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Dynamics of extended-spectrum cephalosporin-resistant *Escherichia coli* in pig farms: A longitudinal study

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ABSTRACT

Objectives: Point prevalence estimates of extended-spectrum cephalosporin-resistant *Escherichia coli* (ESC-R-*Ec*) are important surveillance measures but may not uncover the ESC-R-*Ec* dynamics within pig farms. A longitudinal study was therefore performed by sampling individual pigs, pig farmers and the environment.

Methods: On average, 30 (range 10–46) piglets of 31 Swiss farms were sampled during the suckling, weaning and fattening stages (n= 2437 samples). In addition, stool from pig farmers and environmental samples were obtained and metadata collected by questionnaires. ESC-R-*Ec* was identified by routine culture, and clonal relationships and resistance genes were derived from whole genome sequencing data.

Results: Working on pig farms was not associated with an increased prevalence of ESC-R-*Ec* in humans. ESC-R-*Ec* prevalence significantly decreased from 6.2% to 3.9% and 1.8% for the suckling, weaned and fattening pigs, respectively ($P < 0.001$). Within the 57 ESC-R-positive suckling piglets, persisting carriage was detected in 25 animals at two consecutive time points and one animal at three consecutive time points. Clonal spread (n=7 farms, 22.6%) and horizontal gene transfer (n=1 farm, 3%) within pigs but not between humans and animals was detected. Liquid manure (n=10 samples, 16.7%) was identified as the major environmental reservoir of ESC-R-*Ec* in the pig farm environment.

Conclusions: Pig farming practices like all-in-all-out systems, but not antimicrobial usage, were associated with reduced risk of ESC-R-*Ec* at the farm level. As carriage duration is normally short within the individual pigs, the risk of recolonisation and clonal spread of ESC-R-*Ec* might be reduced by applying appropriate decontamination strategies.

1. Introduction

The spread of extended-spectrum cephalosporin-resistant *Escherichia coli* (ESC-R-*Ec*) constitutes a major public health problem. Modern animal food production depends on antimicrobial treatment and, hence, livestock has been described as a reservoir of antibiotic resistance [1]. In 2009, faecal samples from Danish pigs were collected and 11% (86/786) of slaughter pigs carried ESC-R-*Ec* [2]. In another study applying a sampling strategy representative of the livestock population slaughtered in Switzerland between 2010 and 2011, the prevalence of ESC-R-*Ec* in pigs was 3.3% [3]. Considering data submitted by 28 EU member states, the proportion of presumptive extended-spectrum beta-lactamase (ESBL) and/ or AmpC producers was low among all indicator *E. coli* isolates recovered from fattening pigs (52/4205, 1.2%) [4]; however, large differences among the reporting countries were observed.

Depending on the life stage, pigs are exposed to distinct nourishment, housing and antibiotic treatment [5,6]. This may have consequences on the development of the animals' pool of resistance genes. As longitudinal studies of ESC-R carriage in pigs are scarce, little is known about when pigs acquire resistant bacteria and how long they persist in the animals' gastrointestinal tract. An important contribution of indigenous enterobacteria maternally transmitted along the sow lineage has beenrecently suggested [7].

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A decrease in ESC-R prevalence was reported in a study that longitudinally monitored pig herds from three farms in Germany [8]. Also, ESBL/*ampC*-producing *E. coli* were found in a non-significantly higher prevalence in breeding pig farms, where it was present in nine of 16 (56.3%) as compared with seven of 16 (43.8%) fattening pig holdings in Germany [9]. Collectively, these studies suggest that monitoring of ESC-R-*Ec* should be performed in a longitudinal manner as its presence may fluctuate over a pig's life time.

Contact with livestock has been discussed as a risk factor for human ESBL carriage. Both clonal transmission and horizontal gene transfer in isolates of animal and human host origin have been described [10–12]. Evidence of zoonotic transmission of ESC-R-*Ec* is still scarce for pig farm settings, with routes of transmission unclearly defined.

This study aimed to characterise ESC-R-*Ec* isolates, and its prevalence, persistence and spread in the pig farm environment. A One Health approach with longitudinal sampling of pig faeces combined with a cross-sectional sampling of farm workers and environmental samples was conducted in 31 Swiss maternity pig farms with varying antimicrobial usage (AMU). Animals were sampled during suckling, weaning and fattening periods.

2. Material and Methods

2.1. Study design

A prospective, longitudinal study was conducted in Switzerland from March 2018 through July 2019. Pig farmers and their piglets were recruited from 31 maternity pig farms in western Switzerland, with (n=18) or without (n=13) finishing units. Overall, 15 and 16 farms were reported to have low and high AMU, respectively, by the central service and competence centre for the Swiss pig industry (SUISAG), according to a score including the percentage of animals within a class of age that were treated during the last 4 months. Farm workers were asked to self-collect stool using a sterile cup and send by mail. Concerning the prospective sampling of pigs, samples from the same animal were collected 2 weeks (suckling period), 6 weeks (weaning period) and 16 weeks (fattening period) after birth. Coloured ear tags were used to ensure identification of animals over time. Depending on the farm type (farrow-to-finish vs. maternity farm), post-weaned pigs stayed in their native farm or were transported to one or two different fattening units. In case of transportation, the change in location occurred between weaning and fattening stages. Transportation was tracked in order to ensure sampling of the fattening units. On each sampled maternity unit and fattening unit, environmental samples were collected cross-sectionally.

2.2. Ethics

Written informed consent was obtained from all participating farmers. The participants answered questionnaires comprising information about antimicrobial usage, farm characteristics and health-related data of the pig farm worker. Ethical clearance for this study was obtained from Human Research Ethics Committee of the Canton Vaud (2018-00080) and the Veterinary Ethics Committee of the French Cantons (VD2903).

