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

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Running title: CTX-M in cattle-associated *E. coli*

Abstract

Little is known about the drivers of critically important antibacterial resistance in species with zoonotic potential present on farms (e.g. CTX-M β-lactamase-positive *Escherichia coli*). There is also debate about the influence of farms on the circulation of resistance in local human populations. This was a two-year surveillance study on 53 dairy farms. *E. coli* positive for $bla_{CTX\text{-}M}$ were detected in 224/4145 (5.4%) of all samples from faecally-contaminated sites. *E. coli* positive for $bla_{CTX\text{-}M}$ were more prevalent (98/631; 15.5%) in calf samples and less prevalent (12/630; 1.9%) in samples collected from pastureland, including publicly accessible sites. Multilevel, multivariable logistic regression showed antibiotic dry cow therapeutic choice to be associated with risk of $bla_{CTX\text{-}M}$ positivity, including use of cefquinome or framycetin; 74% of $bla_{CTX\text{-}M}$-positive *E. coli* were framycetin-resistant. Low temperature was associated with low risk of $bla_{CTX\text{-}M}$ positivity. This was additional to the effect of temperature on total *E. coli* density, a finding with profound implications for surveillance. There was no evidence that study farms had a significant impact on circulating $bla_{CTX\text{-}M}$ plasmids in the local human population: across 296 fully sequenced *E. coli* isolates, two cattle isolates shared $bla_{CTX\text{-}M}$ plasmids with eight urinary isolates collected in parallel.

Introduction

Antimicrobial resistance (AMR) - and particularly antibacterial resistance (ABR) - is a significant global challenge. Many countries are implementing plans to reduce the use of antibacterial drugs (ABs) in food-producing animals. For example, the most recent UK five-year National Action Plan includes a target to reduce AB use (ABU) in the treatment of food-producing animals by 25% (1). In Europe, AB sales for food-producing animals fell by 20% from 2011 to 2016 (2). In the UK dairy industry, overall ABU dropped from 24 mg/kg in 2015 to 17 mg/kg in 2017(3, 4). In 2018, additional industry-led policies were enforced in the UK that aim to almost eliminate the use of highest priority critically important antimicrobials (HP-CIAs) such as third- and fourth-generation cephalosporins (3GCs and 4GCs) and fluoroquinolones on dairy farms. One reason for reducing ABU in farming is the belief that such measures will reduce the prevalence of ABR bacteria carried by farm animals. However, there is a need for better data on drivers of ABR in farming. More granularity of understanding is required concerning the risks of using individual ABs and other management practices. This is especially important in terms of drivers of HP-CIA resistance. One key focus is on 3GC resistance in *Escherichia coli*, a species commonly found in animal faeces and considered one of the most significant potential zoonotic threats to humans (5).

3GC-resistance is increasingly common in *E. coli* causing infections in humans (6) and is also found in farmed and domestic animals around the world (7). The production of CTX-M (an extended-spectrum β-lactamase) is the most common mechanism of 3GC-resistance in *E. coli* in humans in the UK; for example, in a recent study of urinary *E. coli* from humans in South West England, 82.2% of 3GC-resistant isolates carried $bla_{\text{CTX-M}}$ (8).

The objective of this study was to describe the prevalence of *E. coli* carrying *bla*$_{CTX-M}$ found in faecally contaminated environments of dairy cattle in a geographically restricted population of UK dairy farms in South West England. Furthermore, this study investigated ABU and management practice risk factors for the presence of such *E. coli.* Finally, this study used extensive molecular epidemiology based on whole genome sequencing (WGS) to help explain risk factors identified and to investigate evidence for transmission of *bla*$_{CTX-M}$-encoding plasmids between farm- and human-associated *E. coli* collected in parallel in a relatively small (50 x 50 km) region.

Materials and Methods

Farm recruitment and ethical approval

A convenience sample of 53 dairy farms was recruited through personal contacts, local veterinary practices and milk processors. Details of the study population are presented in Supplementary. Of these, 43 farms were in a 50 x 50 km area defined based on the locations of 146 general practices that referred routine urine samples from human patients to the microbiology reference lab at Severn Pathology, Southmead Hospital (8). A further 10 study farms were clustered in a separate region in South West England. All farmers gave fully informed consent to participate in the study. Ethical approval was obtained from the University of Bristol's Faculty of Health Sciences Research Ethics Committee (ref 41562).

Farm sampling and sample processing

Farms were visited monthly between January 2017 and December 2018. Samples were collected using sterile overshoes (over-boot socks) traversing farm areas.

