Reduction in antimicrobial resistance prevalence in Escherichia coli from a pig farm following withdrawal of group antimicrobial treatment

Reduction in antimicrobial resistance prevalence in *Escherichia coli* in a pig farm following withdrawal of group antibiotic treatment

De Lucia A.[1,2], Card R.M.[2], Duggett N.[2], Smith R.P.[3], Davies R.[2], Cawthraw S.A. [2], Anjum M.F.[2], Rambaldi M.[1], Ostanello F.[1], Martelli F.[2*]

[1]*Dipartimento di Scienze Mediche Veterinarie. Università di Bologna. Italy;*
[2]*Animal and Plant Health Agency (APHA) Bacteriology Department. UK;*
[3]*Animal and Plant Health Agency (APHA) Department of Epidemiological Sciences. UK*

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[*]**Corresponding author**
current address: Woodham Lane, New Haw, Addlestone, KT15 3NB, United Kingdom.

E-mail addresses: Alessia.delucia3@studio.unibo.it (A. De Lucia), Roderick.Card@apha.gov.uk (R.M. Card), Nick.Duggett@apha.gov.uk (N.A. Dugget), Richard.P.Smith@apha.gov.uk (R.P. Smith), Rob.Davies@apha.gov.uk (R. Davies), shaun.cawthraw@apha.gov.uk, (S.A.Cawthraw), muna.anjum@apha.gov.uk (M. Anjum), margherita.rambaldi@gmail.com (M. Rambaldi), fabio.ostanello@unibo.it (F. Ostanello), Francesca.Martelli@apha.gov.uk (F. Martelli).

[1] Current address: via Tolara di Sopra 50, 40064, Ozzano Emilia, Italy.
[2, 3] Current address: Woodham Lane, New Haw, Addlestone, KT15 3NB, United Kingdom.

Highlights

- Significant increase in fully susceptible *E. coli* after withdrawal of antibiotics
- Multidrug resistant *E. coli* decline significantly after withdrawal of antibiotics
- Resistant commensal *E. coli* remain after withdrawal of antibiotics
- The environmental reservoir of resistant *E. coli* contributes to persistence in pigs
- Plasmids have an important role for the maintenance and dissemination of resistance

Abstract

An important element in the control of antimicrobial resistance (AMR) is reduction in antimicrobial usage. In the veterinary sector individual antimicrobial treatment of livestock, rather than the use of group treatment, can help achieve this goal. The aim of this study was to investigate how cessation of group antimicrobial treatment impacted the prevalence of AMR in commensal Escherichia coli in pigs at one farm over an 11-month period. Minimum inhibitory concentrations of eight antimicrobials were determined for 259 *E. coli* isolates collected during the study. A significant reduction in the prevalence of multidrug resistance and a significant increase in the proportion of full susceptibility to the panel of nine antimicrobials tested was seen after 11 months. Whole genome sequencing of 48 multidrug resistant isolates revealed *E. coli* clones that persisted across multiple visits and provided evidence for the presence of plasmids harbouring AMR genes shared across multiple *E. coli* lineages. *E. coli* were also isolated from on-farm environmental samples. Whole genome sequencing of one multidrug resistant isolate obtained from cleaning tools showed it was clonal to pig-derived *E. coli* that persisted on the farm for 11 months. In this study we provide evidence that withdrawal of group antimicrobial use leads to significant reductions in key indicators for AMR prevalence and the importance of the farm environment as a reservoir of resistant bacteria. These findings support policy makers and producers in the implementation of measures to control AMR and reduce antimicrobial use.

1. Introduction

Antimicrobial resistance (AMR) is a naturally occurring phenomenon resulting from the evolutionary adaptation of bacteria (Magnusson et al., 2019; WHO, 2015). The prevalence of AMR in bacteria has increased dramatically over the past few decades, becoming one of the most important threats to global public and animal health (WHO, 2015). One key factor for the emergence, selection, and dissemination of AMR microorganisms in veterinary and human medicine is antibiotic usage (Magnusson et al., 2019). Additionally, in relation to food-producing animals, resistant microorganisms can be introduced onto farms from outside sources such as new stock, vectors including rodents, birds and insects, or through contaminated feed and water (Davies and Wales, 2019). Furthermore, AMR bacteria can disseminate resistance genes among the diverse microbial communities via mobile genetic elements, such as plasmids and transposons (Davies and Wales, 2019). Commensal bacteria are ubiquitous in the intestinal tract of food-producing animals and are considered good indicators of AMR (EFSA, 2020). In particular, *E. coli* bacteria have been long used as an indicator of faecal pollution and have been chosen as a target bacterial species for AMR surveillance in commensal Gram-negative bacteria from livestock populations (Davies and Wales, 2019; EFSA, 2020). In the United Kingdom (UK), data obtained from the AMR-surveillance programme, based on random sampling of the caecal contents of healthy pigs at slaughter in 2017, showed a reduction in the prevalence of *E. coli* resistant to many antimicrobials including ampicillin, gentamicin, sulphonamide, Veterinary Microbiology 258 (2021) 109125 2tetracycline, and trimethoprim (UK-VARSS, 2018). These reductions in AMR parallel a significant decrease in the use of antimicrobials in UK pig herds. Similar studies in other countries, such as Belgium (Callens et al., 2018) and Netherlands (Dorado-García et al., 2016), and in the European Union (ECDC/EFSA/EMA, 2017), have demonstrated associations between reductions in antimicrobial use and AMR in commensal *E. coli*. Importantly, surveillance data are often not collected or structured in a manner that enables a direct correlation between reduced antimicrobial use and decreases in AMR prevalence on individual farms. On-farm longitudinal studies of resistance and antimicrobial usage can link antimicrobial use directly with temporal trends in AMR within herds and the dynamic development and maintenance of resistance. Several farm-based longitudinal studies have helped establish that antimicrobial administration in pigs is associated with an increased prevalence of the corresponding resistance in *E. coli* (Belloc et al., 2005; Lin et al., 2017; Mathew et al., 2003; Varga et al., 2009). Furthermore, following cessation of antimicrobial use, a restoration to pre-treatment levels of the corresponding resistance in *E. coli* has been reported (Belloc et al., 2005; Mathew et al., 2003). An investigation at a single UK pig farm reported a direct link between the cessation of colistin use and the elimination of colistin resistant *E. coli* (Duggett et al., 2018; Randall et al., 2018).

However, the broader impact of the cessation of antimicrobial use on AMR in indicator bacteria such as *E. coli* at the farm level remains poorly defined. The aim of this study was to help address this evidence gap by investigating the impact of withdrawal of group antimicrobial treatment on the intestinal carriage of AMR in commensal *E. coli* over a period of 11 months at a UK pig farm. Faecal samples were collected over three time points and processed for quantification of commensal *E. coli* abundance and the abundance of *E. coli* sub-populations resistant to apramycin and trimethoprim-sulfamethoxazole, antimicrobials previously used to treat clinical salmonellosis on the farm. Additionally, *E. coli* were purified by culture from the faecal samples for susceptibility testing against eight antimicrobials, to monitor changes in key outcome indicators such as prevalence of multidrug resistance and full susceptibility. The persistence of *E. coli* clones and presence of plasmids harbouring AMR genes was examined using whole genome sequencing. Finally, isolates were obtained from the farm environment to provide insight to the role of the environment as a reservoir of resistant bacteria that may serve as a source for (re)colonisation of pigs.

