Characterisation of AmpC Hyper-Producing *Escherichia coli* from Humans and Dairy Farms Collected in Parallel in the Same Geographical Region

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Abstract

Objectives. To characterise putative AmpC hyper-producing 3rd generation cephalosporin-resistant *E. coli* from dairy farms and their phylogenetic relationships as well as to identify risk factors for their presence; to assess evidence for their zoonotic transmission into the local human population

Methods. Proteomics was used to explain differences in antimicrobial susceptibility. Whole genome sequencing 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 hyper-producers on farms. Expansion of cephalosporin resistance in AmpC hyper-producers was seen in farm isolates with *marR* mutations (conferring cefoperazone resistance) or when AmpC was mutated (conferring 4th generation cephalosporin and cefoperazone resistance). Phylogenetic analysis confirmed the dominance of ST88 amongst farm AmpC hyper-producers but there was no evidence for acquisition of farm isolates by members of the local human population.

Conclusions. In this two-year surveillance study of 53 dairy farms, AmpC hyper-production was the cause of cefotaxime resistance in 46.2% of *E. coli*. There was evidence of recent farm-to-farm transmission and of adaptive mutations to expand resistance. Whilst there was no evidence of isolates entering the local human population, efforts to reduce 3rd generation cephalosporin resistance on dairy farms must address the high prevalence of AmpC hyper-producers. The finding that amoxicillin-clavulanate use was associated with increased risk of finding AmpC hyper-producers is important because this is not currently categorised 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 wild-type cells is low and not enough to confer clinically relevant resistance to β-lactam antibiotics. Many mutations, insertions and gene duplication events have been shown to cause *ampC* hyper-expression, and this leads to varying spectra of β-lactam resistance, dependent on the actual amount of AmpC produced.[1] AmpC hyper-production was first seen in *E. coli* from human clinical samples in 1979,[2] and for a period before the emergence of plasmid-mediated extended spectrum β-lactamases, AmpC hyper-production was a dominant mechanism of 3rd 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 hyper-producers because of their lack of horizontally acquired β- lactamase genes; whole genome sequencing (WGS) confirmed that 13/13 sequenced isolates had *ampC* promoter mutations typical of AmpC hyper-producers.[3]

AmpC is typical of class 1 enzymes in that it does not confer resistance to the 4th generation cephalosporins (4GC).[1] However, *ampC* structural variants of *E. coli*, expanding AmpC activity to include, for example, cefepime, 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] probably because expanded-spectrum activity evolves from existing AmpC hyper-producers, of 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] In an recent paper, we 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 hyper-producers.[11] If this presumption was correct, AmpC hyper-

71 production was the mechanism of resistance in 46.2% of CTX-R *E. coli* from dairy cattle in this
72 region of the UK. This figure is comparable with the 42.9% presumed AmpC hyper-producers
73 seen in CTX-R *E. coli* from dairy cattle in a recent nationwide Dutch study [12] and contrasts
74 with the 3.8% of AmpC hyper-producers seen in CTX-R isolates in our recent study of human
75 urinary *E. coli*.[3]

76 One aim of the work reported here was to characterise putative AmpC hyper-producing *E. coli*
77 from our recent survey of dairy farms [10, 11] and to identify risk factors for the presence of AmpC
78 hyper-producers on these farms. Another aim was to investigate potential zoonotic
79 transmission of AmpC hyper-producers by using WGS-based phylogenetic analysis to
80 compare isolates from farms located within an approximately 50 x 50 km sub-region of the
81 study with human urinary *E. coli* collected in parallel from this same sub-region .[3]

82

83 **Materials and Methods**

84 Bacterial isolates, identification and susceptibility testing

85 Isolates used in this study came from dairy farms located within a sub-region of the wider
86 study area.[10,11] This region was chosen because it also included the locations of 146 GP
87 practices that submitted urine samples for processing at the Severn Pathology laboratory, as
88 described in a recently published survey of human urinary *E. coli;*[3] this laboratory was also the
89 source of the human urinary isolates used in the present study. Disc susceptibility testing and
90 microtiter MIC assays were performed and interpreted according to CLSI guidelines.[13-15]

