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Characterization of AmpC-hyperproducing *Escherichia coli* from humans and dairy farms collected in parallel in the same geographical region

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Objectives: To characterize putative AmpC-hyperproducing third-generation cephalosporin-resistant *E. coli* from dairy farms and their phylogenetic relationships; to identify risk factors for their presence; and to assess evidence for their zoonotic transmission into the local human population.

Methods: Proteomics was used to explain differences in antimicrobial susceptibility. WGS allowed phylogenetic analysis. Multilevel, multivariable logistic regression modelling was used to identify risk factors.

Results: Increased use of amoxicillin/clavulanate was associated with an increased risk of finding AmpC hyperproducers on farms. Expansion of cephalosporin resistance in AmpC hyperproducers was seen in farm isolates with *marR* mutations (conferring cefoperazone resistance) or when AmpC was mutated (conferring fourth-generation cephalosporin and cefoperazone resistance). Phylogenetic analysis confirmed the dominance of ST88 amongst farm AmpC hyperproducers but there was no evidence for acquisition of farm isolates by members of the local human population.

Conclusions: Clear evidence was found for recent farm-to-farm transmission of AmpC-hyperproducing *E. coli* and of adaptive mutations to expand resistance. Whilst there was no evidence of isolates entering the local human population, efforts to reduce third-generation cephalosporin resistance on dairy farms must address the high prevalence of AmpC hyperproducers. The finding that amoxicillin/clavulanate use was associated with an increased risk of finding AmpC hyperproducers is important because this is not currently categorized as a highest-priority critically important antimicrobial and so is not currently targeted for specific usage restrictions in the UK.

Introduction

Escherichia coli typically produce a class 1 cephalosporinase, encoded by the *ampC* gene, which is chromosomally located. Expression of *ampC* in WT cells is low and not enough to confer clinically relevant resistance to β-lactam antibiotics.[1] Many mutations, insertions and gene duplication events have been shown to cause *ampC* hyperexpression and this leads to varying spectra of β-lactam resistance, dependent on the actual amount of AmpC produced.[1] AmpC hyperproduction was first seen in *E. coli* from human clinical samples in 1979[2] and for a period before the emergence of plasmid-mediated ESBLs, AmpC hyperproduction was the dominant mechanism of third-generation cephalosporin (3GC) resistance in *E. coli* from humans.[1] This is no longer the case, however. For example, in a recent survey of cefotaxime-resistant (CTX-R) *E. coli* from urine collected from people living in South West England, only 24/626 isolates (3.8%) were presumed to be AmpC hyperproducers because of their lack of horizontally acquired β-lactamase genes; WGS confirmed that 13/13 sequenced isolates had *ampC* promoter mutations typical of AmpC hyperproducers.[3]

AmpC is typical of class 1 β-lactamases in that it does not confer resistance to the fourth-generation cephalosporins (4GCs).[1] However, *ampC* structural variants in *E. coli*, expanding AmpC activity to include cefepime, for example, have been identified from humans[4–7] and cattle.[8] These are dominated by isolates from the relatively less pathogenic phylogroup A and particularly ST88.[6,8] This is probably because extended-spectrum activity evolves from existing AmpC hyperproducers, among which ST88 isolates are particularly common.[9]

We recently conducted a survey of 4594 samples collected from faecally contaminated sites on 53 dairy farms in South West England. We identified 384 samples, collected across 47 farms, that were positive for the detectable growth of CTX-R *E. coli* isolates.[10] We then reported that 566/1226 of these CTX-R *E. coli* isolates (from 186 samples from 38 farms) were PCR negative for mobile cephalosporinases and so were presumed to be chromosomal AmpC hyperproducers.[11] If this presumption was correct, AmpC hyperproduction was the mechanism of resistance in 46.2% of CTX-R *E. coli* from dairy cattle in this region of the UK. This figure is comparable with the 42.9% presumed AmpC hyperproducers seen in CTX-R *E. coli* from dairy cattle in a recent nationwide Dutch study[12] and contrasts with the 3.8% of AmpC hyperproducers seen in CTX-R isolates in our recent study of human urinary *E. coli*.[3]

One aim of the work reported here was to characterize putative AmpC-hyperproducing *E. coli* from our recent survey of dairy farms[10,11] and to identify risk factors for the presence of AmpC hyperproducers on these farms. Another aim was to investigate potential zoonotic transmission of AmpC hyperproducers by using WGS-based phylogenetic analysis to compare isolates from farms with human urinary *E. coli* collected in parallel from the same 50×50 km region.[3]

Materials and methods

Bacterial isolates, identification and susceptibility testing

The 25 test *E. coli* isolates came from dairy farms located within a 50×50 km region of the South West of England, part of the wider area of our earlier study.[10,11] Isolates variously came from faecally contaminated sites around calves, heifers, cows and the near-farm environment. Samples were collected between January 2017 and December 2018. This 50×50 km region was chosen because it also included the locations of 146 GP practices that submitted urine samples for processing at the Severn Pathology laboratory, as described in a recently published survey of human urinary *E. coli*.[3] This was also the source of the human urinary isolates used in the present study. Isolate Farm-WT is an AmpC-hyperproducing *E. coli* from a dairy farm located outside of the region defined for this study. To select a ceftazidime-resistant derivative, 100 μL of overnight culture of Farm-WT grown in Nutrient Broth was spread onto Mueller–Hinton agar containing 8 mg/L ceftazidime and incubated for 24 h. One representative mutant colony was picked and designated Farm-WT-M1. *E. coli* isolate 17 is a fully susceptible human urinary isolate provided by Dr Mandy Wootton, Public Health Wales. Disc susceptibility testing and microtitre MIC assays were performed and interpreted according to CLSI guidelines.[13–15]