2. Material and methods

2.1. Study farm and sampling strategy

This study was carried out on a farrow to finish farm in the UK consisting of approximately 500 sows, 2000 weaner and grower pigs, and 2000 finisher pigs. Sows and gilts were housed in outdoor radials and pens, while weaners, growers and finishers were housed in indoor pens. No all-in/all-out programme was operated on the farm, instead a cleaning and disinfection program was performed for empty pens when the pigs were transferred to new accommodation based on age class. Salmonella enterica serovar Typhimurium was present on farm at the beginning of the study at a prevalence of approximately 60 % in weaners, resulting in significant clinical disease. For this reason, feed medicated with apramycin (APR) or trimethoprim-sulfamethoxazole (SXT) had been used as group treatment under veterinary advice of weaned pigs when clinical gastrointestinal symptoms occurred, prior to the commencement of this study (Fig. 1). In December 2016 a programme of antimicrobial use reduction was implemented on the farm and antimicrobial group medication was discontinued (antimicrobials were administered individually to treat animals that were clinically sick). The antimicrobial use on this farm was already low (1 mg/kg) compared to the average antimicrobial usage recorded in the pig sector as a whole at this time (131 mg/kg in 2017 and 110 mg/kg in 2018) (UK-VARRS, 2018). After discontinuation of group treatment in weaner pigs, the usage on farm was further reduced, down to 0 mg/kg in the last two quarters of 2018. The farm was followed longitudinally over three visits undertaken at approximately six-month intervals (T1, T2 and T3). The first visit was carried out in May 2017 (T1), the second in September 2017 (T2) and the third in April

2018 (T3). On each visit, six pens were randomly selected for each age class of fattening pigs (weaner, grower, and finisher pigs). Five fresh, individual, faecal samples were collected from the floor of each pen (30 samples per age class) and processed at the laboratory within 24 h (see below).

2.2. Isolation and enumeration of total E. coli and E. coli recovered on culture plates containing apramycin or sulfamethoxazole/trimethoprim in pooled faecal samples

The five individual faecal samples from each pen were pooled (one- gram per sample), resulting in six pools per age group (except for T1 where there were only five pools available). Quantitative bacteriology was performed using the method of Miles et al., 1938 in which a 10-fold dilution series of the pooled faecal samples was prepared in 0.1 M PBS (pH 7.2). The dilutions were plated onto CHROMagar ECC plates for enumeration of total presumptive *E. coli* (i.e. indicator *E. coli*). Dilutions were also plated onto CHROMagar ECC supplemented with either 4 mg/L of trimethoprim-sulfamethoxazole (1:19 ratio) or 32 mg/L of apramycin, to enumerate the *E. coli* sub-populations resistant to these antimicrobials. The EUCAST human clinical breakpoint of resistant > 4 mg/L was used for trimethoprim-sulfamethoxazole to obtain intermediate and clinically resistant isolates (EUCAST, 2021). Apramycin was employed at 32 mg/L to obtain resistant isolates. Presumptive *E. coli* were identified by their chromogenic properties and the CFU/g of faeces determined for each pooled sample on selective and non-selective plates. The proportion of presumptive resistant *E. coli* in each pooled sample was then calculated. For each pooled sample one representative presumptive *E. coli* from each antibiotic-containing plate and three presumptive indicator *E. coli* from non-selective plates were sub-cultured to purity. Species identification was verified by MALDI-ToF MS.

2.3. Environmental samples

Environmental samples were collected during visit T3. Swab samples of cleaning tools (n = 3) and drinkers (n = 3) were collected from the weaner and grower areas. Swab samples (n = 3) were also collected from a cleaned and disinfected weaner pen (empty at the time of the visit). Individual faecal samples from synanthropic animals (rat, n = 3 and wild bird, n = 3) were also collected at the farm premises. Each environmental sample was placed into 225 mL of Buffered Peptone Water (BPW) and incubated for 18 ± 2 h at 37 ± 1 ◦C. Following this overnight enrichment a single pool was prepared for each sample type by mixing equal volumes to obtain seven environmental samples from which *E. coli* were isolated by culture as detailed above.

2.4. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by agar dilution (Andrews, 2001) for the following antimicrobials: ampicillin (AMP), tetracycline (TET), cefotaxime (CTX), florfenicol (FLO), ciprofloxacin (CIP), streptomycin (STR), apramycin (APR) and trimethoprim-sulfamethoxazole (1:5) (SXT). *E. coli* strains ATCC 25922 and NCTC 10418 were used as quality controls. The MIC was recorded as the lowest concentration that prevented visible growth and interpreted using Epidemiological cut-off (ECOFF) values issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021). For apramycin no defined ECOFF value or clinical breakpoint for *E. coli* has been published by EUCAST, and the breakpoint of ≥32 mg/L proposed by the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme was used (DANMAP, 2004). Isolates were differentiated between susceptible wild type (WT) and non-wild type (NWT) strains based on the ECOFF. When the MIC was above the ECOFF value isolates were defined as being non-wild type (NWT) to the corresponding antimicrobial (Schwarz et al., 2010), and as this does not necessarily correspond to clinical resistance the term 'reduced susceptibility' has been used. For subsequent analysis, reduced susceptibility to apramycin and/or streptomycin was scored as reduced susceptibility to the aminoglycoside (AMG) class and multidrug resistance (MDR) was defined as resistance to three or more antimicrobial classes (EFSA, 2020; Schwarz et al., 2010).

2.5. Whole genome sequence analysis

DNA was extracted from fifty MDR *E. coli* isolates using the MagMax core nucleic acid purification kit and the KingFisher flex system (ThermoFisher), according to the manufacturer's protocol. Whole Genome Sequencing (WGS) was carried out using an Illumina NextSeq or Miseq (2 × 150 bp). Sequences were deposited in the European Nucleotide Archive (ENA) under study accession number PRJEB39219. AMR gene presence was established using APHA SeqFinder after a quality control step as described previously (Anjum et al., 2016). Raw reads were assembled with SPAdes 3.11 (Bankevich et al., 2012) and the assembled genomes were analysed with Abricate (https://github.com/tseemann/ abricate) to determine presence of AMR genes and plasmid replicon genes on the same contigs. The sequence types (ST) of the isolates were established using MLST (https://github.com/tseemann/mLst). A core genome SNP alignment was produced with Snippy (https://github. com/tseemann/snippy) using *E. coli* K12 MG1655 (accession: U00096.3) as reference and this was used to build the phylogenetic tree with RAxML (Stamatakis, 2014). The trees were annotated using iTOLv3 (Letunic and Bork, 2016).

2.6. Statistical analyses

Chi-square test was applied for the comparison of the estimated proportion of *E. coli* recovered on antimicrobial plates over time and between age classes. All the CFU counts in pooled faecal samples at specific time points were dichotomized (above or below the median). Temporal trends of AMR and MDR percentages of *E. coli* were analysed using Linear-by-Linear Cochran-Armitage test (Aerts et al., 2011). A Chi-square test was also applied to investigate the percentages of AMR and MDR *E. coli* isolates between two different age classes (weaner vs finisher pigs). Statistical analyses were performed using the software SPSS 25.0 (IBM SPSS Statistics, NY, US) and $p < 0.05$ was set as statistically significant.

3. Results

The farrow to finisher pig farm was followed for 11-months after the suspension of group antimicrobial treatment (Fig. 1) and a total of 53 pooled pig faecal samples, spanning three time points (T1, T2, and T3) and three age groups (weaner, grower, and finisher) were processed for quantification of *E. coli* abundance and purification of bacterial isolates for susceptibility testing and WGS.