Where access was restricted (e.g. for pens containing single or pairs of calves), samples were collected directly from the ground using gloved hands. Details of the six types of samples collected are in Supplementary; these represent faecally-contaminated environments representative of milking cows (Adult), cows between periods of lactation (Dry Cow), heifers after weaning (Heifer), heifers before weaning (Calf). Samples of these types collected from pastureland were designated as such for separate analysis (Pasture) which also included samples collected from publicly accessible land on the farm (Footpath). Samples were refrigerated from collection to processing. They were transferred into individual labelled sterile stomacher bags and suspended in 10 mL/g of phosphate buffered saline (PBS Dulbecco A; Oxoid, Basingstoke, UK). Samples were then mixed for one min in a stomacher (Stomacher 400, Seward, Worthing, UK). Samples were mixed 50:50 with 100% sterile glycerol and aliquots stored at -80°C.

Microbiology and WGS analysis

Twenty microlitres of sample (diluted 1:10) were spread onto tryptone bile X-glucuronide agar (TBX; Scientific Laboratory Supplies); 20 µL of undiluted sample were spread onto TBX agar containing 16 mg/L cephalexin. Plates were incubated at 37°C, and the number of blue colonies (*E. coli*) counted. Samples yielding no *E. coli* colonies on antibiotic-free agar were excluded from further analysis. Up to five *E. coli* isolates from each cephalexin (16 mg/L) TBX agar plate were transferred onto cefotaxime (CTX, 2 mg/L) TBX agar. Concentrations were chosen as those which define clinically relevant resistance in humans according to EUCAST (9). Confirmed CTX-resistant isolates were subjected to multiplex PCR to detect $bla_{\text{CTX-M}}$ (testing for $bla_{\text{CTX-M}}$ groups 1, 2, 8, 9 and 25; [10]). 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 (11), assembled into contigs using SPAdes (12) 3.13.0 (http://cab.spbu.ru/software/spades/) and contigs were annotated using Prokka (13). Resistance genes, plasmid replicon types, sequence types and fim types were assigned using the ResFinder (14), PlasmidFinder (15), MLST (16) 2.0 and FimTyper on the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) platform.

Risk factor data analysis

The risk factors examined fall into four categories: farm management, ABU, sample characteristics and meteorological. Four management practice questionnaires were developed (details provided in Supplementary). ABU was extracted from prescribing and sales data between Jan 2016 to Dec 2018 obtained from veterinary practices servicing the study farms. For two farms, sales data were not available and on-farm records were used. The full method for obtaining, cleaning and processing ABU data can be found in Supplementary. Local meteorological data were extracted from publicly available UK Met Office data (https://www.metoffice.gov.uk/pub/data/weather/uk/climate/stationdata/yeoviltondata.txt).

Sample processing and data analysis workflows are illustrated in **Figure S1**. All data analysis was performed using R (https://www.r-project.org/). Two modelling approaches were used: 1) variable selection via univariable screening and stepwise model selection with a multilevel, multivariable logistic regression model and 2) a regularised Bayesian model. Both were used to analyse risk factors associated with $bla_{CTX\text{-}M}$ *E. coli* positivity. A sensitivity analyses was performed to test for

measurement bias, to account for the fact that *bla*$_{CTX-M}$ is more likely to be found in a sample if there is a higher density of bacterial colony forming units. Further details of variable selection and development of the models and model checking are provided in Supplementary.

Results

Farm- and sample-level risk factors for *bla*$_{CTX-M}$ *E. coli* positivity

4581 samples were collected: 4145 were positive for growth of *E. coli* on non-selective agar. Of these, 384/4145 (9.3%) samples representing 47/53 (88.7%) of farms were positive for growth of *E. coli* that were resistant to cefotaxime. Overall, 5.4% (224/4145) of samples representing 42/53 (79.2%) of farms contained cefotaxime-resistant *E. coli* confirmed to carry *bla*$_{CTX-M}$ using PCR. Positivity for *bla*$_{CTX-M}$ *E. coli* was three times higher in Calf samples (98/631 [15.5%] of samples) than overall (**Table 1**).

A separate risk factor analysis using only Calf data was performed, given the high positivity rate for *bla*$_{CTX-M}$ *E. coli* in these samples. One farm-level fixed effect and three sample-level fixed effects were retained in the final model (**Table S1**, **Table 2**). The use of cefquinome or framycetin dry cow therapies were both associated with increased risk of *bla*$_{CTX-M}$ *E. coli* positivity, as was higher average monthly temperature. Plotting sample-level positivity for *E. coli* carrying *bla*$_{CTX-M}$ versus average monthly temperature revealed that the relationship between positivity and temperature was primarily driven by low *bla*$_{CTX-M}$ *E. coli* positivity rates in months where the average temperature was below 10°C (**Figure 1A**).