Fluorescent Hoescht (H) 33342 dye accumulation assay

Envelope permeability in living bacteria was tested using a standard dye accumulation assay protocol[16] where the dye only fluoresces if it crosses the entire envelope and interacts with DNA. Overnight cultures in CAMHB at 37°C were used to prepare CAMHB subcultures, which were incubated at 37°C until an OD_{600} of 0.6–0.8 was reached. Cells were pelleted by centrifugation (10 min, 4000 **g**, 4°C) and resuspended in 1 mL of PBS. The OD_{600} values of all suspensions were adjusted to 0.1. Aliquots of 180 μL of cell suspension were transferred to a black flat-bottomed 96-well plate (Greiner Bio-One, Stonehouse, UK). Eight technical replicates for each strain tested were in each column of the plate. The plate was transferred to a POLARstar spectrophotometer (BMG Labtech) and incubated at 37°C. Hoescht dye (H33342, 25 μM in water) was added to the bacterial suspensions of the plate using the plate reader's auto-injector to give a final concentration of 2.5 μM per well. Excitation and emission filters were set at 355 nm and 460 nm respectively. Readings were taken in intervals (cycles) separated by 150 s. Thirty-one cycles were run in total. A gain multiplier of 1300 was used. Results were expressed as absolute values of fluorescence versus time.

Proteomics

One millilitre of an overnight CAMHB culture was transferred to 50 mL of CAMHB and cells were grown at 37°C to an OD_{600} of 0.6–0.8. Cells were pelleted by centrifugation (10 min, 4000 **g**, 4°C) and resuspended in 35 mL of 30 mM Tris–HCl, pH 8 and broken by sonication using a cycle of 1 s on, 0.5 s off for 3 min at an amplitude of 63% using a Sonics Vibra-Cell VC 505^{TM} (Sonics and Materials Inc., Newton, CT, USA). The sonicated samples were centrifuged at 7650 **g** for 15 min at 4°C to pellet intact cells and large cell debris. Protein concentrations in all supernatants were quantified using the Bio-Rad Protein Assay Dye Reagent Concentrate according to the manufacturer's instructions. Proteins (1 μg/lane) were separated by SDS-PAGE using 11% acrylamide, 0.5% bis-acrylamide (Bio-Rad) gels and a Bio-Rad Mini-PROTEAN Tetra cell chamber. Gels were resolved at 200 V until the dye front had moved approximately 1 cm into the separating gel. Proteins in all gels were stained with InstantBlue (Expedeon) for 5 min and de-stained in water. LC-MS/MS data was collected as previously described.[17] The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against bacterial genome and horizontally acquired resistance genes as described previously.[18]

WGS and analyses

WGS was performed by MicrobesNG (https://microbesng.uk/) on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA) using 2×250 bp paired-end reads. Reads were trimmed using Trimmomatic[19] and assembled into contigs using SPAdes 3.13.0[20] (http://cab.spbu.ru/software/spades/). Resistance genes, plasmid replicon types and STs (according to the Achtman scheme[21]) were assigned using ResFinder,[22] PlasmidFinder[23] and MLST 2.0 on the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) platform. Contigs were annotated using Prokka 1.2.[24]

Phylogenetic analysis

Sequence alignment and phylogenetic analysis was carried out using the Bioconda software package[25] on the Cloud Infrastructure for Microbial Bioinformatics (CLIMB).[26] All fasta files used are available for download (https://github.com/HannahSchubert1/OH-STAR-modelling-code/tree/nerc_oa/Code%20for%20open%20access/Alzayn%20et%20al%202019) and an NCBI Bioproject has been recorded under accession number PRJNA615796. The reference sequence was *E. coli* strain cq9 complete genome (accession: NZ_CP031546.1). Sequences were first aligned to a closed-read reference sequence and analysed for SNP differences, whilst omitting insertion and deletion elements, using the Snippy alignment program. Alignment was then focused on regions of the genome found across all isolates, using the Snippy-core program, thus eliminating the complicating factors of insertions and deletions.[27] Aligned sequences were then used to construct a maximum-likelihood phylogenetic tree using RAxML, utilizing the GTRCAT model of rate heterogeneity and the software's autoMR and rapid bootstrap to find the best-scoring maximum-likelihood tree and including tree branch lengths, defined as the number of base substitutions per site compared.[28,29] Finally, phylogenetic trees were illustrated using the web-based Microreact program.[30]

Risk factor analysis

Multivariable, multilevel logistic regression analysis was performed to identify risk factors for the presence of AmpC hyperproducers in samples collected from farms.[10] All code is available for download at https://github.

com/HannahSchubert1/OH-STAR-modelling-code/tree/nerc_oa/Code%20for%20open%20access/Alzayn%20et%20al%202019. Positivity for AmpC-hyperproducing *E. coli* in a sample was defined by the growth of *E. coli* on tryptone bile X-glucuronide agar containing 2 mg/L cefotaxime that were PCR negative for known horizontally acquired cefotaxime resistance genes: bla_{CTX-M}, bla_{SHV}, bla_{CMY} and bla_{DHA}.[10,11] The risk factor analysis methodology used has been described previously, including the use of a novel method using a logistic link function to account for measurement error.[10]

Ethics

All farmers gave fully informed consent to participate in the study. Ethics approval was obtained from the University of Bristol's Faculty of Health Sciences Research Ethics Committee (ref 41562).

