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Multi drug and extended spectrum beta-lactamase resistant *Escherichia coli* isolated from a dairy farm.

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Keywords: Cattle slurry, *Escherichia coli*, extended spectrum beta-lactamase resistance, multidrug resistance

Running title: Multi drug and ESBL resistant *E. coli* from a dairy farm

ABSTRACT

Escherichia coli strains were isolated from a single dairy farm as a sentinel organism for the persistence of antibiotic resistance genes in the farm environment. Selective microbiological media were used to isolate 126 *E. coli* isolates from slurry and faeces samples from different farm areas. Antibiotic resistance profiling for 17 antibiotics (seven antibiotic classes), showed 57.9% of the isolates were resistant to between 3 and 15 antibiotics. The highest frequency of resistance was to ampicillin (56.3%), and the lowest to imipenem (1.6%), which appeared to be an unstable phenotype and was subsequently lost. Extended spectrum beta-lactamase resistance (ESBL) was detected in 53 isolates and bla_{CTX-M}, bla_{TEM} and bla_{OXA} genes were detected by PCR in twelve, four and two strains, respectively. Phenotypically most isolates showing resistance to cephalosporins were AmpC rather than ESBL, a number of isolates having both activities. Phenotypic resistance patterns suggested co-acquisition of some resistance genes within subsets of the isolates. Genotyping using ERIC PCR demonstrated these were not clonal, and therefore co-resistance may be associated with mobile genetic elements. These data show a snapshot of diverse resistance genes present in the *E. coli* population reservoir, including resistance to historically used antibiotics as well as cephalosporins in contemporary use.

INTRODUCTION

The use of antibiotics in agriculture and animal husbandry is increasingly being considered a global health issue, both from the animal health and welfare aspect, and because of the development of antibiotic resistance in animal pathogens (Davies 2013; O'Neill 2015). In the UK from 2006-2011, (after the EU ban on antibiotic use as a growth promoter), about 400 tonnes of antibiotics were used annually in treatment of food-producing animals (Wellington *et al.* 2013) which is more than the amount used in humans. Animal manure is a major source of antimicrobial resistant bacteria entering the environment, especially the soil (Aarestrup *et al.* 1996; Binh *et al.* 2007; Ghosh and LaPara 2007), and it has been estimated that annually about 70 million tonnes of animal manure are used as fertilizer on agricultural land in the UK (Hutchison *et al.* 2004). Much of this will contain low levels of antibiotics or antibiotic metabolites/conjugates, and antimicrobial resistant bacteria.

Escherichia coli serves as a sentinel organism for antimicrobial resistance development in different types of animals, because it is a common enteric commensal, can be a pathogen, and easily acquires resistance and therefore can act as a reservoir which can transfer resistance to other species/pathogens (Aarestrup *et al.* 1998; van den Bogaard *et al.* 2000; Allen *et al.* 2010; Ashbolt *et al.* 2013). Cephalosporin antibiotics are commonly used in dairy veterinary medicine because they are effective in treating environmental mastitis caused largely by *E. coli* strains (Shpigel *et al.* 1997). Of particular concern has been the emergence and spread of extended spectrum beta lactamase (ESBL) producing *E. coli* associated with cattle and other farm animals (Bush and Jacoby 2010; Pfeifer *et al.* 2010). Both ESBL and AmpC ß-Lactamases can confer resistance to third generation cephalosporins, penicillins and monobactams. These two groups of enzymes are very distinct and can mainly be differentiated by different susceptibility patterns against β-lactam site-specific inhibitors such as clavulanic acid, their differences in activity against fourth generation cephalosporins such as cefepime, and cephamycins such as cefoxitin (Moritz and Carson 1986; Livermore *et al.* 2001; Bush and Jacoby 2010; CLSI 2013a). To date, more than 220 TEM, 190 SHV,

and 160 CTX-M ESBL enzyme variants, have been described, and more than 230 variants from the different plasmid AmpC families are known, 136 of which belong to the CMY family (www.lahey.org/studies).

In the dairy farm studied, cephalosporins were used for treatment of environmental mastitis caused by *E. coli*, and mastitic milk was discarded into the slurry tank, which may potentially select for *E. coli* strains carrying ESBL. The aim of this study was to determine the prevalence and range of ESBL resistance in *E. coli* within the farm, to understand the diversity of resistance to β-lactam antibiotics, and to determine if co-carriage of other antimicrobial resistance (AMR) was associated with ESBL resistance. This would allow a better understanding of the contributions that farms and farm waste may make to persistence of AMR in the environment, and the reservoir of resistance in agriculture.

Materials and Methods

Dairy Farm Unit

The farm has a herd of approximately 200 dairy cows. The animals are milked using an automated milking system, and consequently the milking cows are housed for the majority of the year inside barns. Each cow produces 60-70 kg of waste per day, which is automatically scraped into a sump, daily. Slurry solids are mechanically separated and deposited into a trailer, where it goes to a heap or is directly used as a fertilizer. The remaining liquid slurry, containing approximately 5% solids, is pumped into an on-site slurry tank and stored for field spreading. The slurry tank has a capacity of 3 million litres and is generally emptied after circa 90 days, either into a slurry lagoon by means of a pipeline, or is pumped into a tanker and taken directly to fields for spreading. The major use of antibiotics on the farm is to treat mastitis infections, which occur at a rate of ~100 incidences per year, i.e. approximately one every 3 days. At the time of this study, these infections were treated

with cephalosporin antibiotics (Cefquinome- 4th generation cephalosporin- veterinary use only) through intramammary infusion directly into the infected quarter; the withheld milk was disposed of into the slurry tank, alongside waste footbath contents, washing water and other cleaning solutions and disinfectants used in the dairy unit. Cefquinome was also used as a prophylactic to prevent mastitis when cows were dried off before entering another lactation cycle. Other antibiotics had been used on the farm for treatment of other bacterial infections common to dairy cattle including: ceftiofur (3rd generation cephalosporin- veterinary use only), cephalexin (1st generation cephalosporin), oxytetracycline, penicillins, aminoglycosides and aminocoumarin antibiotics.

Samples

Slurry samples were collected from different areas in the dairy unit in December 2012 and March 2014. In all cases samples were kept at 4°C and isolation of *E. coli* started within 48 h of collection. Three biological replicates were taken from each sample and individually serially diluted in maximum recovery diluent (MRD; Oxoid, UK), and duplicate 100 µL samples spread onto selective media. In the preliminary study in 2012, samples were taken from the slurry tank (n=2), separated solid slurry (n=1), cow faeces solid (n=1) and liquid exudate (n=1) and *E. coli* isolated by plating on tryptone bile X-glucuronide agar (TBX agar; Merck) with overnight incubation at 37°C. In the 2014 study, samples from liquid slurry taken from the slurry tank (n=3), separated solid slurry (n=3), and cow faeces from animal housing (n=3) were plated on TBX agar, TBX containing cefotaxime (CTX; 2 mg L^{-1}) and CHROMagar ESBL (BioConnections, UK). All plates were incubated overnight at 37°C. *E. coli* NCTC 13353 and *E. coli* ATCC 25922 were used as ESBL positive and negative control strains, respectively. In general, for each sample five colonies were picked from each of the TBX and TBX + CTX plates, and most of the pink colonies from the CHROMagar ESBL plates and were purified using LB agar (Fisher Scientific, USA). In total 155 colonies were selected from different media: in 2012 25 colonies from TBX; in 2014 35 colonies were selected from CHROMagar ESBL, 47 colonies from TBX and 48 colonies

from TBX+CTX. All isolates were tested for indole production (Collins *et al.* 2004) and oxidase activity (Oxidase Detection sticks, Oxoid); and indole positive, oxidase negative strains were considered as *E. coli*. API 20E tests (BioMerieux, France) were used to confirm identification for selected isolates. All confirmed isolates were immediately stored as Microbank (Pro-Lab Diagnotics UK) bead stocks at -80°C, and were grown from frozen stocks for each subsequent characterization.

Antibiotic sensitivity tests

The disc diffusion antibiotic sensitivity tests were carried out according to the National Committee for Clinical Laboratory Standards (NCCLS) and Clinical and Laboratory Standards Institute (CLSI) guidelines with some modifications (NCCLS 2002 ; CLSI 2012). Four or five bacterial colonies were taken from LB plates which had been inoculated and incubated overnight at 37°C. The colonies were suspended in 5 mL of Muller-Hinton broth (Oxoid, UK) and incubated for 16 h at 37°C. Each sample was diluted with sterile water until it reached a 0.5 McFarland standard concentration. Then, 100 µL of the culture was pipetted onto the surface of a 25 mL Muller-Hinton agar plate (Oxoid, UK), and the inoculum was distributed by spreading using an L-shaped spreader. The plates were left to dry at room temperature (for no more than 15 min), and the antibiotic discs were placed on to the plate surface, with 4-5 antibiotic discs on each plate. The plates were then incubated at 37°C for 18-24 h, and the results recorded by measuring the inhibition zone diameter across the disc and then interpreted according to standard measurement tables (CLSI 2013a, b). All antibiotics used in the tests are listed in Table 1; all discs were supplied by Oxoid (UK), except for Cefquinome (Bioconnection, UK): the quality control strains used were *E. coli* ATCC 25922 (ESBL negative) and *E. coli* NCTC 13353 (ESBL positive CTX-M-15).

Phenotypic confirmation of ESBL/AmpC-producing *E. coli*

Phenotypic detection of ESBLs can be obscured by AmpC-producing bacteria. All isolates that gave an inhibition zone indicating resistance or intermediate resistance to cefotaxime, and/or ceftazidime using standard antibiotic discs were further tested using the Total ESBL Confirm Kit (Rosco Diagnostica, France; product code 98014) containing a combination of cefotaxime (CTX), ceftazidime (CAZ), and cefepime (FEP) discs (30 µg of each antibiotic) alone and in combination with clavulanic acid (CA). The test was performed in accordance with the guidelines of the manufacturer. An increase in the diameter of the zone of clearing around the disc by ≥ 5-mm for either antimicrobial agent tested in combination with CA vs the diameter of the zone of clearing around a disc containing the agent when tested alone, indicated ESBL presence (CLSI 2013a; EUCAST 2013). The total ESBL kit is composed of 6 discs, the first 4 are recommended to be used by CLSI while the other two (FEP and FEP+C) are further recommended by EUCAST to confirm that resistance is due to ESBL, and not chromosomal AmpC, as chromosomal AmpC does not confer resistance to cefepime.

