



One Health compartmental analysis of ESBL-producing *Escherichia coli* on Reunion Island reveals partitioning between humans and livestock

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Background: Extended-spectrum β -lactamase-producing *Escherichia coli* (ESBL-Ec) is a major cause of infections worldwide. An understanding of the reservoirs and modes of transmission of these pathogens is essential, to tackle their increasing frequency.

Objectives: We investigated the contributions of various compartments (humans, animals, environment), to human colonization or infection with ESBL-Ec over a 3 year period, on an island.

Methods: The study was performed on Reunion Island (Southwest Indian Ocean). We collected ESBL-Ec isolates prospectively from humans, wastewater and livestock between April 2015 and December 2018. Human specimens were recovered from a regional surveillance system representative of the island's health facilities. These isolates were compared with those from livestock and urban/rural wastewater, by whole-genome sequencing.

Results: We collected 410 ESBL-Ec isolates: 161 from humans, 161 from wastewater and 88 from animals. Phylogenomic analysis demonstrated high diversity (100 STs), with different STs predominating among isolates from humans (ST131, ST38, ST10) and animals (ST57, ST156). The large majority (90%) of the STs, including ST131, were principally associated with a single compartment. The CTX-M-15, CTX-M-27 and CTX-M-14 enzymes were most common in humans/human wastewater, whereas CTX-M-1 predominated in animals. Isolates of human and animal origin had different plasmids carrying *bla*_{CTX-M} genes, with the exception of a conserved IncI1-ST3 *bla*_{CTX-M-1} plasmid.

Conclusions: These molecular data suggest that, despite their high level of contamination, animals are not a major source of the ESBL-Ec found in humans living on this densely populated high-income island. Public health policies should therefore focus primarily on human-to-human transmission, to prevent human infections with ESBL-Ec.

Introduction

Infections caused by multidrug-resistant bacteria (MDRB) are a major cause of morbidity and mortality worldwide.¹ In this context, the WHO has identified seven specific pathogens in its strategy for fighting antibiotic resistance, including, first and foremost, *Escherichia coli* resistant to third-generation cephalosporins (3GC) and fluoroquinolones. The main mechanism of 3GC resistance in this species is the production of extended spectrum β -lactamase (ESBL). *E. coli* is a leading cause of infection in hospitals and in the community.² If we are to tackle the trend towards an increase in MDRB infections in humans, we will need to use One Health approaches to develop an understanding of the global reservoirs and transmission patterns leading to the acquisition of ESBL-producing Enterobacteriales (ESBL-E) in humans.³ Several potential sources of ESBL-producing *E. coli* (ESBL-Ec) for human infection or colonization have been identified, including food-producing animals, in which the prevalence of ESBL-Ec can be very high.⁴ ESBL-Ec can also be transmitted via the food chain.⁵ Moreover, environments contaminated with human and animal faeces can also act as a source of colonization, particularly in developing countries, which tend to have lower health standards than high-income countries.^{6–9} Studies based on this model addressing this question have yielded conflicting results, leaving the specific roles of these reservoirs unclear.^{10,11} The evaluation of exchanges of resistance vectors (genes or bacteria) between humans, animals and the environment is challenging and subject to several difficulties: (i) it involves the evaluation of a nearly closed ecosystem weakly influenced by imports from the exterior; (ii) a population representative of the ecosystem must be selected; and (iii) appropriate highly discriminating phylogenetic methods based on both core and accessory genomes, including mobile genetic elements (MGE), must be used.

In this study, we aimed to clarify the contribution of livestock to human colonization or infection with ESBL-Ec, using a delimited high-income area, the French overseas territory of Reunion Island, as a model. We report here the phylogenomic investigation, by WGS, of ESBL-Ec isolated from humans, livestock and wastewater on Reunion Island.

Materials and methods

Study design

Reunion Island is a French overseas territory located close to Madagascar, with 850 000 inhabitants and a surface area of 2500 km² (340 inhabitants/km²). Together with Mayotte, it forms the French overseas territories of the Southwest Indian Ocean Area (SIOA). Antimicrobial resistance is a major problem on this island, located at the crossroads of Southern Africa and the Indian subcontinent.¹² Reunion Island has the same level of healthcare as mainland France, with the University Hospital of Reunion Island (UHRI) as the referral hospital in SIOA, and the same health infrastructure (including access to drinking water, a sewerage system, modern wastewater treatment plants, etc.).

We prospectively collected ESBL-Ec isolates from humans, human and animal wastewater (considered a proxy for polluted human or animal environments) and livestock, between April 2015 and December 2018. All isolates suspected to have originated off the island (travel, medical evacuation, or imported animals) were excluded from the study.