Results and discussion

Confirmation of AmpC hyperproduction and identification of porin loss and marR mutations in E. coli from dairy farms

Our first aim was to investigate putative AmpC-hyperproducing *E. coli* isolates from dairy farms identified in our recent surveillance study.[10,11] We decided to focus on a 50×50 km subregion of the study area, in which 25 farms were found to be positive for putative AmpC hyperproducers, defined as CTX-R isolates that were PCR negative for known mobile cephalosporinase genes. First, antibiograms were determined for one putative AmpC-hyperproducing isolate from each of four randomly selected farms. All isolates (from Farms 1 to 4) presented a typical AmpC-hyperproducing phenotype: resistance to ampicillin and cefalexin and non-susceptibility to cefotaxime and ceftazidime. The isolate from Farm 1 was clearly different from the others: resistant to ceftazidime, cefotaxime and ceftriaxone and non-susceptible to cefoperazone and cefepime, based on disc testing (Table 1). MIC testing confirmed this difference for ceftazidime and cefepime, extending it into 3GCs/4GCs licensed for use in cattle in the UK (Table 2). Relative to the non-AmpC-hyperproducing control human urinary *E. coli* 17, the four putative AmpC hyperproducers were non-susceptible to ceftazidime and ceftiofur (a 3GC used on several study farms during the period of sample collection) but not generally cefoperazone, cefepime or cefquinome (a 4GC used on some study farms during the period of sample collection). The MICs of the 4GCs cefepime and cefquinome were, respectively, six and seven doublings higher against the isolate from Farm 1 than against the control isolate *E. coli* 17 and five doublings higher for each drug than against the isolate from Farm 2 (Table 2).

Using LC-MS/MS proteomics, AmpC hyperproduction was confirmed in the isolate from Farm 1, relative to the control *E. coli* 17, but AmpC production in this isolate was not more than in the other three confirmed AmpC-hyperproducing farm isolates (Table 3). Sequencing the *ampC* promoter region revealed that all four AmpC hyperproducers had the same mutations, relative to the *E. coli* 17 control (Figure 1), which have previously been shown to cause

Table 1. β-Lactam susceptibility of putative AmpC-hyperproducing *E. coli* isolates from dairy farms

Isolate	Aztreonam	Cefepime	Cefotaxime	Ceftazidime	Ceftriaxone	Cefotetan	Cefoperazone	Cefalexin	Ampicillin
Farm-1	S	**I**	**I**	**R**	**R**	S	**I**	**R**	**R**
Farm-2	S	S	**I**	**R**	**I**	S	S	**R**	**R**
Farm-3	S	S	**I**	**I**	S	S	S	**R**	**R**
Farm-4	S	S	**I**	**R**	S	S	S	**R**	**R**
Farm-5	S	S	**I**	**R**	S	S	S	**R**	**R**
Farm-6	S	S	**I**	S	S	S	S	**R**	**R**
Farm-7	S	S	**I**	**R**	S	S	**I**	**R**	**R**
Farm-8	S	S	**R**	**I**	**I**	S	S	**R**	**R**
Farm-9	S	S	**R**	**R**	S	**R**	**R**	**R**	**R**
Farm-10	S	S	**R**	**R**	**I**	S	**I**	**R**	**R**
Farm-11	S	S	**I**	S	S	S	**I**	**R**	**R**
Farm-12	S	S	**I**	S	S	S	**R**	**R**	**R**
Farm-13	S	S	**R**	**R**	S	S	**I**	**R**	**R**
Farm-14	S	S	**I**	**I**	S	S	S	**R**	**R**
Farm-15	S	I	**R**	**R**	S	S	S	**R**	**R**
Farm-16	S	S	**I**	S	S	S	S	**R**	**R**
Farm-17	S	S	**I**	S	S	S	S	**R**	**R**
Farm-18	S	S	**R**	S	S	S	S	**R**	**R**
Farm-19	S	S	**I**	**R**	S	S	S	**R**	**R**
Farm-20	S	S	**I**	S	S	S	**I**	**R**	**R**
Farm-21	S	**R**	**I**	**R**	S	S	**I**	**R**	**R**
Farm-22	S	**I**	**I**	**R**	**R**	S	**R**	**R**	**R**
Farm-23	S	**I**	**I**	**I**	S	S	S	**R**	**R**
Farm-24	S	S	**I**	**I**	S	S	**I**	**R**	**R**
Farm-25	S	S	**R**	**R**	**I**	S	**I**	**R**	**R**

Bold values represent intermediate (I) or resistant (R) based on CLSI breakpoints, otherwise susceptible (S).