91 Fluorescent Hoescht (H) 33342 dye accumulation assay

92 Envelope permeability in living bacteria was tested using a standard dye accumulation assay
93 protocol [16] where the dye only fluoresces if it crosses the entire envelope and interacts with
94 DNA. Overnight cultures in Cation Adjusted Muller Hinton Broth (CA-MHB) at 37°C were used
95 to prepare CA-MHB subcultures, which were incubated at 37°C until a 0.6-0.8 OD_{600} was

reached. Cells were pelleted by centrifugation (4000 rpm, 10 min; ALC, PK121R) and resuspended in 1 mL of phosphate-buffered saline. The optical densities of all suspensions were adjusted to 0.1 OD_{600}. 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 bacterial suspension 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 seconds (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

1 mL of an overnight CA-MHB culture were transferred to 50 mL CA-MHB and cells were grown at 37°C to 0.6-0.8 OD_{600}. Cells were pelleted by centrifugation (10 min, 4,000×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 amplitude of 63% using a Sonics Vibracell VC-505TM (Sonics and Materials Inc., Newton, Connecticut, USA). The sonicated samples were centrifuged at 8,000 rpm (Sorval RC5B PLUS using an SS-34 rotor) for 15 min at 4°C to pellet intact cells and large cell debris. Protein concentrations in all supernatants were quantified using Biorad Protein Assay Dye Reagent Concentrate according to manufacturer's instructions. Proteins (1 µg/lane) were separated by SDS-PAGE using 11% acrylamide, 0.5% bis-acrylamide (Biorad) gels and a Biorad Min-Protein Tetracell chamber model 3000X1. 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 Instant Blue (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 using as described previously.[18]

Whole genome sequencing and analyses

WGS was performed by MicrobesNG (https://microbesng.uk/) on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA) using 2x250 bp paired end reads. Reads were trimmed using Trimmomatic[19] and assembled into contigs using SPAdes 3.13.0[20] (https://http://cab.spbu.ru/software/spades/). Resistance genes, plasmid replicon types and sequence types (according to the Achtman scheme[21]) were assigned using the 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 on the Bioconda software package[25] on the Cloud Infrastructure for Microbial Bioinformatics (CLIMB).[26] 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, utilising 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 hyper-producers in samples collected from farms. Positivity for AmpC hyper-producing *E. coli* in a sample was defined by the growth of *E. coli* on cefotaxime agar which were PCR-negative for known horizontally-acquired cefotaxime resistance genes.[11] The

148 risk factor analysis methodology used has been described previously, including the use of a
149 novel method using a logistic link function to account for measurement error.[10]

150

151 **Results and Discussion**

152 Confirmation of AmpC hyper-production and identification of porin loss and *marR* mutations
153 in *E. coli* from dairy farms

154 In order to further investigate putative AmpC hyper-producing *E. coli* isolates from dairy farms
155 identified in our recent surveillance study,[10,11] antibiograms were determined for one putative
156 AmpC hyper-producing isolate from each of 5 randomly selected farms. All isolates presented
157 a typical AmpC-hyper-producing phenotype: resistance to ampicillin and cefalexin, and non-
158 susceptibility to cefotaxime and ceftazidime. The isolate from Farm 1 was clearly different from
159 the others - resistant to ceftazidime, cefotaxime, ceftriaxone, and non-susceptible to
160 cefoperazone and cefepime based on disc testing (**Table 1**). MIC testing confirmed this
161 difference for ceftazidime and cefepime, extending it into 3GC/4GCs licenced for use in cattle
162 in the UK (**Table 2**). Relative to a non-AmpC hyper-producing control *E. coli* 17, all putative
163 AmpC hyper-producers were non-susceptible to ceftazidime and ceftiofur (a 3GC used on
164 several study farms during the period of sample collection) but not generally cefoperazone,
165 cefepime or cefquinome (a 4GC used on some study farms during the period of sample
166 collection). The MICs of the 4GCs cefepime and cefquinome were, respectively, 6 and 7
167 doublings higher against the isolate from Farm 1 than against the control isolate, and 5
168 doublings higher for each drug than against the isolate from Farm 2 (**Table 2**).