Human specimens

The study was based on the regional antimicrobial resistance surveillance system of Reunion Island, which collects ESBL-Ec from six healthcare facilities: two private laboratory groups and four public hospitals (Figure 1). These four public hospitals reflected a total of 2500 beds and 250 000 hospital admissions accounting for >90% of complete-hospitalization days and 100% of medical, surgical and obstetric (MSO) hospitalizations on Reunion Island. The bacterial isolates were collected between January and December 2018 from clinical or rectal screening samples and from non-duplicate patients. Isolates from patients who had a history of off-island travel within the last 6 months, medical evacuation or a home address (postcode) located off the island were excluded. We then randomly selected three ESBL-Ec isolates per centre and per month for further analysis. Anonymized data were recorded for each isolate: gender, age, origin of patient (community or healthcare facility), specimen source, infection/colonization and postcode.

Wastewater

Wastewater samples were collected from four sites (Figure 1). The northern sites corresponded to two sewage effluent discharge points (one from a hospital and the other from the community) and one wastewater treatment plant (WWTP) in the major town of the island (separate inflow and outflow sampling). The site in the centre of the island is the only animal waste treatment plant (AWTP) on the island (runoff water and outflow into the lagoon). Each collecting point was sampled (2 × 500 mL) during the first week of each month, between January and December 2018. ESBL-Ec isolates were identified by (i) the serial dilution method for sewage and untreated inflow, and (ii) the filtration method (membranes with 0.45 μ m pores) for treated outflow, as previously described.¹³

Animal specimens

The sampling procedure was adapted from previous studies on the prevalence of ESBL-producing Enterobacteriales, to ensure that a collection of samples representative of all the cattle, pig, poultry, rabbit, and small ruminant (sheep and goat) farms on the island was obtained.¹⁴ Samples were collected with Sterisox® boot swabs (Sodibox, Nevez, France) or rectal swabs (for cattle). For the boot swabs, the number of samples depended on area considered, with one Sterisox® swab per 100 m² of building. All samples were maintained at 4°C until analysis, which took place within 48 h of sample reception. In total, 114 farms were sampled, and 566 animal samples (124 swabs from cattle; 177 pig, 176 poultry, 39 rabbit and 50 small-ruminant boot swabs) were obtained between April 2015 and December 2018. Sterisox® boot swabs were introduced into a sterile recipient with 100 mL of saline enriched with brain-heart infusion broth (BHI-T, bioMérieux, Marcy l'Etoile, France). The mixture was incubated overnight at room temperature, and secondly, plated (10 μ L) on selective chromogenic agar plates (ChromID-ESBL agar, bioMérieux, Marcy l'Etoile, France) as previously described.¹⁵

Collection of ESBL-producing *E. coli*

The characterization of presumptive ESBL-Ec isolates took place at a single site, at the northern site of the UHRI. Each isolate was identified by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) and its ESBL production was assessed by the combined disc method, in accordance with the 2019 EUCAST recommendations (https://www.sfm-microbiologie.org/wp-content/uploads/2019/05/CASFM2019_V2.0_MAI.pdf). For the collection of bacteria from animals or environment, only one ESBL-producing isolate for each different resistance profile from each farm or sampling point was retained for further analysis.