Risk factor analysis was next performed for the full dataset. One farm-level fixed effect and three sample-level fixed effects were retained in the final model (**Table S2**, **Table 3**). Interestingly, this model revealed that *bla*$_{\text{CTX-M}}$ *E. coli* was less likely to be found in samples obtained from pastureland, which includes publicly accessible farmland (Footpaths) compared with other sample types. Analysis of the full dataset confirmed what was seen with the Calf dataset: that higher average monthly temperature was associated with an increased risk of *bla*$_{\text{CTX-M}}$ *E. coli* positivity. Again, visualisation of the data confirmed that this was primarily driven by a reduction in *bla*$_{\text{CTX-M}}$ *E. coli* positivity rate in months with an average temperature below 10°C (**Figure 1B**).

A Bayesian logistic regression model was also constructed in which the effect of total farm ABU and specifically total 3GC and 4GC use were tested as predictors for *bla*$_{\text{CTX-M}}$ *E. coli* positivity in the total dataset, with 102 potential confounders. The impacts of temperature (Odds Ratio 1.71 [1.42, 2.08]) and of samples being collected from pastureland (Odds Ratio 0.51 [0.22, 1.02]) on *bla*$_{\text{CTX-M}}$ *E. coli* positivity were also retained in this alternative model (**Table S3**).

Defining sample-level positivity for *bla*$_{\text{CTX-M}}$ *E. coli* depends on finding *bla*$_{\text{CTX-M}}$ using PCR in *E. coli* colonies that have grown on agar containing cefotaxime. If *bla*$_{\text{CTX-M}}$ *E. coli* in a sample exist at such a low density that they are not detected using selective agar, the sample will be falsely identified as negative for *bla*$_{\text{CTX-M}}$ *E. coli*. This impact of bacterial density on assay sensitivity/specificity is an important consideration in the context of the finding that *bla*$_{\text{CTX-M}}$ positivity is low at low temperatures. To account for this, the logistic link function was adjusted (see Supplementary). This only modestly altered the effect sizes or the p values for the risk factors (**Figure S2**) confirming that the effect of low temperature on *bla*$_{\text{CTX-M}}$ *E. coli* positivity was

additional to its effect on *E. coli* prevalence. All values in **Tables 2**, **3** and **S3** come from models with this adjusted logistic link function applied.

Molecular analysis and evidence of limited zoonotic transmission of *bla*$_{CTX-M}$-encoding plasmids

Based on WGS for 115 *bla*$_{CTX-M}$ *E. coli* isolates from farm samples, 77.4%, 84.3% and 81.7% also carried genes encoding resistance to framycetin, streptomycin or tetracycline, respectively. In contrast, 14.7% had trimethoprim resistance genes, 11.3% carried phenicol resistance genes and only 8.7% had plasmid-mediated fluoroquinolone resistance genes. Thirty-seven out of 107 *bla*$_{CTX-M}$-positive *E. coli* isolates from farm samples were found to carry *bla*$_{CTX-M}$ variants also seen amongst 189 *bla*$_{CTX-M}$-positive urinary *E. coli* cultured from people living in the same 50 x 50 km region during the same time period (8). By filtering sequenced isolates by their *bla*$_{CTX-M}$ variants and plasmid replicon types, plasmids that were almost identical in farm and human isolates were identified. One plasmid type, found in a single sequence type (ST)345 farm isolate, harboured *bla*$_{CTX-M-1}$ carried on an IncI1-ST3 plasmid. BLAST analysis of the *bla*$_{CTX-M-1}$ contig showed that it was most similar to part of an unpublished ~106 kb plasmid - pTC_N40607 (GenBank Accession No. CP007651) - found in *E. coli* obtained from meat/cattle isolates in the USA. Mapping of the ST345 farm isolate sequencing reads against pTC_N40607 showed it exhibited 100% coverage and 97.5% sequence identity. Six human urinary *E. coli* isolates representing STs 23, 127, 131, 141 and 2015 harboured *bla*$_{CTX-M-1}$ on an IncI1-ST3 plasmid that exhibited 99.4-100% coverage and 96.4-98.7% identity when sequence reads were mapped to pTC_N40607.