169 Using LC-MS/MS proteomics, AmpC hyper-production was confirmed in the isolate from Farm
170 1, relative to a control *E. coli* 17, but AmpC production in this isolate was not more than in the
171 other 4 confirmed AmpC hyper-producing isolates (**Table 3**). Sequencing the *ampC* promoter
172 region revealed that all 5 AmpC hyper-producers had the same mutations, relative to the *E.*
173 *coli* 17 control (**Figure 1**), which have previously been shown to cause *ampC* hyper-

174 expression.[1] Proteomics showed that, unlike the other 4 AmpC hyper-producers, the
175 cefepime-resistant isolate from Farm 1 did not produce the OmpF porin (**Table 3**), and WGS
176 revealed a loss of function mutation in *ompF* caused by the insertion of IS*4* at nucleotide 625.
177 OmpF porin loss did not noticeably affect envelope permeability in the Farm 1 isolate relative
178 to the other 4 isolates or the *E. coli* 17 control (**Figure 2**). Indeed, the isolate from Farm 4 had
179 markedly reduced permeability, reminiscent of an efflux hyper-production phenotype (constant
180 reduced accumulation of the fluorescent dye; **Figure 2**) and yet it was not resistant to cefepime
181 (**Table 2**). Proteomics confirmed hyper-production of AcrAB-TolC in the Farm 4 isolate and
182 down regulation of OmpF porin (**Table 3**). This was reminiscent of a Mar phenotype and
183 suspected loss of function mutation in *marR* was confirmed by WGS (causing a Pro57Thr
184 change in MarR). As expected of a Mar isolate, the Farm 4 isolate was non-susceptible to
185 minocycline and chloramphenicol, which are known AcrAB-TolC substrates. Other isolates
186 resistant to these agents, were shown by WGS to carry specific mobile resistant genes (**Table**
187 **1**). Interestingly, the Farm 4 isolate was cefoperazone-resistant (**Table 2**). It would seem,
188 therefore, that a combination of AmpC plus AcrAB-TolC hyper-production and/or OmpF down
189 regulation leads to cefoperazone resistance in *E. coli*. Cefoperazone has been, albeit rarely,
190 used as a therapy for mastitis in dairy cows in the UK.

191 First identification of expanded-spectrum AmpC variants in *E. coli* from UK dairy farms and
192 phylogenetic analysis of AmpC hyper-producers showing recent transmission between farms

193 Having ruled out additional AmpC hyper-production as the cause of 4GC and cefoperazone
194 resistance in the isolate from Farm 1, we next looked at the *ampC* gene sequence. There were
195 several sequence nucleotide polymorphisms from one *ampC* gene to the next amongst our 5
196 representative isolates, but only one in the Farm 1 isolate stands out: causing a His312Pro
197 change (His296Pro when considering the mature AmpC protein following removal of the signal
198 peptide), a mutation previously shown to enhance the spectrum of AmpC hydrolytic activity.[31]

199 WGS showed that isolate Farm-WT had an identical *ampC* open reading frame and promoter
200 sequence to that carried by the isolate from Farm 1, but without the single mutation predicted

to cause expanded-spectrum AmpC activity. We therefore selected a mutant (Farm-WT-M1) using ceftazidime at its agar dilution MIC (8 mg/L) using Muller 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 identical His296Pro mutation to that seen in the isolate from Farm 1, and the mutant had the same expanded-spectrum antibiogram as the isolate from Farm 1 (**Table 2**). Since Farm-WT-M1, like its parent, has wild type *ompF*, this confirmed that the insertional inactivation of *ompF* seen in the isolate from Farm 1 had little impact on the MICs of expanded-spectrum cephalosporins in the presence of an expanded-spectrum AmpC variant (**Table 2**).

We next performed WGS analysis of putative AmpC hyper-producers identified in our molecular epidemiology survey [11] from 25 farms across the core portion of our study area;[10] this area also included the locations of 146 GP practices involved in a parallel survey of human urinary *E. coli*.[3] All 25 representative isolates had the same *ampC* promoter mutation reported above (**Figure 1**). **Table 4** shows the spread of *E. coli* STs. 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 expanded-spectrum AmpC variant, in this case with the same His296Pro mutation as seen in the isolate from Farm 1. This isolate had the same expanded spectrum antibiogram as that from Farm 1 (**Table 2**). These 2 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 6 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 separation of the isolates. Measurement of MICs against the isolates provided further evidence that loss of *ompF* was not important for 3GC/4GC resistance conferred by the expanded-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 expanded-