Table 2. MICs of 3GCs/4GCs against putative AmpC-hyperproducing *E. coli* isolates from dairy farms

	MIC (mg/L)				
Isolate	ceftazidime[h]	ceftiofur[c]	cefepime[h]	cefquinome[c]	cefoperazone[h,c]
EC17	0.25	0.5	0.125	0.03	0.25
Farm-1	**256**	16	**8**	4	**64**
Farm-2	**16**	4	0.25	0.125	4
Farm-3	**16**	4	0.125	0.125	4
Farm-4	**32**	4	0.5	0.5	**32**
Farm-WT	**8**	8	1	2	8
Farm-WT-M1	**128**	8	**8**	8	**32**
Farm-22	**128**	4	**8**	4	**32**

Bold values represent resistant according to CLSI breakpoints. EC17, control *E. coli* strain 17.
[h]These cephalosporins are used in humans.
[c]These cephalosporins are licensed for use in cattle in the UK.

Table 3. Abundance of key resistance proteins in putative AmpC-hyperproducing *E. coli* from dairy farms and human urinary tract infections

Accession	Description	EC17	Farm-1	Farm-2	Farm-3	Farm-4	Farm-WT	Farm-WT-M1	UTI-8	UTI-9
P02931	OmpF	0.69±0.36	**0.02±0.03**	0.99±0.36	1.03±0.34	**0.12±0.08**	1.54±1.34	0.81±0.24	0.86±0.18	0.43±0.31
P00811	AmpC	ND	**0.79±0.19**	**0.86±0.20**	**0.89±0.16**	**0.96±0.20**	**1.13±0.77**	**0.76±0.24**	**2.13±0.37**	**1.35±0.34**
P0AE06	AcrA	0.10±0.04	0.13±0.05	0.18±0.15	0.11±0.03	**0.20±0.01**	0.13±0.07	0.12±0.02	0.14±0.02	0.16±0.03
P31224	AcrB	0.07±0.01	0.07±0.06	**0.14±0.03**	0.08±0.08	**0.11±0.02**	0.04±0.01	0.05±0.01	0.07±0.02	0.07±0.02
P02930	TolC	0.12±0.06	0.08±0.07	0.13±0.02	0.12±0.02	**0.39±0.09**	0.19±0.10	0.19±0.05	0.16±0.03	0.10±0.04

Protein abundance is reported relative to the average abundance of ribosomal proteins in a cell extract and is a mean ± SEM, ($n = 3$). Proteins whose abundance is significantly ($P < 0.05$) up or downregulated at least 2-fold relative to the *E. coli* strain 17 (EC17) control (see Materials and methods) are in bold. ND, not detected.

ampC hyperexpression.[1] Proteomics showed that, unlike the other three AmpC hyperproducers, the cefepime-resistant isolate from Farm 1 did not produce the OmpF porin (Table 3) and WGS revealed a loss-of-function mutation in *ompF* caused by the insertion of IS*4* at nt 625. OmpF porin loss did not noticeably affect envelope permeability in the Farm 1 isolate relative to the other three isolates or the *E. coli* 17 control (Figure 2). Indeed, the isolate from Farm 4 had markedly reduced permeability, reminiscent of an efflux hyperproduction phenotype (constant reduced accumulation of the fluorescent dye; Figure 2) and yet it was not resistant to cefepime (Table 2). Proteomics confirmed hyperproduction of AcrAB-TolC in the Farm 4 isolate and down-regulation of the OmpF porin (Table 3). This was reminiscent of a Mar phenotype and suspected loss-of-function mutation in *marR* was confirmed by WGS (causing a Pro57Thr change in MarR). As expected of a Mar isolate, the Farm 4 isolate was non-susceptible to minocycline and chloramphenicol, which are known AcrAB-TolC substrates, but according to WGS the isolate did not carry any relevant mobile resistance genes. Interestingly, the Farm 4 isolate was cefoperazone resistant (Table 2). It would seem, therefore, that a combination of AmpC plus AcrAB-TolC hyperproduction and/or OmpF down-regulation leads to cefoperazone resistance in *E. coli*. Cefoperazone has been used, albeit rarely, as a therapy for mastitis in dairy cows in the UK.

First identification of extended-spectrum AmpC variants in E. coli from UK dairy farms and phylogenetic analysis of AmpC hyperproducers showing recent transmission between farms

Having ruled out additional AmpC hyperproduction as the cause of 4GC and cefoperazone resistance in the isolate from Farm 1, we next looked at the *ampC* gene sequence. There were several nucleotide sequence polymorphisms from one *ampC* gene to the next amongst our four representative isolates, but only one in the Farm 1 isolate stands out, causing a His312Pro change (His296Pro when considering the mature AmpC protein following removal of the signal peptide), a mutation previously shown to enhance the spectrum of AmpC hydrolytic activity.[31]