In addition, a cefoxitin (FOX) disc (30 µg, Oxoid, UK) was added to the test, to detect AmpC phenotypes, as it is known that AmpC producers are resistant to cefoxitin, one of the cephamycin group.

The presence of hyper-produced and de-repressed/plasmid mediated AmpC beta-lactamase was detected using the AmpC Confirm Kit (Rosco Diagnostica; product code 98007), where a ≥ 5-mm increase in the inhibition zone diameter of cefotaxime 30 µg + cloxacillin (CTXCX) or ceftazidime 30 µg + cloxacillin (CAZCX) compared with the inhibition zone for the antibiotics alone indicated the presence of de-repressed/plasmid encoded AmpC, inhibited by cloxacillin. Control strains used were *E. coli* ATCC 29522 (ESBL negative control), *E. coli* NCTC13353 (ESBL positive) and *Enterobacter cloacae* NCTC 13406 (AmpC β-lactamase de-repressed).

Clustering of resistance profiles

For consistency, antibiotic resistance scores of less than 6 (nominal disc size in mm) were replaced with 6 as a minimum score. Clustering of antibiotic sensitivity profiles was carried out using the heatmap.2 function in the gplots library (version 2.17.0) in the R software package (version 3.2.0). Euclidean distance was used for both row (bacterial strain) and column (antibiotic) dissimilarity. Clustering used complete linkage. Clustering was used for data visualization, so no formal statistics were run.

Genotyping of isolates

DNA extraction

Bacterial DNA was extracted by dispersing one colony of *E. coli* from an overnight culture on LB agar into 100 µL of sterile 1 x TE buffer (10 mM Tris-Cl, 1 mM EDTA buffer, pH 7.6). The suspension was heated to 100°C for 30 min to rupture bacterial cells (Eppendorf Thermomixer Comfort, Germany) and then centrifuged (Heraeus Pico17 centrifuge, UK) at 13 000 *x g* for 15 min. The crude DNA in the supernatants was transferred into sterile microcentrifuge tubes and stored at -20 ˚C until use (total DNA).

PCR detection of ESBL genes

All phenotypically ESBL isolates were screened for bla_{SHV}, bla_{TEM}, bla_{OXA-1}, bla_{OXA-2} and bla_{CTX-M} genes using PCR as described by Dierikx and co-workers (2012). Total DNA (2 µL) was used in a 25 µL reaction mixture which contained 12.5 µL of DreamTaq Green PCR master mix (2X) (ThermoFisher Scientific, UK) (containing DreamTaq™ DNA polymerase, optimized DreamTaq Green buffer, 0.4 mM of each of the dNTPs, 4 mM $MgCl_2$), 8.5 µL of nuclease free water, and 1 µL (10 µM) of each primer. The primers and expected PCR product sizes are given in Table 2. *E. coli* NCTC 13353 was used as

positive control for CTX, *E. coli* NCTC 13352 was used as a positive control for TEM, and *Klebsiella pneumoniae* NCTC 13368 was used as a positive control for SHV. *E. coli* ATCC 25922 was used as a negative control for PCR.

All oligonucleotides were synthesized by Eurofins MWG Operon, Germany. A programmable C1000™ Thermal cycler (Bio-Rad, UK) was used to carry out the reactions under the following conditions: one cycle of denaturation for 5 min at 94˚C followed by 30 cycles of 30 s at 94˚C, 30 s at 55˚C and 60 s at 72˚C, with a final extension of 7 min at 72˚C. The PCR product (7 µL) was loaded onto a 2% w/v agarose gel, containing ethidium bromide (0.4 µg mL^{-1}) in 1x TAE running buffer (40 mM Tris-acetate, 1 mM EDTA) and electrophoresed at 120 V for 1 h. A 100 bp DNA size marker (Invitrogen, UK) was used in each gel.

PCR detection for plasmid *ampC*

A multiplex PCR assay was carried out as described in Dallenne *et al.* (2010) to detect plasmid *ampC* genes from six families: ACC, FOX, MOX, DHA, EBC and CIT. The PCR reactions were carried out in a 50 µL PCR mixture including 25 µL of DreamTaq Green PCR master mix (2X), 1 µL of each forward and reverse primer for all families, except for FOX and DHA where 2.5 µL of each of the forward and reverse primers were used, 5 µL of nuclease free water and 2 µL of DNA. The PCR cycle started with an initial denaturing step of 10 min at 94°C and then 30 cycles of 94°C (40 s), 60°C (40 s) and 72°C (60 s). The PCR reaction ended with a final extension step of 72°C for 7 min. All primers are listed in Table 2.

ERIC PCR

Genotypes were investigated using the ERIC-PCR method (Versalovic *et al.* 1991). The PCR mixture (25 µL) contained 12.5 µL of DreamTaq Green PCR master mix (2X), 9.5 µL nuclease free water, 1 µL DNA and 1 µL (10 µM) of each primer: ERIC1 (forward) and ERIC2 (reverse) (Eurofins MWG Operon, Germany) (Table 2). A programmable C1000™ Thermal cycler was used to carry out the reactions, using the following conditions: one cycle for 3 min at 94˚C, 35 cycles of: 30 s at 94˚C, 1 min at 52˚C, and 4 min at 65˚C. The final cycle was for 8 min at 65˚C. The PCR product (7 µL) was loaded onto a 2% w/v agarose gel, as described above and electrophoresed at 120 V for 2 h. A 1 kb plus DNA size marker (Invitrogen, UK) was included on every gel to allow cross gel comparison. After electrophoresis, the gel was imaged using a Gel-Doc XR system (Bio-Rad, UK) and the image analysed using FPQuest gel analysis software V4.5 (Bio-Rad, UK). A dendrogram was obtained from the comparison of ERIC-PCR profiles, using the Dice coefficient, and clustered by the unweighted pair group method with arithmetic averages (UPGMA) with 1.5% of optimization and 1.5% of tolerance to display the dendrogram. Molecular variance framework (AMOVA; Excoffier *et al.* 1992) was used to analyse the confidence of the selected similarity threshold and the significance of clusters. The AMOVA calculation was carried out using GenAlEx v 6.5b5 software (Peakall and Smouse 2006). The significance was examined with the calculation of ΦPT, a measure of population differentiation that suppresses intra-individual variation. In the case of AMOVA, the null hypothesis (H0; ΦPT = 0) meant that there was no genetic difference among the populations and the alternative hypothesis (H1; ΦPT >0) meant there are genetic differences amongst the populations.

Results

Samples

E. coli strains were isolated from all of the farm samples using the different selective media; 126 out of 155 isolates were confirmed as *E. coli*. Many of the colonies isolated on the ESBL CHROMagar™

gave typical colony characteristics for *E. coli*, but the confirmation tests did not confirm this identification and they were excluded from further analysis. Strain numbering is indicative of source and medium of isolation: A designates a 2012 strain isolated on TBX; B designates 2014 isolates: BL liquid slurry, BS slurry solids, BC cattle faeces from the cow barn; a further suffix C designates CTX agar, suffix E is CHROMagar ESBL, no suffix is TBX without antibiotic selection. For the 2012 strains, numbers following A indicate sampling site: 1 and 2 designate the slurry tank, 3 solid cow faeces, 4 liquid exudates from solid faeces, and 5 separated solid slurry.

Antibiotic Resistance

One hundred and twenty six (2012/14) confirmed *E. coli* isolates from TBX, TBX + CTX and CHROMagar ESBL were tested against 17 antibiotics (representing seven antibiotic groups) using standard disc diffusion antibiotic sensitivity tests (Table 1). Only 7.9% of isolated strains were sensitive to all antibiotics whereas 92% showed resistance to at least 1 antibiotic of which 27.8%, (35) of the isolates were isolated on TBX and showed resistance to at least one antibiotic. 57.9% (73) of the isolates were multidrug resistant (MDR- as defined in Magiorakos *et al.* 2012) and showed resistance to three or more antibiotics from three different antibiotic groups. The resistance profile of MDR strains ranged from 3-15 antibiotics Figure 1 and 2). The isolate with the highest number of resistances (BCC2) (Figure 3) was sensitive only to imipenem and ceftazidime. The highest percentages for MDR isolates were from solid slurry, 26.1% (33 isolates); then cow accommodation slurry, 19.8% (25 isolates) and from liquid slurry 11.9% (15 isolates).

With regard to the prevalence of resistance to each antibiotic, Figure 2 shows the percentage of strains resistant to each tested antibiotic. Amongst the strains tested, the highest percentage resistance was to ampicillin (56.3%), followed by oxytetracycline, streptomycin, and sulphonamide (41.1%, 39.6%, and 37.3% respectively). Dihydrofolate reductase inhibitors, such as trimethoprim

were tested in combination with sulfamethoxazole and 12.6% of the *E. coli* isolates were resistant to this combination, while the resistance to chloramphenicol and nitrofurantoin was 8.7% for both. It is worth noting that ampicillin was one of the most widely used antibiotics for veterinary use in the UK between 2006-2011, as well as tetracycline and trimethoprim/sulphonamides (DEFRA 2012). Furthermore, the isolates showed a range of resistance to different antibiotics from the cephalosporin family: 38%, 14.2% and 23% were resistant to cefotaxime, ceftazidime and ceftiofur, respectively, which are all third generation cephalosporins, while 11.1% of the isolates were resistant to cefquinome, which is a fourth generation cephalosporin. This is not surprising as approximately 48% (59 isolates were from selective media: 15 from CHROMagar ESBL and 44 from TBX+CTX) of the isolates were directly selected on media containing beta-lactam antibiotic supplements. Quinolone antibiotics were still largely effective against these strains, with only 3.1% of the *E. coli* isolates resistant to both ciprofloxacin (CIP) and enrofloxacin (ENR). 1.6% (2) of the *E. coli* isolates were phenotypically resistant to imipenem (IMP), with 3.2% (4) showing intermediate sensitivity, but subsequent characterization of the imipenem resistant strains showed this resistance was not stable. The highest intermediate resistance was against streptomycin (S10) (36.5%) and aztreonam (ATM) (23.8%).