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 survey,[11] show that 46.2% of CTX-R *E. coli* from dairy cattle across the 53 farms enrolled in our study were AmpC hyper-producers. 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 hyper-producers. In order to identify factors associated with an increased risk of finding CTX-R, AmpC hyper-producing *E. coli* in a sample from farms in our study, we performed risk factor analyses. Three farm-level fixed effects and 2 sample-level fixed effects were identified as important (**Table 5**). As seen with our risk factor analysis for *bla*$_{\text{CTX-M}}$-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 hyper-producing *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 hyper-producers. Interestingly, however, the total usage of amoxicillin-clavulanate was associated with a higher risk of finding AmpC hyper-producing *E. coli* on a farm ($p=0.001$). This association can be explained by direct selection since AmpC hyper-production 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 World Health Organisation,[32] and, whilst great strides have been made within the UK farming industry to reduce antibiotic use,[33] 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 hyper-producing *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*$_{CTX-M}$-positive *E. coli* in this same survey of dairy farms,[10] was not identified as a risk factor for finding AmpC hyper-producing *E. coli*. This may be an issue of power, but the numbers of *bla*$_{CTX-M}$ *E. coli* positive and AmpC hyper-producing *E. coli* positive samples in the survey were similar (224 vs 186). It may be hypothesised, therefore, that carriage of (i.e. because of some fitness cost) or transmission rate for the horizontally acquired *bla*$_{CTX-M}$ is specifically affected by temperature, whereas the presence of chromosomal mutations in the *ampC* promoter leading to AmpC hyper-production is not.

No evidence for recent human/farm transmission of AmpC hyper-producing *E. coli* isolates collected in parallel in a 50 x 50 km region

We next looked at WGS data for 20 human urinary *E. coli* presumed to hyper-produce AmpC, collected during the same timeframe from people living in the same geographical range as the 25 farms for which WGS data of AmpC hyper-producing *E. coli* had been obtained.[3] Proteomics confirmed AmpC hyper-production in 2 representative isolates: UTI-8 and UTI-9, with almost double the amont of AmpC in UTI-8 than in UTI-9 (**Table 3**). There were 9 different *ampC* promoter types seen across the 20 AmpC hyper-producing human isolates, though 11/20 isolates carried the same promoter mutation seen in all 25 farm isolates, including UTI-8 and UTI-9, though UTI-8 also has an attenuator mutation at +37, which probablhy explain the higher level of AmpC seen in UTI-8 that UTI-9 (**Figure 4, Table 3**). None of the human isolates had mutations suggestive of an expanded spectrum AmpC variant, which was confirmed phenotypically using cefepime disc susceptibility testing.

Our final aim was to identify if there was any evidence of sharing AmpC hyper-producing *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 2 other examples where isolates from the same ST were found in farm and human samples gave the same story (**Figure 3**): for ST75, the 2 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 (**Table 4**), with 8/20 human isolates falling into the highly pathogenic phylogroup B2, including a cluster of ST73 isolates of which 3 were only 2 SNPs apart.

Conclusions

AmpC hyper-production is a remarkably common mechanism of 3GC resistance in *E. coli* from dairy farms in our study - similar to a national survey in The Netherlands.[12] We have shown an association between amoxicillin-clavulanate use and the risk of finding AmpC hyper-producers on dairy farms and would caution against a blanket switch from 3/4GCs to amoxicillin-clavulanate in response to justifiable action to reduce HP-CIA use. However, our comparison between AmpC hyper-producing farm and human urinary *E. coli* in the same region provided no evidence of local sharing of AmpC hyper-producers between farms and the local human population. Accordingly, whilst reducing the on-farm prevalence of AmpC hyper-producing *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.

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Transparency declaration

The authors declare no conflict of interests. 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.

References

1. Jacoby GA. AmpC beta-lactamases. Clin Microbiol Rev. 2009 22:161-82.
2. Bergström S, Normark S. Beta-lactam resistance in clinical isolates of *Escherichia coli* caused by elevated production of the *ampC*-mediated chromosomal beta-lactamase. Antimicrob Agents Chemother. 1979 16:427-33.
3. Findlay J, Gould VC, North P et al. Characterisation of cefotaxime-resistant urinary *Escherichia coli* from primary care in South-West England 2017-2018. J Antimicrob Chemother. 2019 In Press. pii: dkz397. doi: 10.1093/jac/dkz397

