Implementation of the WHO Tricycle protocol for surveillance of extended-spectrum β-lactamase producing *Escherichia coli* in humans, chickens, and the environment in Madagascar: a prospective genomic epidemiology study

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Summary

Background Antimicrobial resistance (AMR) is a major public health threat, affecting not only people but also animals and the environment. The One Health dimension of AMR is well known; however, data are lacking on the circulation of resistance-conferring genes, particularly in low-income countries. In 2017, WHO proposed a protocol called Tricycle, focusing on extended-spectrum β-lactamase (ESBL)-*Escherichia coli* surveillance in the three sectors (humans, animals, and the environment). We implemented Tricycle in Madagascar to assess ESBL-*E coli* prevalence and describe intrasector and intersector circulation of ESBL-*E coli* and plasmids.

Methods In this prospective study, we collected blood culture data from hospitalised patients with a suspected bloodstream infection processed from May 1, 2018, to April 30, 2019, and rectal swabs from healthy pregnant women from July 30, 2018, to April 27, 2019, both from three hospitals in Antananarivo, Madagascar; and caeca from farm chickens and surface waters from the Ikopa river, wastewater, and slaughterhouse effluents in the Antananarivo area, Madagascar, from April 9, 2018, to April 30, 2019. All samples were tested for ESBL-*E coli*. The genomes of all isolates were sequenced using a short-read method on NextSeq 500 and NovaSeq 6000 platforms (Illumina, San Diego, CA, USA) and those carrying plasmid replicons using an additional long-read method on a MinION platform (Oxford Nanopore Technologies, Oxford, UK). We characterised genomes of isolated strains (sequence type, resistance and virulence gene content, and plasmid replicons). We then compared isolates using the variant calling method (single-nucleotide polymorphism).

Findings Data from 1056 blood cultures were collected and 289 pregnant women, 246 chickens, and 28 surface waters were sampled. Of the blood cultures, 18 contained *E coli*, of which seven (39%) were ESBL. ESBL-*E coli* was present in samples from 86 (30%) of 289 pregnant women, 140 (57%) of 246 chickens, and 28 (100%) of 28 surface water samples. The wet season (November to April) was associated with higher rates of carriage in humans (odds ratio 3·08 [1·81–5·27]) and chickens (2·79 [1·65–4·81]). Sequencing of 277 non-duplicated isolates (82 from pregnant women, 118 from chickens, and 77 from environmental samples) showed high genetic diversity (90 sequence types identified) with sector-specific genomic features. Single nucleotide polymorphism (SNP) analysis revealed that 169 (61%) of 277 isolates grouped into 44 clusters (two or more isolates) of closely related isolates (<40 SNPs), of which 24 clusters contained isolates from two sectors and five contained isolates from all three sectors. ESBL genes were all $bla_{\text{CTX-M}}$ variants (215 [78%] of 277 being $bla_{\text{CTX-M-15}}$) and were located on a plasmid in 113 (41%) of 277 isolates. These ESBL-carrying plasmids were mainly IncF (63 [55%] of 114; one strain carried two plasmids) and IncY (42 [37%] of 114). The F31/36:A4:B1 (n=13) and F-:A-:B53 (n=8) pMLST subtypes, and the IncY plasmids, which were all highly conserved, were observed in isolates of differing genetic backgrounds from all sectors and were transferable in vitro by conjugation.

Interpretation Despite sector-specific population structures, both ESBL-*E coli* strains and plasmids are circulating among humans, chickens, and the environment in Antananarivo, Madagascar. The Tricycle protocol can be implemented in a low-income country and represents a powerful tool for investigating dissemination of AMR from a One Health perspective.

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Introduction

For decades, antimicrobial resistance (AMR) was considered exclusively from a human health perspective, but has now come to be regarded as a global problem also involving animal health, food production, and environmental conditions.[1] The importance of a holistic One Health approach with epidemiological surveillance is now acknowledged as a key element in the fight against AMR.[2] In this context, in 2017, WHO developed a simplified, integrated, multisectoral surveillance protocol called Tricycle,[3] which uses a One Health approach to survey AMR in three major sectors: human (community carriage and bloodstream infections), the food chain (live poultry), and the environment (surface water, slaughterhouse effluents, and wastewater). The Tricycle protocol focuses on a single key indicator: the prevalence of extended-spectrum β-lactamase (ESBL) producing *Escherichia coli*. ESBL-*E coli* was chosen as the target organism because ESBL producing *Enterobacterales* are viewed as one of the greatest threats in terms of AMR, particularly because it is one of the species most often isolated from invasive infections in humans.[4] In addition, *E coli* is a commensal of the human gut, a wide variety of warm-blooded animals, and is also widespread in the environment.[5,6] AMR surveillance studies, aimed at investigating links between antibiotic resistant bacteria in humans, animals and the environment within the same temporal and geographical scale, are still rare. Moreover, such studies rarely include the investigation of AMR plasmids.

The Tricycle protocol was implemented in 2018 in Antananarivo, Madagascar. Here, we report the results of the first year of the Tricycle project describing the prevalence of ESBL-*E coli* in blood cultures, healthy pregnant women, the food chain, and in the environment. We aimed to compare the genomic characteristics of the strains isolated from the three sectors, and to investigate the intrasectoral and intersectoral circulation of ESBL-*E coli* and ESBL-carrying plasmids.

Methods

Study design

In this prospective study, sampling and microbiological methods were performed according to the Tricycle protocol.[3]

Research in context

Evidence before this study

Surveillance is a key element in the fight against antimicrobial resistance and must be conducted from a One Health perspective. In this context, WHO released Tricycle 2017, a standardised surveillance protocol that addresses the prevalence and circulation of extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* in the three sectors involved in the One Health approach: human, animal, and the environment. To find evidence of the circulation routes of ESBL-*E coli* from a One Health perspective, we searched PubMed for studies published in English before Oct 25, 2022, without start date restriction, using the following search terms: ("ESBL"[Title] OR "extended spectrum beta lactamase"[Title] OR "extended spectrum β-lactamase") AND ("*Escherichia coli*"[Title/Abstract] OR "*E. coli*"[Title/Abstract] OR "coliform"[Title/Abstract]) AND ("Human" OR "farmers" OR "patients") AND ("animal" or "poultry" OR "chicken" OR "livestock" OR "farms" OR "food") AND ("environment" OR "water" OR "wastewater" or "sewage"). 202 unduplicated articles (excluding reviews [n=12]) were identified using this search strategy. Of these, only one study reported the prevalence results of the WHO Tricycle protocol and only six compared ESBL-*E coli* strains isolated from the three sectors (human, animal, and the environment). None of these studies were conducted in Madagascar. Among the six studies comparing strains, three used methods with low discriminatory power to compare them. These methods include pulse-field gel electrophoresis, multi-locus sequence typing, ESBL enzyme, or plasmids replicons. Three studies used whole-genome sequencing but with different thresholds to consider isolates as genetically related. These studies were conducted mostly in high-income countries and rarely with sampling performed within the same temporal scale and in a limited geographical area. Finally, none of these studies explored precisely the intersectoral circulation of plasmids carrying ESBL encoding genes. In all, studies investigating the circulation of ESBL-*E coli* were conducted and analysed with heterogeneous protocols, making it difficult to compare them.

Added value of this study

In this study, we implemented the Tricycle protocol in Antananarivo, Madagascar. By collecting high-quality samples within the same temporal scale in a limited geographical area, according to the Tricycle protocol, we were able to reveal substantial circulation of ESBL strains and plasmids within and between the three sectors in Antananarivo, Madagascar. To our knowledge, our study is the first to address the circulation of resistance-conferring genes from a One Health perspective, in humans, farm chickens, and the environment, using a well-designed and standardised protocol, and taking into account both ESBL-*E coli* and plasmids carrying ESBL encoding genes. Our study is also the first to thoroughly analyse the results of the implementation of a WHO Tricycle protocol.

Implications of all the available evidence

Previous studies on the circulation of ESBL-*E coli* from a One Health perspective have identified genetically distinct populations of human, animal, and environmental isolates, with human-to-human transmission being the main source of community colonisation and, to a lesser extent, environmental-to-human transmission. In contrast, our study shows that in Madagascar, circulation of ESBL-*E coli* strains and plasmids carrying resistance markers takes place among the three sectors.