Based on WGS of AmpC-hyperproducing isolates from other dairy farms in the South West of England in our collection, another isolate was identified that had an identical *ampC* ORF and promoter sequence to that carried by the isolate from Farm 1, but without the single mutation predicted to cause extended-spectrum AmpC activity. For reference, we named this isolate Farm-WT and selected a mutant (Farm-WT-M1) using ceftazidime at its CLSI agar dilution breakpoint MIC (8 mg/L) using Mueller–Hinton Agar. The mutant did not have altered production of key resistance proteins relative to its parent, Farm-WT (Table 3). Sequencing of the *ampC* gene from Farm-WT-M1 revealed an

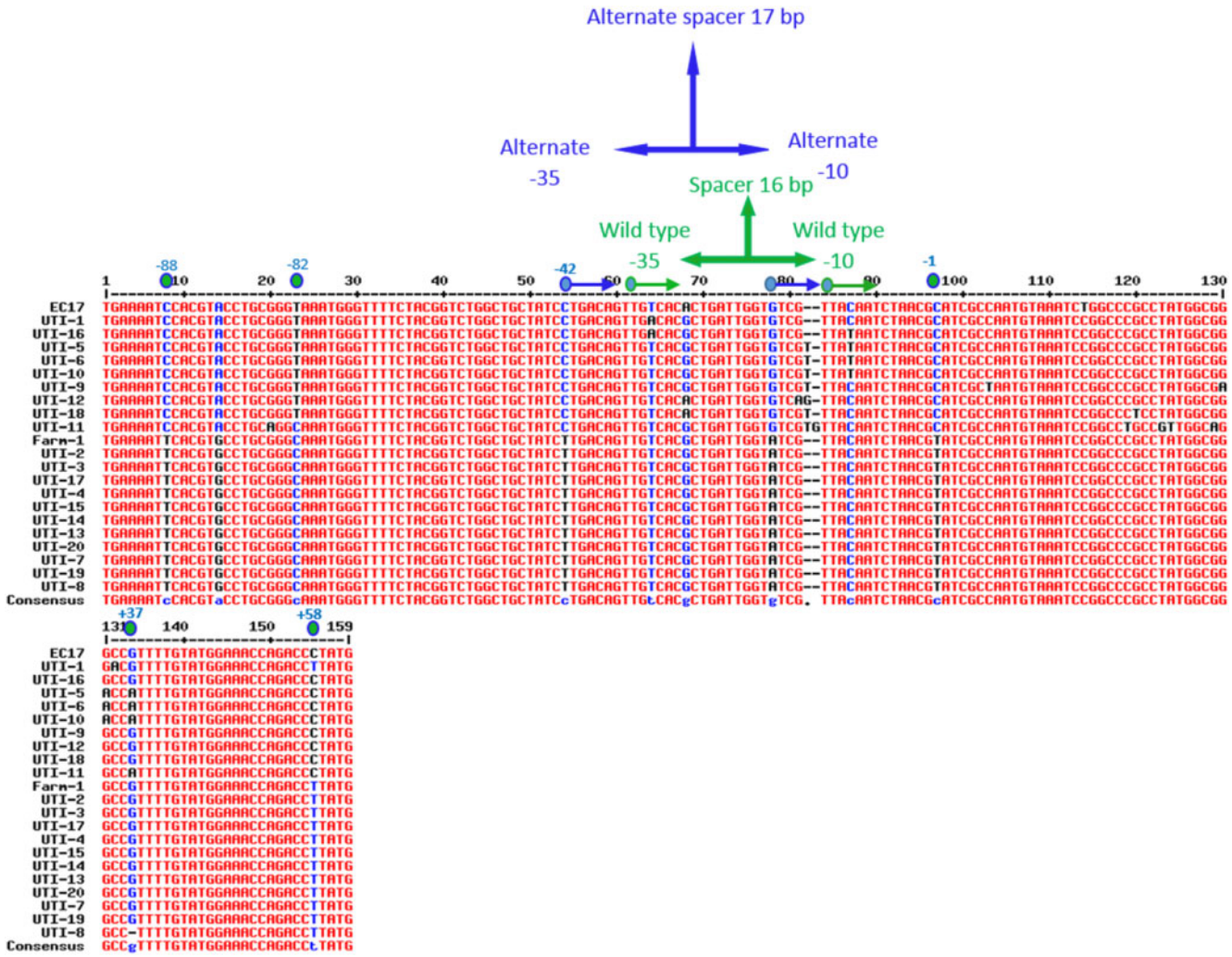

Figure 1. Promoter/attenuator sequences for *ampC* from *E. coli* AmpC-hyperproducing isolates in comparison with a WT *E. coli*. Modified residues, relative to the control *E. coli* strain (EC17), seen in AmpC-hyperproducing *E. coli* from farms (Farm-1 to Farm-25) and human urinary *E. coli* (UTI-1 to UTI-20) are noted, with their positions relative to the transcriptional start site. Novel promoter(s) created are annotated. All 25 farm isolates had an identical sequence in this region, represented by the isolate from Farm 1.

identical His296Pro mutation to that seen in the isolate from Farm 1 and the mutant had the same extended-spectrum antibiogram as the isolate from Farm 1 (Table 2). Since Farm-WT-M1, like its parent, had WT *ompF* sequence, according to WGS, and expression, according to proteomics (Table 3), this confirmed that the insertional inactivation of *ompF* seen in the isolate from Farm 1 had little impact on the MICs of extended-spectrum cephalosporins in the presence of an extended-spectrum AmpC variant (Table 2).