4. Doi Y, Wachino J, Ishiguro M et al. Inhibitor-sensitive AmpC beta-lactamase variant produced by an *Escherichia coli* clinical isolate resistant to oxyiminocephalosporins and cephamycins. Antimicrob Agents Chemother. 2004 48:2652-8.
5. Mammeri H, Poirel L, Nordmann P. Extension of the hydrolysis spectrum of AmpC beta-lactamase of *Escherichia coli* due to amino acid insertion in the H-10 helix. J Antimicrob Chemother. 2007 60:490-4.
6. Crémet L, Caroff N, Giraudeau C et al. Occurrence of ST23 complex phylogroup A *Escherichia coli* isolates producing extended-spectrum AmpC beta-lactamase in a French hospital. Antimicrob Agents Chemother. 2010 54:2216-8.
7. Bogaerts P, Rodriguez-Villalobos H, Laurent C et al. Emergence of extended-spectrum-AmpC-expressing *Escherichia coli* isolates in Belgian hospitals. J Antimicrob Chemother. 2009 63:1073-5.
8. Haenni M, Châtre P, Madec JY. Emergence of *Escherichia coli* producing extended-spectrum AmpC β-lactamases (ESAC) in animals. Front Microbiol. 2014 5:53.
9. Guillouzouic A, Caroff N, Dauvergne S et al. MLST typing of *Escherichia coli* isolates overproducing AmpC {beta}-lactamase. J Antimicrob Chemother. 2009 63:1290-2.
10. Schubert H, Findlay J, Morley K et al. Evidence for reduced CTX-M carriage in cattle-associated *Escherichia coli* at low temperatures and on publicly accessible farmland: implications for surveillance and potential for farm-to-human transmission. BioRxiv 2019 doi: https://doi.org/10.1101/778407.
11. Findlay J, Schubert H, Morley K et al. Molecular epidemiology of cefotaxime-resistant *Escherichia coli* from dairy farms in South West England identifies a dominant plasmid encoding CTX-M-32. Submitted.
12. Ceccarelli D, Kant A, van Essen-Zandbergen A et al. Diversity of Plasmids and Genes Encoding Resistance to Extended Spectrum Cephalosporins in Commensal *Escherichia coli* From Dutch Livestock in 2007-2017. Front Microbiol. 2019 10:76.

13. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Disk Susceptibility Tests – Thirteenth Edition: Approved Standard M2-A13. CLSI, Wayne, PA, USA. 2018.
14. Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically – Tenth Edition: Approved Standard M7-A10. CLSI, Wayne, PA, USA. 2015.
15. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Ninth Informational Supplement M100-S29. CLSI, Wayne, PA, USA. 2019.
16. Coldham NG, Webber M, Woodward MJ et al. A 96-well plate fluorescence assay for assessment of cellular permeability and active efflux in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. J Antimicrob Chemother 2010; 65:1655–63.
17. Wan Nur Ismah WAK, Takebayashi Y, Findlay J et al. Prediction of fluoroquinolone susceptibility directly from whole genome sequence data using liquid chromatography-tandem mass spectrometry to identify mutant genotypes. Antimicrob Agents Chemother 2018;62:e01814-17.
18. Takebayashi Y, Wan Nur Ismah WAK, Findlay J et al. Prediction of cephalosporin and carbapenem susceptibility in multi-drug resistant gram-negative bacteria using liquid chromatography-tandem mass spectrometry. BioRxiv 2017. https://doi.org/10.1101/138594.
19. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014; 30: 2114-20.
20. Bankevich A, Nurk S, Antipov D et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. Journal of computational biology. Journal of Computational Molecular Cell Biology 2012; 19: 455-77.
21. Wirth T, Falush D, Lan R et al. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol 2006; 60: 1136-51.

22. Zankari E, Hasman H, Cosentino S et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 2012; 67: 2640-4.
23. Carattoli A, Zankari E, Garcia-Fernandez A et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 2014; 58: 3895-903.
24. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014; 30: 2068-9.
25. Grüning B, Dale R, Sjödin A et al. Bioconda: sustainable and comprehensive software distribution for the life sciences. Nature Methods. 2018; 15:475-6.
26. Connor TR, Loman NJ, Thompson S et al. CLIMB (the Cloud Infrastructure for Microbial Bioinformatics): an online resource for the medical microbiology community. Microbial genomics. 2016;2(9):e000086-e.
27. Seemann T. Snippy: fast bacterial variant calling from NGS reads. 3.2-dev ed2015.
28. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics (Oxford, England). 2006;22(21):2688-90.
29. Stamatakis A, Meier H, Ludwig T. RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. Bioinformatics (Oxford, England). 2004;21(4):456-63.
30. Argimon S, Abudahab K, Goater RJ et al. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. Microbial genomics. 2016;2(11):e000093.
31. Mammeri H, Poirel L, Fortineau N et al. Naturally occurring extended-spectrum cephalosporinases in *Escherichia coli*. Antimicrob Agents Chemother. 2006 50:2573-6.
32. World Health Organisation. Critically important antimicrobials for human medicine: Ranking of medically important antimicrobials for risk management of antimicrobial resistance due to non-human use. 5th Revision 2016. http://apps.who.int/iris/bitstream/handle/10665/255027/9789241512220-eng.pdf

411 33. UK Veterinary Antibiotic Resistance and Sales Surveillance Report (UK-VARSS 2017).
412 New Haw, Addlestone: Veterinary Medicines Directorate.