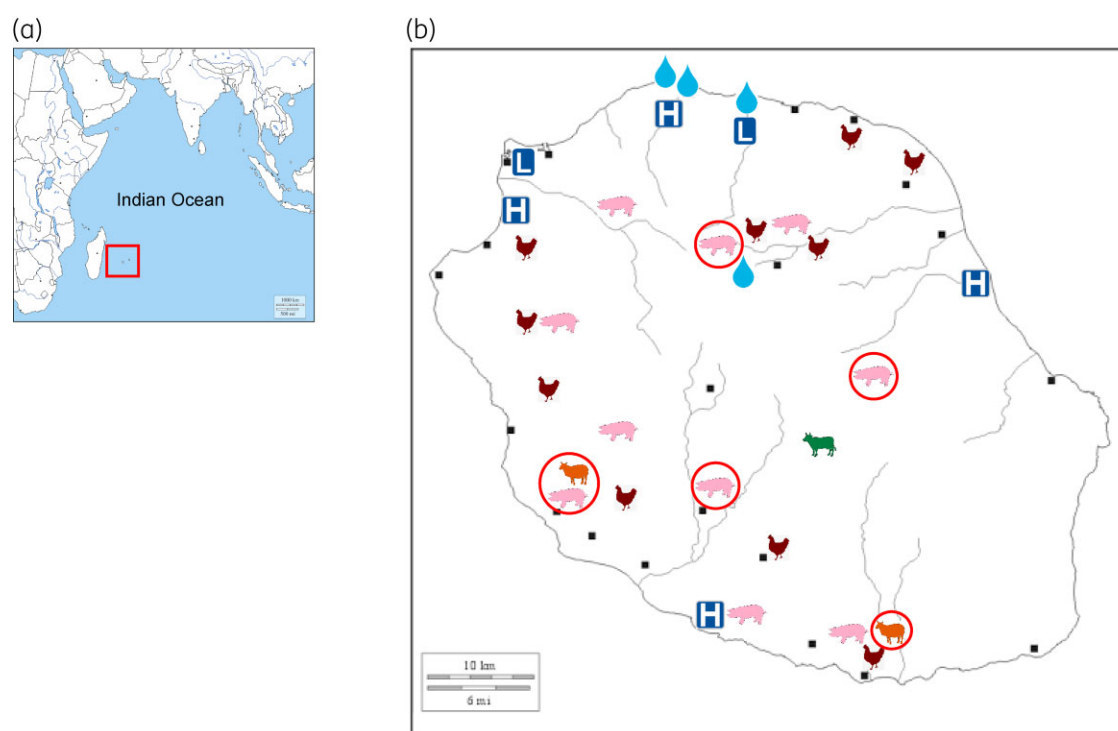


Figure 1. Map of Reunion Island, showing the locations from which the ESBL-producing *E. coli*-positive samples were obtained. (a) Map indicating the position of Reunion Island in the Southwest Indian Ocean. (b) Detailed map of Reunion Island showing the locations of the healthcare facilities (L, private laboratories; H, hospitals) at which the human ESBL-*Ec* isolates were obtained. The wastewater sampling sites are indicated by a teardrop shape; those in the north correspond to the sewage and wastewater treatment plant of Saint-Denis, the largest city on the island. The teardrop in the centre corresponds to the Camp Pierrot animal waste treatment plant in Grand Ilet. The animal symbols indicate the farms of the various livestock sectors. The symbols surrounded by a red circle represent farms with clonally related isolates, belonging to ST156, in common.

Whole-genome sequencing and data analysis

Whole-genome sequencing was performed on an Illumina NextSeq high-output system generating 2×150 paired-end reads with a median coverage of $88\times$. Reads were trimmed with sickle (v1.33) to a minimum length of 50, and were then subsampled to a coverage of at least $80\times$. Assembly was performed with Spades (v3.13).

STs were determined *in silico* with pyMLST, using the Enterobase and Achtman schemes. Phylogroup and *fimH* typing were assigned with ClermonTyping and FimTyper, respectively. We searched for resistance genes with BLAT software and the ResFinder database.

We performed core genome MLST analysis (cgMLST) with pyMLST software and the Enterobase v2 scheme.¹⁶ Phylogenetic analysis was performed with bioNJ, using cgMLST distances, and the results were visualized with ItoL. Isolates were clustered if their cgMLST differed by fewer than 10 alleles.¹⁷ A network was built with the igraph package (v1.2.5), to determine the connections between different sampling sites.

The proximity of mobile genetic elements between strains was evaluated by a method derived from that described by Ludden et al.¹⁸ Briefly, mlplasmids (v1.0) was used to classify contigs as potentially plasmid-borne or on the chromosome. Genes were detected with prodigal on contigs that were considered to be plasmid-borne and contained a *bla*_{ESBL}. These genes were then clustered with cd-hit (v4.7) to obtain a non-redundant list of mobile genes associated with *bla*_{ESBL}. We then searched all the genomes for these mobile elements, and their presence/absence was used to build a dendrogram based on a binary distance, with bioNJ.

Long-read sequencing was performed on the PacBio Sequel system (SMRT cell v2.1 chemistry). The raw data generated were assembled with flye (v2.8.1). Plasmids were extracted and polished with pilon

(v1.23). BLAST was used to align complete plasmid sequences with the assembled genomes containing *bla*_{CTX-M-1} or *bla*_{CTX-M-15}.

The sequences of the plasmids and the genomes (raw reads) obtained were deposited on NCBI under the accession numbers OM105937 to OM105940 and BioProject PRJNA795027, respectively.