2.3. Sample collection from humans, animals and the environment

The collection of human stool samples was initiated at the first visit by asking the farm workers to self-collect and send material. Upon arrival, samples were refrigerated at 4°C. The sample processing occurred within seven days after collection, at the latest. From each pig, one rectal swab sample was collected per time point. The obtained faecal material was kept in liquid Cary Blair transport medium (Copan), and transported and stored at 4°C until culture identification. As part of sampling the environment, air samples were collected at the second visit on maternity farms and during the third visit (fattening unit), as previously described [13,14]. In brief: airborne particles from a volume of 3000 L were collected in 15 mL of Triton X 0.005% solution using a Coriolis® μ air sampler (Bertin Technologies, Montigny-le-Bretonneux, France). Settled dust was collected using CultureSwab EZ from surfaces of the sheds that were inaccessible to the pigs. Swabbing occurred on a length of 15 cm and the swabs were always saturated with dust after the sampling. If present, one to four flies per farm visit were collected using a sterile swatter and transferred into Eppendorf tubes at the first, second and third visits. At any visit, if present, mouse excrement was collected from the floor and transferred into Eppendorf tubes. If accessible, liquid manure was collected at the first and third visits using a sterile cup on each visited farm unit (maternity and fattening). On farm 24, manure was not accessible and faeces from a mother sow was collected instead.

2.4. Sample processing and ESC-R screening

The protocol for ESBL detection in porcine rectal swabs followed the protocol issued by the European Reference Laboratory for Antimicrobial Resistance. In brief: pools of up to 10 pig swabs were transferred to 10 mL buffered peptone water (BPW) and vortexed. The BPW was aerobically incubated at 37°C ± 1°C for 18–22 hours. Environmental samples were aerobically incubated individually in 9 mL of BPW at 37°C ± 1°C for 18–22 hours. One loopful (10 µL) of the mixed enrichment was streaked out on MacConkey Agar containing 1 µg/mL cefotaxime (CTX, Tritium) and incubated at 44°C ± 1°C for 18–22 hours. For human faecal samples, 20 mg of stool was aerobically enriched in 10 mL BPW containing 3 µg/mL cefuroxime (CTM) for 18–22 hours at 37°C, as described [15]. To detect carbapenemase producers, 10 µL of the mixed enrichment was plated on ChromID ESBL (BioMérieux) and Carba Smart and incubated at 37°C for 24 hours.

Escherichia coli (*E. coli*) isolates were identified using Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass-Spectrometry (MALDI-TOF MS) using the direct transfer protocol according to the manufacturer's instructions (Biotyper 3.0, Bruker Daltonics GmbH, Bremen, Germany). In case of ESC-R *E. coli* detection in pooled samples, enrichment and plating were repeated for each individual sample of the pool by pipetting 1 mL of the remaining Cairy Blair medium of each sample to 9 mL BPW. After vortexing, the procedure was the same as for the pooled samples. Per sample, one suspected ESC-R *E. coli* isolate was phenotypically confirmed by broth microdilution using Sensititre™ MIC Plates EUVSEC and EUVSEC2 (Thermo Scientific™) following the CLSI guidelines. In brief: *E. coli* isolates were grown on trypticase soy agar with 5% sheep blood (TSA SB, Becton, Dickinson, Franklin Lakes, NJ) for 18–24 hours at 37°C ± 1°C in an aerobic atmosphere, and the obtained colonies were used to achieve an inoculum of approximately 1×10^5 cfu/mL in cation-adjusted Mueller-Hinton broth (Thermo Fisher Scientific). Inoculated EUVSEC and EUVSEC2 plates were aerobically incubated for 18–24 hours at 35°C ± 2°C in a humidified chamber. MIC values were interpreted according to epidemiological cut-off values (ECOFFs) and tentative ECOFFs for azithromycin, ertapenem and temocillin, respectively, issued by EUCAST (www.eucast.org).

2.5. Whole genome sequencing (WGS) and molecular analyses

Genomic DNA of phenotypically-confirmed ESC-R *E. coli* was extracted using QIAamp DNA Mini Kit. For the first batch, Illumina sequencing was conducted using Nextera XT library preparation. Sequencing was performed on a HiSeq3000 with 300 cy-

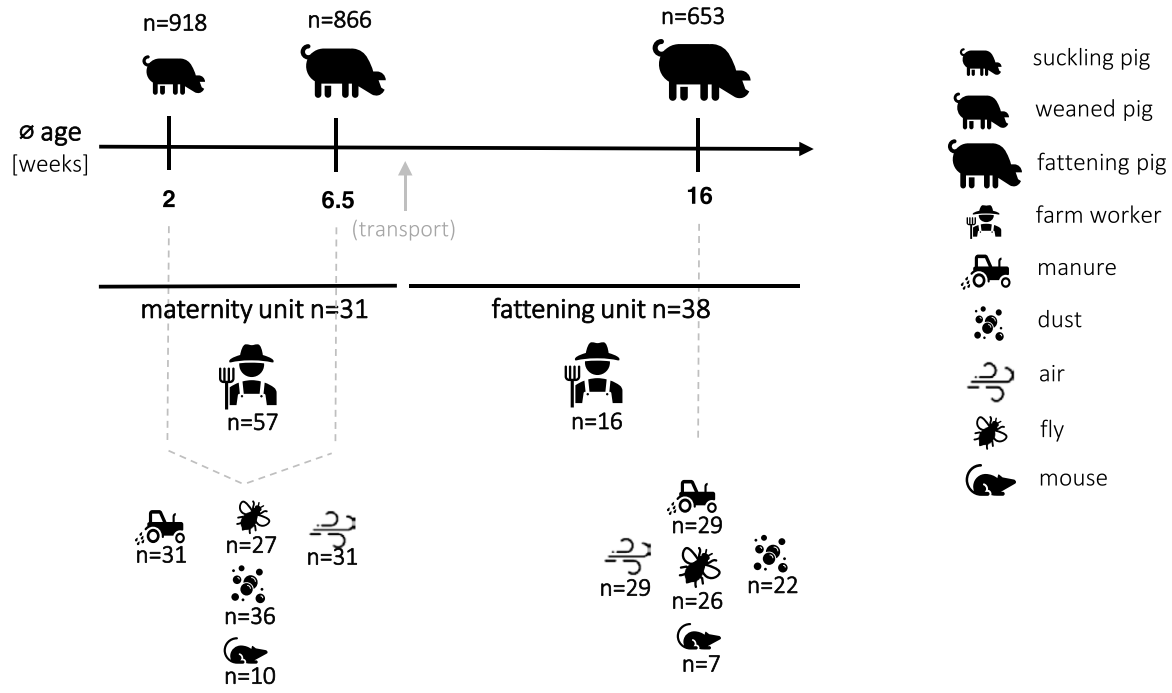

Figure 1. Longitudinal collection of pig faecal material, received cross-sectional human stool samples from farm workers and environmental samples. The majority of pigs were transported to one or multiple distinct fattening units after weaning (indicated as transport). N, number of samples; Ø, average; n, number