3.1. Intestinal carriage of antimicrobial resistant E. coli in pigs differs by age class

Quantitative bacteriology was performed on all pig faecal samples to estimate the abundance of total *E. coli* and the abundance of *E. coli* subpopulations able to grow on the APR- and SXT-containing plates. All samples were positive for growth of indicator *E. coli* and SXT-resistant sub-populations. All samples were also positive for the presence of APR-resistant sub-populations, except four samples from finishers. Indicator *E. coli* were present at an overall geometric mean of 4.7×10^6 CFU/g and the abundance of APR- and SXT-resistant populations had overall geometric means of 4.5 × 105 CFU/g and 5.2 × 104 CFU/g respectively (Supplementary Table 1). The proportion of *E. coli* recovered from APR- and SXT-containing plates varied between samples, although SXT-resistant *E. coli* were consistently more abundant than the APR-resistant sub-population (Supplementary Fig. 1; Supplementary Table 1). Between-sample variation in abundance was highest in weaner pigs and lowest in finisher pigs (Supplementary Fig. 1). Furthermore, there was a significant difference in the proportion of APR-resistant and STX-resistant *E. coli* between weaners and finishers ($p < 0.001$ and $p = 0.005$ respectively). Although a visual trend for reduced abundance of resistant *E. coli* can be discerned between visits T1 and T3 this was not statistically significant.

3.2. The proportion of antimicrobial susceptible E. coli isolates increased in pigs following withdrawal of group antimicrobial treatment

To further examine changes in antimicrobial susceptibility, a total of 259 *E. coli* isolates were cultured from the pig faecal samples across the three farm visits: T1 (n = 82), T2 (n = 89), and T3 (n = 88). Isolates were obtained from weaners (n = 85), growers (n = 89), or finishers (n = 85); and comprised indicator *E. coli* (n = 159), and *E. coli* recovered from APR selective plates (n = 48) and from SXT selective plates (n = 52) (Supplementary Table 2). All were verified as *E. coli* by MALDI-ToF and tested by agar dilution for susceptibility towards nine antimicrobials (trimethoprim-sulfamethoxazole in combination). In the 159 indicator *E. coli*, recovered from non-selective agar a high proportion were susceptible to streptomycin (82 %), apramycin (87 %), florfenicol (91 %) and ciprofloxacin (98 %) and all were susceptible to cefotaxime (Fig. 2). A lower proportion had susceptibility to tetracycline (45 %), ampicillin (67 %), and trimethoprim-sulfamethoxazole (67 %). Fifty indicator *E. coli* (31 %) were susceptible to all antimicrobials tested and there was a significant increase in fully susceptible isolates between T1 and T3 ($p = 0.001$) (Fig. 3A). Additionally, there was a significant increase in the proportion of indicator *E. coli* susceptible to ampicillin ($p = 0.01$) and trimethoprim-sulfamethoxazole ($p = 0.007$) between visits T1 and T3 (Fig. 2). A similar increasing temporal trend was also observed for apramycin and streptomycin, but these differences were not statistically significant.

All isolates purified from APR- and SXT-containing plates were NWT, showing a reduced susceptibility to these respective compounds, except isolate ADL131 which had an apramycin MIC of 16 mg/L (Supplementary Table 3). Comparing visits T1 and T3, the *E. coli* from selective APR-containing plates showed a significant increase in the proportion of susceptible strains to four antimicrobial agents: ampicillin ($p = 0.01$), streptomycin ($p = 0.02$), trimethoprim-sulfamethoxazole ($p = 0.03$) and tetracycline ($p = 0.03$) (Fig. 2). A similar increase between T1 and T3 in the subpopulation of *E. coli* from SXT-containing plates was noted for only streptomycin ($p = 0.006$) (Fig. 2). When examined by pig age class, the proportion of indicator *E. coli* susceptible was higher in finishers compared to weaners for all eight antimicrobials tested; with significant differences for ampicillin, apramycin, florfenicol, SXT, and streptomycin ($p < 0.05$) (Supplementary Fig. 2). There was no significant change in the proportion of susceptible isolates by age class for *E. coli* obtained from APR-containing plates, however *E. coli* from SXT-containing plates had a significant increase in proportion of susceptible strains to ampicillin (Supplementary Fig. 2).

3.3. Intestinal carriage of multidrug-resistant E. coli in pigs decreased following withdrawal of group antimicrobial treatment

Having observed significant reductions in resistance to individual antimicrobials we next examined how withdrawal of group antimicrobial treatment affected multidrug resistance (MDR) in the *E. coli*.

For this we defined multidrug resistance as reduced susceptibility to three of more antimicrobial classes. Of the 259 pig-derived *E. coli* isolates 46 % (118/259) were MDR. The proportion of MDR isolates was significantly higher ($p < 0.001$) in *E. coli* from APR-containing plates (69 %) and SXT-containing plates (73 %) compared to the indicator *E. coli* (30 %), which were isolated in the absence of antimicrobials (Supplementary Table 2). When examined by age class the percentage of MDR indicator *E. coli* from weaner pigs was significantly higher ($p < 0.001$) than finisher pigs (Table 1). Furthermore, between visits T1 and T3 there was a significant decrease ($p = 0.023$) in the proportion of MDR *E. coli* isolates (Fig. 3B and Supplementary Table 2). A total of 14 different MDR phenotypes were observed (Supplementary Table 4). The most commonly observed MDR phenotype (48/ 118; 41 %) was resistance to ampicillin, trimethoprim-sulfamethoxazole, tetracycline and aminoglycosides (ASTTeA) (Supplementary Table 4). As a proportion of total *E. coli* there was a significant reduction in isolates with this predominant phenotype between T1 and T3 ($p = 0.005$), but not as a proportion of total MDR isolates ($p = 0.64$).

3.4. Multidrug resistant E. coli are present in the farm environment

Environmental samples were collected during the T3 farm visit, as evidence emerged for the persistence of resistance following preliminary observation of results from T1 and T2. This enabled an investigation into the potential role for the farm environment to act as a reservoir of resistant bacteria that may serve as a source for (re)colonisation of pigs. Seven environmental sample types were examined (see methods) and, following overnight enrichment in BPW, they all yielded growth of *E. coli* on antibiotic-free plates and plates containing APR or SXT. A single colony was purified from each plate (total isolates = 21). Two isolates were fully susceptible to the antimicrobials tested, while the remaining 19 isolates were NWT with reduced susceptibility to at least one antimicrobial (Supplementary Table 3). All the environmental *E. coli* obtained from APR-containing plates (n = 7) and SXT-containing plates (n = 7) had reduced susceptibility (i.e. NWT) to apramycin and trimethoprim-sulfamethoxazole respectively (Supplementary Table 3). Twelve of the 21 environmental isolates (57 %) had an MDR phenotype (Supplementary Table 3). Isolate ADL629 had the ASTTeA phenotypic resistance pattern common in pigs on the farm, and was isolated from farm equipment used for cleaning weaner pens. Additionally, the wild bird sample yielded an *E. coli* isolate (ADL648) with reduced susceptibility to seven of the eight antimicrobials tested (ampicillin, ciprofloxacin, florfenicol, tetracycline, trimethoprim-sulfamethoxazole, streptomycin, and apramycin). Three *E. coli* were obtained from swabs of the empty weaner pen after it had been cleaned and disinfected, of which one (strain ADL636) showed reduced susceptibility to four antimicrobials (ampicillin, trimethoprim-sulfamethoxazole, florfenicol, and apramycin).