For the human sector, three hospitals in Antananarivo and their respective laboratories participated in the study: Joseph Raseta Befelatanana hospital, Mère-Enfant Tsaralalana hospital, and Joseph Ravoahangy Andrianavalona hospital. In brief, the results of all blood cultures (patients with a suspected bloodstream infection), processed from May 1, 2018, to April 30, 2019, were collected. Rectal swabs from healthy pregnant women were collected from July 30, 2018, to April 27, 2019, during pregnancy check-ups in the three hospitals and sent to each laboratory.[7]

Animal (chicken caeca) and environmental samples (surface waters) were collected from April 9, 2018, to March 19, 2019, and processed at the Charles Mérieux Center of Infectious Disease (CICM) in Antananarivo. Live chickens from 19 different farms were purchased at markets in Antananarivo. 41 market visits were conducted, each involving the purchase of a batch of six chickens from the same farm, with farms alternating at each visit. Each batch of six chickens was transported in the same cage to the CICM. Environmental sampling was conducted every 2 months from April 9, 2018, to April 30, 2019 (seven sampling campaigns) on surface water from the Ikopa river, which crosses the city. Samples were collected at sites upstream (5·5 km from downtown), downstream (5·4 km from downtown), from a wastewater channel (in the city centre), and from a slaughterhouse sewage (5·4 km from downtown; appendix 1 p 4).

The study was approved by the Ethics Committee of Ministry of Health, Madagascar (038-MSANP/CERBM). Written informed consent was obtained from all pregnant women included in the study.

Procedures

Blood cultures were processed by routine practices in each laboratory. Rectal swabs and chicken caeca were plated on MacConkey agar with 4 mg/L cefotaxime. Environmental samples were plated quantitatively on tryptone bile X-glucuronide agar with and without 4 mg/L cefotaxime. For each positive plate, three (human and animal) or five (environment) ESBL-*E coli* presumptive colonies were selected and tested for indole production. ESBL production was confirmed using double disc synergy testing on antibiotic susceptibility testing.[3] For environmental sampling, total *E coli* and ESBL-*E coli* colonies were counted, and respective concentrations calculated. Strains were shipped to France where an identification and an extended antibiogram (32 antibiotics) were performed in the Fondation Mérieux laboratory (Lyon, France; appendix 1 p 2).

Transfer frequencies of plasmids of interest were determined in the Infection Antimicrobials Modelling Evolution (IAME) unit, Université Paris Cité (Paris, France), by conjugation in a rifampicin resistant *E coli* K-12 strain, after incubation at 37°C for 24 h and selection on lysogeny broth with rifampicin (250 mg/L) and cefotaxime (4 mg/L).

For each sample, all isolates with distinct antimicrobial susceptibility profiles were selected for whole-genome sequencing (WGS). DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) and sequencing was performed, at Oxford Genomics Centre (Oxford, UK) on NextSeq 500 and NovaSeq 6000 platforms (Illumina, San Diego, CA, USA).

To avoid over-representation of clonal strains in chicken samples, similar isolates (<10 single-nucleotide polymorphisms [SNPs]) from different chickens within the same batch were considered as duplicates and removed from the analysis after sequencing.

Isolates carrying at least one plasmid replicon were also sequenced using Nanopore technology in the IAME unit (Paris, France). DNA was extracted using a NucleoMag 96 Tissue kit (Macherey Nagel, Hœrdt, France) and sequencing was performed on a MinION platform (Oxford Nanopore Technologies, Oxford, UK).

Plasmid sequences were reconstructed using Illumina and Nanopore reads with Unicycler v0.4.9b software.[8] Further details of laboratory methods including genome assembly, antibiotic resistance and virulence gene detection, replicon detection, phylogenetic group, serotype, multilocus sequence typing (MLST), core genome MLST, phylogenetic analysis, and isolate genome and plasmid comparison are in appendix 1 (pp 2–3).

Statistical analysis

See Online for appendix 1

Factors associated with ESBL-*E coli* carriage in pregnant women (age, seasonality [May to October is the dry season and November to April is the wet season], rainfall, environmental temperature, education level, sanitation, electricity and drinking water access, animal ownership, and recent antibiotic intake in the past 3 months), and in chickens (weight, seasonality, rainfall, and environmental temperature) were identified with univariable logistic regressions with carriage as dependent variable. ESBL-*E coli* concentrations between different environmental samples were compared using the Kruskal–Wallis test. Association between seasonality and ESBL-*E coli* concentrations in environmental samples was analysed using the Wilcoxon–Mann–Whitney test. Mean genome size, number of resistance and virulence genes, and plasmid replicons per isolate were also compared between sectors using the Kruskal–Wallis test. Proportions of phylogenetic groups, sequence types (ST), clonal complexes (STc), resistance and virulence genes, plasmid types and $bla_{CTX\text{-}M}$ chromosomal integration within each sector were compared using the Fisher exact test, and the reported p value was corrected for multiple comparisons using the Benjamini–Hochberg method. Genetic diversity within and between sectors was evaluated by computing pairwise nucleotide diversity (π)[9] and compared using *t* tests with Benjamini–Hochberg correction for multiple tests (appendix 1 p 3). Statistical analyses were performed using R software v4.2.2.

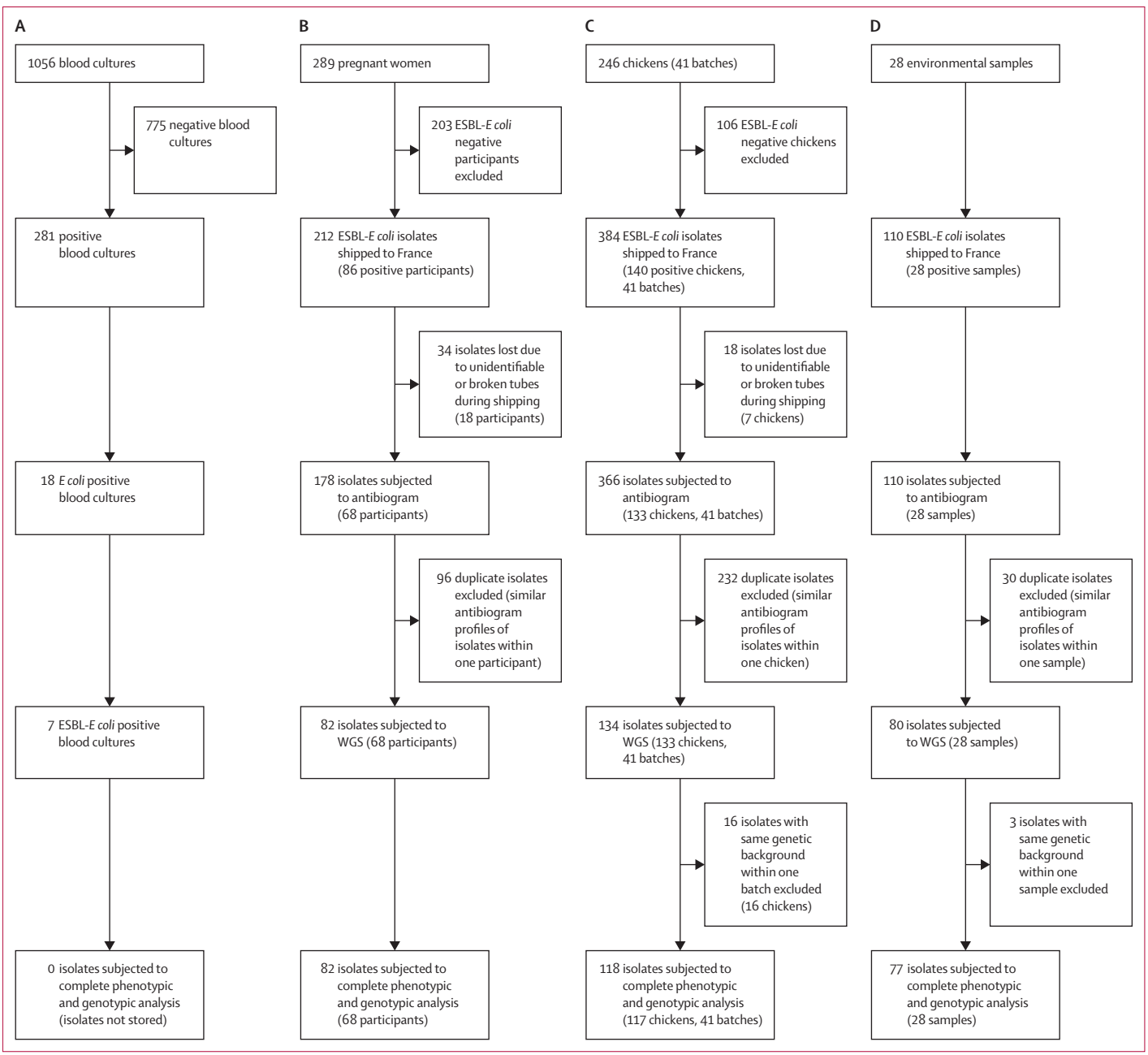


Figure 1: **Flow chart of the number of isolates from blood cultures (A), pregnant women (B), chickens (C), and environmental samples (D) throughout the study**
E coli=Escherichia coli. ESBL=extended-spectrum β-lactamase. WGS=whole-genome sequencing.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Of the 1056 collected blood cultures, 281 were positive for any bacteria and 18 contained *E coli*, of which seven (39%) of 18 were ESBL. Due to a communication oversight, blood culture isolates were not stored and so did not undergo complete phenotypic and genotypic analysis (figure 1). Samples from 289 pregnant women, 246 chickens, and 28 environmental samples were analysed in the study (figure 1; table 1). ESBL-*E coli* was present in samples from 86 (30%) of 289 pregnant women and 140 (57%) of 246 chickens. All 28 environmental samples contained ESBL-*E coli*, with concentrations ranging from 1·11 log colony forming units per 100 mL in upstream surface water, to 2·37 in downstream water, 4·06 in wastewater, and 4·09 in slaughterhouse effluents (p<0·0001; appendix 1 p 5). The