cles paired-end. For a second batch, Illumina Nextera Flex library preparation and sequencing on a NovaSeq6000, 300 cycles paired-end was performed (due to replacement of the first-named sequencing machine). Reads were assembled using SPAdes [16]. Resistance genes were identified using ResFinder [17]. A phylogenetic neighbour-joining tree based on core-genome multilocus sequence type (cgMLST) was created using Ridom seqsphere (v6.0.2) and annotated using iTol [18,19]. According to the curator, the Ridom SeqSphere+ *E. coli* core genome MLST scheme makes use of the identical loci and reference gene sequences as the EnteroBase (http://enterobase.warwick.ac.uk) *Escherichia/Shigella* cgMLST v1 scheme. However, the SeqSphere+ allele calling procedure is slightly different and uses an independent allele numbering that is incompatible with the EnteroBase allele nomenclature. For better comparability the current study also received the cgMLST and MLST from CGE (https://cge.cbs.dtu.dk/services/cgMLSTFinder/).

A selection of 22 isolates underwent Nanopore sequencing to reveal ESBL-gene location. The isolates were chosen based on unique cgMLST and/or resistance gene profiles. If there were several isolates originating from the same farm with identical cgMLST and resistance profile, only one *E. coli* was selected. DNA extracts from eight isolates were multiplexed on one flow cell, libraries were prepared using the rapid barcoding protocol and sequencing occurred on a GridIon. Barcode demultiplexing was performed using porechop [20], hybrid assembly was carried out using unicycler [21]. By performing hybrid assembly, the sequence of the plasmid carrying the ESBL gene was identified [22]. PubMLST was then used to obtain information on plasmid MLST of the mobile genetic element that contains the ESBL gene [23]. Gene annotations were based on prokka [24] and BLAST. Plasmid comparison was performed using BLAST Ring Image Generator [25]. The sequence reads were submitted to the European Nucleotide Archive (accession number: PRJEB40496). Nanopore sequencing of the 22 isolates was performed in three runs: ERS5806373 (SAMEA8119393) run 1; ERS5806374 (SAMEA8119394) run 2; ERS5806375 (SAMEA8119395) run 3.

2.6. Statistical analysis

To test for correlation of ESBL status of maternity farms and farm management practices or AMU, a two-tailed Fisher's exact test was carried out online (https://www.graphpad.com/quickcalcs/contingency1/). All other analyses were performed in R (v3.6.1) using functions from R base or 'epiDisplay' and 'lme4' packages.

3. Results

3.1. Characteristics of samples and isolates

Figure 1 summarises the numbers of collected samples by source. On average, rectal swabs of 30 (range 10–46) piglets were collected per farm (31 farms) and the individual animals were tracked throughout the different farm units, resulting in samples from three time points (2437 samples in total). On average, 1.3 (range 0–5) human stool samples per farm were received and environmental samples were collected at 'first visit' and screened for ESC-R-*Ec.* The average age of the farmers was 46.3 years (SD 14.1), with an average of 20.6 (SD 15) years of working experience and 21.3 (SD 12.7) hours of pig contact per week (Supplementary Table 1). Information about the number of samples per farm and transport was recorded (Supplementary Table 2).

Overall, the ESC-R phenotype was detected in 121 *E. coli* isolates. Genotypic resistance was confirmed for all 121 isolates by WGS (Table 1). ESBL genes were present in 111 (91.7%) isolates from 12 farms, of which CTX-M-14 was most prevalent (56.8%), followed by SHV-12 (19.8%), CTX-M-15 (13.5%), CTX-M-1 (6.3%), CTX-M-24 (1.8%), CTX-M-27 (0.9%) and TEM-52B (0.9%). In 10 isolates (8.3%) originating from six farms, a previously described chromosomal promoter mutation (-42C>T) leading to upregulation of *ampC* was identified [26]. Microbiological resistance to additional antimicrobials was found: tetracycline (75.2%), sulfamethoxazole (74.4%), trimethoprim (69.4%), azithromycin (3.3%), nalidixic acid (20.7%), ciprofloxacin (83.4%), chloramphenicol (53.7%), and gen-

Table 1
Number, origin and characteristics of ESC-R-*Ec* isolates.