Clustering of Antibiotic Sensitivity

When the antibiotic resistance profile of each *E. coli* strain was clustered (Figure 3) using the heatmap.2 function of the R statistical package, the analysis showed that, with some exceptions, the isolates mainly clustered together based on the selective medium which was used to isolate them. The strains isolated on TBX medium and then tested for antibiotic resistance were more diverse in their resistance patterns than those isolated on TBX+CTX. The latter clustered in two groups with the majority of isolates showing clustered resistance to ampicillin (AMP), streptomycin (S10), oxytetracycline (OT) amoxicillin-clavulanic acid (AMC) and sulphonamide (S300). The majority of

those isolated on CHROMagar ESBL formed a closely related resistance cluster, with ESBL resistance to cefquinome (CFQ), ceftiofur (EFT), ceftazidime (CAZ), cefotaxime (CTX) and aztreonam (ATM) (Figure 3). Several strains that were isolated on TBX +CTX (BCC1, BSC22) or TBX (BS9) clustered with the CHROMagar isolates, and showed this same cluster of resistances while some CHROMagar ESBL isolates which clustered in the TBX cluster (BSE1, 7, 3, 5, 9, 4) lacked them.

Phenotyping and PCR typing of ESBL resistances and genes

ESBL/AmpC phenotypes were tested in the 53 strains that were found to be resistant to cefotaxime/ ceftazidime in the initial antibiotic sensitivity assays (Table 3). These tests showed that 25/53 (47.1%) strains were phenotypically ESBL using the confirmatory test kits. The strains were also tested by PCR for the presence of $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{OXA1} and $_{OXA2}$, or bla_{SHV} beta lactamases. No bla_{SHV} beta-lactamases were detected, but $bla_{CTX\text{-}M}$ (22.6%; 12 positive) and bla_{TEM} (7.5%; 4 positive) were both detected in the ESBL isolates, including examples of both in the same isolate (BCE3 and BCC2), bla_{OXA1} was present in two (3.7%) isolates, but not in the ESBL isolates, as well as two of the isolates that were positive for TEM but were not phenotypically ESBL: BS7 and A2.5 (Figure 3).

Phenotypic AmpC resistance was identified in 36 isolates, initially by the lack of activity of cefepime against these strains. This was further confirmed when the cefoxitin disc assay showed no activity against the strain. However, cefoxitin resistance can be due to decreased cell wall permeability (Brenwald *et al.* 2005); so full confirmation was if the inhibition zone diameter for CTX and CAZ discs compared to CTX + cloxacillin, and CAZ + cloxacillin was ≥ 5-mm larger than in the discs containing antibiotic alone, as AmpC activity against cephalosporins is inhibited by cloxacillin. Eight isolates (15.0%) had both ESBL and AmpC activity; those strains were BCC12, BCC7, BLC5, BSC14, BSC19, BSC3, BSC4 and BSC9 (Figure 4 and Table 3). PCR tests for the presence of plasmid *ampC* genes from the six major families: ACC, FOX, MOX, DHA, EBC and CIT, were negative for all 36 of the

phenotypically AmpC strains, suggesting that the AmpC phenotype in the farm *E. coli* isolates was chromosomal gene hyperproduction.

Genotyping of *E. coli* strains using ERIC PCR

The similarity of the ERIC-PCR profiles of the *E. coli* isolates from different sampling sites was analysed, and cluster analysis divided strains into six main groups at a 50% similarity threshold which cluster significance analysis demonstrated were non-overlapping and hence genomically independent groups (cluster significance ΦPT=0.48; P<0.001). There was no significant association of clusters with source of isolation, with most groups containing isolates from all three sources: cow barns, slurry tank and solid slurry. Some solid slurry isolates did associate closely in separate clusters in groups IV and V, suggesting this distinct genotype may be partitioning with the solids. Noticeably there was little correspondence of ERIC clusters with the clusters generated in the antibiotic resistance heat map (Figures 3 and 5). Groups of strains with identical ERIC profiles such as BC12, BC14, BC15, BC7, BL15, BLC4, BS10, BS9, were widely dispersed in the resistance dendrogram. Similarly strains showing close similarity in their resistance profile were not always related at the genomic level. Hence the multidrug resistant strains that were resistant to AMP, S10, OT, S300 and AMC do not represent a genomically clonal group. This suggests that this co-resistance may be due to a mobile genetic element which has transferred between multiple strains. A further example is seen in strains BSC7, BSC18, BSC20, all from solid slurry samples and all with the same ERIC profile; strain BSC20 is sulphonamide-trimethoprim (SXT) resistant, which the other two strains are not and could demonstrate gene acquisition e.g. through a resistance plasmid. There were many similar examples of strains with identical (ie 100%S) ERIC profiles showing variability in their resistances. This suggests that in this environment there is a genomically variable *E. coli* population in which there is conservation of antimicrobial resistance carrying genetic elements. This was further substantiated by comparison of the 2014 isolates with the 20 *E. coli* strains from the

preliminary study in 2012. In comparison to the 2014 isolates, the ERIC profiles of the 2012 isolates (A-strains) partitioned them into a separate group on the dendrogram (Figure 5), showing them to be a quite distinct genetic population; all these strains were isolated on TBX agar without antibiotic selection, and whilst there was some subclustering of this group of isolates on the resistance heat map (Figure 3), not all isolates clustered together. Strain A2.5 from 2012 (VI group) isolated from the slurry tank showed multiresistance to 11 antibiotics including the AMP, S10, OT, S300 and AMC multiresistance phenotype seen amongst the 2014 strains and this isolate clustered together with these 2014 strains on the resistance heat map (Figure 3). This again is suggestive of conservation of the genetic determinant(s) over 2 years, rather than conservation of a clonal line.

Discussion

Escherichia coli is a common enteric commensal, specific strains of which can cause human and animal disease. It is one of the group of seven species that the WHO has highlighted as of key AMR concern and serves as a sentinel organism for antimicrobial resistance development. Of particular concern has been the emergence and spread of ESBL producing *E. coli* associated with cattle and other farm animals (Liebana *et al.* 2013). The recent report of transferable colistin resistance in commensal *E. coli* from farm animals and the presence of the same resistance genes in isolates from meat and *E. coli* and *Klebsiella pneumoniae* from patients has highlighted the threat that resistance genes originating in animals can subsequently be spread to humans (Liu *et al.* 2015).

In this study we determined the antibiotic resistance pattern of *E. coli* strains isolated from different cattle slurry sources in a dairy farm unit, with a focus on ESBL mediated resistance. On this farm (as will be the case in others worldwide) different types of antibiotics are being given to treat the cattle, and this will make a contribution to antibiotic resistance gene selection and gene horizontal transfer among different species of bacteria. To our knowledge, similar studies on MDR *E. coli* from cattle

slurry within one dairy farm have not been performed, or have been performed with different methodology or materials, such as the antibiotics that were used to challenge the strains. Comparison with other studies would not necessarily give the same patterns of resistance, as each farm has a different management regime regarding housing the cattle, the type of cattle, their nutrition and veterinary treatments, disposal of antibiotic-contaminated mastitic milk, use of disinfectants and other antimicrobials, and slurry handling. Studying resistance to different groups of antibiotics in a sentinel species (*E. coli*) has given us an indication of the reservoir of resistance that is present in the farm bacterial population. It is worth mentioning that the source of the isolates is a well-managed farm with complete records of the antibiotics that are being used for disease treatment.

This study showed that MDR *E. coli* were present in all samples. However, the faecal samples from the barns and the solid slurry carried more resistant isolates compared to samples from the liquid slurry. This might be due to the dilution that happens to the bacterial levels contained in the slurry tank, as wastewater from cleaning the barns and the milking equipment is disposed of into the slurry tank, and the slurry tank is open-topped and receives rain water.

The total percentage of MDR strains resistant to 3 or more antibiotics was 57.9% (73 isolates) (including ESBL resistance and resistance to other antibiotics) of which 43.6% (55 isolates) were strains that were isolated using extend spectrum cephalosporin ESC selective media (TBX + CTX and CHROMagar ESBL), whilst the percentage of MDR E. *coli* isolated on TBX without antibiotic selection was 14.2% (18 isolates) (to all tested antibiotics). This difference will reflect the effectiveness of the direct selection method, and may also be due to the difference in prevalence of MDR *E. coli* between different sampling times. This was in line with another study which found that ESBL-producing *E. coli* can be isolated from cattle farms more than twice as often using selective enrichment procedures (Schmid *et al.* 2013). What was surprising was that the different isolation media isolated different subpopulations of resistant *E. coli* from the same samples. The same multiple antibiotic resistances

were more frequently seen when an extended spectrum cephalosporin antibiotic (ESC) was used for selective isolation.

The presence of ESBL-mediated resistance in the isolates was not surprising given the use of cephalosporins for mastitis treatment in the herd. What was more unexpected was the level of resistance carriage to older antibiotics, with resistance to oxytetracycline, streptomycin and sulphonamide seen in over a third of strains. Co-acquisition of these resistance genes with ampicillin resistance was strongly indicated in TBX+CTX isolates (Figure 3) and may indicate the presence of a multi-resistance genetic element present in these isolates. Schmid *et al.* (2013) found ESBL *E. coli* from some farms which did not use β-lactam antibiotics and they suggested that the presence of such isolates is due to using other classes of antibiotics that can select for ESBLs as well. In a previous study of *E. coli* strains isolated from cattle, most strains were resistant to ampicillin (64%), tetracycline (74%), streptomycin (60%), sulphonamide (76%) with low occurrence (1%) of enrofloxacin resistance (EMEA 1999). In a later study by Nováková *et al.* (2009), all the *E. coli* isolates from dairy calves and lambs showed multi-resistance to tetracycline, streptomycin and compound sulphonamides with less resistance to enrofloxacin (Nováková *et al.* 2009). The resistance patterns observed in the current study mirror these findings, and resemble a historical record of antibiotic use and development of resistance to them (Toleman and Walsh 2011), and may represent a genetic archaeology of the use of veterinary antimicrobials.