We next selected one putative AmpC-hyperproducing isolate from each of the remaining 21 dairy farms in the 50×50 km region of our wider study.[10,11] This area also included the locations of 146 GP practices involved in a parallel survey of human urinary *E. coli*.[3] The additional 21 putative AmpC-hyperproducing farm isolates expressed typical AmpC-hyperproducing phenotypes (Table 1) and all had the same *ampC* promoter mutation reported above (Figure 1). In addition to the isolate from Farm 1, four others were found to be non-susceptible to cefepime. The isolate from Farm 22 is discussed below; the other three isolates were found by WGS to also carry a bla_{OXA-1} gene. They were the only isolates in this study that carried this gene. The contribution of OXA-1 to cefepime non-susceptibility in *E. coli* has been reported previously.[32] Table 4 shows the spread of *E. coli* STs amongst the 25 study isolates. Similar to a reported cattle study in France,[8] ST88 was dominant (10/25 isolates). Based on analysis of *ampC* sequence, only one other isolate (from Farm 22) was found to carry a known extended-spectrum AmpC variant, in this case with the same His296Pro mutation as seen in the isolate from Farm 1. This isolate had the same extended-spectrum antibiogram as that from Farm 1 (Table 2). These two isolates, from farms 40 km apart, were both ST641 and only 64 SNPs apart in the core genome, based on phylogenetic analysis (Figure 3). This can be compared with SNP distances of 1–13 SNPs across six sequenced isolates collected from Farm 1 over a 12 month period. Interestingly, the *ompF* porin gene was intact in the isolate from Farm 22 so *ompF* disruption must have occurred following

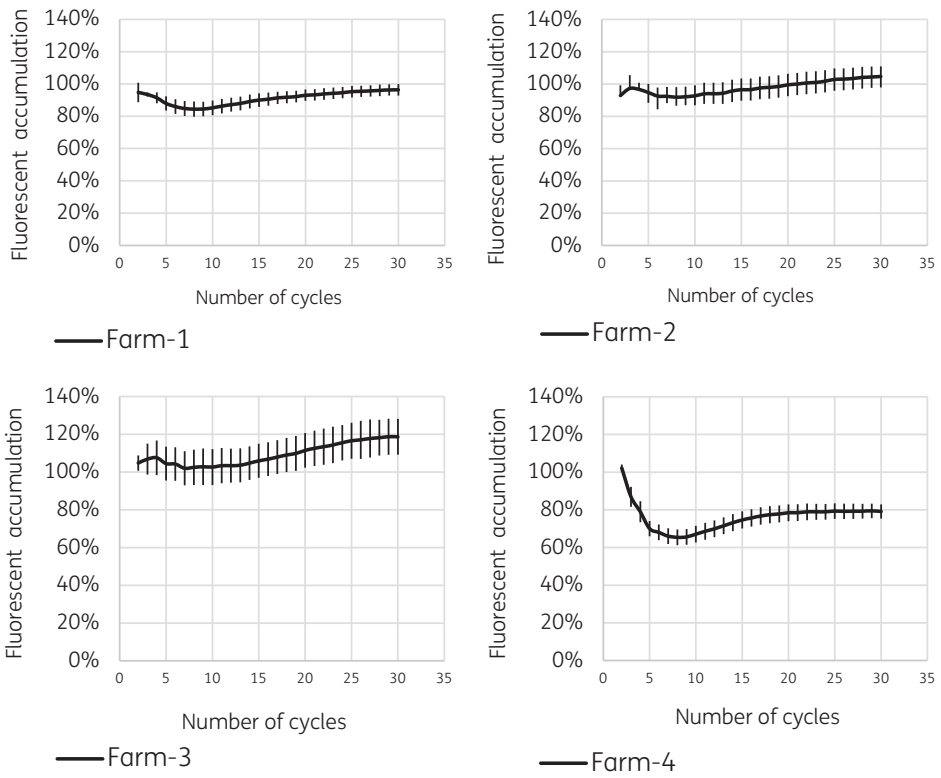

Figure 2. Envelope permeability of AmpC-hyperproducing *E. coli* determined using fluorescent dye accumulation assays. In each case, fluorescence of an AmpC-hyperproducing isolates (Farm-1, -2 etc.) incubated with the dye is presented relative to that in the control *E. coli* strain (EC17) after each cycle. Each line shows mean data for three biological replicates with eight technical replicates in each. Error bars define the SEM.

separation of the isolates. Measurement of MICs for the isolates provided further evidence that loss of *ompF* was not important for 3GC/4GC resistance conferred by the extended-spectrum AmpC in the isolate from Farm 1 (Table 2). Interestingly, another ST641 isolate, from Farm 7 (which is 7 km from Farm 1), had 1520 SNPs different from the isolate from Farm 1 (Figure 3) and did not have the extended-spectrum AmpC mutation or an *ompF* mutation; this isolate shared these properties with the isolate from Farm 14, which was only 35 SNPs (Figure 3) but 45 km away from Farm 7.