only factor associated with ESBL-*E coli* carriage was a sampling during the wet and warm season in both humans (odds ratio 1·53 [95% CI 1·21–1·96] per 100 mm rainfall and 1·26 [1·11–1·44] per °C, p=0·0004) and chickens (1·40 [1·21–1·65] per 100 mm rainfall and 1·22 [1·11–1·35] per °C, p<0·0001; table 2). Variations in ESBL-*E coli* concentrations in the environment were not related to seasonality (appendix 1 p 6).

Among the 706 isolates received in France (212 from pregnant women, 384 from chickens, and 110 from environmental samples), 654 were subjected to identification and antibiotic susceptibility testing (178 from pregnant women, 366 from chickens, and 110 from environmental samples). Then, 296 isolates with non-similar resistance patterns were selected for WGS, among which 19, considered as duplicates, were excluded. In all, 277 isolates (82 from pregnant women, 118 from chickens, and 77 from environmental samples) from 213 samples were included for further analysis (figure 1).

Analysis of the 277 *E coli* genomes revealed a core genome of 2 522 760 bp containing 2623 genes. Pangenome calculations indicated that *E coli* genomic diversity represents an open pangenome model containing a reservoir of more than 17 200 genes. Comparison of the core genome and pangenomes showed quicker decay in the pangenomic gene content of chicken isolates than in human and environmental isolates (appendix 1 p 7). The genetic diversity of chicken isolates was lower than that of human isolates, which was in turn lower than that of environmental isolates (p<0·0001). The mean genome size of chicken isolates (4·77 Mbp [SD 0·27]) was smaller than that of human isolates (4·82 Mbp [0·20]) and of environmental isolates (4·92 Mbp [0·23]; p<0·0001; appendix 1 p 7). Intersectoral genetic diversity was higher than intrasectoral genetic diversity (p<0·0001; appendix 1 pp 7–8).

Isolates from commensalism-associated phylogenetic groups A and B1 were predominant in all sectors, accounting for 108 (92%) of 118 chicken, 71 (87%) of 82 human, and 54 (70%) of 77 environmental isolates (p=0·0013; appendix 1 p 9). Isolates were highly diverse as MLST identified 90 different STs among the 277 isolates including 48 STs in 118 chicken isolates, 52 STs in 82 human isolates, and 39 STs in 77 environmental isolates (p=0·020). Among the 90 different STs, 22 STs were observed in chickens, 20 in pregnant women, and 10 in environmental samples only, whereas 27 STs were identified in two sectors and 11 STs in all three sectors (appendix 1 p 10). The extra-intestinal, virulent-associated, B2 phylogenetic group-ST131 was detected only in human and environmental sectors. The clonal complex STc10 was the most prevalent, being present in 54 (46%) of 118 chicken isolates, 29 (35%) of 82 human isolates, and 24 (31%) of 77 environmental isolates (p=0·29). Interestingly, the five most frequent STc identified in the human sector (STc10, ST450 [no STc], STc46, STc155, and STc38) that accounted for 49 (60%) of 82 human isolates, were detected in all three sectors (appendix 1 p 11). A phylogenetic tree based on the core-genome SNPs shows the high genetic diversity of ESBL *E coli* isolates (figure 2; appendix 1 p 12).

	Pregnant women (n=289)	Chickens (n=246)	Environmental samples (n=28)
Season of sampling*			
Dry	199 (69%)	141 (57%)	16 (57%)
Wet	90 (31%)	105 (43%)	12 (43%)
Rainfall, mm	119·4 (185·2)	138·9 (197·5)	151·1 (221·3)
Temperature, °C	19·8 (2·2)	19·6 (2·9)	19·6 (2·7)
Age, years†			
≤20	31 (11%)	NA	NA
21–25	98 (34%)	NA	NA
26–30	98 (34%)	NA	NA
>30	61 (21%)	NA	NA
Education			
Absent or elementary school	18 (6%)	NA	NA
High school	149 (52%)	NA	NA
University or graduate	122 (42%)	NA	NA
Toilet access‡			
Shared	143 (49%)	NA	NA
Private	146 (51%)	NA	NA
Electricity access			
Have access	269 (93%)	NA	NA
Do not have access	20 (7%)	NA	NA
Drinking water access‡			
Shared	209 (72%)	NA	NA
Private	80 (28%)	NA	NA
Animal ownership			
Yes	129 (45%)	NA	NA
No	160 (55%)	NA	NA
Recent antibiotic intake§			
Yes	36 (12%)	NA	NA
No	253 (88%)	NA	NA
Weight, g	NA	795 (700–860)	NA
Sampling site			
Upstream	NA	NA	7 (25%)
Downstream	NA	NA	7 (25%)
Wastewater	NA	NA	7 (25%)
Slaughterhouse	NA	NA	7 (25%)

Data are n (%), median (IQR), or mean (SD). NA=not applicable. *Dry season runs from May to October and wet season runs from November to April. †Age information for one participant missing. ‡Private means used by a single household; shared means used by more than one household. §Antibiotic intake in the past 3 months.

Table 1: **Characteristics of human, chicken, and environmental samples and of weather conditions during sampling in Antananarivo, Madagascar, from April 9, 2018, to April 30, 2019**

The 277 isolates were resistant to all β-lactams, except to piperacillin plus tazobactam (14%, n=38), cefoxitin (6%, n=17), and imipenem (1%, n=2). All 277 isolates were frequently resistant to fluoroquinolones (ciprofloxacin [77%, n=213]), co-trimoxazole (63%, n=175), and less resistant to aminoglycosides (gentamicin [40%, n=110] and amikacin [10%, n=27]) with slight variation between sectors (appendix 1 p 13). One chicken isolate and one environmental isolate were resistant to imipenem.

The mean number of resistance genes per isolate was higher in the environment (8·2 [SD 3·9]) than in the human

	Non-carriers of ESBL-*E coli*	Carriers of ESBL-*E coli*	OR (95% CI)	p value
Pregnant women (N=289)				
Number of participants	203	86	..	..
Age category, years*				
≤20	22 (11%)	9 (10%)	1 (ref)	..
21–25	67 (33%)	31 (36%)	1·13 (0·48–2·85)	0·79
26–30	72 (36%)	26 (30%)	0·88 (0·37–2·24)	0·79
>30	41 (20%)	20 (23%)	1·19 (0·47–3·16)	0·71
Age, years*	26·57 (5·57)	26·88 (5·87)	1·01 (0·97–1·06)	0·67
Wet *vs* dry season	48 (24%)	42 (49%)	3·08 (1·81–5·27)	<0·0001
Rainfall, mm†	47·78 (93·48)	98·15 (121·82)	1·53 (1·21–1·96)	0·0004
Temperature, °C‡	18·67 (1·96)	19·59 (1·95)	1·26 (1·11–1·44)	0·0004
Education				
Absent or elementary school	12 (6%)	6 (7%)	1 (ref)	..
Secondary	108 (53%)	41 (48%)	0·76 (0·28–2·3)	0·61
University or graduate	83 (41%)	39 (45%)	0·94 (0·34–2·87)	0·91
Private toilets§	101 (50%)	45 (52%)	1·11 (0·67–1·84)	0·69
Electricity access	189 (93%)	80 (93%)	0·99 (0·38–2·87)	0·98
Drinking water access§				
Private	61 (30%)	19 (22%)	1 (ref)	..
Shared	142 (70%)	67 (78%)	1·32 (0·72–2·5)	0·38
Pets or farm animals	95 (47%)	34 (40%)	0·74 (0·44–1·24)	0·26
Recent antibiotic intake¶	23 (11%)	13 (15%)	1·39 (0·65– 2·86)	0·37
Chicken (N=246)				
Number of participants	106	140	..	..
Weight, g	804·11 (155·86)	788·57 (164·57)	1 (1–1)	0·45
Wet *vs* dry season	31 (29%)	74 (53%)	2·79 (1·65–4·81)	0·0002
Rainfall, mm†	66·41 (142·63)	179·74 (216·77)	1·40 (1·21–1·65)	<0·0001
Temperature, °C‡	18·77 (2·71)	20·24 (2·63)	1·22 (1·11–1·35)	<0·0001

Data are n, n (%), or mean (SD) unless indicated otherwise. *E coli=Escherichia coli.* ESBL=extended-spectrum β-lactamase. OR=odds ratio. *Age information missing for one participant in the non-carriers of ESBL-*E coli* group. †OR per 100 mm. ‡OR per °C. §Private means used by a single household; shared means used by more than one household. ¶Antibiotic intake in the past 3 months.