According to Jacoby and Sutton (1991), resistance determinants against aminoglycosides, tetracycline, sulphonamides and cephalosporins, are often situated on the same plasmid. Metagenomics, PCR and exogenous plasmid isolation studies of cow manure have also detected diverse resistance genes (Jacoby and Sutton 1991; van Overbeek *et al.* 2002; Wichmann *et al.* 2014). Plasmids and transposons that carry multi antimicrobial resistance genes can also carry genes mediating resistance to toxic metals, virulence, and metabolic functions, for example Tn*1691* specifies resistance to mercury as well as to some antibiotics: streptomycin, sulphonamides,

chloramphenicol (Barbosa and Levy 2000). This could indicate that there are factors other than veterinary medicines leading to retention of antibiotic resistances within the dairy herd and the farm environment.

The finding that two strains were resistant to imipenem was unexpected and of concern. Confirmatory testing of these isolates showed initial retention of imipenem resistance but on further retesting the strains reverted to imipenem sensitivity, a phenomenon which has been reported elsewhere (Villa *et al.* 2013) and which can be associated with loss of plasmid carriage of genes.

The most frequently encountered ESBL genes in Enterobacteriaceae belong to the TEM, SHV and CTX-M families (Paterson and Bonomo 2005), and the number of novel gene variants is still increasing as different genes are being described (http://www.lahey.org/studies/). This study showed the presence in ESBL *E. coli* in one dairy farm of bla_{CTX-M} (12 strains), and bla_{TEM} (4 strains), bla_{OXA} (2 strains) but not bla_{SHV} and presence of *E. coli* carrying chromosomal *ampC* resistance. Nine of the 12 bla_{CTX-M} positive, and one of the four bla_{TEM} positive *E. coli* strains were isolated on CHROMagar ESBL, although six of the other strains isolated on this medium were not positive for bla_{CTX-M} and bla_{TEM}. In two cases *E. coli* isolates (BCE3 and BCC2) were shown to carry both bla_{CTX-M}, and bla_{TEM}. Thirty six isolates were confirmed as having resistance phenotypes consistent with hyperproduction of AmpC. The phenotypic differentiation between plasmid-borne and chromosomal AmpC resistance in *E. coli* is difficult. Although the AmpC β-lactamase enzyme is normally encoded by a non-inducible chromosomal gene (Honore *et al.* 1986; Philippon *et al.* 2002; Jacoby and Munoz-Price 2005; Jacoby 2009), hyper-production can arise, either by mutations at the *ampC* attenuator or promoter regions, or by gene amplification (Caroff *et al*. 1999; Nelson and Elisha 1999; Philippon *et al*. 2002). In bacteria that lack chromosomal *ampC,* phenotypic differentiation is easier, as the difference in beta-lactamase activity between ESBL and AmpC strains can be seen in the difference in sensitivity towards cefoxitin (to which AmpC is resistant) and Beta lactamase inhibitor combinations: clavulanic acid and EDTA (to which ESBL is sensitive)(Black *et al.* 2005; EUCAST 2013).

However, because no plasmid AmpC was detected in these farm isolates, resistance is likely to be due to mutations in the *E. coli* chromosomal *ampC* attenuator or promoter regions. In a study which used different phenotypic confirmation kits to differentiate between ESBL and AmpC, it was found that, out of 66 isolates, 58 isolates showed activity against cefepime and cefepime plus clavulanate, and 38 isolates showed CAZ activity; none of the AmpC strains showed activity against cefepime (Hansen *et al.* 2012). Besides, class D (OXA-type) ESBLs are poorly inhibited by clavulanic acid and therefore cannot be detected by the methods described here (Drieux *et al.* 2008; Naas *et al.* 2008).

Complicated ESBL gene carriage patterns have been found elsewhere. In a study performed on a farm suffering from calf scour it was found that amongst *E. coli* isolates that were resistant to cefotaxime, in addition to the isolates that were positive for CTX and TEM, 5/48 calf and 28/60 cow isolates were bla_{CTX}- and bla_{TEM}-negative, and sequence analysis confirmed that these had mutations in the promoter region of the chromosomal *ampC* gene (Liebana *et al.* 2006).

Dissemination of CTX-M genes via the food chain may also happen. In another study, on *E. coli* harbouring CTX-M genes and quinolone resistance genes, detected in healthy animals from a Czech zoological garden, the authors imply that the reason that zoo animals are carrying these genes may be due to spreading of MDR *E. coli* via the food chain according to their molecular analysis of the isolates (Dobiasova *et al.* 2013). In a study carried out on *E. coli* from farms in the UK, it was found that *E. coli* transconjugants harbouring $bla_{CTX\text{-}M}$ genes from cattle, chicken and turkey isolates were carrying 10, 8 and 7 other antimicrobial resistance genes respectively, and these mainly encoded resistances to older antibiotics. This study suggested that chicken and cattle CTX-M *E. coli* represent different populations (Toszeghy *et al.* 2012), and in a separate study, where there was the use of 3rd or 4th generation cephalosporin (ceftiofur and cefquinome) in livestock for 12 months prior to the analysis, *E. coli* isolates were nearly four times more likely to be ESBL positive (Snow *et al.* 2012). Samples from different sources have also been used to detect only ESBL genes, in this case the samples were faecal samples from pigs, cattle, chicken and sheep which were investigated at

slaughter. Only 13.7% of the bovine faeces samples showed positive ESBL producers (Geser *et al.* 2012). Each farm has different policies, which can affect the prevalence of antimicrobial resistance. Watson and co-workers found that the increased prevalence of the CTX-M-15 *E. coli* in certain cattle groups and farm environments including calving pens was related to the husbandry, antimicrobial usage and hygiene (Watson *et al*. 2012).

ERIC PCR has been used as a reliable method to differentiate between different genotypes of *E. coli* (Duan *et al*. 2009). There was no absolute distinction between isolate sources using ERIC PCR, as different isolates from different sources are found in most of the ERIC groups, except for group II and V and subgroup A of group IV that do not include any isolates from the slurry tank (Figure 5). This result is not surprising as all tested materials ultimately originated from the same source i.e. the cows. However, there was a significant difference ($P \leq 0.001$ using the AMOVA test) between the isolates from different sampling times, i.e. between group VI (2012) and the others (2014). Previous studies of *E. coli* from cattle faeces and farm environments showed strains were clonally related and carried the same CTX-M genes which were also seen in cultivated soil that was amended with manure one year before (Hartmann *et al.* 2012). In the current study genotyping showed that there was genomic diversity in the multi-resistant *E.coli* isolates with no evidence of clonal lines. In addition identical genotypes had different antibiotic resistance profiles. Both these findings suggest the presence of mobile resistance determinants in the population.

In conclusion the data from our study reflect a complex picture of resistance gene carriage within the farm *E. coli* population with evidence of multiple antibiotic resistance gene carriage and ESBL activity caused by different beta-lactamases, of which there are examples of co-carriage in individual strains. There are common antibiotic resistance patterns with resistance to AMP, S10, OT, S300 and AMC, and a subgroup of ESBL producing strains are also present. The strains we have isolated represent a snapshot of the reservoir of resistance genes within the dairy herd and the farm environment, carried in one species of Enterobacteriaceae (*E. coli*) on one farm. The resistance

profile of the isolates may reflect a historical accumulation of resistance genes, and represents the antibiotic "resistome" present as an environmental reservoir of bacteria, mobile elements and genes. This may aid the understanding of the environmental drivers which lead to the retention of a reservoir of resistance genes in the commensal bacterial population and hence control their spread. Further studies on the molecular basis of resistance amongst those isolates and the mobility of them are now needed.

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References

Aarestrup FM, Ahrens P, Madsen M, Pallesen LV, Poulsen RL & Westh H (1996) Glycopeptide susceptibility among Danish *Enterococcus faecium* and *Enterococcus faecalis* isolates of animal and human origin and PCR identification of genes within the VanA cluster. *Antimicrob Agents Chemother* **40**: 1938-1940.

Aarestrup FM, Bager F, Jensen N, Madsen M, Meyling A & Wegener HC (1998) Resistance to antimicrobial agents used for animal therapy in pathogenic-, zoonotic-and indicator bacteria isolated from different food animals in Denmark: a baseline study for the Danish Integrated Antimicrobial Resistance Monitoring Programme (DANMAP). *Apmis* **106**: 745-770.

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J & Handelsman J (2010) Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology* **8**: 251-259.

Ashbolt N J, Amézquita A, Backhaus T, Borriello P, Brandt KK, Collignon P, Coors A, Finley R, Gaze W. H & Heberer T (2013) Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environ Health Perspect* **121**: 993-1001.

Barbosa TM & Levy SB (2000) The impact of antibiotic use on resistance development and persistence. *Drug Resist Update* **3**: 303-311.

513 Binh CT, Heuer H, Gomes NC, Kotzerke, A, Fulle, M, Wilke, BM, Schloter, M & Smalla, K (2007) Short-
514 term effects of amoxicillin on bacterial communities in manured soil. *FEMS Microbiol Ecol* **62**: 290-
515 302.

516 Black JA, Moland ES & Thomson KS (2005) AmpC disk test for detection of plasmid-mediated AmpC
517 β-Lactamases in Enterobacteriaceae lacking chromosomal AmpC β-Lactamases. *J Clin Microbiol* **43**:
518 3110-3113.

519 Brenwald NP, Jevons G, Andrews J, Ang L & Fraise AP (2005) Disc methods for detecting AmpC β-
520 lactamase-producing clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *J Antimicrob*
521 *Chemother* **56**: 600-601.

522 Bush K & Jacoby GA (2010) Updated functional classification of beta-lactamases. *Antimicrob Agents*
523 *Chemother* **54**: 969-976.

524 Caroff N, Espaze E, Berard I, Richet H & Reynaud A (1999) Mutations in the ampC promoter of
525 *Escherichia coli* isolates resistant to oxyimino-cephalosporins without extended spectrum beta-
526 lactamase production. *FEMS Microbiol Lett* **173**: 459-465.

527 CLSI (2012) Performance standards for antimicrobial disk susceptibility tests; approved standard—
528 Eleventh Edition. Document M02-A11. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

529 CLSI (2013a) Performance standards for antimicrobial susceptibility testing; Twenty-third
530 Informational Supplement. CLSI document M100-S23.

531 CLSI (2013b) (M31-A4) Performance standards for antimicrobial disk and dilution susceptibility tests
532 for bacteria isolated from animals; draft, 2013, and M31-S2 (2nd Informational Supplement),
533 provided by NEO-SENSITABS™ Document: 9.1.0 Veterinary practice according to CLSI breakpoints.