Results

Collection

In total, we collected 410 ESBL-*Ec* isolates: 161 from humans, 161 from wastewater and 88 from animals. The mean age of the human patients was 61.1 years (SD 26.2 years) and the sex ratio was 0.96 (Table S1, available as [Supplementary data](#) at JAC Online). Most of the isolates (80.7%) were of community origin or were detected in patients during the first 48 h of hospitalization. Most of the isolates were obtained from the urinary tract (59.0%) or rectal colonization (26.1%), whereas bacteraemia and surgical site infections accounted for 6.8% and 5.0% of isolates, respectively. Infections accounted for 67.1% of cases. This bacterial collection included 15% of all the human ESBL-*Ec* isolates obtained on the island during the study period. For wastewater (Table S2), 70 isolates were obtained from community effluent ($n=29$; 18.0%) or hospital effluent ($n=41$; 25.4%). For WWTPs, 85 isolates were recovered from inflow ($n=56$; 34.8%) and outflow ($n=29$; 18.0%). For the AWTP, six isolates were retrieved

from runoff water ($n=3$; 1.9%) or outflow destined for the lagoon ($n=3$, 1.9%).

We collected 88 ESBL-*Ec* from animals (Table S3): 50 from poultry, 33 from pigs, 2 from cattle, and 3 from small ruminants. No ESBL-*Ec* strains were detected in rabbits. The rate of ESBL positivity by type of farm was: 70% for poultry farms, 50% for pig farms, 18.5% for small ruminant farms and 8.3% for cattle farms. The overall positivity rate per farm was 36.8%.

Comparative phylogenomics

Whole-genome sequencing was used to type and cluster the isolates. Among the 410 ESBL-*Ec*, we identified 100 different STs, 42 of which were common to at least two isolates (Figure S1). The top three STs were ST131 ($n=93$; 22.7%), ST38 ($n=39$; 9.5%) and ST10 ($n=24$; 5.9%). We then compared samples from two compartments: those of animal origin (livestock and animal wastewater) and those of human origin (clinical samples and human wastewater). Ten STs were common to these two compartments: ST10, ST38, ST48, ST58, ST88, ST117, ST155, ST162, ST361 and ST3489; however, ST10 was the only ST clustering at least five isolates in both compartments. For isolates of human origin, the top three STs were identical to those for the general population; ST131 was found exclusively in this compartment. Conversely, the predominant animal-related STs (ST57, ST156) were absent from the samples of human origin. Most STs (90%) were associated with only one compartment.

cgMLST analysis demonstrated a high degree of genetic diversity (Figure 2) and showed that the strains belonging to the STs common to human and animal sources, such as ST10, were not clonally related. Multiple clusters were observed (in red, Figure 2), but each corresponded to isolates of animal origin (e.g. ST57 or ST156) or of human origin (e.g. ST38, ST44 or ST131).

Phylogroups varied with the source, with B2/D predominating among human/human wastewater isolates (B2 was not identified in animal samples and D was identified in only one sample from poultry), whereas B1/A predominated among isolates of animal origin. Phylogroups B2 and D accounted for 40.4% and 9.9% of human isolates, and 60.2% and 14.8% of infection source isolates, respectively. The clonality network (Figure 3) revealed connections between private laboratories and public hospitals, and between these establishments and sewage/WWTPs (cluster B); whereas farms formed isolated networks or were connected to the AWTP (cluster A). Cluster A3 highlighted connections, via ST156 isolates, between pig and small-ruminant farms, which were located in geographically remote sites of the island (symbols surrounded by a red circle in Figure 1).

Assessments of the occurrence of genetic determinants of resistance revealed that *bla*_{CTX-M-15} was the predominant *bla*_{ESBL} gene ($n=177$; 43.2%), followed by *bla*_{CTX-M-1} ($n=94$; 22.9%), *bla*_{CTX-M-27} ($n=69$; 16.8%) and *bla*_{CTX-M-14} ($n=28$; 6.8%) (Figure 4). The CTX-M-15, CTX-M-27 and CTX-M-14 enzymes were most common in humans/human wastewater, whereas CTX-M-1 predominated in ESBL-*Ec* of animal origin (Figure 4 and Figure S2b). An analysis of ST131 isolates ($n=93$) revealed a separation into two clades: (i) a *fimH30* clade ($n=49$), comprising a C2-M-15 subclade ($n=31$) and a C1-M27 subclade ($n=16$) (Figure 2); and (ii) a *fimH41* clade ($n=37$) mostly associated with *bla*_{CTX-M-27} ($n=27$; 73.0%), corresponding to an ancestral clade A

(Figure 2). CTX-M-1 was mostly associated with the animal-related ST57, ST155, ST156 and ST3489. The CTX-M-1 enzyme was found in only 10 (6.2%) of 161 human isolates and CTX-M-15 in only eight (9.1%) of 88 animal isolates. Screening for non-ESBL resistance genes revealed that nine isolates coproduced an acquired cephalosporinase (*bla*_{CMY} or *bla*_{DHA}) and five coproduced a carbapenemase (*bla*_{NDM-1}, *bla*_{NDM-5}, *bla*_{OXA-181} or *bla*_{OXA-232}) (Figure S2a). The carbapenemase-encoding *bla*_{NDM} and *bla*_{OXA-48-type} genes were detected only in isolates from human wastewater.