Table 2: **Risk factors associated with ESBL-*E coli* carriage in pregnant women and chickens**

See Online for appendix 2

(7·8 [3·3]) and animal sectors (6·2 [3·0]; p=0·0002; appendix 1 p 7). In all isolates, the ESBL phenotype was caused by $bla_{CTX\text{-}M}$ genes, the most prevalent being $bla_{CTX\text{-}M\text{-}15}$ (215 [78%]), followed distantly by $bla_{CTX\text{-}M\text{-}27}$ (34 [12%]), with variation between sectors (appendix 1 p 14). Prevalence of other resistance genes also varied slightly between sectors (appendix 1 p 13). The carbapenem resistant chicken isolate carried the $bla_{NDM\text{-}5}$ gene and the environmental isolate carried both the $bla_{NDM\text{-}5}$ and $bla_{OXA\text{-}181}$ genes.

The mean number of plasmid replicons was higher in environmental isolates (2·8 [SD 1·8]) than in human (1·8 [1·4]) and in chicken (1·3 [1·3]) isolates ($p<10^{-6}$; appendix 1 p 7). IncF was the most prevalent replicon in all sectors (36 [31%] of 118 chicken, 41 [50%] of 82 human, and 54 [70%] of 77 environmental isolates, p<0·0001), followed by IncY in humans (21 [26%] of 82) and chickens (27 [23%] of 118), and IncI in the environment (18 [23%] of 77).

The $bla_{CTX\text{-}M}$ gene was located on the chromosome in 159 (57%) of 277 isolates without difference between sectors, and on a plasmid in 113 (41%) isolates, including two isolates with both locations (chromosome and on the IncF-$bla_{CTX\text{-}M\text{-}15}$ plasmid) and one isolate with two ESBL carrying plasmids (IncN-$bla_{CTX\text{-}M\text{-}1}$ and IncY-$bla_{CTX\text{-}M\text{-}15}$). The location of $bla_{CTX\text{-}M}$ could not be confirmed in seven isolates. At least 58 different integration sites were identified, located mostly in the regions corresponding to 0–65 min of the *E coli* K-12 linkage map (appendix 1 pp 15–16).[10]

The 114 ESBL carrying plasmids (one strain carried two plasmids) were mostly IncF (63 [55%]) and IncY (42 [37%]). Other ESBL-plasmids were IncN (n=3), IncI (n=2), and IncB/O/K/Z (n=2), IncP (N=1), and p0111 (n=1). Prevalence of the ESBL-IncF plasmid was 19% (n=22) in chicken, 21% (n=17) in human, and 31% (n=24) in environmental isolates, and prevalence of the ESBL-IncY plasmid was 14% (n=17) in chicken, 22% (n=18) in human isolates, and 9·1% (n=7) in environmental isolates, with no significant difference between sectors (appendix 1 p 13). The 63 ESBL-IncF plasmids harboured various $bla_{CTX\text{-}M}$ genes (43 [68%] were $bla_{CTX\text{-}M\text{-}15}$, eight [13%] were $bla_{CTX\text{-}M\text{-}55}$, eight [13%] were $bla_{CTX\text{-}M\text{-}27}$, and four [6%] were $bla_{CTX\text{-}M\text{-}14}$), whereas ESBL-IncY harboured exclusively $bla_{CTX\text{-}M\text{-}15}$ (appendix 1 p 18).

A total of 65 different virulence associated gene (VAG) clusters or operons were detected in the 277 isolates. The mean number of VAG clusters per isolate was significantly higher in environmental (21·1 [SD 5·0]) than in human (19·9 [3·6]) and in chicken isolates (18·7 [2·5]; p<0·0001; appendix 1 p 7). Globally, isolates carried mostly genes involved in iron acquisition, with a mean of 15·9 (SD 4·5) genes per isolate, followed by adhesins (13·3 [6·0]), toxins (5·5 [3·0]), and protectins and invasins (2·8 [1·8]) with slight variations among sectors (appendix 1 p 13). Details of isolates characteristics and statistical analyses results are in appendix 2.

We first investigated the intrasector and intersector strain circulation. The global phylogenetic tree and the phylogenetic trees constructed by sector showed isolate clustering, especially in the animal sector (figure 2; appendix 1 pp 12, 19–20). Based on the distribution of SNPs within the genome-based similarity matrix, a threshold of 40 SNPs was chosen to consider isolates as genetically closely related (figure 3A). All isolates differing by fewer than 40 SNPs had the same STc, ST (except two, due to a single mutation in the *fumC* and *gyrB* gene, respectively), serotype, *fimH* allele (except one). Five isolates had a different CTX-M variant. A heat map of SNP differences showed intrasectoral and intersectoral dissemination of genetically related isolates (figure 3B). A more detailed force-directed layout, representing isolates differing by fewer than 40 SNPs and harbouring the same ESBL enzyme, showed 169 isolates (38 [46%] of 82 human, 82 [69·5%] of 118 chicken, and 49 [63·6%] of 77 environmental isolates) gathered in 44 clusters, 15 being monosectoral (figure 3C). The two biggest monosectoral clusters (ST716 and ST3489), containing seven to 12 isolates were observed among chickens, coming from the same, but also from different farms. Three clusters contained four to five environmental isolates, sampled in different sites (upstream, downstream, and wastewater). Human clusters all had fewer