534 Collins C, Lyne P, Grange J & Falkinham J (2004) Identification methods. *Collins and Lyne's*
535 *Microbiological Methods. 8th ed. Arnold*.

536 Dallenne C, Da Costa A, Decre D, Favier C & Arlet G (2010) Development of a set of multiplex PCR
537 assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. *J*
538 *Antimicrob Chemother* **65**: 490-495.

539 Davies S (2013) Annual report of the Chief Medical Officer, Volume Two, 2011, Infections and the
540 rise of antimicrobial resistance. *Department of Health, London*.

DEFRA (2012) Sales of antimicrobial products authorised for use as veterinary medicines in the UK in 2011. URL http://www.vmd.defra.gov.uk/pdf/salesanti11.pdf.

Dierikx CM, Van Duijkeren E, Schoormans AH, Van Essen-Zandbergen A, Veldman K, Kant A, Huijsdens XW, Van Der Zwaluw K, Wagenaar JA & Mevius DJ (2012) Occurrence and characteristics of extended-spectrum-beta-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses. *J Antimicrob Chemother* **67**: 1368-1374.

Dobiasova H, Dolejska M, Jamborova I, Brhelova E, Blazkova L, Papousek I, Kozlova M, Klimes J, Cizek A & Literak I (2013) Extended spectrum beta-lactamase and fluoroquinolone resistance genes and plasmids among *Escherichia coli* isolates from zoo animals, Czech Republic. *FEMS Microbiol Ecol* **85**: 604-611.

Drieux L, Brossier F, Sougakoff W & Jarlier V (2008) Phenotypic detection of extended-spectrum beta-lactamase production in Enterobacteriaceae: review and bench guide. *Clin Microbiol Infect* **14 Suppl 1**: 90-103.

Duan H, Chai T, Liu J, Zhang X, Qi C, Gao J, Wang Y, Cai Y, Miao Z, Yao M & Schlenker G. (2009) Source identification of airborne *Escherichia coli* of swine house surroundings using ERIC-PCR and REP-PCR. *Environ Res* **109**: 511-517.

EMEA (1999) Antibiotic resistance in the European Union associated with the therapeutic use of veterinary medicines. European Agency for the Evaluation of Medicinal Products. Report EMEA/CVMP/342/99-Final. Veterinary Medicines Evaluation Unit, London.

EUCAST (2013) EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. *EUCAST, Basel, Switzerland: http://www. eucast. org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_v1. 0_20131211. pdf*.

Excoffier L, Smouse PE & Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**: 479-491.

Geser N, Stephan R & Hachler H (2012) Occurrence and characteristics of extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae in food producing animals, minced meat and raw milk. *BMC Vet Res* **8**: 21.

570 Ghosh S & LaPara TM (2007) The effects of subtherapeutic antibiotic use in farm animals on the
571 proliferation and persistence of antibiotic resistance among soil bacteria. *ISME J* **1**: 191-203.

572 Hansen F, Hammerum AM, Skov RL, Giske CG, Sundsfjord A & Samuelsen O (2012) Evaluation of
573 ROSCO Neo-Sensitabs for phenotypic detection and subgrouping of ESBL-, AmpC- and
574 carbapenemase-producing Enterobacteriaceae. *APMIS* **120**: 724-732.

575 Hartmann A, Locatelli A, Amoureux L, Depret G, Jolivet C, Gueneau E & Neuwirth C (2012)
576 Occurrence of CTX-M producing *Escherichia coli* in soils, cattle, and farm environment in France
577 (Burgundy Region). *Front Microbiol* **3**: 83.

578 Honore N, Nicolas MH & Cole ST (1986) Inducible cephalosporinase production in clinical isolates of
579 *Enterobacter cloacae* is controlled by a regulatory gene that has been deleted from *Escherichia coli*.
580 *EMBO J* **5**: 3709-3714.

581 Hutchison ML, Walters LD, Moore A, Crookes KM & Avery SM (2004) Effect of length of time before
582 incorporation on survival of pathogenic bacteria present in livestock wastes applied to agricultural
583 soil. *Appl Environ Microbiol* **70**: 5111-5118.

584 Jacoby GA (2009) AmpC beta-lactamases. *Clin Microbiol Rev* **22**: 161-182.

585 Jacoby GA & Sutton L (1991) Properties of plasmids responsible for production of extended-
586 spectrum beta-lactamases. *Antimicrobi Agents Chemother* **35**: 164-169.

587 Jacoby GA & Munoz-Price LS (2005) The New β-Lactamases. *New England J Med* **352**: 380-391.

588 Liebana E, Batchelor M, Hopkins KL, Clifton-Hadley FA, Teale CJ, Foster A, Barker L, Threlfall EJ &
589 Davies RH (2006) Longitudinal farm study of Extended-Spectrum β-Lactamase-Mediated Resistance.
590 *J Clin Microbiol* **44**: 1630-1634.

591 Liebana E, Carattoli A, Coque TM, Hasman H, Magiorakos AP, Mevius D, Peixe L, Poirel L,
592 Schuepbach-Regula G, Torneke K, Torren-Edo J, Torres C & Threlfall J (2013) Public health risks of
593 enterobacterial isolates producing extended-spectrum beta-lactamases or AmpC beta-lactamases in
594 food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk
595 factors, and control options. *Clin Infect Dis* **56**: 1030-1037.

596 Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu L-F, Gu D,
597 Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu J-H & Shen J (2015) Emergence of plasmid-mediated
598 colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and
599 molecular biological study. *The Lancet Infect Dis, DOI: 10.1016/S1473-3099(15)00424-7*.

Livermore DM, Winstanley TG & Shannon KP (2001) Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. *J Antimicrob Chemother* **48 Suppl 1**: 87-102.

Magiorakos AP, Srinivasan A, Carey R, Carmeli Y, Falagas M, Giske C, Harbarth S, Hindler J, Kahlmeter G & Olsson-Liljequist B (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* **18**: 268-281.

Moritz VA & Carson PB (1986) Cefoxitin sensitivity as a marker for inducible beta-lactamases. *J Med Microbiol* **21**: 203-207.

Naas T, Poirel L & Nordmann P (2008) Minor extended-spectrum beta-lactamases. *Clin Microbiol Infect* **14 Suppl 1**: 42-52.

NCCLS (2002) Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard, 2nd ed. NCCLS document M31-A2. National Committee for Clinical Laboratory Standards.Wayne,USA.

Nelson EC & Elisha BG (1999) Molecular basis of AmpC hyperproduction in clinical isolates of *Escherichia coli*. *Antimicrob Agents Chemother* **43**: 957-959.

Nováková I, Kačániová M, Haščík P, Pavličová S & Hleba L (2009) The resistance to antibiotics in strains of *E. coli* and *Enterococcus* sp. isolated from rectal swabs of lambs and calves. *Scientific Papers Animal Science and Biotechnologies* **42**: 322-326.

O'Neill J (2015) Antimicrobials in agriculture and the environment: reducing unnecessary use and waste. *Rev Antimicrob Resist*. http://amr-review.org/

Paterson DL & Bonomo RA (2005) Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev* **18**: 657-686.

Peakall ROD & Smouse PE (2006) Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes* **6**: 288-295.

Peter-Getzlaff S, Polsfuss S, Poledica M, Hombach M, Giger J, Böttger E, Zbinden R & Bloemberg G (2011) Detection of AmpC beta-lactamase in *Escherichia coli*: comparison of three phenotypic confirmation assays and genetic analysis. *J Clin Microbiol*. **49**: 2924-2932.

628 Pfeifer Y, Cullik A & Witte W (2010) Resistance to cephalosporins and carbapenems in Gram-negative
629 bacterial pathogens. *Int J Med Microbiol* **300**: 371-379.

630 Philippon A, Arlet G & Jacoby GA (2002) Plasmid-determined AmpC-type beta-lactamases.
631 *Antimicrob Agents Chemother* **46**: 1-11.

632 Schmid A, Hörmansdorfer S, Messelhäusser U, Käsbohrer A, Sauter-Louis C & Mansfeld R (2013)
633 Prevalence of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* on Bavarian dairy and
634 beef cattle farms. *Appl Environ Microbiol* **79**: 3027-3032.

635 Shpigel N, Levin D, Winkler M, Saran A, Ziv G & Böttner A (1997) Efficacy of cefquinome for
636 treatment of cows with mastitis experimentally induced using *Escherichia coli*. *J Dairy Sci* **80**: 318-
637 323.

638 Snow LC, Warner RG, Cheney T, Wearing H, Stokes M, Harris K, Teale CJ & Coldham NG. (2012) Risk
639 factors associated with extended spectrum beta-lactamase *Escherichia coli* (CTX-M) on dairy farms in
640 North West England and North Wales. *Prev Vet Med* **106**: 225-234.

641 Toleman MA & Walsh TR (2011) Combinatorial events of insertion sequences and ICE in Gram-
642 negative bacteria. *FEMS Microbiol Rev* **35**: 912-935.

643 Toszeghy M, Phillips N, Reeves H, Wu G, Teale C, Coldham N & Randall L (2012) Molecular and
644 phenotypic characterisation of extended spectrum beta-lactamase CTX-M *Escherichia coli* from farm
645 animals in Great Britain. *Res Vet Sci* **93**: 1142-1150.

646 van den Bogaard AEJM, London N & Stobberingh EE (2000) Antimicrobial resistance in pig faecal
647 samples from The Netherlands (five abattoirs) and Sweden. *J Antimicrob Chemother* **45**: 663-671.

648 van Overbeek LS, Wellington EM, Egan S, Egan S, Smalla K, Heuer H, Collard JM, Guillaume G,
649 Karagouni AD, Nikolakopoulou TL & van Elsas JD (2002) Prevalence of streptomycin-resistance genes
650 in bacterial populations in European habitats. *FEMS Micro Ecol* **42**: 277-288.

651 Versalovic J, Koeuth T & Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and
652 application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**: 6823-6831.

653 Villa L, Capone A, Fortini D, Dolejska M, Rodriguez I, Taglietti F, De Paolis P, Petrosillo N & Carattoli A
654 (2013) Reversion to susceptibility of a carbapenem-resistant clinical isolate of *Klebsiella pneumoniae*
655 producing KPC-3. *J Antimicrob Chemother* **68**: 2482-2486.