We investigated whether *bla*_{CTX-M-1} and *bla*_{CTX-M-15}, the two major ESBL resistance genes, were carried by the same or different plasmids. For *bla*_{CTX-M-1}, the analysis identified multiple clusters (with >98% identity), some of which contained isolates of both human and animal origin and from different STs (Figure 5 and Figure S3). For *bla*_{CTX-M-15}, there was considerable variability between isolates, with no clustering between human and animal isolates (Figure 5 and Figure S4). We then cross-checked this information with MGE data. Two triplets of human-animal-wastewater isolates positive for *bla*_{CTX-M-1} and *bla*_{CTX-M-15} were selected for long-read sequencing. We searched the complete plasmid sequences (obtained by long-read sequencing) on the assembled genomes. The *bla*_{CTX-M-1}-positive human isolate carried an Inc11-I *bla*_{CTX-M-1} plasmid (109 992 bp) that was highly similar to the plasmids carrying the *bla*_{CTX-M-1} gene in the animal and wastewater isolates selected (Figure 5). This conserved Inc11-I (or Inc11-ST3) *bla*_{CTX-M-1} plasmid was found in 6 human isolates and 38 animal isolates. In contrast, the *bla*_{CTX-M-15} gene in animal isolates was located on the chromosome and probably resulted from the integration of a *bla*_{CTX-M-15} IncI/Y plasmid. The region harbouring the *bla*_{CTX-M-15} gene in the human, animal and wastewater isolates was less conserved and had undergone multiple recombinations, indicating an absence of recent horizontal transfer (Figure 5).

Discussion

We compared 410 genomes of ESBL-*Ec* isolates from humans or human-polluted environments (i.e. human wastewater) with those of isolates from livestock or animal-polluted environments (i.e. animal wastewater) in a circumscribed island area. The main finding of this study was that the populations of these ESBL-*Ec* isolates from these two reservoirs were genetically different, generally harbouring different *bla*_{ESBL} genes located in different genetic environments. This strongly suggests that livestock have very little effect on the acquisition of ESBL-*Ec* by humans on Reunion Island, despite all the food produced from the livestock kept on the island being consumed locally.

Phylogenomic and resistance gene analyses revealed the existence of very little connection between the human and animal compartments, which had different major STs and predominant ESBL-encoding genes. In contrast, human and human wastewater isolates were much more strongly connected, indicating that wastewater can be used as a proxy for human gut colonization.¹⁹ As expected, phylogroups B2 and D were associated with most of the human infections (75.0%).²⁰ For the predominant ST131 clone, the high proportion of H41-M27 isolates within the ancestral clade A (already described in Thailand and Cambodia) suggests an influence of South-East Asia on the epidemiology of ESBL-*Ec* on Reunion Island.^{5,21} ST10 was the only