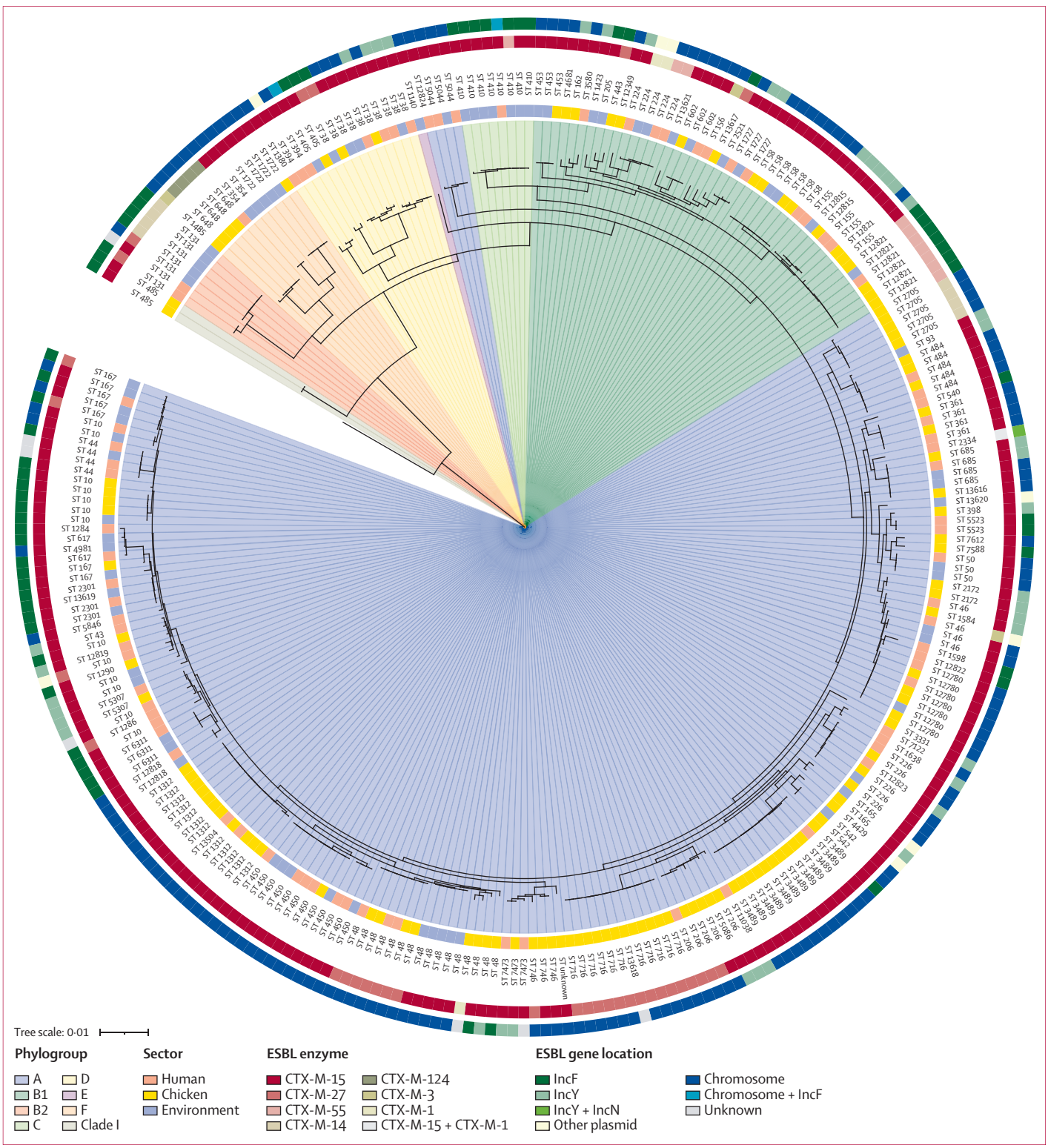
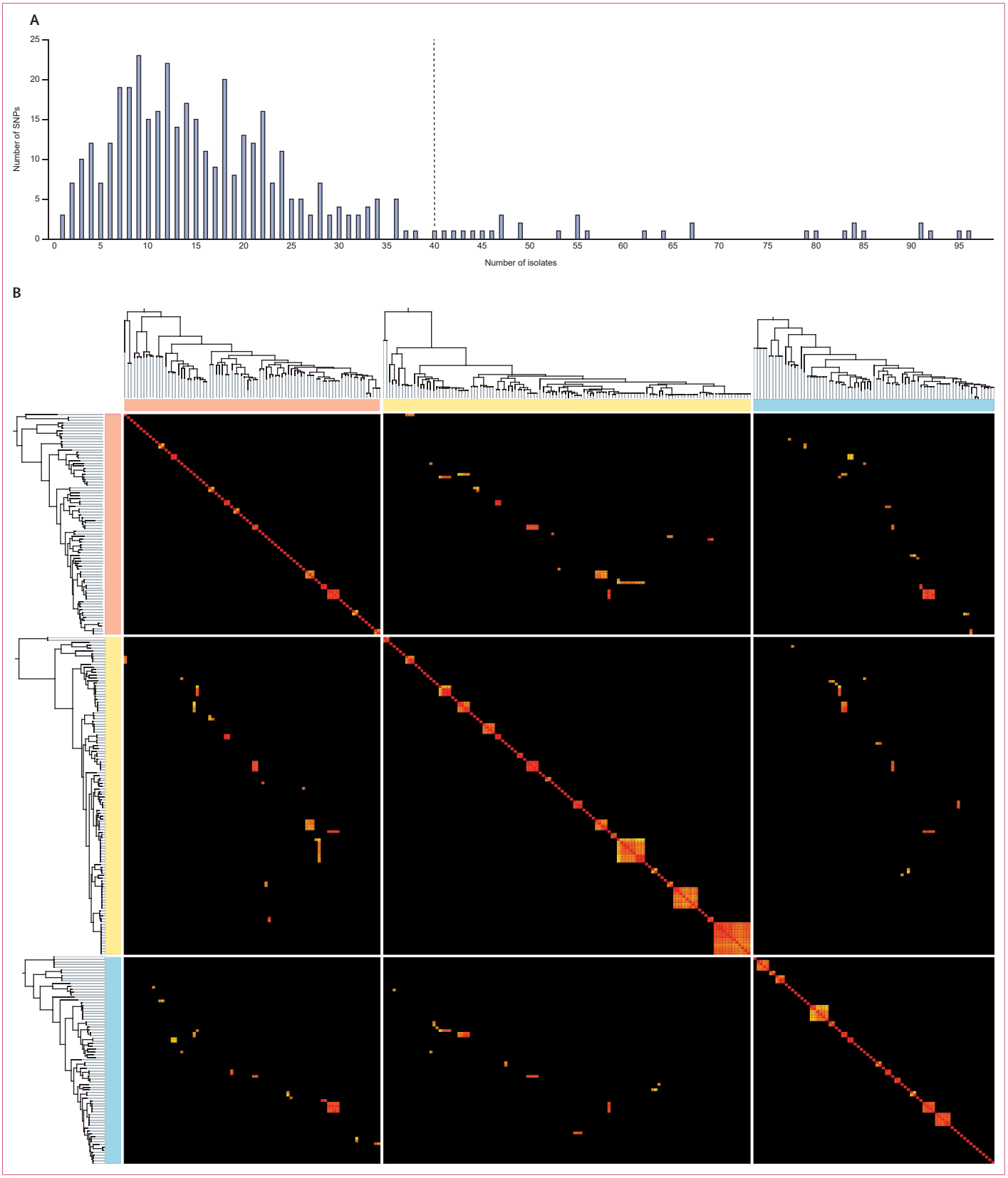


Figure 2: **Phylogenetic tree based on core genome sequences of the 277 ESBL-*E coli* isolates**

The tree was rooted on *Escherichia fergusonii*. Isolates with their terminal branches are highlighted depending on their phylogenetic group. Inner ring represents isolate origin; ring two indicates the ST of each isolate; ring three represents the CTX-M variant and the outer ring represents the location (plasmid or chromosome) of the bla_{CTX-M} genes. Isolates identification and chromosomal insertion sites are detailed in appendix 1 (p 12). *E coli*=*Escherichia coli*. ESBL=extended-spectrum β-lactamase. ST=sequence type.



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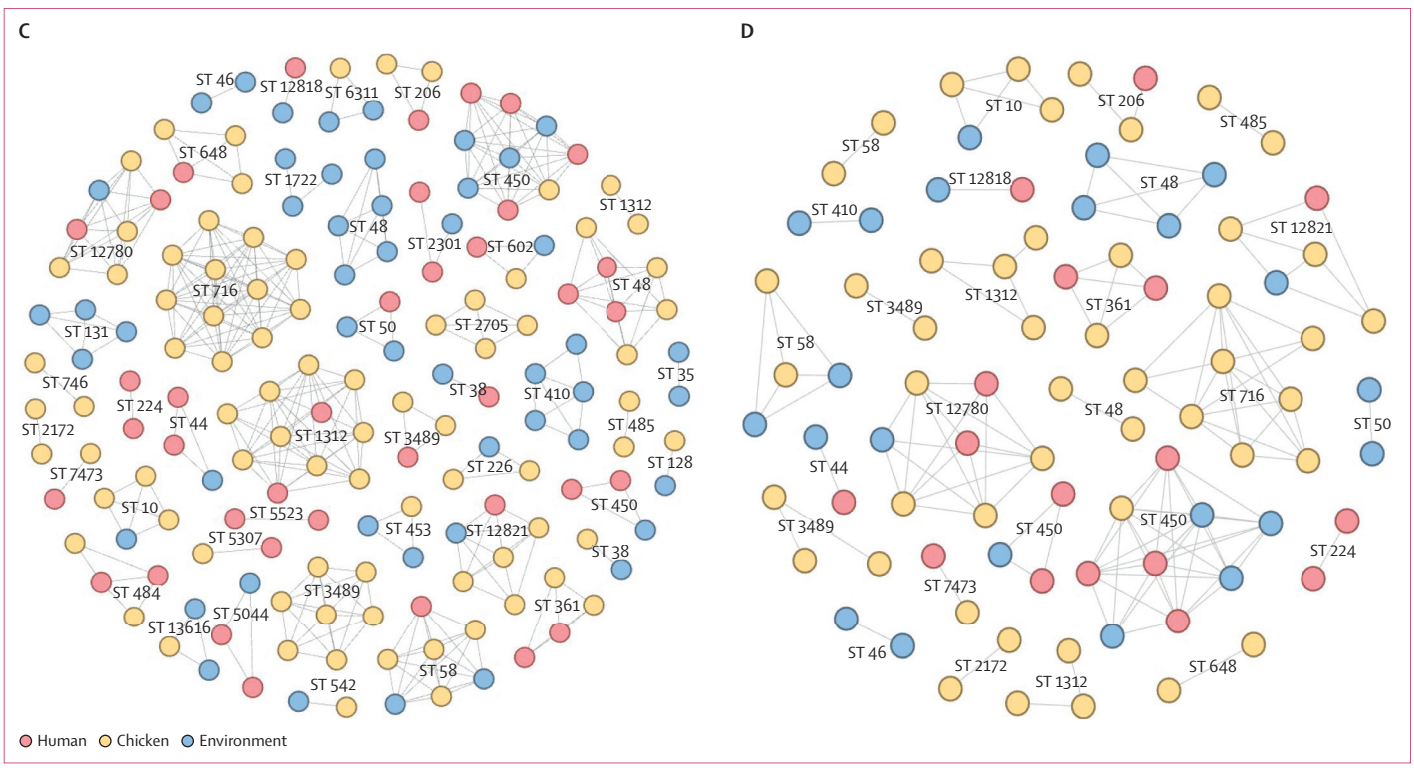