Watson E, Jeckel S, Snow L, Stubbs R, Teale C, Wearing H, Horton R, Toszeghy M, Tearne O, Ellis-Iversen J & Coldham N (2012) Epidemiology of extended spectrum beta-lactamase *E. coli* (CTX-M-15) on a commercial dairy farm. *Vet Microbiol* **154**: 339-346.

Wellington EMH, Boxall ABA, Cross P, Feil EJ, Gaze WH, Hawkey, PM, Johnson-Rollings AS, Jones DL, Lee NM, Otten W, Thomas CM. & Williams AP (2013) The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *The Lancet Infect Dis* **13**: 155-165.

Wichmann F, Udikovic-Kolic N, Andrew S & Handelsman J (2014) Diverse antibiotic resistance genes in dairy cow manure. *MBio* **5**: e01017-01013.

Multidrug and extended spectrum beta-lactamase resistant *Escherichia coli* isolated from a dairy farm.

Figure 1**. Percentages of MDR *E.coli* resistant to different numbers of antibiotics.**

Figure 2. **Percentage sensitivity to 17 antibiotics for 126 *E. coli* farm isolates.**

Blue bar indicates sensitive, red bar indicates intermediate sensitivity, green bar indicates lack of sensitivity, using CLSI (2013a,b) definitions. The Y axis represents the percentage of isolates.

Figure 3. **Heatmap representation of zone of growth inhibition surrounding antibiotic assay discs to 17 antibiotics for 126 *E. coli* isolates.**

The size of the zone of clearing around the antibiotic discs is represented by the colour spectrum in the diagram, with red representing no zone of clearing (not sensitive), red through to orange (resistant- zone of clearing 10-22mm depending on antibiotic), orange to yellow (sensitive-zone of clearing 17-27mm depending on antibiotic) and yellow to white representing a zone of clearing of 30-37mm). A zone of >37mm indicates highly sensitive -white. Presence of $bla_{\text{CTX-M}}$ or bla_{TEM} or bla_{OXA} is indicated by solid black lines, dotted blue lines, or solid red lines respectively. Notation of the antibiotics is as described in Table 1. The strain designation is described in the text. TBX, TBX + CTX (cefotaxime) and CHROMagar ESBL represent the media used to isolate the strains. Strain numbers in blue typeface indicate strains which were not isolated on the same medium as other strains in the cluster.

Figure 4. **Percentages for ESBL and AmpC phenotypes amongst *E. coli* isolates resistant to cefotaxime.**

Figure 5. **Dendogram of ERIC PCR profiles using Dice similarity coefficient and UPGMA cluster analysis for *E. coli* isolates.**

Clustering of ERIC-PCR profiles for *E. coli* isolates from the same farm using the Dice coefficient and UPGMA as described in Methods. Clusters I-VI were formed at 50% similarity as designated using AMOVA analysis. Subclusters A, B and C of cluster IV were designated at 60%. Strain designation is described in the text. Antibiotic notation is as in Table 1.The horizontal blue line divides each main group I-VI which clustered at 50% similarity threshold, which is indicated by the vertical blue line. The red line subdivides group IV into its subclusters.

Table 1: Antibiotic assay discs, abbreviations, and amount of antibiotic contained in each disc.

ANTIBIOTIC Discs	Content
Beta-lactams	
Ampicillin (AMP)	10µg
Amoxicillin-clavulanic acid (AMC)	20/10 µg
Cefotaxime (CTX)	30 µg
Ceftazidime (CAZ)	30 µg
Aztreonam (ATM)	30 µg
Imipenem (IMP)	10 µg
Cefquinome (CFQ)	30 µg
Ceftiofur (EFT)	30 µg
Aminoglycoside	
Streptomycin (S10)	10µg
Quinolones	
Ciprofloxacin (CIP)	5 µg
Enrofloxacin (ENR)	5 µg
Nalidixic acid (NA)	30 µg
Sulphonamide/complex	
Trimethoprim-sulfamethoxazole (SXT)	1.25/ 23.75 µg
Sulfonamide (S300)	300 µg
Phenicol	
Chloramphenicol (C)	30 µg
Tetracycline	
Oxytetracycline (OT)	30 µg
Nitrofuran derivative	
Nitrofurantoin (F)	300 µg

Table 2: PCR primers used for detection of beta-lactamase genes.

Oligonucleotide name	Sequence	Product size (bp)	Reference
CTX-M-F	ATGTGCAGYACCAGTAARGTKATGGC	529[a]	(Dierikx *et al*. 2012)
CTX-M-R	TGGGTRAARTARGTSACCAGAAYSAGC GG		
TEM-F	GCGGAACCCCTATTT G	964	
TEM-R	ACCAATGCTTAATCAGTGAG		
SHV-F-	TTATCTCCCTGTTAGCCACC	796	
SHV-R-	GATTTGCTGATTTCGCTCGG		
OXA-1-F-	ATGAAAAACACAATACATATCAACTTC GC	820	
OXA-1-R-	GTGTGTTTAGAATGGTGATCGCAT T		
OXA-2-F-	ACGATAGTTGTGGCAGACGAAC	601	
OXA-2-R-	ATYCTGTTTGGCGTATCRATATTC		
MultiACC-F-	CACCTCCAGCGACTTGTTAC	346	(Dallenne *et al*. 2010)
MultiACC-R-	GTTAGCCAGCATCACGATCC		
MultiMOX-F-	GCAACAACGACAATCCATCCT	895	
MultiMOX-R-	GGGATAGGCGTAACTCTCCCAA		
MultiDHA-F-	TGATGGCACAGCAGGATATTC	997	
MultiDHA-R-	GCTTTGACTCTTTCGGTATTCG		
MultiCIT-F-	CGAAGAGGCAATGACCAGAC	538	
MultiCIT-R-	ACGGACAGGGTTAGGATAGYb		
MultiEBC-F-	CGGTAAAGCCGATGTTGCG	683	
MultiEBC-R-	AGCCTAACCCCTGATACA		
MultiFOX-F-	CTACAGTGCGGGTGGTTT	162	
MultiFOX-R-	CTATTTGCGGCCAGGTGA		
AmpC-F-	GATCGTTCTGCCGCTGTG	271	(Peter-Getzlaff *et al*. 2011)
AmpC-R-	GGGCAGCAAATGTGGAGCAA		
ERIC-F-	ATGTAAGCTCCTGGGGATTCAC	variable	(Versalovic *et al*. 1991)
ERIC-R-	AAGTAAGTGACTGGGGTGAGCG		

[a](R is a purine; Y is a pyrimidine; S is G or C)

List of primers used in this study for detection of ESBL genes (CTX-M TEM, SHV, and OXA), plasmid-mediated *ampC* (ACC, MOX, DHA, CIT, EBC and FOX). The correct PCR product sizes are shown for each primer pair. The ERIC PCR primer sequences are also shown; PCR products from amplifications using ERIC are variable in size.

Table 3: Phenotypic ESBL resistance in *E. coli* isolates

	ANTIBIOTICS															
sample ID	CTX	CTX+C	D	CAZ	CAZ+C	D	FE	FE +C	D	CTX	CTCCX	D	CAZ	CAZCX	D	FOX
BC13	20	23	3	20	25	5	30	30	0	20	26	6	20	25	5	15
BCC1	0	30	30	16	26	10	16	30	14	0	3	3	16	17	1	25
BCC11	21	21	0	19	20	1	26	30	4	22	26	4	19	26	7	18
BCC12	22	24	2	19	24	5	25	28	3	22	30	8	20	30	10	20
BCC14	24	25	1	21	24	3	30	31	1	25	31	6	22	29	7	18
BCC15	20	20	0	18	20	2	23	25	2	20	28	8	15	29	14	20
BCC16	20	22	2	18	22	4	25	25	0	20	30	10	18	30	12	20
BCC2	15	28	13	25	26	1	20	30	10	15	18	3	25	28	3	28
BCC3	24	26	2	21	24	3	30	31	1	24	29	5	20	25	5	16
BCC4	23	25	2	20	22	2	29	29	0	23	30	7	20	29	9	16
BCC5	23	24	1	21	23	2	29	29	0	22	27	5	20	26	6	16
BCC6	22	25	3	20	25	5	30	31	1	22	24	2	20	20	0	22
BCC7	23	24	1	20	25	5	30	30	0	23	30	7	20	30	10	18
BCC8	23	24	1	21	22	1	28	28	0	25	30	5	23	29	6	16
BCC9	22	24	2	19	24	5	27	28	1	23	25	2	21	25	4	18
BCE1C	0	30	30	15	30	15	18	30	12	0	3	3	15	17	2	25
BCE2	0	25	25	15	25	10	17	30	13	0	3	3	16	17	1	25
BCE3	14	25	11	23	25	2	16	28	12	14	18	4	23	27	4	25
BCE4	10	29	19	15	25	10	17	30	13	10	12	2	15	16	1	26
BCE5	11	29	18	16	25	9	15	29	14	11	15	4	16	19	3	35
BLC1	21	24	3	20	21	1	26	27	1	21	31	10	20	30	10	20
BLC2	21	23	2	18	22	4	27	29	2	21	27	6	18	25	7	17
BLC3	20	24	4	18	21	3	25	25	0	21	26	5	19	25	6	15
BLC4	28	29	1	21	28	7	25	25	0	28	28	0	21	25	4	20
BLC5	19	25	6	20	25	5	30	30	0	20	28	8	20	26	6	17
BLC6	20	20	0	18	20	2	25	25	0	20	24	4	19	27	8	20
BLC8	20	24	4	19	21	2	25	25	0	20	24	4	21	27	6	19
BLC9	21	21	0	18	21	3	25	27	2	21	30	9	20	30	10	20
BS9	10	30	20	17	28	11	19	32	13	10	14	4	17	18	1	22
BSC1	23	27	4	20	23	3	30	34	4	23	30	7	20	30	10	15
BSC10	24	26	2	21	25	4	31	31	0	26	33	7	23	31	8	16
BSC13	22	24	2	20	22	2	28	28	0	22	26	4	20	26	6	17
BSC14	23	26	3	20	25	5	28	28	0	23	31	8	20	30	10	16
BSC15	22	24	2	20	20	0	28	30	2	20	25	5	18	24	6	20
BSC16	23	25	2	20	22	2	30	30	0	24	32	8	22	30	8	16
BSC17	23	24	1	20	24	4	28	28	0	23	30	7	21	28	7	14
BSC18	22	24	2	20	23	3	30	30	0	22	30	8	20	29	9	17
BSC19	20	25	5	20	24	4	26	28	2	20	30	10	20	30	10	18
BSC2	22	24	2	20	23	3	28	30	2	21	30	9	20	27	7	15
BSC20	24	27	3	20	25	5	30	30	0	25	30	5	20	29	9	14
BSC21	19	21	2	17	21	4	25	25	0	19	30	11	19	29	10	15
BSC22	10	30	20	15	21	6	22	30	8	10	12	2	15	17	2	25
BSC3	22	24	2	20	25	5	30	30	0	23	29	6	20	28	8	15
BSC4	22	25	3	20	25	5	28	31	3	24	32	8	20	31	11	15
BSC5	23	24	1	20	24	4	30	31	1	23	31	8	20	30	10	16
BSC6	22	25	3	20	24	4	30	30	0	22	27	5	20	24	4	18
BSC7	23	25	2	22	26	4	29	30	1	23	30	7	22	29	7	15
BSC9	21	25	4	20	24	4	24	28	4	21	30	9	21	29	8	16
BSE2	14	30	16	17	28	11	20	30	10	14	15	1	17	19	2	25
BSE3	10	27	17	14	25	11	19	27	8	10	14	4	14	18	4	27
BSE6	17	30	13	17	27	10	16	27	11	15	15	0	17	18	1	25
BSE8	20	30	10	26	28	2	30	32	2	20	24	4	26	30	4	24
BSE9	28	32	4	30	31	1	34	36	2	28	32	4	30	31	1	18
NCTC13353	0	28	28	13	30	17	14	30	16	0	0	0	13	13	0	25
E.cloacae	9	11	2	9	11	2	25	25	0	9	24	15	9	24	15	6