Unit of farm*	N**	Origin	*E. coli* ST***	ESC-R gene	Gene location****
ID 1					
m	1	suckling pig	ST-1011	*CTX-M-15*	IncI1 (ST-37)
m	1	manure	ST-1011	*CTX-M-15*	IncI1 (ST-37)
m	1	human	ST-38	*CTX-M-15*	chromosomal
f	1	fattening pig	ST-3497	*ampC*	chromosomal
f	2	fattening pig	ST-88	*ampC*	chromosomal
ID 3					
f	1	manure	ST-165	*CTX-M-1*	IncI1 (ST-3)
ID 4					
f	5	fattening pig	unknown	*CTX-M-1*	IncFIB
ID 9					
f	1	manure	ST-5177	*ampC*	chromosomal
ID 14					
f	1	manure	ST-58	*ampC*	chromosomal
ID 16					
f	1	manure	ST-345	*ampC*	chromosomal
ID 17					
f	3	fattening pig	ST-23	*ampC*	chromosomal
ID 19					
f	1	manure	ST-167	*CTX-M-15*	IncFIB
ID 20					
m	7	suckling pig	ST-58	*CTX-M-15*	IncFIB
m	1	'weaned pig	ST-58	*CTX-M-15*	IncFIB
m	1	manure	ST-58	*CTX-M-15*	IncFIB
f	1	dust	ST-58	*CTX-M-15*	IncFIB
ID 21					
m	1	human	ST-131	*CTX-M-24*	chromosomal
ID 24					
m	38	suckling pig	ST-10	*CTX-M-14*	IncFII
m	1	mother sow	ST-10	*CTX-M-14*	IncFII
m	1	fly (suckling unit)	ST-10	*CTX-M-14*	IncFII
m	18	weaning pig	ST-10	*CTX-M-14*	IncFII
m	1	weaning pig	ST-2325	*CTX-M-1*	IncN
m	1	fly (weaning unit)	ST-752	*TEM-52B*	IncX1
f	1	fattening pig	ST-10	*CTX-M-14*	IncFII
ID 25					
m	1	human	ST-405	*CTX-M-27*	IncFII
ID 27					
m	7	suckling pig	ST-1114	*SHV-12*	IncX3
m	1	suckling pig	ST-906	*SHV-12*	IncX3
m	12	weaning pig	ST-1114	*SHV-12*	IncX3
m	1	weaning pig	ST-48	*SHV-12*	IncX3
m	1	manure	ST-1114	*SHV-12*	IncX3
ID 28					
m	3	suckling pig	ST-744	*CTX-M-14*	IncFII
m	1	weaning pig	ST-744	*CTX-M-14*	IncFII
ID 30					
m	1	human	ST-131	*CTX-M-24*	chromosomal
ID 32					
f	1	manure	ST-453	*ampC*	chromosomal
f	1	human	ST-3877	*CTX-M-15*	IncFIB

* maternity (m) and fattening (f) units were sampled from individual (ID) farms
** Number of isolates (N) with identical sequence type (ST) and ESC-R gene
*** Multilocus sequence types (ST) were derived from whole-genome sequencing data from the website of the Center for Genomic Epidemiology (CGE).
**** Gene location was assigned by inclusion of both, long and short read sequencing data (see text for details).

tamicin (56.2%) (Supplementary Table 3). In the majority of the ESC-R isolates (93.4%), resistance genes towards at least three antimicrobial classes were identified; the isolates were therefore classified as multidrug-resistant. Carbapenem resistance was not detected (Supplementary Table 3).

3.2. ESC-R-Ec prevalence in animals, humans and the environment of pig farms

ESC-R-*Ec* were found in all pig age groups, pig farmers and environmental samples. Prevalence of having at least one ESC-R-*Ec* was 16.1% (95% CI 5.5–33.7%), 12.9% (95% CI 3.6–29.8%) and 31.6% (95% CI 17.5–48.6%) for the suckling, weaning and fattening compartments, respectively ($P = 0.1$) (Supplementary Table 4). Excluding chromosomal *ampC*, prevalence of ESBL-*Ec* was 16.1% (95% CI 5.5–33.7%), 12.9 % (95% CI 3.6–29.8%) and 13.2% (95% CI 4.4–28.1%) for the suckling, weaning and fattening sections, respectively ($P = 0.7$) (Supplementary Table 4). There were four farms (ID 21, 25, 30, 32) with ESC-R-*Ec*-positive farm workers that had no positive pigs, although positive manure was detected for ID32 (Table 1).

ESC-R-*Ec* prevalence significantly decreased from 6.2% to 3.9% and 1.8% for the 31 suckling, weaned and fattening pigs, respectively ($P < 0.001$) (Table 2). When studying the individual animals, ESC-R-*Ec* and ESBL prevalence significantly decreased with increasing age of the pigs ($P = 1.887e\text{-}05$; Table 2; $P = 1.371e\text{-}07$; Supplementary Table 5). ESC-R-*Ec* were detected in five human stool samples (6.8%). Prevalence did not differ between workers being

Table 2
ESC-R-*Ec* prevalence in the pig farm environment.

Type of sample group	Samples, n	ESC-R genotype, n (%)	95% CI	*P*-value
Rectal swabs from pigs (n = 2439)				
suckling piglet	918	57 (6.2%)	4.6-7.8%	1.86E-
weaned piglet	868	34 (3.9%)	2.6-5.2%	05*
fattening pig	653	12 (1.8%)	0.8-2.9%	
human stool samples (n = 73)				
Farmer with maternity (and fattening) unit contact	57	4 (7%)	1.9-17%	1*
fattening unit contact	16	1 (6.2%)	0.2-30.2%	
environmental samples				
manure/dung (n = 59)**				
maternity unit	31	4 (12.9%)	3.6-29.8%	0.44***
fattening unit	29	6 (20.7%)	8.0-39.7%	
Dust (n = 58)				
maternity unit	36	0	0	0.8019**
fattening unit	22	1 (4.5%)	01-22.8%	
Air (n = 60)				
maternity unit	31	0	0	NA
fattening unit	29	0	0	
Fly (n = 53)				
maternity unit	27	2 (7.4%)	0.9-24.3%	0.48**
fattening unit	26	0	0	
mouse faeces (n = 17)				
maternity unit	10	0	0	NA
fattening unit	7	0	0	

* χ^2 test for trend in proportions
** One sample was collected from the mother sow rather than manure
*** two-sample test for equality of proportions with continuity correction; NA, not applicable

exposed to maternity sections (7%) or fattening sections (6.2%; $P = 1$) (Table 2). In the environmental samples, ESC-R-*Ec* were more often identified in liquid manure from fattening (20.7%) compared with maternity units (12.9%), but the difference was non-significant ($P = 0.6$). ESC-R-*Ec* were present in one dust and two fly samples, but absent from the air and mouse faecal samples.