3.5. Multidrug resistant E. coli clones are present in both pigs and the farm environment

Forty six of 48 pig-derived *E. coli* with the predominant ASTTeA pattern and the environmental isolates ADL629 (cleaning tool) and ADL648 (wild bird faeces) were subjected to WGS and investigated for their genetic diversity and relationship. There was good correspondence between the AMR phenotype and AMR genes, with all isolates having at least one gene encoding resistance to each component of the MDR pattern (Fig. 4); except isolate ADL171 that had reduced susceptibility to ampicillin but no beta-lactamase AMR gene. Ampicillin resistance was associated with the beta-lactamase genes blaTEM-1b, blaTEM-1 or blaOXA-1. Isolate ADL282 harboured blaTEM-1b and a C to T mutation at position -42 in the chromosomal ampC promoter region, and although resistant to ampicillin was susceptible to cefotaxime. All isolates harboured representatives of the sul and dfrA AMR genes, which confer sulphonamide and trimethoprim resistance respectively. Tetracycline resistance was associated with tetA(B) and tetA(4), with 25/48 isolates harbouring both genes. Streptomycin resistance was conferred by ant3-Ia, strAB, aadA2, aadA12, or aadA24 and apramycin resistance by aac(3)-IVa. WGS also revealed the presence of further AMR genes, which can confer resistance to antimicrobials not in the panel tested in this study. For example, the chloramphenicol AMR genes cml and catA1 were present in 36 and 1 isolate respectively; notably these genes have not been associated with resistance to florfenicol (Stubberfield et al., 2019); two isolates harboured floR (ADL152 and ADL648). Isolates ADL6 and ADL263 harboured mphB, which confers resistance to azithromycin. The aminoglycoside resistance genes aph(3′)-Ia, aph(3′)-IIa and aph(4)-Ia were present in 28, 24 and 38 isolates respectively, and 14 isolates possessed the streptothricin AMR gene sat2A. Considerable genetic diversity was observed within the 48 examined MDR *E. coli* with a total of 22 different STs identified; ST10 was the most common (13/48; 27 %), and seven isolates had a new MLST (Fig. 4). The maximum likelihood phylogenetic tree based on core genome single nucleotide polymorphisms (SNPs) illustrated this diversity and additionally allowed identification of eight sub-clusters of *E. coli*, which we have called "clones" due to the high sequence similarity in the core genome between members of a clone (≤ 10 SNP difference, in accordance with (Schürch et al., 2018)) (Fig. 4). Seven of the eight clones were present in different age classes and/or at different time points, indicating their persistence on-farm. These included clone 1 (ST10), clone 4 (ST165) and clone 5 (ST1112), which were isolated from different age classes and from different time points. Clone 6 (ST2705), clone 7 (ST57) and clone 8 (ST925) were detected in one visit each, but from different age groups. Clone 3 (ST unknown) comprised the environmental isolate ADL629 (obtained from a cleaning tool at visit T3) and four isolates from grower pigs (two each at visit T1 and T3). The two isolates that comprised clone 2 were detected only in weaner pigs at visit T1. Each

clone harboured a different repertoire of AMR genes (Fig. 4). However, all isolates in a single clone harboured the same AMR genes (except for clones 3 and 8, in which a single isolate harboured 4 or 1 additional genes respectively). AMR gene carriage in clone 3 was identical between the environmental isolate ADL629 and three of the four pig *E. coli* isolates, each harboured ten AMR genes: aadA2, ant(3″)-Ia, strA, strB, cml, dfrA12, sat2A, sul3, blaTEM-1b and tetA(B) (Fig. 4). Analysis of sequence assemblies provided evidence for linkage between AMR genes and also their co-location with plasmid markers, and four exemplars are presented in Table 1. A contig with an IncHI2 plasmid marker and the AMR genes strA and strB was present in all isolates from clones 1 and 4, and five further isolates. The clone 2 isolates both carried a ColRNAI plasmid contig with the AMR genes strA, strB, sul2, and dfrA14. This candidate ColRNAI plasmid was also present in two more *E. coli* of different lineages, although in one isolate (ADL467) the contig was shorter and carried only strB and sul2. A potential IncQ1 plasmid harbouring strA, strB and sul2 was present in three isolates from different lineages. A candidate IncFIA/B plasmid was present in the two clone 3 isolates and one additional isolate. Interestingly each of these four plasmid contigs harbouring AMR genes were present at more than one time-point.

4. Discussion

In this study we examined if the withdrawal of group antimicrobial treatment results in changes to the occurrence of AMR in commensal *E. coli* of healthy pigs. Quantitative bacteriology showed the persistence of *E. coli* sub-populations resistant to apramycin and trimethoprim-sulfamethoxazole on the farm and no significant reduction in the proportion of resistant isolates over the 11 month study period. Both antimicrobials had been used to treat clinical salmonellosis in weaners on the farm in the period prior to the study. It would have been informative to investigate the impact of this treatment regime on AMR in commensal *E. coli* and establish an earlier AMR baseline, before cessation of group treatment, but unfortunately due to practical restrictions we were not able to collect samples during this preceding period. To provide a greater resolution into the effect of antimicrobial withdrawal we undertook susceptibility testing of 259 *E. coli* isolates purified from the pig faecal samples. Reduced susceptibility to tetracycline, ampicillin, and trimethoprim-sulfamethoxazole was most common, and these antimicrobials have the greatest use in the pig sector (EFSA, 2020; UK-VARSS, 2018), although of these only trimethoprim-sulfamethoxazole had been used recently on this farm. Importantly, amongst the isolates studied, reduced susceptibility towards ciprofloxacin was very low and no isolates were resistant to cefotaxime, as both are highest priority critically important antimicrobials. Examination of isolates by time-point revealed several important changes in AMR prevalence. Of particular note is the significant decrease in the proportion of MDR isolates and

355 significant increase in isolates susceptible to the antimicrobial panel used over the 11 months. These
356 key outcome indicators provide a useful correlation with antimicrobial use and suggest that a change
357 from group to individual treatment is a practicable approach for reducing the burden of AMR in pigs.
358 There was also a significant increase in the proportion of isolates susceptible to ampicillin and
359 trimethoprim-sulfamethoxazole, but not to the other six antimicrobials tested. This highlights the
360 complexity and challenges presented by AMR, with many factors not yet fully elucidated, especially
361 regarding the mechanism of dissemination and maintenance of resistance on farms (Davies and
362 Wales, 2019). Indeed, AMR bacteria may be isolated repeatedly from subsequent groups of animals,
363 even in the face of little external selection pressure and frequent cleaning and disinfection (Davies
364 and Wales, 2019). A longer study period or an increased sampling and testing effort might have
365 helped uncover smaller effects of withdrawal on AMR prevalence. However, power calculations
366 based on our sample size of 53 indicates that there was an approximately 15 % chance that a decrease
367 of 10 % per time point from an initial prevalence ranging from 30% to 50% would be detected as
368 statistically significant. Furthermore, although harbouring AMR provides a clear selective advantage
369 for *E. coli* in the presence of antimicrobials, it may impose little or no selective disadvantage in their
370 absence (Davies and Wales, 2019). Indeed bacterial adaptions such as compensatory mutations and
371 plasmid addiction systems are recognised drivers for the maintenance of AMR in bacterial
372 populations (Davies and Wales, 2019) The farm environment plays a critical role as potential
373 reservoir of resistant microorganisms that can re-colonise livestock after the cessation of
374 antimicrobial use (Davies and Wales, 2019). Farm biosecurity (e. g. controlling access by wildlife)
375 and effective cleaning and disinfection are therefore important tools in the control of AMR (Davies
376 and Wales, 2019; Magnusson et al., 2019; WHO, 2015). The farm involved in this study did not
377 employ an all-in/all out management system but did have a generally good routine of cleaning and
378 disinfection after depopulation of the pig pens. However, in the weaner building cleaning and
379 disinfection was done on a pen basis and there was the potential for seepage of contaminated material
380 through poorly fitting wooden panels to adjacent pens. Environmental sampling at this farm
381 demonstrated the presence of AMR *E. coli* not only in recently cleaned and disinfected pens, but also
382 on cleaning tools, drinkers, and in wildlife faeces collected from animal pens. During this study we
383 provided advice on good management practices such as all-in/all-out, pen segregation using concrete
384 walls, effective biosecurity measures, and pest control to help minimize the spread of pathogens (such
385 as Salmonella) and AMR bacteria. Implementation of similar interventions would be applicable more
386 broadly to all pig farms (Davies and Wales, 2019; Magnusson et al., 2019). This study highlighted
387 the importance of the environment and good biosecurity through the analysis of the WGS data, which
388 showed that an *E. coli* clone harbouring ten AMR genes (clone 3; Fig. 4) was present on cleaning