Inhibition zone sizes for ESBL *E. coli* isolates using the ESBL (left columns: CTX to Cefepime +C) and AmpC confirm (right columns CTX to FOX) test kits. D is the difference between the 2 previous values (or the difference between antibiotic alone and antibiotic with supplement), 5 mm or greater difference for D indicates positive results for the test, which has been highlighted in red. Strain numbers in red typeface indicate strains with both ESBL and AmpC activity.

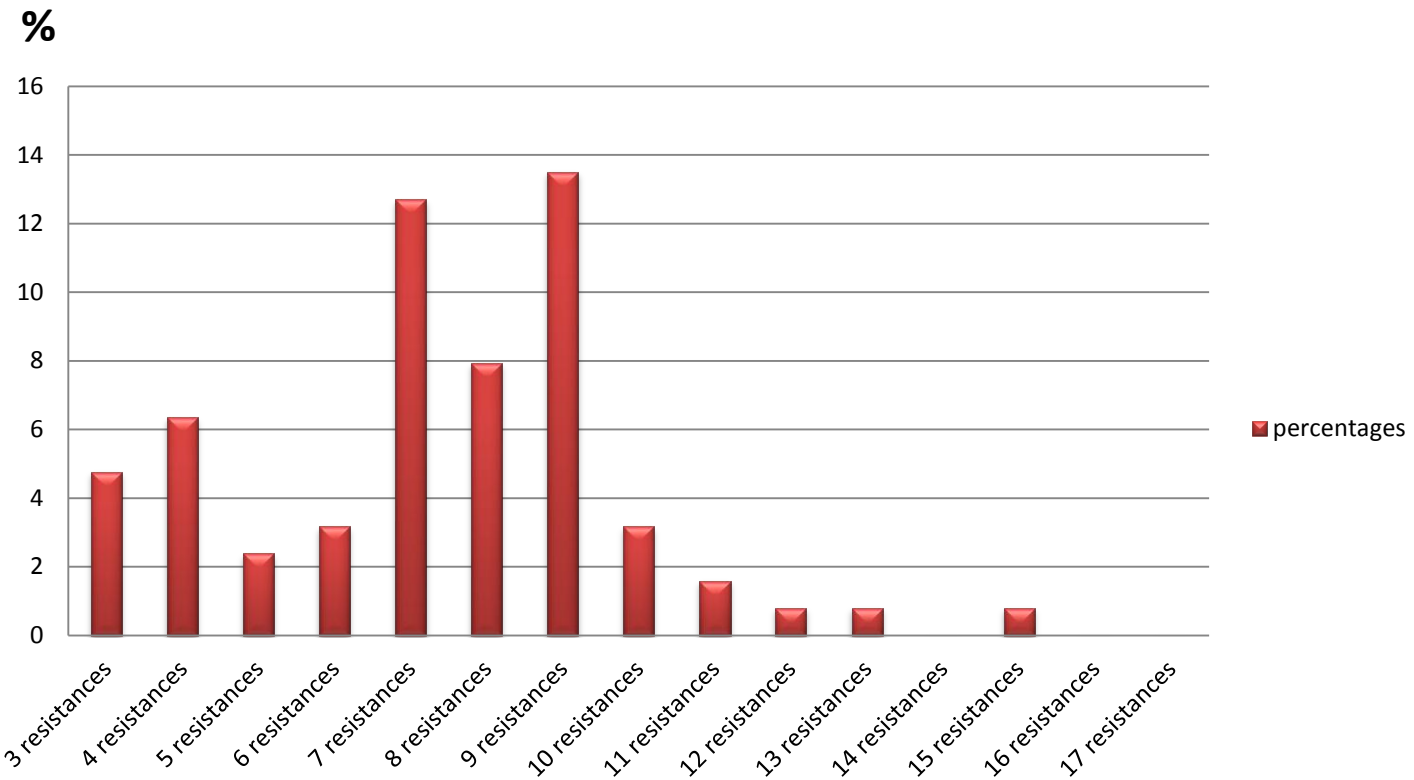

Figure 1: Percentage of MDR *E.coli* resistant to different numbers of antibiotics

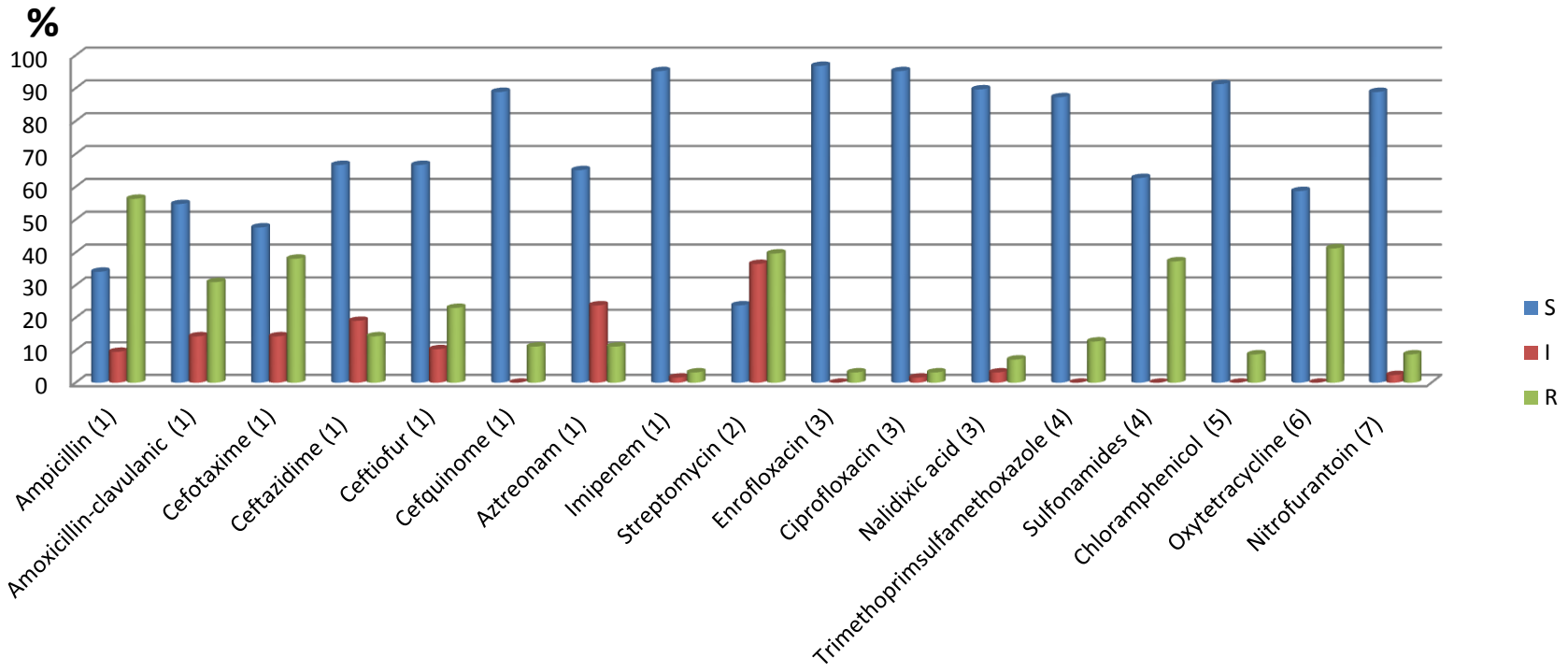

Figure 2. Percentage sensitivity or resistance to 17 antibiotics for 126 *E. coli* farm isolates. (1) Beta-lactams; (2) Aminoglycoside; (3) Quinolones; (4) Sulphonamide/complex; (5) Phenicol; (6) Tetracycline, and (7) Nitrofuran derivative