3.3. Antimicrobial usage and farm management practices

High and low AMU did not correlate with the presence or absence of ESC-R on maternity farms ($P = 0.3$, Fisher's exact test) (Supplementary Table 6). Usage of beta-lactams within the last six months before and during sampling was reported on 22 maternity farms. However, there was no significant correlation between exposure to beta-lactams and ESBL status of maternity farms ($P = 1$, Fisher's exact test). In addition, none of the visited farms reported usage of cephalosporins. The breeding farms housed between 12 and 497 mother sows, in which the number of sows did not correlate with ESBL detection in piglets ($P = 0.9$; linear regression model). All five ESBL-positive maternity farms did not follow all-in-all-out systems in their breeding sections ($P = 0.04$; Fisher's exact test) (Supplementary Table 6).

3.4. Carriage duration of ESC-R-Ec within pigs

Figure 2 shows changes in the ESC-R *E. coli* carriage status over the pigs' lifespan. ESC-R-*Ec* colonisation was found during all life stages of the pigs. Overall, 32 (57.1%) of the 57 pigs that had an ESBL-positive status during the suckling phase were no longer positive at weaning and one pig tested positive at all three time points. Furthermore, 24% of the ESBL-positive weaning pig samples constituted new cases (i.e. were found negative for ESBL-producing *E. coli* during the suckling period). New positive cases in weaned pigs were only present in farms that already had animals with a positive ESBL status during the suckling period. By contrast, carriage in formerly ESC-R-negative fattening pigs was detected in units that were not related to positive maternity farms. Chromosomal *ampC* was only detected in fattening pigs. In addition, the cgST from CGE was included for the isolates to further investigate the diversity and persistence of ESC-R-*Ec* over time. It was found that – with the exception of one single pig from farm 24, which had a clonally differing ESBL-positive isolate during suckling than during weaning – no pig had two or more clonally different isolates during the three time points.

3.5. Clonal spread and horizontal gene transfer

Figure 3 displays the cgMLST tree, isolation source and resistance profile of the ESC-R-*Ec* isolates. As for the human samples, the ESBL gene was located on a plasmid and on the chromosome for two (F32H1E and S25H2E) and three pig farmers (S1H2E, S21H1E and S30H1E), respectively (Figure 3). For the latter three samples, the genetic context of the resistance was additionally identified using long-read sequencing (Figures 4A and 4B). For one human sample (S1H2E), *CTX-M-15* was surrounded by transposon and insertion sequence elements (Figure 4A). For the remaining two epidemiologically independent samples (S21H1E and S30H1E), *CTX-M-24* was flanked by IS elements (Figure 4B). Both human isolates belonged to the highly virulent clone ST-131 with a fumC-fimH type 40-89. No ST-131 isolates were found in animals or the environment.

Generally, when multiple positive isolates were detected on the same farm, one cgMLST and one ESC-R gene dominated per farm unit. On farm 27, two isolates (Mbl2148 and Mbl2690) differed in (cg)MLST type compared with the other 20 isolates. Hybrid assembly revealed that the IncX3 plasmids carrying the *SHV-12* gene shared identical sequences with the plasmid in Mbl2690, except from a 231 bp long segment that was missing in Mbl2162, indicating horizontal gene transfer (Figure 4C). The segment of 231 bp matched with plasmid sequences from other Enterobacteriaceae but no genes were identified within the short region. Farm 24 was the only farm on which multiple ESBL genes (*CTX-M-14, CTX-M-1* and *TEM-52B*) were detected in animal-derived samples. Moreover, ESBL-producing *E. coli* were isolated from two flies, of which isolate Mbl1957 was identified to be clonally related to the *E. coli* found in the majority of pigs (ST-10, *CTX-M-14*), indicating clonal spread within farm 24. The other isolate Mbl2353 was of a differing MLST (ST-752) and carried an ESBL gene (*TEM-52B*) that was

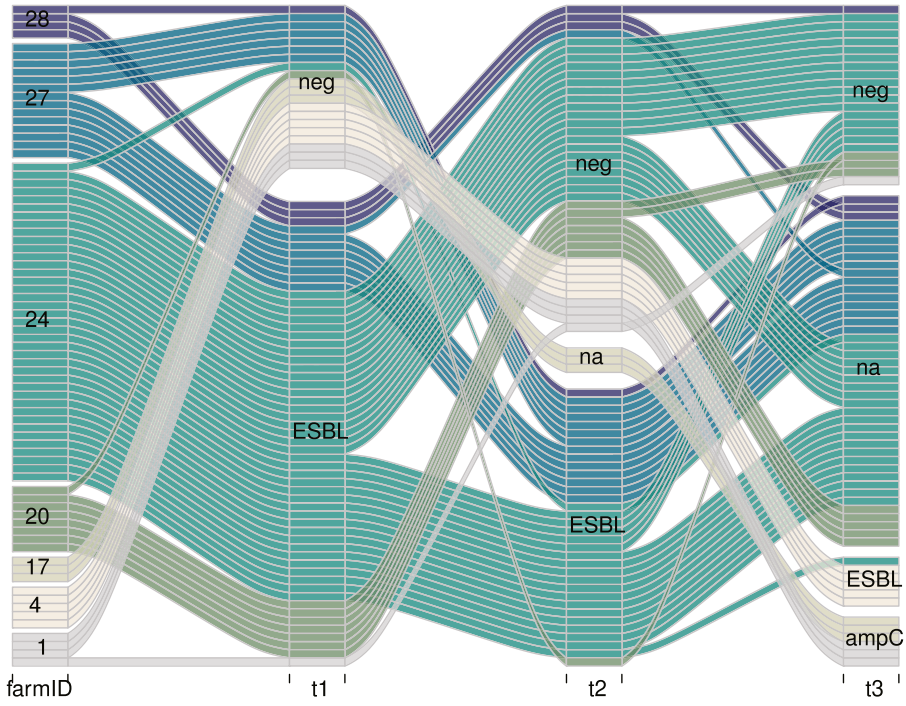

Figure 2. Alluvial plot with dynamics of ESC-R carriage.
Each line illustrates an individual pig from a specific farm (the different farm IDs are indicated). Carriage status from pigs that were at least ESC-R positive at one time point are shown (this was the case in seven farms). ESC-R were either ESBL-positive or *ampC*-positive isolates. Colours distinguish pigs originating from different farms. Samples were taken at three different time points during suckling (t1), weaning (t2) and fattening (t3) periods. Carriage of ESC-R *E. coli* within pigs was dynamic and short-term. With the exception of a single pig from farm 24, which had a differing ESBL-positive isolate during suckling than during weaning, no pig had two or more clonally different isolates during the three time points. 'Na' indicates sample not available, while 'neg' means negative for ESC-R.