Figure 3: **Genetic relatedness of isolates based on SNP analysis**

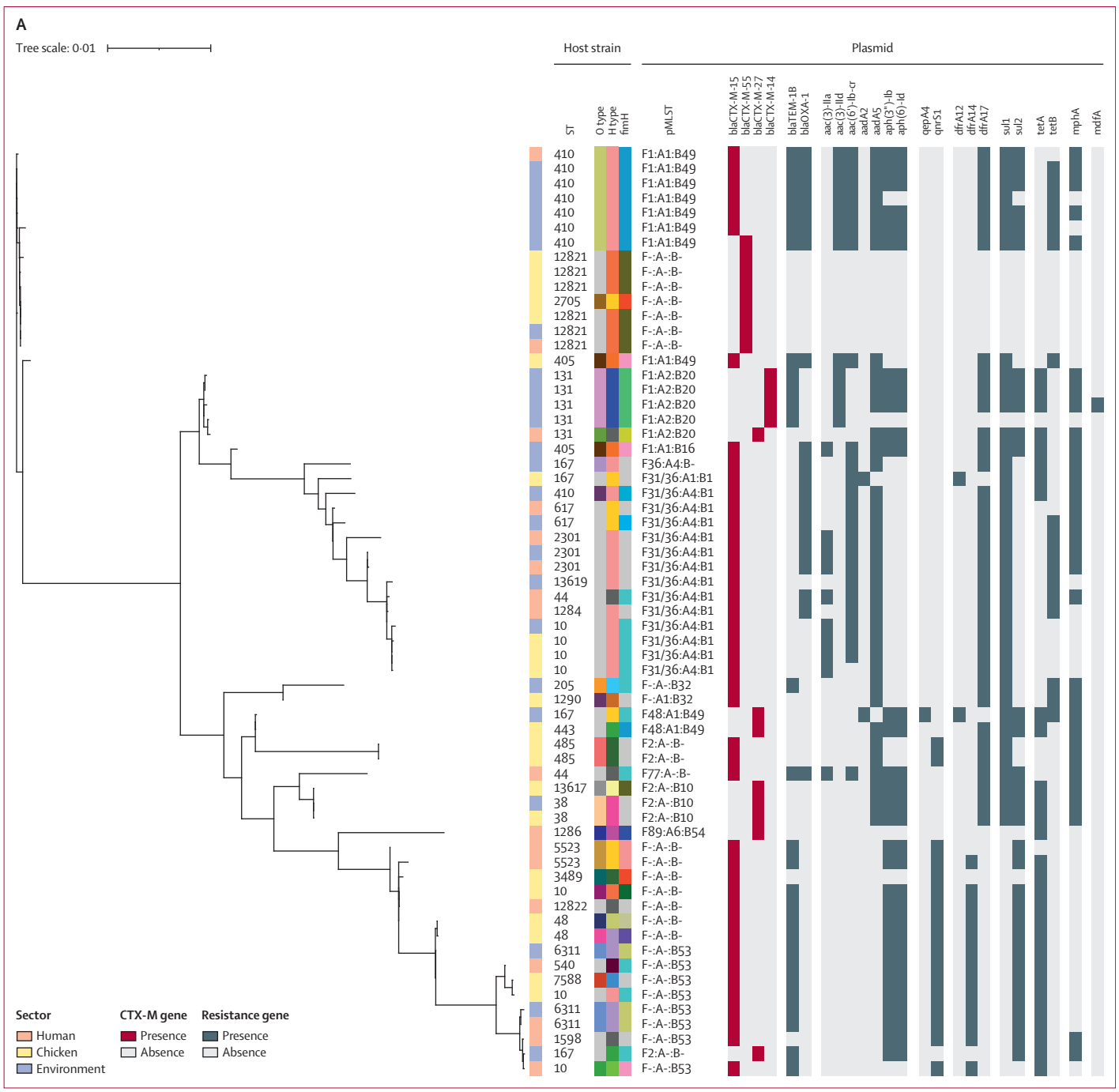
(A) Distribution of SNPs comparing core genome of ESBL *E coli* isolates from the three sectors differing of up to 100 SNPs and cutoff chosen (40 SNPs) to consider isolates as genetically related. (B) Heat map of SNP differences (0–40 SNPs) between ESBL-*E coli* isolates grouped by sector. Horizontal and vertical phylogenetic trees are based on core genome sequence per sector (human in red, chicken in yellow, and environmental in blue). Numbers of SNPs (0–40) in the core genome between different sequenced isolates were interpreted as a distance matrix. Red to yellow colours in the heat map represent SNP differences, respectively 0 to 40 SNPs. The diagonal line corresponds to the intrasector comparison with the three main clusters in the chicken sector. Force-directed Fruchterman–Reingold layout representing intrasectoral and intersectoral dissemination of isolates differing by fewer than 40 SNPs (C) and up to 10 SNPs (D). Branch lengths are not representative of SNP distances. *E coli=Escherichia coli*. ESBL=extended-spectrum β-lactamase. SNP=single-nucleotide polymorphism. ST=sequence type.

than four isolates. 24 clusters contained isolates from two sectors (seven human or environment, eight chicken or environment, and nine human or chicken). Five clusters (from ST58, ST450, ST602, ST12780, and ST12821) contained 32 (12%) of 277 isolates from the three sectors. Using the threshold of 10 SNPs, frequently used in the literature to consider *E coli* isolates as similar,[11] we constructed another force-directed layout still showing multiple bisectoral and three trisectoral clusters (figure 3D). The core genome MLST analysis was consistent with the SNP analysis and confirmed the presence of multiple clusters containing genetically related isolates from different sectors (appendix 1 pp 21–22).

We next investigated intrasectoral and intersectoral circulation of plasmids carrying ESBL genes. Because a small number of isolates harboured other plasmid types, we focused the analysis on IncF and IncY plasmids.

The 63 ESBL-IncF plasmids were distributed into 15 different pMLST subtypes, the primary being F31/36:A4:B1 (13 [21%]), followed by F-:A-:B53 (eight [13%]), and F1:A1:B49 (eight [13%]). IncF plasmids with undetermined pMLST F-:A-:B-, were the most frequent (14 [22%]) but were distributed in two distinct groups, including a highly conserved phage plasmid carrying $bla_{CTX\text{-}M\text{-}55}$. All plasmid subtypes identified in two or more isolates (eight [53%] of 15), were observed in at least two different sectors, carried by *E coli* isolates with different genetic backgrounds. The F31/36:A4:B1 (99% sequence homology) subtype was harboured by seven genetically different *E coli* isolates and the F-:A-:B53 (95% sequence homology) subtype was harboured by six genetically different *E coli* isolates, and both subtypes were observed in all three sectors (figure 4A; appendix 1 pp 23–24).

The ESBL-IncY plasmids showed more than 99% sequence homology (with 70–100% coverage) and a highly conserved resistance gene content (figure 4; appendix 1 pp 23–24). With the exception of *tet(A)* and *dfrA14*, lost by deletion in two and five plasmids, respectively, all other ESBL-IncY plasmids carried *qnrS1*, $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, *aph(6)-Id*, *aph(3")-Ib*, *sul2*, and *dfrA14* genes and two copies of *tet(A)*. Alignment of the IncY genomes confirmed this homology and highlighted the presence of two common unstable zones, one starting with an IS26 and containing the *xerC* (tyrosine recombinase) and *betU* genes and the other starting with an IS1R and affecting the *virB* gene cluster involved in conjugation (appendix 1 p 25). ESBL-IncY plasmids were observed in all three sectors within 34 *E coli* isolates with different genetic backgrounds.



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The mean conjugation frequencies of the F31/36:A4:B1 plasmid subtype was $1{\cdot}70\times10^{-7}$, the F-:A-:B53 plasmid subtype was $4{\cdot}99\times10^{-8}$, and the IncY plasmid was $2{\cdot}07\times10^{-7}$, without significant difference (appendix 1 p 26).