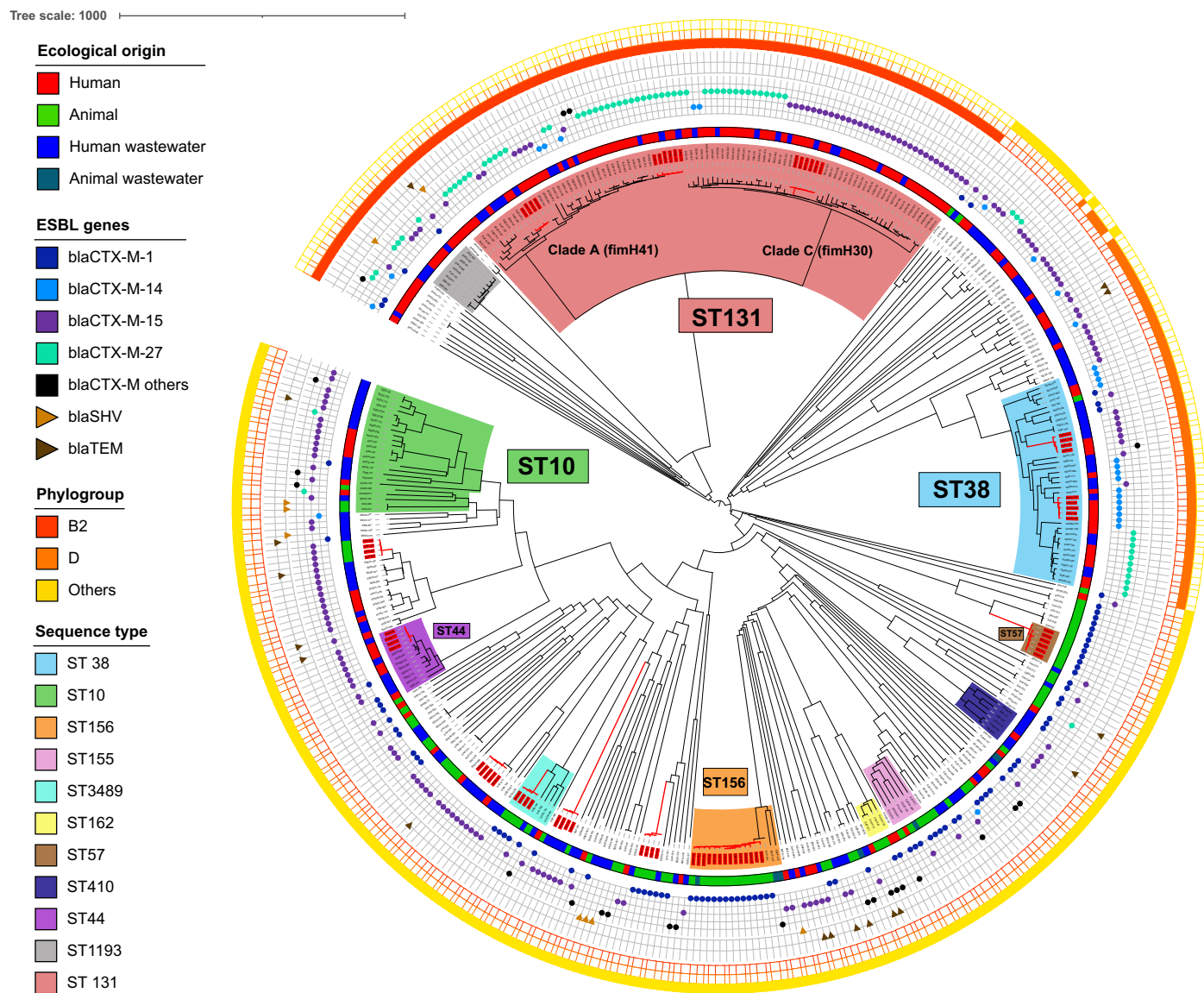


Figure 2. Phylogenetic tree illustrating the relationship between ESBL-producing *E. coli* isolates from humans, animals and wastewater. Neighbour-joining phylogenetic tree of 410 ESBL-*Ec* based on cgMLST data. Clonally related isolates are shown in red on the dendrogram. The innermost coloured ring indicates the ecological origin of the isolates, whereas the outermost ring indicates the phylogroup. The symbols located between these two rings (round, triangle) represent the β -lactam resistance-encoding *bla*_{BLSE} genes. The main STs are represented by a specific colour on the dendrogram, as defined in the legend.

top-three ST common to both compartments, but phylogenomic analysis nevertheless highlighted the presence of different sub-clones that appeared to be adapted to human or animal hosts.^{18,22} The clonality networks established between the different structures (farms or hospitals) revealed the partitioning between the human and animal compartments, each of which was connected to its own wastewater treatment circuit (Figure 3). This finding supports the hypothesis that human-to-human transmission accounts for most cases of infection or colonization in humans. This clonality network connected previously described spatially remote care facilities and farms on the island.²³ In animals, this clonal relationship between farms

remained unclear and could be explained by the exchange of some animals (especially male breeding stock) between farms. The finding of separate compartments was confirmed by MGE analysis, as the animal compartment was not identified as a progenitor of MGEs for humans; with the exception of the *bla*_{CTX-M-1} gene carried by Inc11-ST3 plasmids, which may represent a minor source of horizontal transfer between compartments.²⁴ CTX-M-15 was the main ESBL found in humans (47.8%), whereas CTX-M-1 accounted for only 6.2% of human ESBL isolates. We can therefore estimate that <5% of human isolates carry antimicrobial resistance genes closely related to those found in livestock, consistent with previous findings.¹⁸ A limitation of this analysis

