-protocol-letter of the isolate, Ch (chicken meat), T (turkey meat); [3]Phylogroup (PG) was designated by PCR according to Clermont scheme (Clermont et al., 2013); [4]Serotype: O antigen: non-typeable isolates were designated as ONT; H antigen: HNM for non-motile isolates, HNT for those which did not react with any antisera and HAA for self-agglutinating isolates; [5]Sequence type (ST) and clonal complex (CC) were assessed following the Achtman scheme (Wirth et al., 2006), NR for isolates where this was not performed; [6] Clonotype based on the internal 469-nucleotide (nt) and 489-nt sequence of the fumC (allele obtained from MLST) and fimH genes, respectively (Weissman et al., 2012), isolates what did not amplified by PCR were designated as negative (neg.), NR for isolates where this was not perfromed; [7] ExPEC status + (highlighted in black): *E. coli* strains considered with higher capacity of developing extraintestinal pathologies when positive for two or more of five markers, including *papAH* and / or *papC, sfa/focDE, afa/draBC, kpsM II* and *iutA*; ExPEC -: strains negative for those markers (Johnson et al., 2003b); [8]UPEC status + (highlighted in black): strains considered with higher capacity of developing UTI pathologies when positive for three or more of four markers, including *chuA, fyuA, vat* and *yfcV*; UPEC -: strains negative for those markers (Spurbeck et al., 2012); [9]*eae*: 1 for positive isolates and 1* for isolates typed as *eae*-β1; [10]Virulence profile: NR for isolates where this characterization was not performed; [11]Antibiotic profile: Phenotypic resistance interpreted according to the CLSI standard guidelines (The Clinical and Laboratory Standards Institute, 2020): ampicillin (AMP), amoxicillin-clavulanic acid (AMC), ceftazidime (CAZ), cefotaxime (CTX), cefoxitin (FOX), aztreonam (ATM), imipenem (IMP), gentamicin (GEN), tobramycin (TOB), amikacin (AMK), fosfomycin (FOF), doxycycline (DOX), chloramphenicol (CHL), nitrofurantoin (NIT), sulfamethoxazole-trimethoprim (SXT), ciprofloxacin (CIP), nalidixic acid (NAL), tigecycline (TGC) and colistin (CST).

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