Finally, to visualise links among plasmids, strains, and sectors, we constructed a Sankey diagram, which confirmed the significant intrasectoral and intersectoral circulation of both ESBL-*E coli* strains and ESBL-plasmids (figure 5; appendix 1 p 27).

Discussion

To our knowledge, the present study is the first to address the circulation of antibiotic resistance from a One Health perspective in a low-income country, considering both

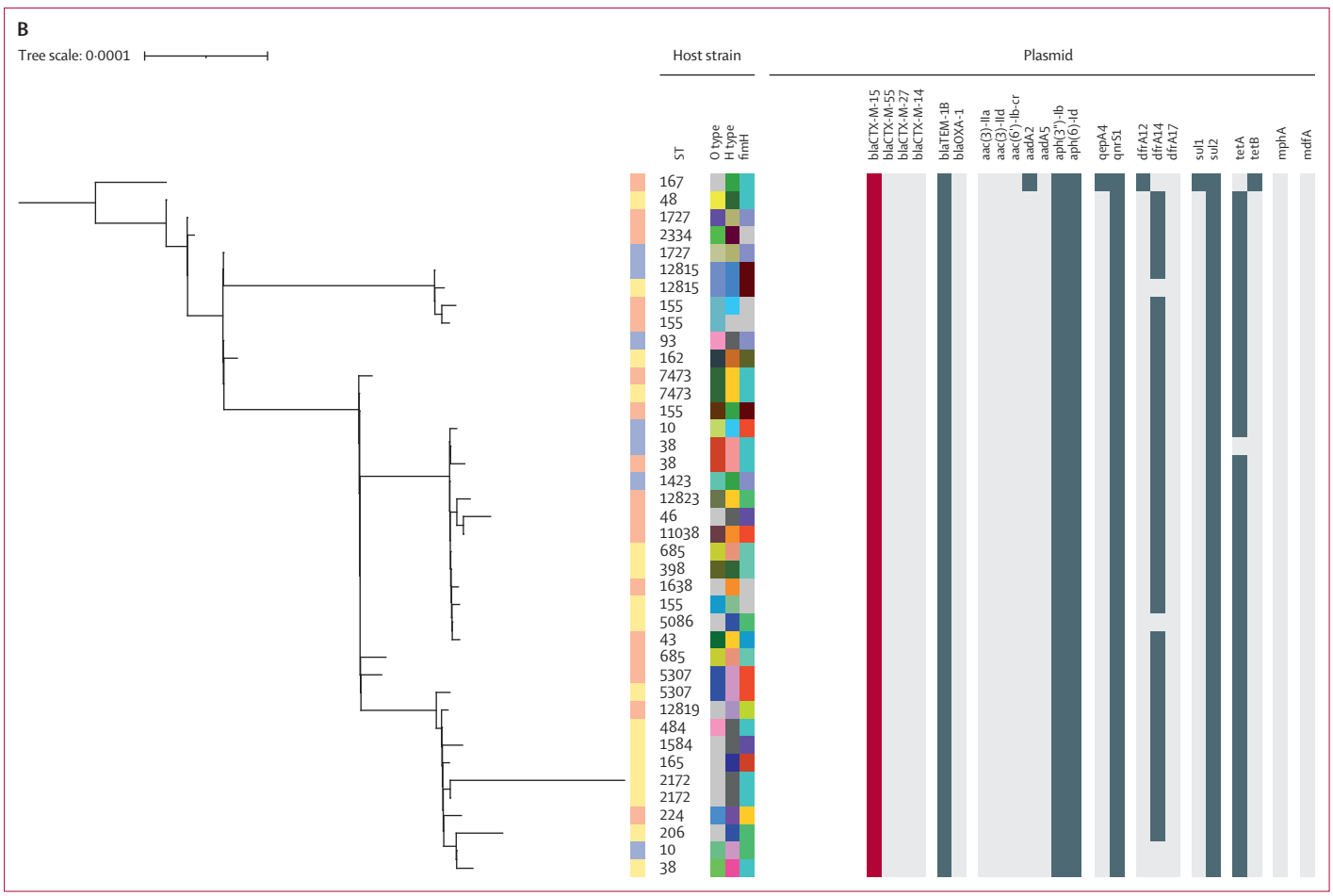

Figure 4: **Pangenome trees of IncF and IncY plasmids**

(A) Pangenome tree of IncF plasmids. (B) Pangenome tree of IncY plasmids. The trees were constructed from the presence or absence matrix of genes. Scales differ between the two trees. Strip one represents isolate origins (sectors). The strains are characterised by their ST, serotype (O type and H type) and *fimH* allele (strips two to five). Strip six represents the plasmid MLST type (for IncF plasmids only). Strips seven to ten represent the CTX-M variant detected. Strips 11 to 30 represent plasmid-borne resistance genes. Two isolates (one harbouring IncF and one IncY) were excluded from the alignment due to insufficient sequencing quality. Isolates identification, serotypes, and *fimH* alleles are indicated in appendix 1 (pp 23–24). ST=sequence type.

ESBL-*E coli* strains and ESBL-carrying plasmids in humans, farm chickens, and the environment. Our study is also the first to include genomic analysis of the results of the implementation of the WHO Tricycle protocol. Through high-quality sampling, performed within the same temporal scale and in a limited geographical area, we were able to evidence substantial circulation of ESBL-*E coli* strains and ESBL-plasmids within and between all three sectors despite sector-specific population structure features.

In our study, ESBL-*E coli* prevalence in healthy pregnant women was 30%, which is consistent with previous studies that reported prevalence of ESBL producing *Enterobacterales* in the Malagasy community of 19% and 30%.[12,13] Interestingly, the proportion of ESBL-*E coli* in bloodstream infections was similar to human commensal prevalence, indicating a burden of antibiotic resistance in invasive *E coli* infections. In the animal sector, ESBL-*E coli* prevalence in chickens reached 57%, much higher than a previous study from Madagascar, reporting a prevalence of 35%.[13] Finally, all environmental samples contained ESBL-*E coli*, with increasing concentrations from upstream and downstream surface water to wastewater and slaughterhouse effluents, underscoring the effect of dense human populations and of untreated wastewater on ESBL-*E coli* spread in the environment. Poultry in Madagascar is mostly raised through traditional, non-extensive farming, without the use of antibiotics for treatment or growth promotion. However, these animals are free roaming, feeding from unregulated sources and drinking contaminated water, which makes the environment a key factor in the transmission between farm animals and humans.

The only risk factor identified for human and animal ESBL-*E coli* carriage was the wet season (November to April), characterised by high temperatures and rainfall. We have already reported a correlation between ESBL-*E coli* prevalence carriage in humans and the wet season in a previous study in Madagascar, as well as other studies.[7,14,15] In contrast, we did not find any publications reporting a positive