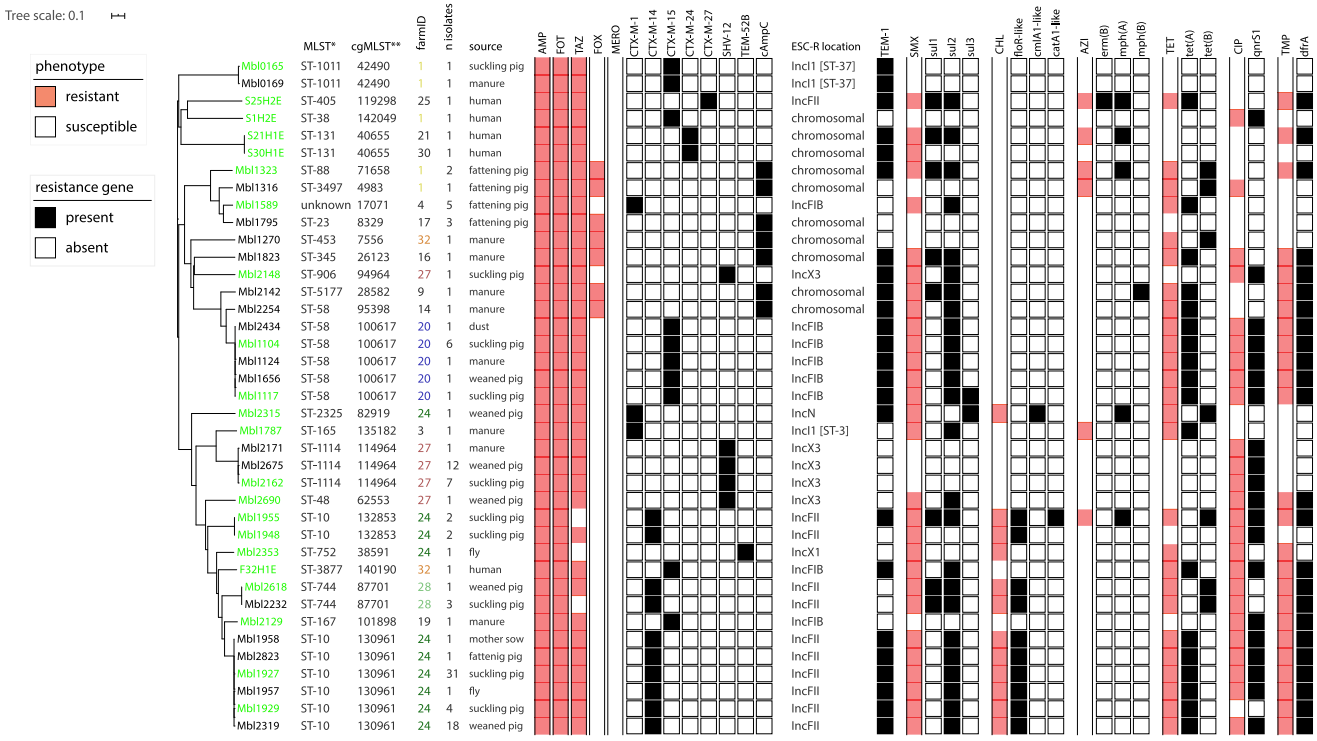

Figure 3. Neighbour-joining tree based on core-genome multilocus sequence type (cgMLST), including 2513 alleles of ESC-R *E. coli*.
Additional information including farm ID, isolation source, microbiological resistance and genotype, as well as Warwick sequencing type (ST) and cgMLST from Center for Genomic Epidemiology (CGE) are included. Of the 121 isolates, one representative *E. coli* per farm ID and source is displayed. The total number of *E. coli* with the same cgMLST and resistance profile is indicated by 'n isolates'. Isolates (n = 22) are written in light-green if hybrid assembly was applied on the isolate; for the remaining isolates, location was not confirmed but plausible based on mapping of Illumina reads on hybrid assembly genomes. The isolates that underwent Nanopore sequencing are also listed in Supplementary Table 7.
AMP, ampicillin; FOT, cefotaxime; TAZ, ceftazidime; FOX, cefoxitin; MERO, meropenem; SMX, sulfamethoxazole; CHL, chloramphenicol; AZI, azithromycin; TET, tetracycline; CIP, ciprofloxacin; TMP, trimethoprim