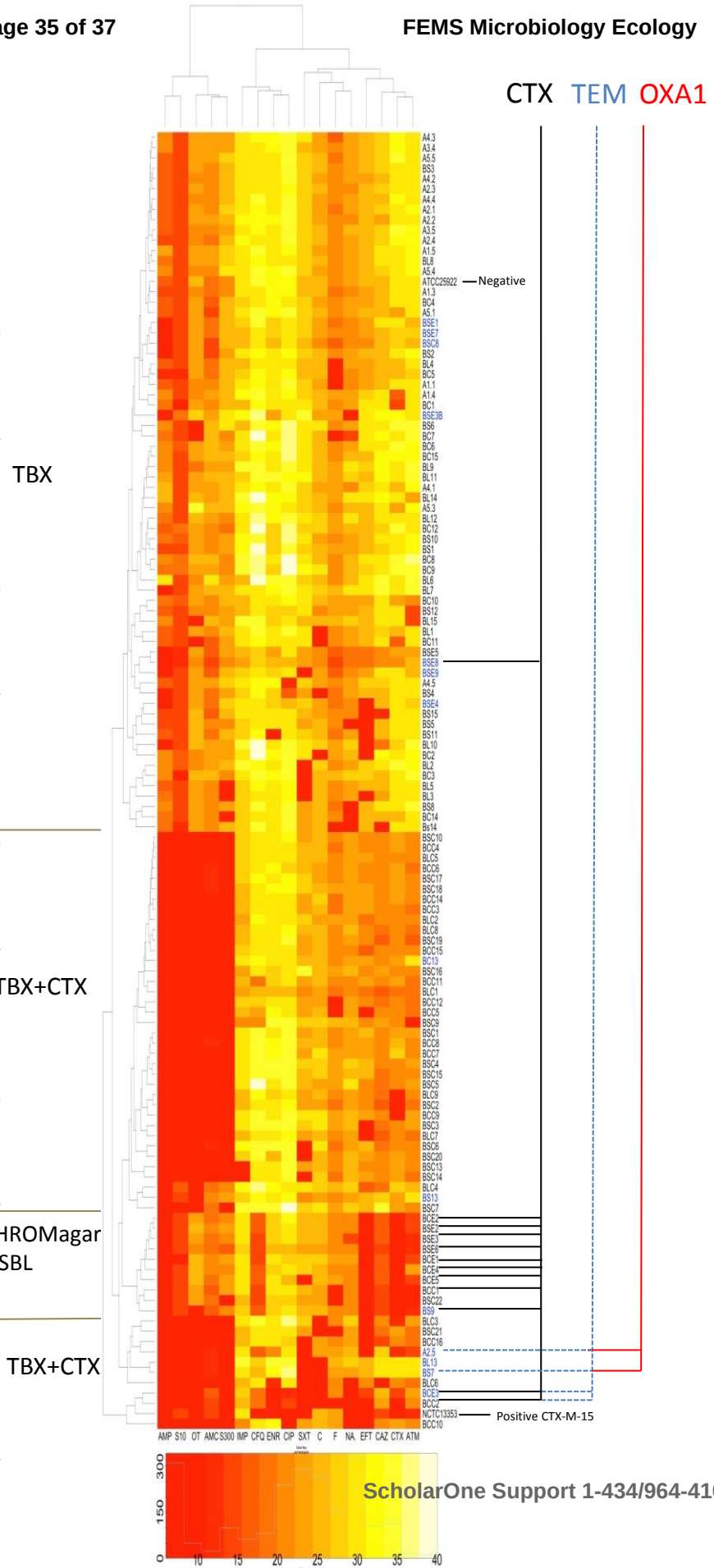

Figure 3
Heatmap

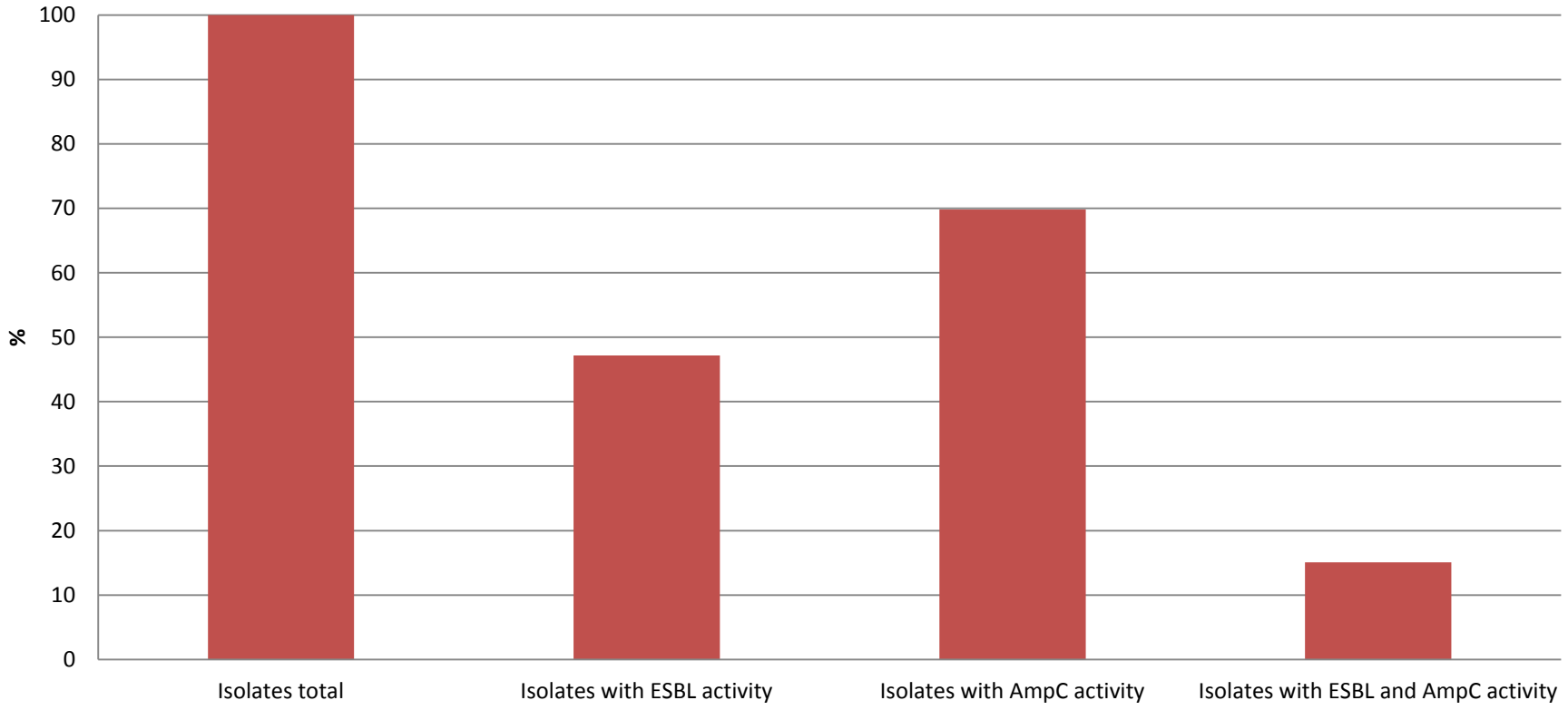

Figure 4. Percentage of ESBL and AmpC phenotypes amongst *E. coli* isolates resistant to cefotaxime and/ or ceftazidime.

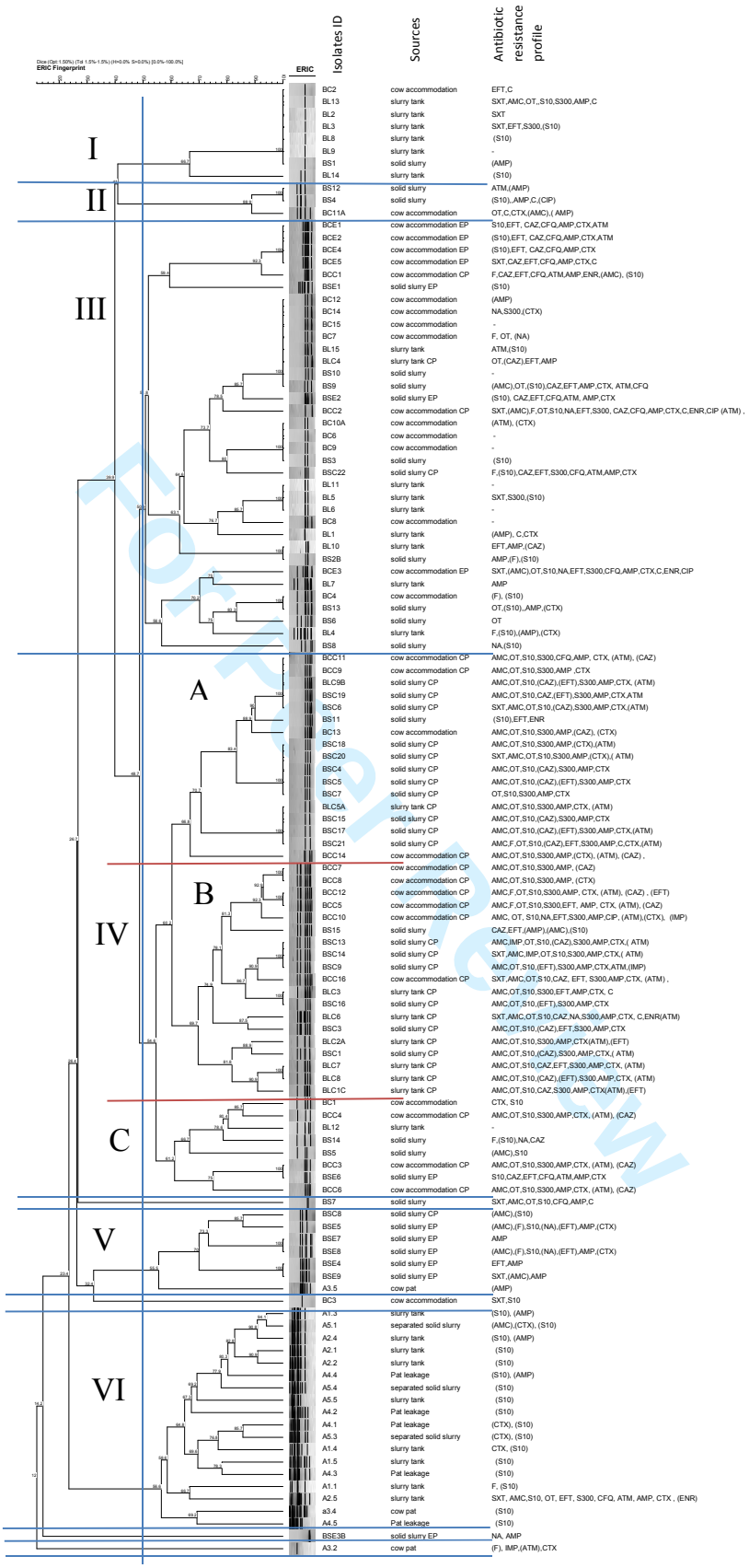

Figure 5. ERIC-PCR dendogram