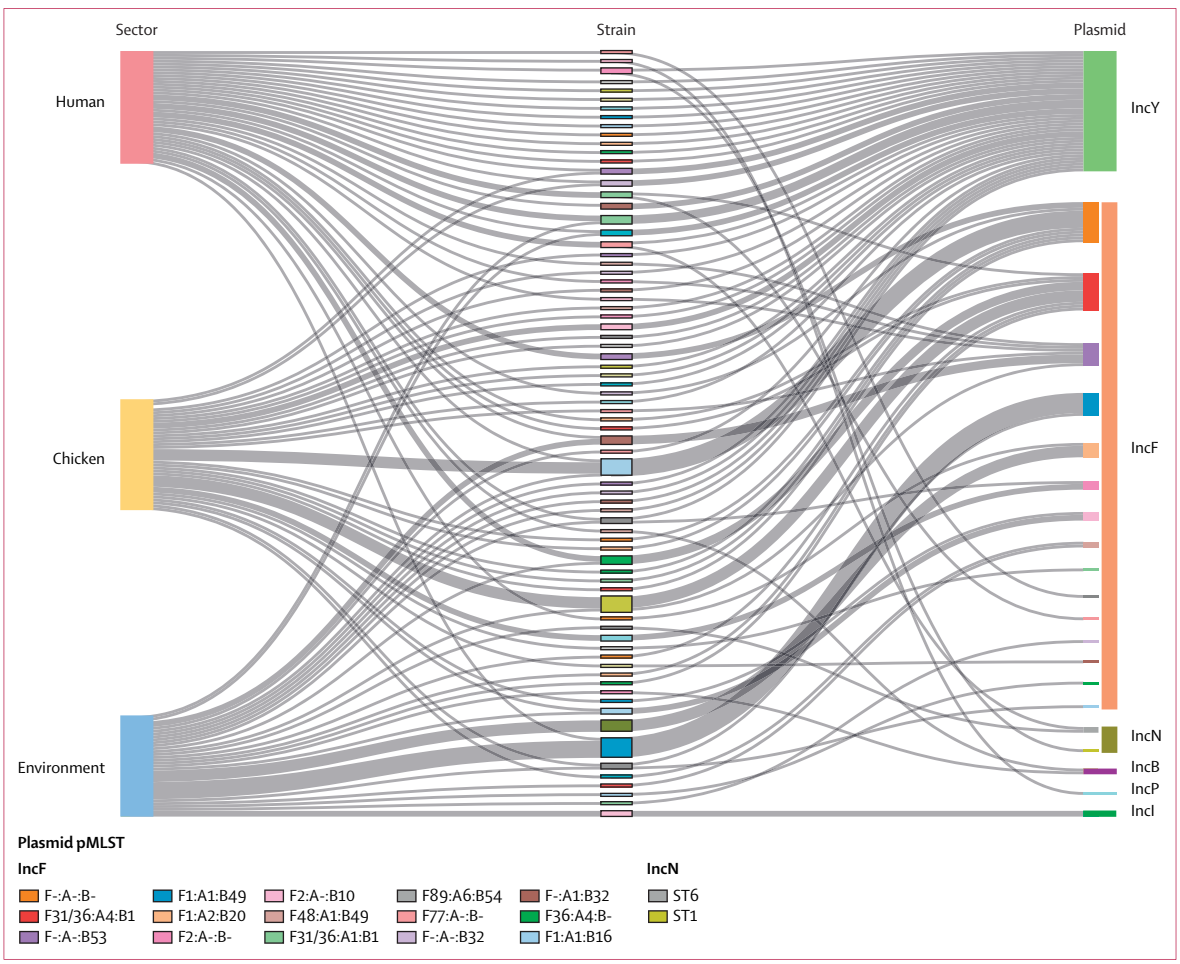

Figure 5: **Sankey diagram representing relationships between sampling sectors, ESBL-*E coli* isolates and ESBL carrying plasmids**
The width of the coloured boxes and their connecting grey bands are directly proportional to the frequency of sector samplings (left), ESBL-*E coli* isolates (middle), and ESBL carrying plasmids (right). Coloured rectangles in the middle represent ESBL-*Ecoli* clones based on the same ST, serotype, and *fimH* allele (ST, serotypes, and *fimH* alleles are indicated in appendix 1 (p 27). *E coli=Escherichia coli*. ESBL=extended-spectrum β-lactamase. ST=sequence type.

correlation between seasonality and ESBL-*E coli* carriage in farm animals. The wet season is well known for favouring faecal-oral transmission of enteric bacteria.[16] In vitro models also suggest that growth rates and horizontal gene transfer might be enhanced by higher temperatures and humidity,[17,18] making global warming an additional threat to the dissemination of AMR.

In all isolates, the ESBL phenotype was due to a $bla_{\text{CTX-M}}$ gene, and $bla_{\text{CTX-M-15}}$ being the most frequent in each sector, which is consistent with the global ESBL epidemiology.[19] Surprisingly, we observed chromosomal integration of $bla_{\text{CTX-M}}$ genes in 57% of isolates. High rates of $bla_{\text{CTX-M}}$ chromosomal integration are being increasingly reported, reaching, for example, 64% in a Japanese study.[20] Moreover, we detected multiple chromosomal insertion sites over a large part of the genome suggesting frequent and repeated events. Chromosomal integration is regarded as providing stabilisation of $bla_{\text{CTX-M}}$ genes at a lower fitness cost in comparison to a plasmid, which enables vertical transmission and $bla_{\text{CTX-M}}$ persistence even in the absence of selective pressure.[20] However, the impact of chromosomal integration on the $bla_{\text{CTX-M}}$ dissemination in the future, is difficult to predict.

As already described in the literature,[5] WGS analysis confirmed different population structures in the three sectors with an increasing gradient of genomic diversity, genome size, number of VAGs, antibiotic resistance genes, and replicons from poultry to human and then to the environment. Despite these ecological peculiarities, we observed an intersectoral circulation of strains. We performed an SNP analysis of strains differing by fewer than 40 SNPs, a threshold based on SNPs distribution in the entire collection. This analysis revealed multiple clusters of closely related isolates, containing isolates from two and from all three sectors, reinforcing the hypothesis of intersectoral transmission. SNP cutoffs for considering genetically related isolates are highly variable in the literature, ranging from 10 to 100.[21,22] Therefore, we performed a more stringent

analysis, retaining only isolates differing by up to 10 SNPs, the threshold frequently used to consider *E coli* isolates as similar,[22] and we still observed clusters of closely related isolates belonging to the three sectors, evidencing intersectoral ESBL-*E coli* dissemination in the Antananarivo area. Most studies analysing the intersectoral circulation of ESBL-*E coli* have identified rather genetically distinct populations of human, animal and environmental isolates, with interhuman transmission being the major source for community colonisation.[23–26] However, these studies have rarely investigated isolates collected at the same temporospatial scale.[23,24,26] Moreover, these studies included human strains, isolated from invasive infections, and were mainly conducted in high-income countries, whereby *E coli* strains from B2 and D phylogroups have progressively outpaced commensal phylogroups A and B1.[5,26] The discrepancy in relation to our results could be explained by different behaviours in low-income countries, driven by local socioeconomic factors, differences in selection pressure from antimicrobial use, or insufficient sanitation. This hypothesis is supported by the few studies performed in low-income countries, showing closely related strains, shared by humans and animals.[13,21,27] A study conducted in Malawi, showed the key role of environmental health infrastructure, direct human or animal contact and interactions with contaminated environment in the carriage of ESBL-*E coli* in humans, particularly in urban areas.[14]

Additionally, we investigated the intrasectoral and intersectoral circulation of ESBL carrying plasmids. Predominant ESBL-plasmids were of IncF type, well known for their high dissemination ability, especially F31/36:A4:B1 and F-:A-:B53, which we identified in *E coli* of different genetic backgrounds and from different sectors.[28,29] In a previous study we described an unusual prevalence of a highly conserved ESBL-IncY plasmid in *E coli* isolates from pregnant Malagasy women.[7] Here, we confirmed the substantial dissemination of the same ESBL-IncY plasmid also in chickens and in the environment. IncY plasmids are usually considered as phage plasmids, but the IncY plasmid described here do not carry genes coding for phage proteins and contain a *virB* conjugation system.[30] We have also confirmed the in-vitro transferability of these plasmids by conjugation from a selected set of isolates. The detection of the same IncY plasmid and some IncF subtypes in the three sectors, harboured by *E coli* of various genetic background, showed another pathway of dissemination of ESBL resistance in a One Health context in the Antananarivo area.

The present study has several limitations. First, our sampling slightly differed from the original Tricycle protocol. Due to logistic issues, it was not possible to purchase and transport six chickens from different farms for each campaign. Chickens were therefore purchased in batches of six from the same farm, but a different farm was chosen each week. Water sampling points could not be duplicated. Second, unfortunately, ESBL-*E coli* strains isolated from blood cultures were not stored and were excluded from the rest of the study. Third, prevalence in pregnant women does not give an exact estimate for ESBL-*E coli* carriage in the community because it specifically targets an age group and sex category with particular behaviour.[3] Finally, although our results showed circulation of ESBL-*E coli* and ESBL-plasmids between sectors, the direction of the transmission routes remains undetermined.

By implementing the One Health Tricycle surveillance project in Madagascar, we detected a high ESBL-*E coli* prevalence in healthy pregnant women, farm chickens and the environment and evidenced the circulation of both ESBL-*E coli* strains and ESBL-carrying plasmids, between all three sectors, despite specific sectoral population structures. To tackle antibiotic resistance, measures related to global antibiotic consumption must be accompanied by actions to limit the transmission of resistance between the three sectors.

Contributors

AA, ED, and LA-L designed the study. HE, FK-P, and LA-L obtained funding for the study. MM, FK-P, HE, and LHS implemented and supervised the protocol in Madagascar. LR, SR, VLR and LHS coordinated laboratory participation in Madagascar. TLR, FAR, SR, VLR, CR, ZR, and IT'HR collected and processed the samples. MM performed antimicrobial susceptibility testing and DNA extraction. CP performed plasmid sequencing. EW and MP performed bioinformatical analysis. MM, OC, and CP performed sequence analysis. JMa performed genetic diversity analysis. LH and JMu performed statistical analysis. VB performed conjugation experiments. MM and LA-L did data verification and curation, formal analysis, visualisation and wrote the first draft. All authors read and approved the final version of the manuscript. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

The original contributions presented in the study are publicly available and can be found at: https://www.ebi.ac.uk/ena/browser/home (accession number PRJEB56633).

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Editorial note: The Lancet Group takes a neutral position with respect to territorial claims in published maps.

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