Risk factor analysis

The data presented above, when considered in conjunction with that in our recent PCR survey,[11] show that 46.2% of CTX-R *E. coli* from dairy cattle across the 53 farms enrolled in our study were AmpC hyperproducers. This compares with 52.9% that were CTX-M producers, the remainder being plasmid AmpC producers.[11] Accordingly, attempts to reduce the prevalence of 3GC resistance on dairy farms must address the specific factors that are driving the accumulation of AmpC hyperproducers. In order to identify factors associated with an increased risk of finding CTX-R and AmpC-hyperproducing *E. coli* in a sample from farms in our study, we performed risk factor analyses. Three farm-level fixed effects and two sample-level fixed effects were identified as important (Table 5). As seen with our risk factor analysis for $bla_{CTX\text{-}M}$-positive CTX-R *E. coli* on the same farms,[10] samples collected from the environment of young calves were much more likely to be positive for AmpC-hyperproducing *E. coli* ($P < 0.001$) and samples collected from pastureland, including publicly accessible sites, were much less likely to be positive ($P = 0.005$). We found no association between cephalosporin use (including 3GC use) and increased risk of finding AmpC hyperproducers. Interestingly, however, the total usage of amoxicillin/clavulanate was associated with a higher risk of finding AmpC-hyperproducing *E. coli* on a farm ($P = 0.009$). This association can be explained by direct selection since AmpC hyperproduction confers amoxicillin/clavulanate resistance in *E. coli*.[1] This finding is important because amoxicillin/clavulanate is not currently identified as a highest-priority critically important antimicrobial (HP-CIA) by the WHO[33] and whilst great strides have been made within the UK farming industry to reduce antibiotic use,[34] there is a particular focus on reducing HP-CIA, e.g. 3GC use. The associations identified in our risk factor analysis suggest that reducing HP-CIAs without also reducing amoxicillin/clavulanate use may not impact on the prevalence of CTX-R, AmpC-hyperproducing *E. coli* on farms. Indeed, a bigger concern is that reducing 3GC use on farms may drive up amoxicillin/clavulanate use, providing additional co-selective pressure for 3GC-resistant *E. coli*.

A final observation from this analysis is that average monthly temperature, which was identified as a strong risk factor for finding $bla_{CTX\text{-}M}$-positive *E. coli* in this same survey of dairy farms,[10] was not identified as a risk factor for finding AmpC-hyperproducing *E. coli*. This may be an issue of power, but the numbers of $bla_{CTX\text{-}M}$-

Table 4. STs of AmpC-hyperproducing isolates representing 25 dairy farms and 20 human urine samples

Isolate	ST	Phylogroup
Farm-1	641	B1
Farm-2	88	C
Farm-3	88	C
Farm-4	388	B1
Farm-5	88	C
Farm-6	75	B1
Farm-7	641	B1
Farm-8	23	C
Farm-9	162	B1
Farm-10	88	C
Farm-11	2522	B1
Farm-12	88	C
Farm-13	278	B1
Farm-14	641	B1
Farm-15	88	C
Farm-16	278	B1
Farm-17	661	B1
Farm-18	88	C
Farm-19	88	C
Farm-20	278	B1
Farm-21	345	B1
Farm-22	641	B1
Farm-23	88	C
Farm-24	278	B1
Farm-25	88	C
UTI-1	141	B2
UTI-2	75	B1
UTI-3	200	B1
UTI-4	155	B1
UTI-5	73	B2
UTI-6	73	B2
UTI-7	200	B1
UTI-8	54	B1
UTI-9	73	B2
UTI-10	73	B2
UTI-11	405	D
UTI-12	131	B2
UTI-13	1499	C
UTI-14	200	B1
UTI-15	75	B1
UTI-16	73	B2
UTI-17	200	B1
UTI-18	428	B2
UTI-19	88	C
UTI-20	448	B1

positive *E. coli* and AmpC-hyperproducing *E. coli* samples in the survey were similar (224 versus 186). It may be hypothesized, therefore, that carriage of (i.e. because of some fitness cost) or transmission rate for the horizontally acquired $bla_{CTX\text{-}M}$ is specifically affected by temperature, whereas the presence of chromosomal mutations in the *ampC* promoter leading to AmpC hyperproduction is not.

No evidence for recent human/farm transmission of AmpC-hyperproducing E. coli isolates collected in parallel in a 50×50 km region

We next looked at WGS data for 20 human urinary *E. coli* presumed to hyperproduce AmpC, collected during the same time frame from people living in the same geographical range as the 25 farms for which WGS data of AmpC-hyperproducing *E. coli* had been obtained.[3] STs for these isolates are reported in Table 4. Proteomics confirmed AmpC hyperproduction in two representative isolates: UTI-8 and UTI-9 (Table 3). There were nine different *ampC* promoter types seen across the 20 AmpC-hyperproducing human isolates, though 11/20 isolates carried the same promoter mutation seen in all 25 farm isolates (Figure 1). None of the human isolates had mutations suggestive of an extended-spectrum AmpC variant, which was confirmed phenotypically using cefepime disc susceptibility testing.