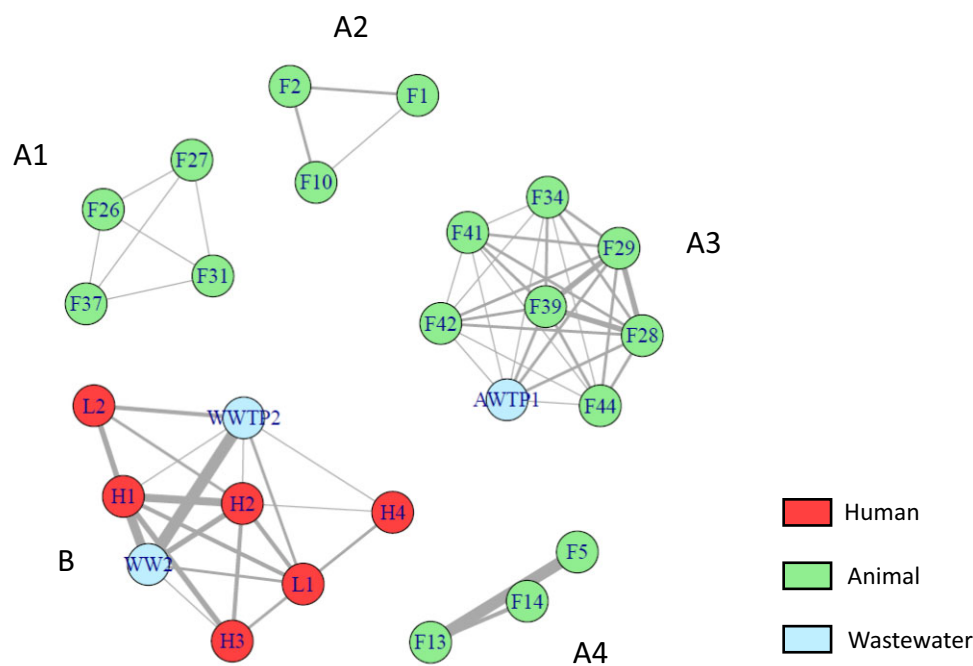


Figure 3. Clonality network representing the healthcare facilities, farms and wastewater sources at which clonally related ESBL-producing *E. coli* isolates were detected. After phylogenetic analysis, each clonally related isolate was assigned to its sampling site. Farms (F), laboratories (L), hospitals (H), waste-water from sewage (WW), wastewater treatment plant (WWTP) and the animal waste treatment plant (AWTP) are symbolized by circles, whereas clonal links are indicated by the lines connecting the circles. The more clonal strains connecting two sites, the wider the line between the two sites.

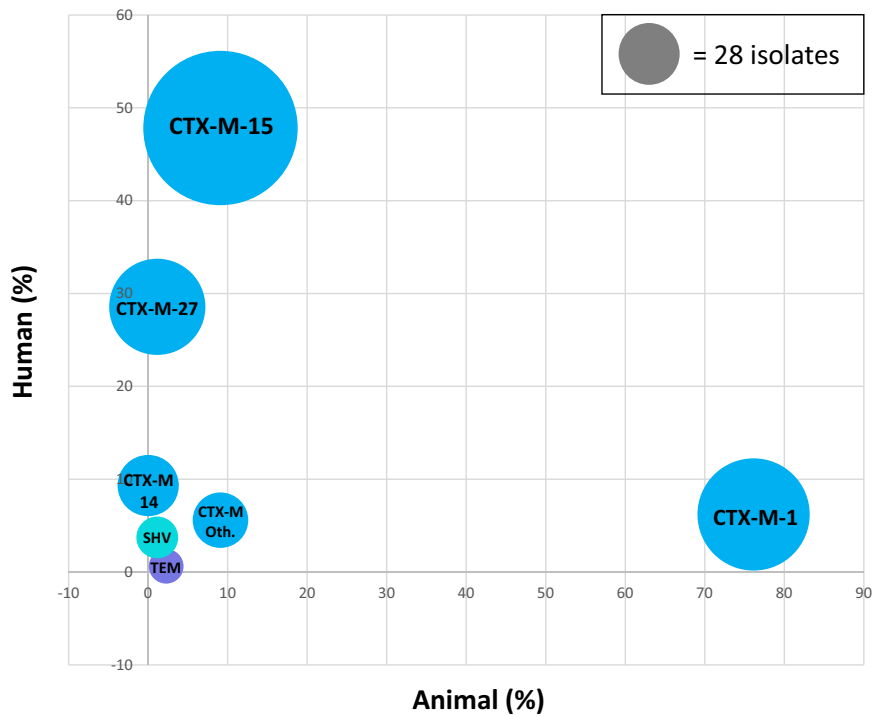
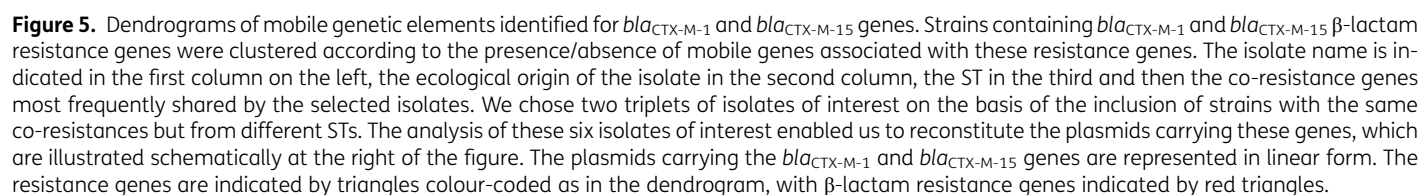