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Table 1. Disc susceptibility for putative AmpC hyper-producing *E. coli* isolates from dairy farms

	Farm 1	Farm 2	Farm 3	Farm 4	Farm WT
Ampicillin	R	R	R	R	R
Cefalexin	R	R	R	R	R
Cefotetan	S	S	S	S	S
Cefotaxime	R	I	I	I	I
Ceftazidime	R	I	I	I	I
Ceftriaxone	R	S	S	S	S
Cefoperazone	I	S	S	S	S
Cefepime	I	S	S	S	S
Aztreonam	S	S	S	S	S
Meropenem	S	S	S	S	S
Chloramphenicol	S	S	S	I	R
Minocycline	R	R	R	I	R
tetA gene	Y	Y	Y	N	Y
floR gene	N	N	N	N	N

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

Isolate	MIC µg/ml				
	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

Cephalosporins highlighted “h” are used in humans and those highlighted with “c” are licenced for use in cattle in the UK.

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

Protein abundance is reported relative to the average abundance of ribosomal proteins in a cell extract and is a mean +/- standard error of the mean, (n=3). Proteins whose abundance is significantly ($p<0.05$) up or downregulated at least 2-fold relative to the EC17 control (see methods) are shaded in red or green, respectively.

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

452 **Table 4. Sequence types of representative AmpC hyper-producing isolates from 25**
453 **dairy farms**

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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

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459 **Table 5**. Significant associations (p<0.05) with AmpC hyper-producing *E. coli* from dairy

460 farms from the multilevel, multivariable logistic regression model

Risk factor	Odds ratio [95% confidence interval]	p
Sample taken from the environment of pre-weaned heifers	3.93 [2.72, 5.67]	<0.001
Total usage of amoxicillin-clavulanate on the farm	1.16 [1.06, 1.27]	0.001
Routine use of vaccination against respiratory disease in calves	2.72 [1.34, 5.56]	0.005
Samples taken from pastureland	0.32 [0.14, 0.72]	0.005
Calving all-year-round as opposed to in seasonal blocks	3.97 [1.45, 10.86]	0.006