389 tools and in pigs at the beginning and end of the 11 month study. Even though the sequencing effort
390 was small (n = 46 isolates from pigs), we identified three more *E. coli* clones (each with a different
391 ST; Fig. 4) that were present in pigs at more than one time point, illustrating the persistence of AMR
392 on farms even when antimicrobial use is very low. Indeed, Birkegård et al., 2017 suggest that
393 antimicrobial exposure is not the only important determinant of AMR gene levels. We have
394 previously demonstrated the persistence of AMR *E. coli* in humans (Card et al., 2015; Kirchner et
395 al., 2014) and this finding in pigs was not unexpected. It is possible that clones circulated in pigs and
396 repeatedly contaminated the environment, leading to transfer between batches. These persistent
397 clones may be well adapted to survival in the pig gut and be spread between animals via direct contact,
398 fomites, or wildlife vectors (Ahmed et al., 2017; Davies and Wales, 2019). The antimicrobial
399 resistance genes found in the isolates examined by WGS have been commonly reported in similar
400 studies on resistance genes in *E. coli* isolates from pigs in Canada, Denmark and UK (AbuOun et al.,
401 2020; Ahmed et al., 2017; Boerlin et al., 2005; DANMAP, 2018; Gerzova et al., 2015). Whole
402 genome sequencing also showed that AMR genes were often linked and potentially located on
403 plasmids, such as the putative ColRNAI plasmid that harboured trimethoprim, sulphonamide, and
404 streptomycin resistance genes (Table 1). Linkage of AMR genes may have contributed to the
405 significantly higher proportions of MDR *E. coli* obtained from APR- and SXT-containing plates.
406 The four exemplar putative plasmids harbouring AMR genes possessed Incompatibility types
407 previously described in Enterobacteriaceae (AbuOun et al., 2020; Ahmed et al., 2017) and each was
408 present in two or more different *E. coli* lineages and persisted across multiple visits to the farm. This
409 provides further evidence that plasmids present a mechanism for the maintenance and dissemination
410 of resistance in the commensal *E. coli* population, especially given the small sequencing effort
411 employed in this project. In future, the use of long read sequencing would provide valuable additional
412 insight into plasmid structure, maintenance, and changes over time (Duggett et al., 2018). Overall,
413 examination of resistance by age class showed that weaners harboured greater proportions of resistant
414 *E. coli* than finishers. Finisher pigs had significantly fewer APR- and SXT-resistant *E. coli*
415 determined by quantitative bacteriology; significantly less *E. coli* isolates with reduced susceptibility
416 (i.e. NWT) towards ampicillin, apramycin, florfenicol, trimethoprim-sulfamethoxazole, and
417 streptomycin; and significantly less MDR *E. coli*. Weaner pigs were housed in pens where individual
418 animals were previously treated with antimicrobials and, as noted above, faced a potential for cross-
419 contamination between pens. In contrast finisher pigs were housed in pens where antimicrobials had
420 not been used. The prevalence of resistant *E. coli* is often higher in young animals because they are
421 more prone to enteric disease and stressed by weaning and mixing with other litters and thus more
422 likely to be treated with antimicrobials (Akwar et al., 2008). Our findings regarding the association

of age class with resistance prevalence correlate with previous studies (Akwar et al., 2008) and help inform risk models assessing the potential transfer of resistance to humans via the food chain. In conclusion, although antimicrobial use is strongly associated with an increased abundance of commensal AMR bacteria in humans (Card et al., 2015; Kirchner et al., 2014) and animals (EFSA, 2020), few studies have examined the impact of reduced antimicrobial use on AMR prevalence at the farm level. A core objective of the Global Action Plan on AMR is the prudent and efficient use of antimicrobials in public and veterinary medicine (WHO, 2015). To promote responsible antimicrobial use in the livestock sector the FAO recommends individual antimicrobial treatment of pigs, based on a diagnosis of disease, over the use of group treatment (Magnusson et al., 2019). In this study, we provide strong evidence that implementation of these recommendations can lead to significant reductions in key indicators for AMR prevalence, although some residual level of AMR was found to remain. Our findings therefore provide evidence which may assist producers and policy makers in the implementation of Action Plans on AMR. In future it would be informative to undertake a detailed, longer term, economic cost/benefit analysis, encompassing the cost of farm inputs and income obtained, as well as capturing any adverse effects, to further support the implementation of these treatment practices.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2021.109125.

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Fig. 1. Farm history and experimental design. Graphic timeline of the administration and withdrawal of antimicrobials and schedule of the sampling visits on-farm over the 11 months of the study period.

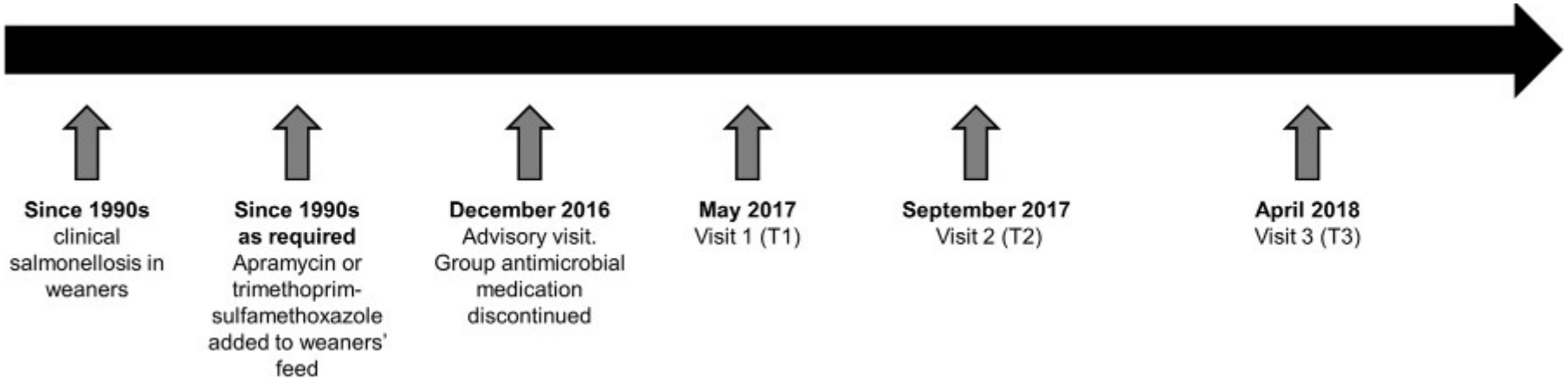


Fig. 2. Proportion of wild type (WT) and non-wild-type (NWT) *E. coli* isolates at farm visits T1, T2, and T3. Antimicrobials tested were ampicillin (AMP), tetracycline (TET), cefotaxime (CTX), florfenicol (FLO), ciprofloxacin (CIP), streptomycin (STR), apramycin (APR) and trimethoprim-sulfamethoxazole (SXT). Interpretative criteria to define reduced susceptibility are shown. Significant differences between visits T1 and T3 were determined using Linear-by-Linear. Cochran-Armitage test; * indicate respectively $p < 0.05$