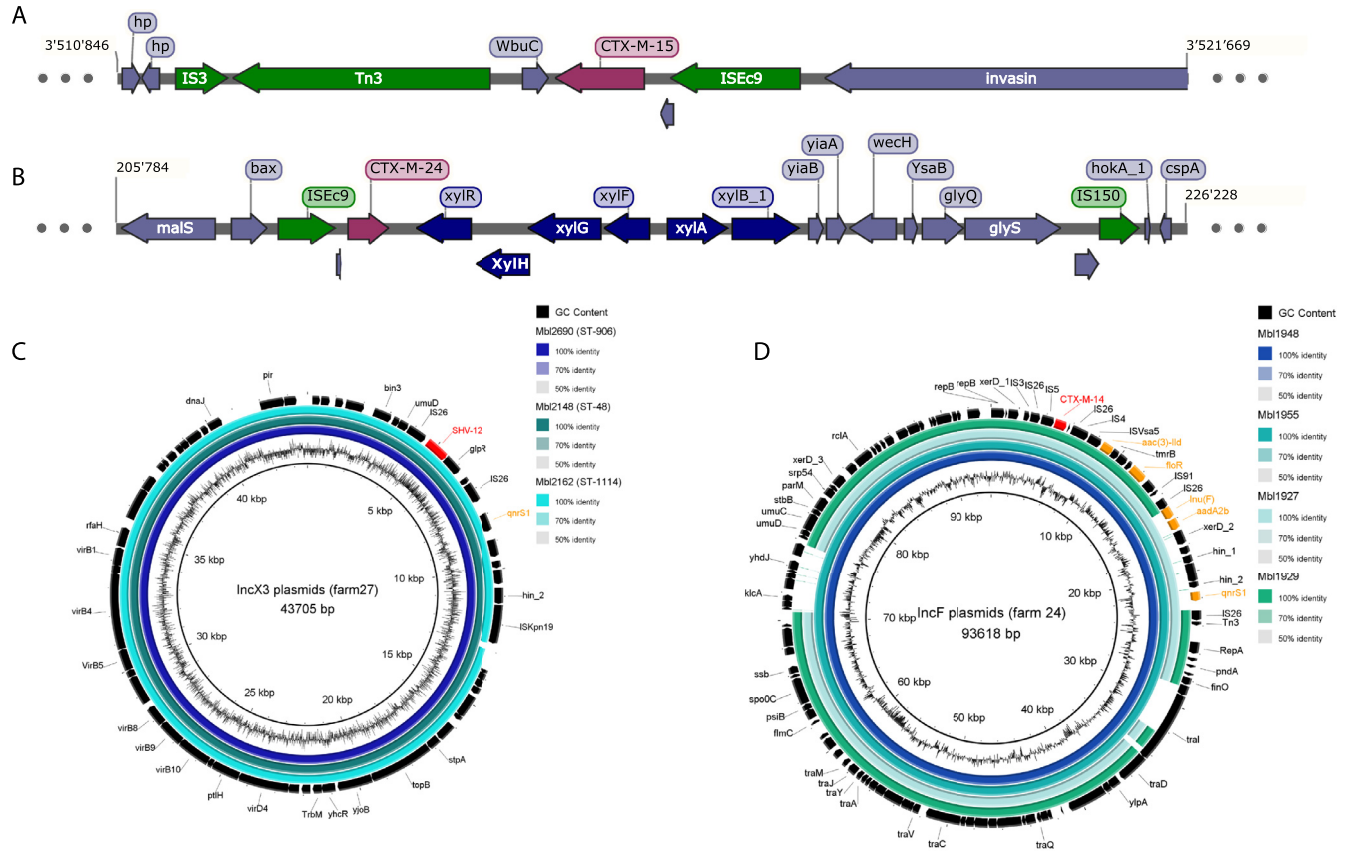

Figure 4. Genetic context of ESBL genes based on hybrid assembly.
Genetic environment of chromosomally inserted *CTX-M-15* from isolate S1H2E (A) and *CTX-M-24* from isolates S21H1E and S30H1E (B) is shown. Comparison of SHV-12 carrying IncX3 plasmids indicates horizontal gene transfer between three isolates of differing MLST types originating from the same farm (C); *CTX-M-14* carrying IncFII plasmids show sequence diversity within farm 24 (D).
Hp, hypothetical protein

not found in any other sample. Overall, neither clonal transmission nor horizontal gene transfer was observed between pigs and pig farmers (Figure 4D).

4. Discussion

This longitudinal rectal pig sampling approach with concurrent collection of samples from farm workers and the environment gave unique and detailed insights into the epidemiology of ESC-R-*Ec* of pig farms. An increased ESC-R-*Ec* prevalence in suckling was observed compared with fattening pigs. ESC-R-*Ec* carriage in piglets was short-term and prevalence was generally low. In the absence of cephalosporin treatment, farm management like all-in-all-out practices rather than AMU seemed to be associated with occurrence and persistence of ESC-R-*Ec*. No clonal transmission between humans and pigs was identified.

The Swiss annual surveillance report of 2018 reported an ESC-R-prevalence of 17.6% in fattening pigs at slaughter [personal communication]. However, the prevalence at slaughter may not be directly comparable with the current results, as gathering during transport and at the abattoir waiting areas may contribute to increased ESC-R-*Ec* [27]. Overall, the current study did not find a correlation between ESC-R status of maternity farms and exposure to beta-lactams or other AMU within the last six months. Additionally, the presence of ESBL-*Ec*-carrying pigs was not related to total AMU. Of relevance, a Danish study detected ESBL-producing *E. coli* in 79% of pigs on farms with high usage of cephalosporins as opposed to 20% of pigs on farms with no administration [28]. In Switzerland, third-generation and fourth-generation cephalosporins have not been handed out in stock to farmers since 2016; this practice may explain the low prevalence values found in the current study. In line with this finding, the presence of ESBL-*Ec*-carrying pigs was not related to total AMU, but it was strongly determined by the presence or absence of cephalosporin use at the farm in a study from the Netherlands (OR = 46.4, $P = 0.006$) [29].

Rectal swabs were used as opposed to pooling faeces collected from the stable ground, which enabled tracing of individual animals during the study. With this design, newly acquired ESC-R isolates were detectable in weaning pigs, which were found to be tested negative during the suckling period. Importantly, new cases in weaning pigs were only found in farms that already had a positive ESBL status during the suckling period in other animals. Overall, five positive suckling units were identified. WGS analysis revealed clonal and/or plasmidic relations between the isolates, indicating transmission from animal to animal or via contaminated stable surfaces rather than introduction of new strains. Although samples from the mother sows were not collected, a contribution of enterobacteria maternally transmitted along the sow lineage is probable, as has recently been suggested [7].