Our final aim was to identify whether there was any evidence of sharing AmpC-hyperproducing *E. coli* between humans and cattle, since dominance of ST88 has previously been reported in humans in Northern Europe[9] and since we found an over-representation of ST88 on our farms (Table 4). A phylogenetic tree drawn based on core genome comparison showed that the cattle and human isolates were intermixed only to a small extent, with only one human ST88 isolate found (Figure 3). Importantly, all 10 ST88 cattle isolates were 15 or fewer SNPs apart, suggesting very recent farm-to-farm transmission; the human ST88 isolate (UTI-19) was, at its closest distance, 1279 SNPs different from the cattle isolates. The two other examples where isolates from the same ST were found in farm and human samples painted the same picture (Figure 3): for ST75, the two human isolates (UTI-2 and UTI-15) were 60 SNPs apart, but the cattle isolate (Farm-6) was 1972 SNPs different at best. For ST23, the human and cattle isolates (UTI-13 and Farm-8, respectively) were 2754 SNPs different. Otherwise, there was no ST sharing and all cattle isolates fell into phylogroups B1 and C, with 8/20 human isolates falling into the highly pathogenic phylogroup B2, including a cluster of ST73 isolates (Table 4), of which three were only two SNPs apart (Figure 3).

Conclusions

AmpC hyperproduction is a remarkably common mechanism of 3GC resistance in *E. coli* from dairy farms in our study—a finding similar to that of a national survey in The Netherlands.[12] We have shown an association between amoxicillin/clavulanate use and the risk of finding AmpC hyperproducers on dairy farms and would caution against a blanket switch from 3GCs/4GCs to amoxicillin/clavulanate in response to justifiable action to reduce HP-CIA use. However, our comparison between AmpC-hyperproducing farm and human urinary *E. coli* in the same region provided no evidence of local sharing of AmpC hyperproducers between farms and the local human population. Accordingly, whilst reducing the on-farm prevalence of AmpC-hyperproducing *E. coli* should be an important aim, the primary reason for achieving this would be to reduce the likelihood of difficult-to-treat infections in cattle rather than because of any direct zoonotic threat.

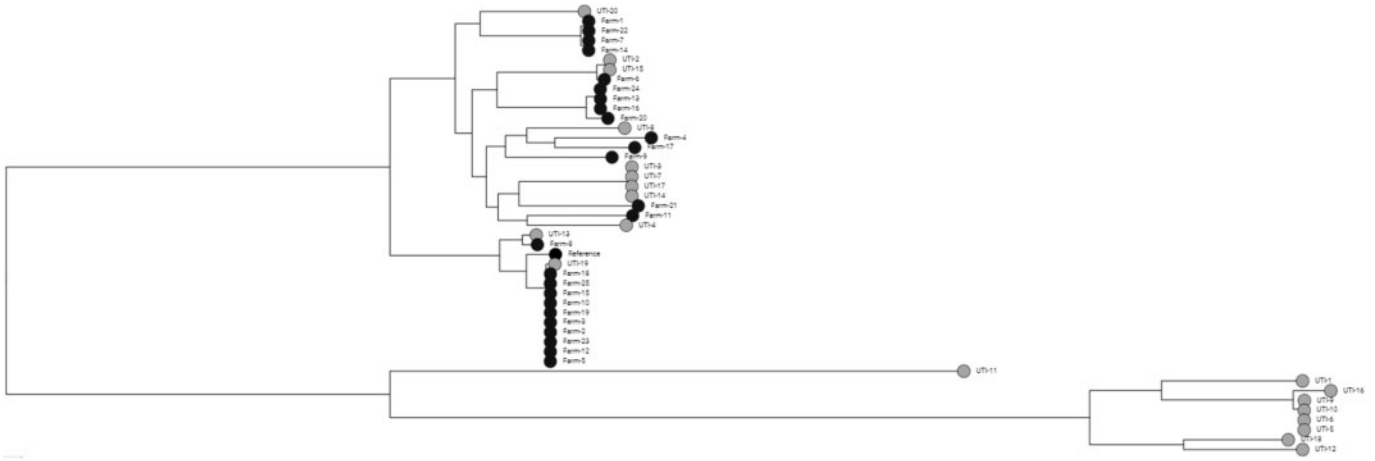

Figure 3. Phylogenetic tree of farm and human urinary AmpC-hyperproducing *E. coli*. The phylogenetic tree was illustrated using the Microreact program using a maximum-likelihood tree generated from core genome alignments as described in Materials and methods. Isolates are coloured light grey (human urinary) and black (farm). The ST88 finished reference genome (Accession: NZ_CP031546.1) used to generate the alignments is noted.

Table 5. Significant associations ($P < 0.05$) with AmpC-hyperproducing *E. coli* from dairy farms from the multilevel, multivariable logistic regression model

Risk factor	OR (95% CI)	*P* value
Sample taken from the environment of pre-weaned heifers	3.92 (2.72–5.67)	<0.001
Total usage of amoxicillin/clavulanate on the farm	1.41 (1.08–1.84)	0.009
Routine use of vaccination against respiratory disease in calves	2.58 (1.22–5.47)	0.012
Samples taken from pastureland	0.33 (0.15–0.73)	0.005
Calving all year round as opposed to in seasonal blocks	4.2 (1.49–11.8)	0.005

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Transparency declarations

The authors have none to declare. Farming and veterinary businesses who contributed data and permitted access for sample collection were not involved in the design of this study or in data analysis and were not involved in drafting the manuscript for publication.

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