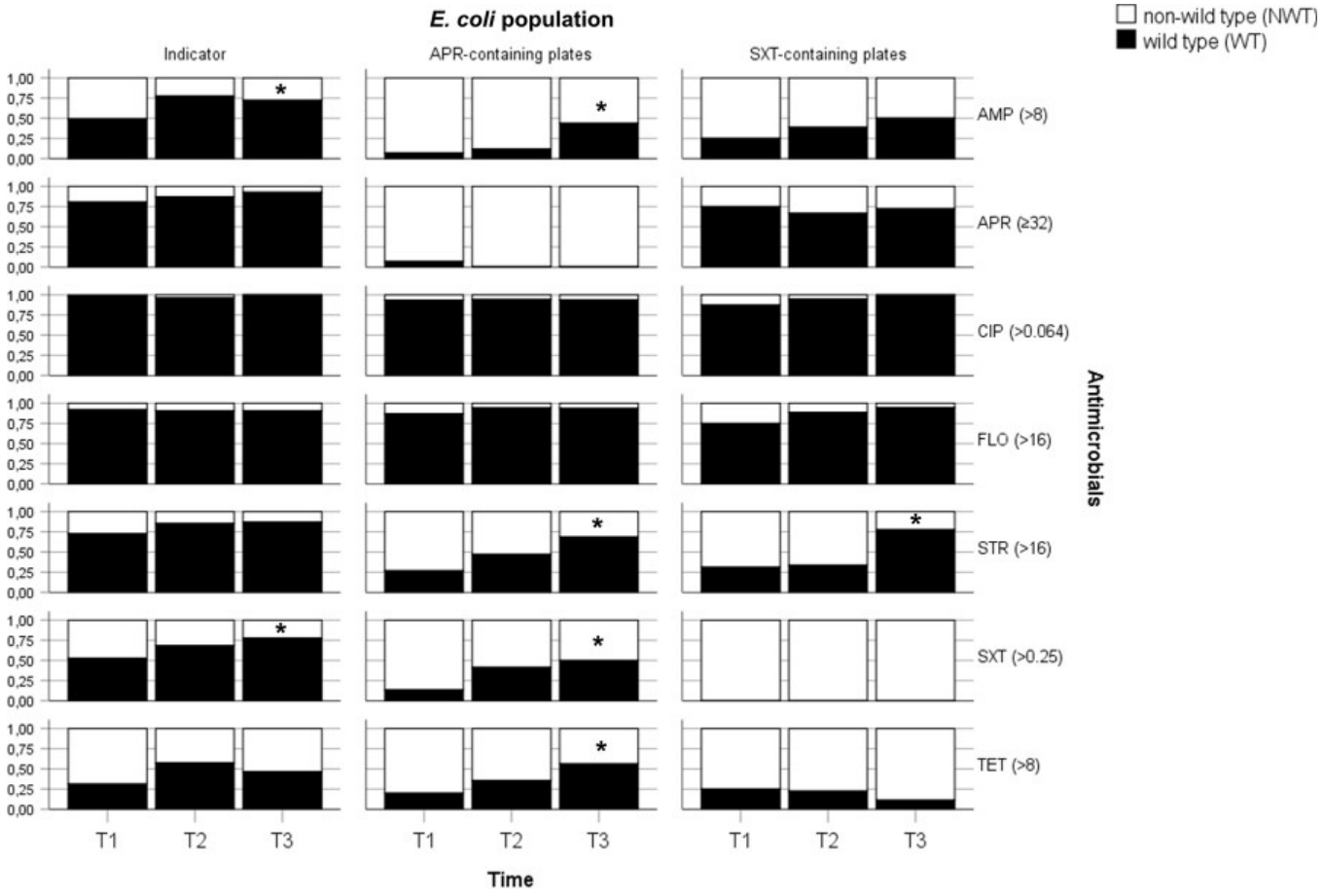


Fig. 3. Proportion of A) fully susceptible indicator and B) total of Multidrug Resistant *E. coli* isolates at farm visits T1 and T3. Full susceptibility was defined as susceptibility to all nine antimicrobials tested and multidrug resistance was defined as resistance to three or more antimicrobial classes; * and ** indicate respectively $p < 0.05$ and $p < 0.001$ respectively.