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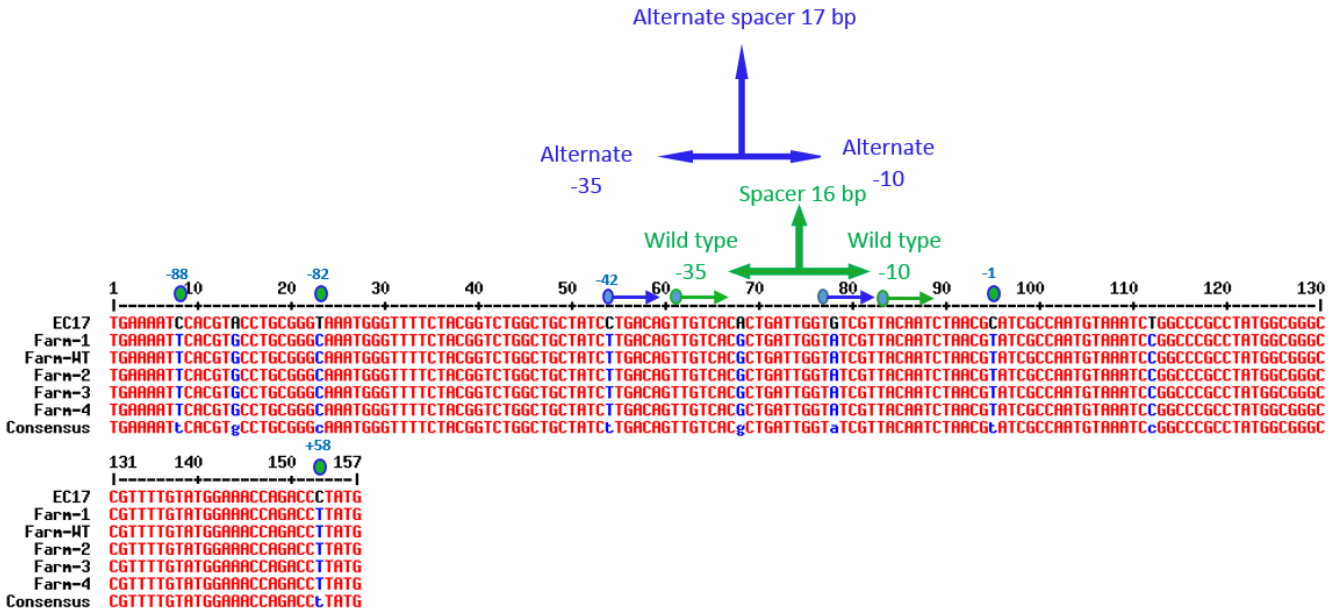
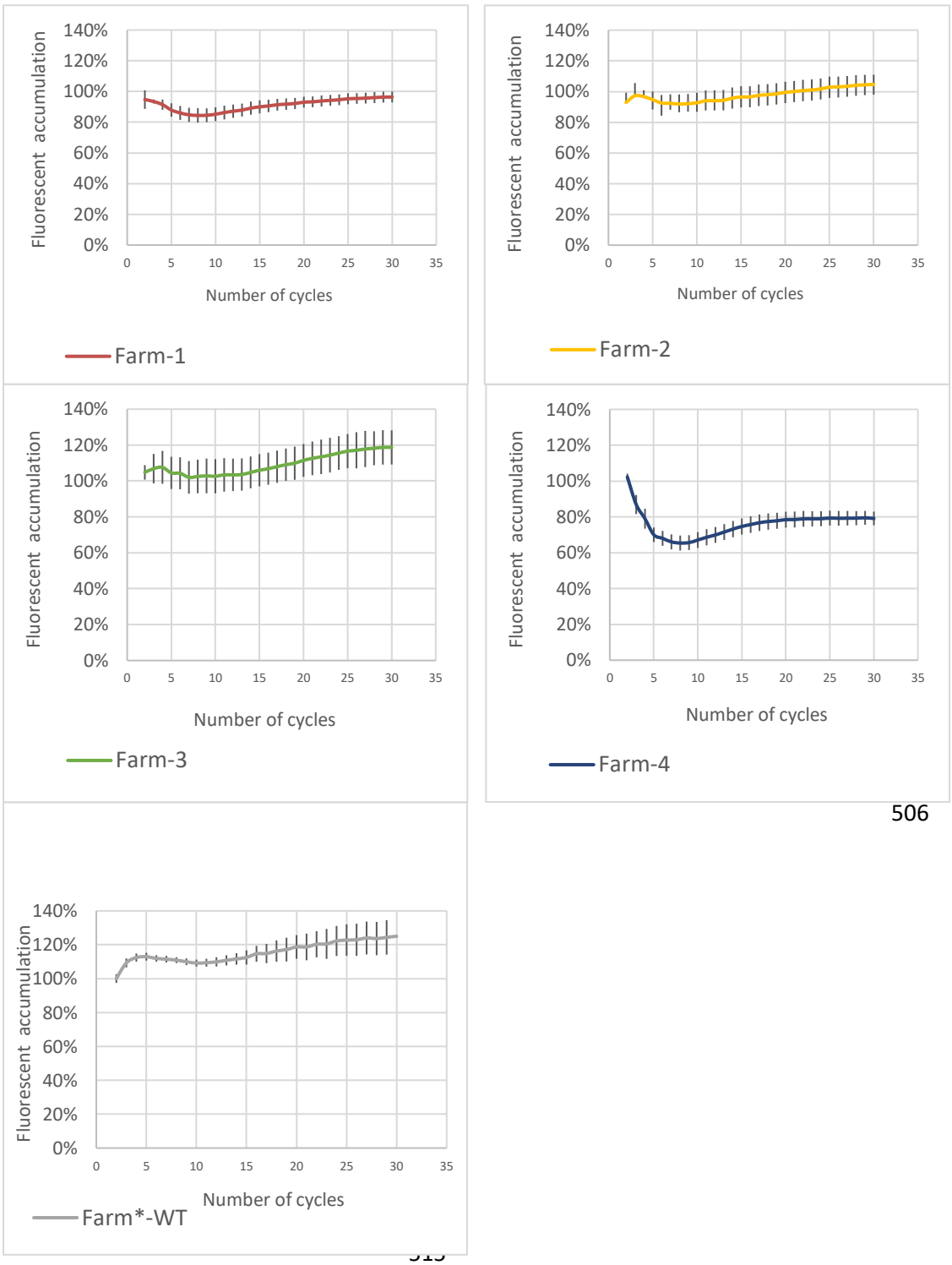
Figures