Furthermore, none of the positive suckling units followed all-in-all-out management, indicating that good hygiene practices might be crucial to reduce transmission or re-introduction of resistant strains in the farm units. An earlier study revealed that ESBL-*Ec* is frequently found in the nose of pigs [15]. Taken together, it was speculated that the nose of pigs gets into contact with potentially

ESBL-*Ec*-positive faeces, possibly facilitating clonal spread. Importantly, it has been demonstrated that decontamination protocols can lead to successful elimination of ESBL-*Ec* in pigs and contaminated stable environments [30]. This observation is of special importance in light of the findings that carriage duration of resistant strains was short term. One single pig with an ESBL-*Ec* carriage duration of three months was identified, although it could not be distinguished between long-term colonisation or recolonisation by identical strain.

The detected ESBL carriage of 6.8% in the participating farm workers was comparable with a prevalence of 5.8% in healthy humans living in Switzerland reported in 2012 [31]. Thus, working on a pig farm does not seem to introduce a higher risk for ESC-R-*Ec* carriage. Interestingly, using long-read sequencing, this study identified two human ST-131 isolates for which the *CTX-M-24* gene was inserted in the chromosome. Chromosomally inserted CTX-M within isolates of the highly virulent clone ST-131 have been described [32–34]. However, it is believed that a chromosomally inserted *CTX-M-24* gene within ST-131 has so far not been reported. Overall, this study detected a large number of isolates of ST-10 and closely related STs. In line with these observations, previous studies have shown that ST-10 isolates have a higher prevalence of plasmid-carried AMR genes, including CTX-M ESBL genes, compared with other STs [35,36]. ST-10 is also known to be ubiquitous in human and avian faeces. Further, ST-10 isolates have been characterised as avian pathogenic *E. coli* (APEC) and the zoonotic potential was recently discussed [37].

Occurrence of clonal transfer of ESBL-*Ec* between pigs and pig farmers has been strongly suggested [10,38]. However, neither clonal spread nor horizontal gene transfer from animal to farm worker was detected in the current study. In 2015, a Danish study suggested clonal spread, but the prevalence of ESBL-producing Enterobacteriaceae was much higher compared with the current study, potentially increasing the probability of such transmission events [10].

It has been hypothesied that pigs are a reservoir of ESBL genes, which may subsequently be transferred to more pathogenic *E. coli* strains [28]. The current study hypothesized that horizontal gene transfer of an IncX3 plasmid occurred between ST-1114, ST-906 and ST-48 isolates from pigs on farm 27 (Figure 4B). However, it did not investigate if the distinct *E. coli* strains showed different pathogenic behaviour. For *E. coli* ST-48 the possibility of transfer of ESBL-producing bacteria to humans through drinking water has been discussed, as an ST-48 *E. coli* was recovered from drinking water in France [39].

As found in the current study, manure has previously been identified as the main environmental reservoir for ESC-R [40]. Flies carrying ESBL-producing *E. coli* in farm environments have been described before and their occurrence was discussed as an indication of environmental contamination [41,42]. In the current study, flies from a single farm constituted potential vectors shedding ESBL genes from the stable into the environment or vice versa. A recent study described stable air exposure as a possible route of ESBL transmission to pig farmers, as ESBL carriage was associated with the presence of CTX-M group 1 in dust [10]. ESC-R-*Ec* was not detected in air samples (and only one dust sample contained ESC-R-*Ec*). It was therefore concluded that exposure to stable air is not a likely route of transmission in this sampling setting.

The major strengths of this study were its longitudinal and concurrent sampling of faeces from individual animals and humans as well as environmental samples. The importance of analysing longitudinal data has recently been underscored by the fact that ESC-R-*Ec* carriage is temporary and the importance of some sources might fluctuate over time [43]. In addition, the latest short- and long-read sequencing technologies were used for characterisation of isolates. Using the latter enabled identification of horizontal gene transfer within one farm and a more in-depth molecular characterisation of isolates like the detection of the chromosomal insertion of a *CTX-M-24* gene within the virulent clone ST-131.

There were also some limitations. A participation bias might have occurred, as inclusion was based on voluntary participation of the farmers. All the farrowing units were members of a central service and competence center for the Swiss pig industry. Except for one maternity and one fattening unit, all the contacted pig farmers agreed to participate in this study. Furthermore, despite collecting over 2000 samples, the number of ESC-R-*Ec* isolates was low (mainly due to the low prevalence within the chosen farms), which may have impeded a more in-depth analysis of risk factors for increased ESC-R-*Ec* carriage (including AMU). For this type of analyses, the reader is referred to other point-prevalence studies [44]. Due to logistical reasons, human samples had to be processed in a different laboratory using a slightly different protocol, as compared with the animal and environmental samples. However, the ESC-R-*Ec* prevalence for human and animal samples was in the expected range for Switzerland (https://www.anresis.ch/wp-content/uploads/2020/11/Swiss-Antibiotic-Resistance-Report-2020_def_WEB.pdf) and the high sensitivity of both protocols was proven in previous studies [4,15,45]. Despite a lot of effort, some pigs were lost during longitudinal tracking; this was due to the fact that distinctly ear-tagged pigs are extremely difficult to detect in large fattening units, especially when the marked animals are not kept together. Finally, as only one suspected ESBL *E. coli* isolate per sample was further analysed, no possible diversity of ESBL within one sample could be observed. However, it was hypothesized that this diversity is neglectable in a low prevalence setting.

5. Conclusion

The results indicate that prevalence of ESC-R-*Ec* changes over time and ESC-R carriage in individual pigs is short term. Future studies need to investigate whether distinct STs are associated with longer carriage duration in pigs. A deeper understanding of the duration of ESC-R-*Ec* in pigs will allow the design of more accurate decontamination protocols in order to reduce ESC-R prevalence in pig farms and prevent shedding into a close environment.

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Declarations

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Competing Interests: None declared.

Ethical Approval: Ethical clearance for this study was obtained from Human Research Ethics Committee of the Canton Vaud (2018-00080) and the Veterinary Ethics Committee of the French Cantons (VD2903).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2021.106382.

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