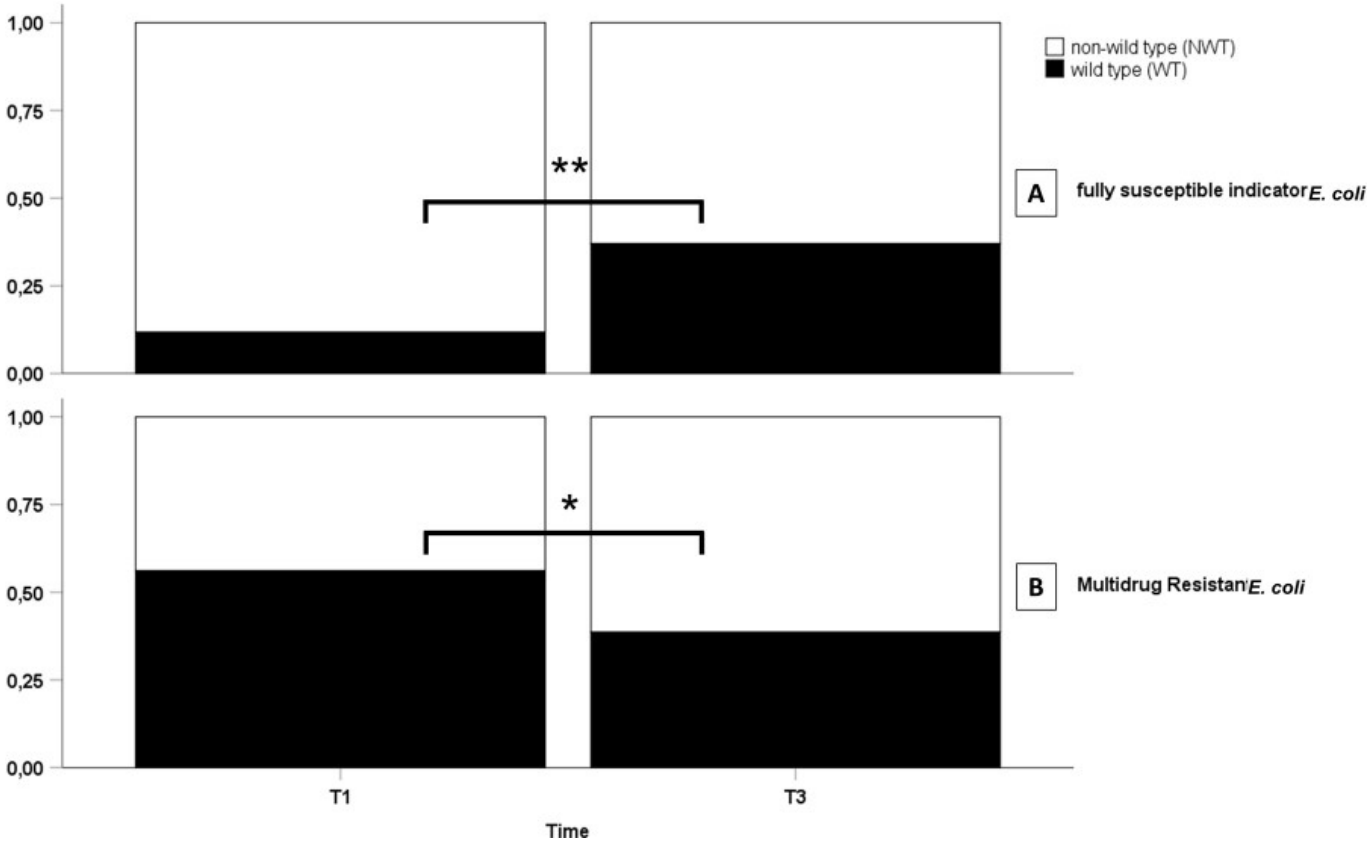
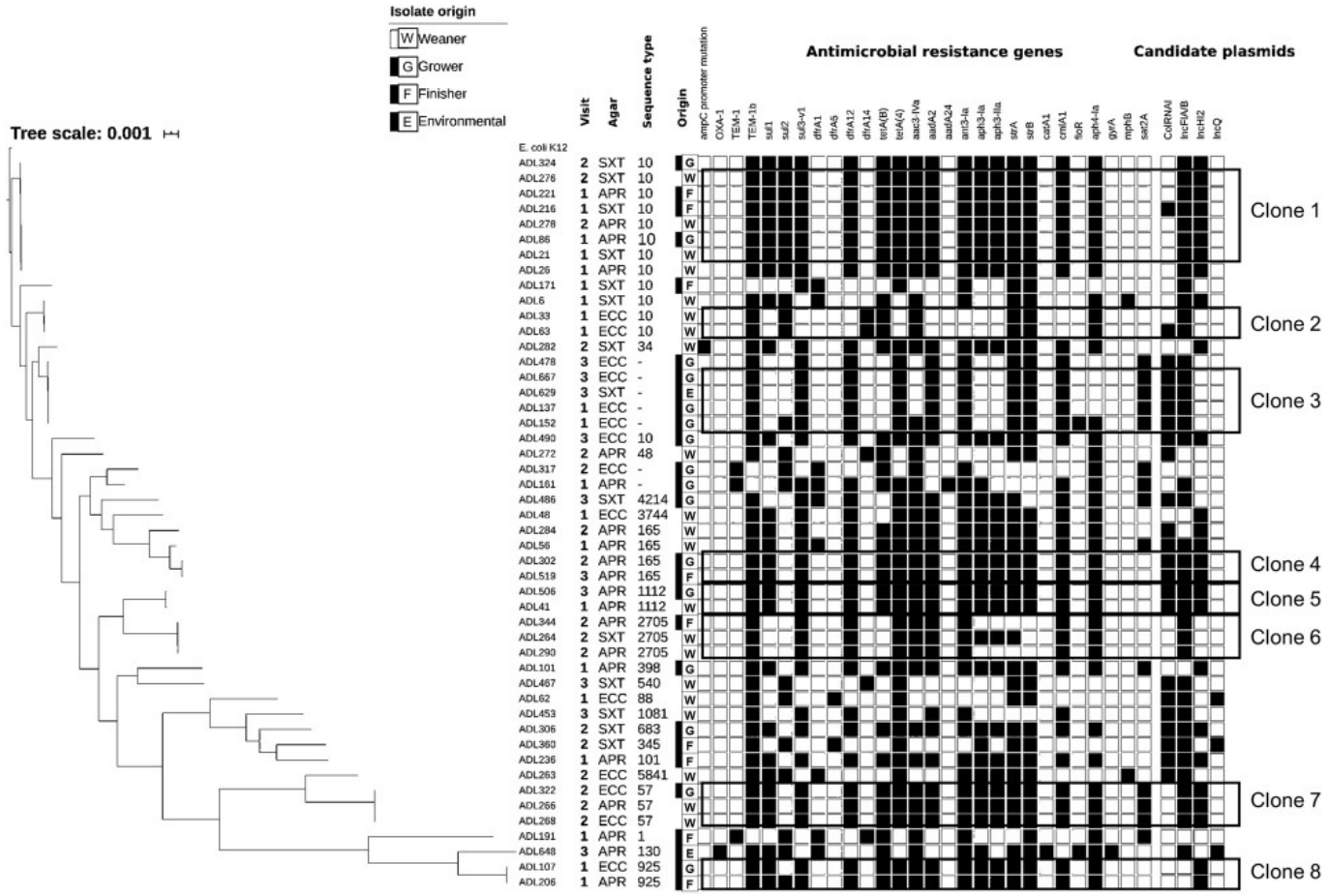


Table 1. Summary of four different candidate plasmids detected in assemble whole genome sequences that harboured AMR genes and were present in multiple *E. coli* lineages and time points. Where relevant *E. coli* clones is indicated (n/a = isolated not assigned to a clone).

Plasmid incompatibility type	Linked AMR genes	*E. coli* clone	*E. coli* ST	Isolate ID	Plasmid contig size (bp)	Visit
IncHI2	*strA*; *strB*	1	10	ADL21	109,551	1
		1	10	ADL26	109,755	1
		1	10	ADL56	109,555	1
		1	10	ADL86	109,755	1
		1	10	ADL216	109,551	1
		1	10	ADL221	109,551	1
		1	10	ADL276	109,552	2
		1	10	ADL278	109,552	2
		4	165	ADL302	100,023	2
		4	165	ADL519	99,865	3
		5	1112	ADL41	108,510	1
		n/a	10	ADL490	97,758	3
		n/a	34	ADL282	109,555	2
		n/a	398	ADL101	109,555	1
		n/a	683	ADL306	89,493	2
ColRNAI	*strA*; *strB*; *sul2*; *dfrA14*	2	10	ADL33	6,951	1
		2	10	ADL63	6,895	1
		n/a	48	ADL272	6,895	2
	strB; *sul2*	n/a	540	ADL467	4,740	2
IncFIA/B	$bla_{\text{TEM-1b}}$	3	new	ADL137	1,943	2
		3	new	ADL629	2,183	3
		n/a	new	ADL478	1,947	3
IncQ1	*strA*; *strB*; *sul2*	n/a	345	ADL360	4,638	2
		n/a	130	ADL648	4,640	2
		n/a	88	ADL62	4,639	1

Fig. 4. Maximum likelihood phylogenetic tree generated from core genome single nucleotide polymorphisms of *E. coli* from pigs harbouring the prevalent MDR resistance pattern (n ¼ 46) and the environment (n ¼ 2). The origin of the isolate (weaner, grower, finisher, environment), visit number, agar used for isolation (CHROMagar ECC (ECC), CHROMagar ECC with apramycin (APR) and CHROMagar ECC with sulfamethoxazole/trimethoprim (SXT)) and Sequence Type (-indicates a new ST) is given. AMR gene presence is indicated by black filled squares. Clones discussed in the text are boxed.