Figure 4. Bubble graph showing the proportion of *bla*_{BLSE} resistance genes common to ESBL-producing *E. coli* from humans and animals. Each *bla*_{BLSE} resistance gene is positioned according to its prevalence in the human and animal compartments. The size of each bubble represents the number of isolates in which the gene was identified. Bubbles are coloured according to the type of ESBL enzyme: blue for CTX-M, turquoise for SHV and purple for TEM enzymes. With the exception of the *bla*_{TEM} gene, bubbles are relatively close to the axes, with no bubble in a central position on the bisector. CTX-M Oth, bubble representing the other CTX-M variants.



The partitioning of ESBL-Ec between the human and animal compartments observed here goes against the findings of several previous studies.^{25,26} However, these previous studies often involved less-thorough analyses or focused on sporadic transmission in specific exposed populations (farmers, in particular), and the results obtained were sometimes overturned by subsequent WGS analyses.²⁷ In contrast, our results are consistent with two models that have recently emerged from major studies published on the subject: a One Health model in low-income countries, in which people are much more closely linked to their environment,^{5,8,28} and a partitioned model in high-income

The key strengths of our study are: (i) the representativeness of the human ESBL-*Ec* isolate collection with respect to the

population of strains in the community; (ii) the prospective sampling of these strains over a period of 3.5 years; and (iii) the optimal geographic context of collection in a well-defined, densely populated island environment. The large collection of human isolates ($n=161$, 39.3% of all the isolates) originated mostly from the community (80.7%) and corresponded to both infections (two-thirds) and colonization (one-third). This collection was complemented by 155 urban wastewater isolates (sewage and WWTP), which can be considered a proxy for the gut flora of the population living in the community.^{13,18,22} The animal isolates were also highly representative, with recruitment covering all the livestock sectors present on the island. Samples were also taken from an AWTP, although few isolates were obtained from this source. A recent study on the island of Zanzibar (Tanzania) also highlighted the benefits of One Health studies of the interconnection between different compartments in a circumscribed territory.²⁸ One potential limitation of this study is the lack of exploration of the food chain, but several studies have shown that the ESBL-*Ec* populations from livestock and the food chain are genetically similar.^{22,28,32,33} This pathway should nevertheless be considered as a potential source of direct transmission to humans.

ESBL-*Ec* was isolated from 36.8% of livestock farms, with particularly high rates of isolation for poultry (70.0%) and pig (50.0%) farms. These rates are higher than those for mainland France during the same period but consistent with data for other European countries, such as Germany, the Netherlands or the United Kingdom.^{18,34–37}

As a consequence, national and European public health policies aiming to reduce antibiotic use in livestock are likely to have only a limited and delayed impact on human infections over time.³⁸ In contrast with the rapid decline in *mcr-1*-linked colistin resistance in China, limiting the prevalence of ESBL-*Ec* in livestock will have only a limited impact on the rate of *bla*_{ESBL} transmission for human-adapted *E. coli* clones.³⁹ The priority should therefore be to limit the selection pressure imposed by antibiotic use in humans, to maximize the impact of measures.

This study on the exchange of ESBL-*Ec* between human and animal compartments is the first to demonstrate a clear sectorization of animal and human isolates on a densely populated, high-income island with a high prevalence of ESBL-*Ec* in livestock. Our findings suggest that livestock is not a major source of ESBL-*Ec* acquisition in humans, either through direct contact with animals or indirectly, via the food chain.

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Transparency declarations

None to declare.

Supplementary data

Information on the whole genome sequencing and data analysis, plus Tables S1 to S3 and Figures S1 to S4 are available as [Supplementary data](#) at JAC Online.

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