Figure 1. Promoter/attenuator sequences for *ampC* from 5 farm *E. coli* AmpC hyper-producing isolates in comparison with a wild-type *E. coli* EC17

Modified residues relative to the transcriptional start site are noted and the novel promoter created is annotated.



488 **Figure 2. Envelope permeability of AmpC hyper-producing *E. coli* determined using**
489 **fluorescent dye accumulation assays**



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516 In each case, fluorescence of an AmpC hyper-producing isolates (Farm-1, -2, etc.) incubated
517 with the dye is presented relative to that in the control *E. coli* strain EC17 after each cycle.
518 Each line shows mean data for 3 biological replicates with 8 technical replicates in each.
519 Error bars define the standard error of the mean.

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Figure 3. Phylogenetic tree of farm and human urinary AmpC hyper-producing *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 green (human urinary) and blue (farm). The ST88 finished reference genome used to generate the alignments is noted in black.

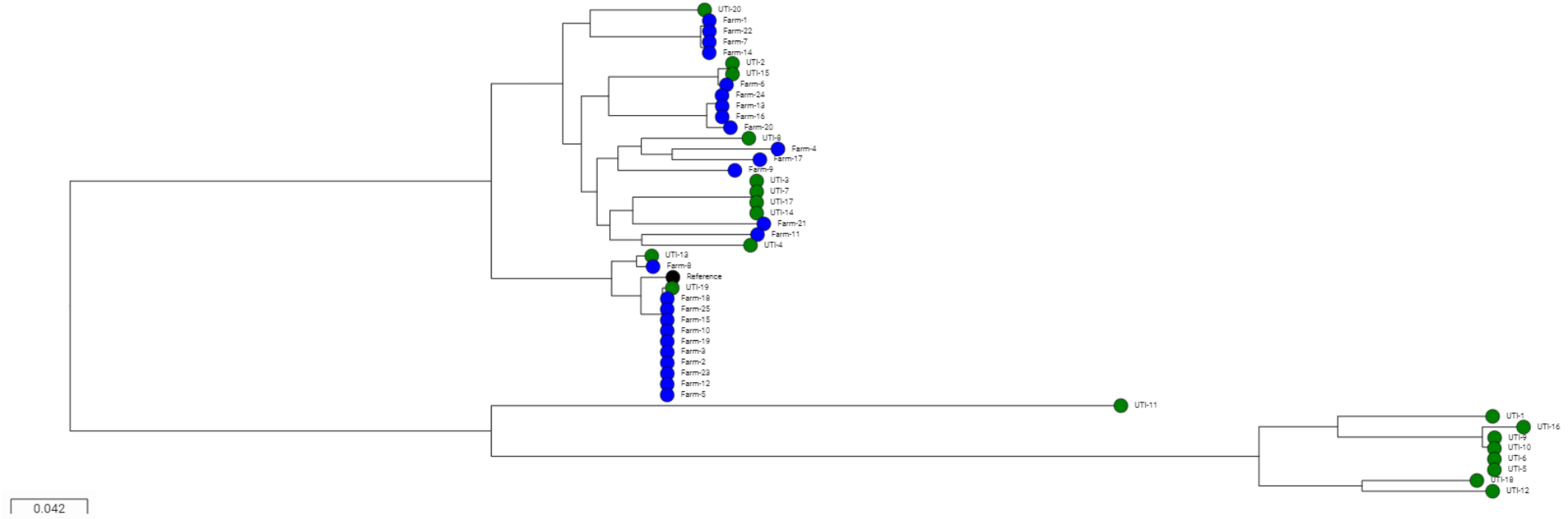

Figure 4. Promoter/attenuator sequences for *ampC* from 20 human urinary *E. coli* AmpC hyper-producing isolates in comparison with a wild-type *E. coli* EC17 and the isolate from Farm 1

Modified residues relative to the transcriptional start site are noted and the novel promoter created is annotated.