Supplementary Table 1. Bacterial counts of presumptive *E. coli* isolated from different pig age classes, recovered from CHROMagar ECC with and without apramycin and sulfamethoxazole/trimethoprim. Number of samples collected per visit and per age classes during each visit are shown in brackets.

Age class	CHROMagar	Farm visit (no. of samples)		
		T1 (17)	T2 (18)	T3 (18)
Weaners (6)	ECC [a]	5.1×10^6	1.5×10^7	8.4×10^6
	SXT [b]	6.4×10^5	2.7×10^6	8.8×10^5
	APR [c]	2.7×10^5	1.7×10^6	2.7×10^5
Growers (6)	ECC	3.6×10^6	5.3×10^7	2.2×10^6
	SXT	6.6×10^5	4.9×10^6	2.8×10^5
	APR	3.0×10^5	2.3×10^6	5.5×10^4
Finishers (6)	ECC	2.3×10^5	1.7×10^7	9.8×10^5
	SXT	1.3×10^4	1.3×10^6	3.6×10^4
	APR	2.5×10^2	2.3×10^4	1.4×10^2
Total	ECC	-	4.7×10^6	-
	SXT	-	4.5×10^5	-
	APR	-	5.2×10^4	-

[a] ECC. CHROMagar ECC

[b] STX. CHROMagar ECC + 4 mg/L of trimethoprim-sulfamethoxazole (1:19)

[c] APR. CHROMagar ECC + 4 mg/L of 32 mg/L of apramycin

Supplementary Table 2: Commensal *E. coli* isolates obtained by culture in the presence or absence of antimicrobials. Data is presented grouped by age-class and by isolate susceptibility: fully susceptible, reduced susceptibility to 1 or 2 antimicrobial classes, and multidrug resistance (reduced susceptibility to ≥ 3 antimicrobial classes). The asterisk (*) indicated significant difference ($p < 0.05$) in the frequency of MDR *E. coli* isolated from finisher and weaner pigs

E. coli population	Age class	All sensitive				1 or 2 Abs				MDR class			
		T1 (51)	T2 (54)	T3 (54)	T1+T2+T3 (159)	T1 (51)	T2 (54)	T3 (54)	T1+T2+T3 (159)	T1 (51)	T2 (54)	T3 (54)	T1+T2+T3 (159)
Indicator	Weaners	3 (20%)	5 (27,8%)	4 (22,2%)	12 (23,5%)	4 (26,7%)	6 (33,3%)	6 (33,3%)	16 (31,4%)	8 (53,3%)	7 (38,9%)	8 (44,4%)	23 (45,1%)*
	Growers	1 (5,6%)	9 (50%)	7 (38,9%)	17 (31,5%)	9 (50%)	4 (22,2%)	7 (38,9%)	20 (37%)	8 (44,4%)	5 (27,8%)	4 (22,2%)	17 (31,5%)
	Finishers	2 (11,1%)	10 (55,6%)	9 (50%)	21 (38,9%)	12 (66,7%)	7 (38,9%)	7 (38,9%)	26 (48,1%)	4 (22,2%)	1 (5,6%)	2 (11,1%)	7 (13%)*
	Tot Age class	6 (11,8%)*	24 (44,4%)	20 (37%)*	50 (31,4%)	25 (49%)	17 (31,5%)	20 (37%)	62 (39%)	20 (39,2%)	13 (24,1%)	14 (25,9%)	47 (29,6%)
APR-containing plates		T1 (15)	T2 (17)	T3 (16)	T1+T2+T3 (48)	T1 (15)	T2 (17)	T3 (16)	T1+T2+T3 (48)	T1 (15)	T2 (17)	T3 (16)	T1+T2+T3 (48)
	Weaners	0	0	0	0	0	0	4 (66,7%)	4 (23,5%)	5 (100%)	6 (100%)	2 (33,3%)	13 (76,5%)
	Growers	1 (16,7%)	0	0	1 (5,9%)	1 (16,7%)	2 (40%)	2 (33,3%)	5 (29,4%)	4 (66,7%)	3 (60%)	4 (66,7%)	11 (64,7%)
	Finishers	0	0	0	0	0	3 (50%)	2 (50%)	5 (35,7%)	4 (100%)	3 (50%)	2 (50%)	9 (64,3%)
	Tot Age class	1 (6,7%)	0	0	1 (2,1%)	1 (6,7%)	5 (29,4%)	8 (50%)	14 (29,2%)	13 (86,7%)	12 (70,6%)	8 (50%)	33 (68,8%)
SXT-containing plates		T1 (16)	T2 (18)	T2 (18)	T1+T2+T3 (52)	T1 (16)	T2 (18)	T2 (18)	T1+T2+T3 (52)	T1 (16)	T2 (18)	T2 (18)	T1+T2+T3 (52)
	Weaners	0	0	0	0	0	2 (33,3%)	1 (16,7%)	3 (17,6%)	5 (100%)	4 (66,7%)	5 (83,3%)	14 (82,4%)
	Growers	0	0	0	0	2 (33,3%)	1 (16,7%)	2 (33,3%)	5 (27,8%)	4 (66,7%)	5 (83,3%)	4 (66,7%)	13 (72,2%)
	Finishers	0	0	0	0	1 (20%)	2 (33,3%)	3 (50%)	6 (35,3%)	4 (80%)	4 (66,7%)	3 (50%)	11 (64,7%)
	Tot Age class	0	0	0	0	3 (18,8%)	5 (27,8%)	6 (33,3%)	14 (26,9%)	13 (81,3%)	13 (72,2%)	12 (66,7%)	38 (73,1%)

Supplementary Table 4: Frequencies of the most common multidrug-resistant (resistance to ≥ 3 antimicrobial classes) patterns among the 259 *E. coli* commensal isolates tested.

Reduced susceptibility profile	**T1**		**T2**		**T3**	
AMP/SXT/CIP/FLO/TET/AMINO	4	8.7%	1	2.6%	1	2.9%
AMP/SXT/FLO/TET/AMINO	5	10.9%	3	7.9%	0	0.0%
AMP/SXT/CIP/FLO/AMINO	0	0.0%	1	2.6%	0	0.0%
AMP/SXT/TET/AMINO	22	47.8%	17	44.7%	9	26.5%
AMP/FLO/TET/AMINO	1	2.2%	0	0.0%	0	0.0%
AMP/SXT/FLO/AMINO	0	0.0%	1	2.6%	0	0.0%
AMP/SXT/FLO/TET	0	0.0%	2	5.3%	6	17.6%
AMP/SXT/CIP/TET	0	0.0%	1	2.6%	0	0.0%
AMP/CIP/TET/AMINO	0	0.0%	1	2.6%	0	0.0%
AMP/SXT/TET/	5	10.9%	2	5.3%	7	20.6%
AMP/SXT/AMINO	5	10.9%	2	5.3%	3	8.8%
AMP/TET/AMINO	2	4.3%	1	2.6%	4	11.8%
SXT/TET/AMINO	2	4.3%	6	15.8%	4	11.8%
AMP/FLO/TET	0	0.0%	0	0.0%	0	0.0%
Total of MDR*	46	56.1%	38	42.7%	34	38.6%

Antimicrobial tested: Ampicillin (AMP), tetracycline (TET), cefotaxime (CTX), florfenicol (FLO), ciprofloxacin (CIP), trimethoprim-sulfamethoxazole (SXT) and apramycin and/or streptomycin (AMINO).