Another plasmid type - again obtained from a single farm isolate, in this case of ST58 - exhibited 100% coverage and 98.5% identity by read mapping to a published IncK plasmid pCT (GenBank Accession No. NC_014477). pCT is ~94 kb and is known to harbour *bla*$_{CTX-M-14}$; pCT-like plasmids have been reported in both human and veterinary *E. coli* isolates across three continents (17, 18). Amongst human urinary *E. coli* isolates found in this study, two also carried pCT-like plasmids. Both isolates were the pandemic clone ST131, and their pCT-like plasmids exhibited 96.4 or 97.2% identity and 100% coverage to pCT.

Discussion

Prevalence of *bla*$_{CTX-M}$ *E. coli* and impact of temperature - implications for surveillance studies

This study is unique in its scale: extensive management practice and ABU data along with multiple samples from multiple farms were collected over multiple time points across a two-year period. Overall, 224/4145 (5.4%) of samples were positive for *E. coli* carrying *bla*$_{CTX-M}$ with considerable farm- and sample-level variation (**Tables 1-3**). Of studies using similar methodology (phenotypic selection followed by PCR analysis), Snow et al. (2012) (19) found 17/48 (35%) of randomly selected UK dairy farms were positive for *bla*$_{CTX-M}$ *E. coli*, whereas Mollenkomf et al. (2012) (20) found 5/25 (20%) of farms in Ohio to be positive. This study found 42/53 (79%) of farms to be positive, although many samples were collected each month over two years, hence the chances of finding a positive sample on each farm may have been greater than in these earlier point-prevalence studies. To further elucidate this, farm-

level positivity for *bla*$_{CTX-M}$ *E. coli* was plotted on a month-by-month basis (**Figure 2**) and revealed the highest prevalence for a single monthly survey to be 22.5%. The prevalence of *bla*$_{CTX-M}$ *E. coli* varied across sample types in this study: although prevalence was low overall (5.4%), prevalence was higher (15.5%) in Calf samples. In contrast, Horton et al. 2016 (21) reported a very high prevalence (>90%) of *bla*$_{CTX-M}$ *E. coli* in samples taken from faecal pats, even from adult milking cows. Brunton et al. (2014) (22) reported a prevalence of 50% in calves. The finding of relatively low prevalence in this study could be due to the large number of samples collected, particularly over winter, given low temperature was associated with low *bla*$_{CTX-M}$ *E. coli* positivity (**Fig. 1A; 1B**). Indeed, the observation that average monthly temperature had a significant effect on *bla*$_{CTX-M}$ *E. coli* positivity highlights problems with studies where a single time-point or sampling season is used. This has potentially profound implications for surveillance studies performed to identify risk factors, to identify general trends in ABR levels, to benchmark farms or to test the effects of policy interventions. **Figure 2** shows the stark impact of this in real terms: *bla*$_{CTX-M}$ *E. coli* positivity at farm level was zero and 1.9%, respectively in February and March, the coldest months of the year (based on average temperature). Whilst average annual temperature found at locations across an entire continent has previously been shown to impact average ABR levels at those locations (23), the finding that periods of low temperatures were associated with lower prevalence of a dominant cause of HP-CIA resistance at a given location during the course of a year is particularly important; this observation also leads to concern about the impact of climate change - and especially increasing temperatures - on attempts to reduce ABR. Whilst temperature was associated with the total number of *E. coli* found in each sample, this was accounted for using a novel measurement error method

incorporated into the model; as such, the effect of temperature on *bla*$_{CTX-M}$ *E. coli*, whilst in part driven by the effect on total *E. coli* number, also had an independent association suggestive of a temperature-dependent fitness burden of carrying *bla*$_{CTX-M}$.

In terms of sample-level effects, there were clear differences in the risk of encountering *bla*$_{CTX-M}$ *E. coli* at different sites on a farm (e.g. 15.5% in Calf samples, 4.1% in Adult samples). Watson et al. (2012) (24) also found that CTX-M prevalence was much higher in calves, but Horton et al. (2016) (21) did not report such a difference; however, *bla*$_{CTX-M}$ status in this latter study was presumptive based on the phenotypic identification of cefotaxime resistance, which would make these results less comparable with those presented here. In other farmed species, Agerso et al. (2011) (25) found a prevalence of *bla*$_{CTX-M}$ *E. coli* carriage of approximately 7% in Danish slaughter pigs and Randall et al. (2010) (26) found a *bla*$_{CTX-M}$ *E. coli* prevalence of 3.6% in UK broiler chickens and turkeys. Various studies have identified much higher prevalence in chicken meat, but this could be due to cross-contamination at slaughter and in the food chain (27, 28).

Studies examining the prevalence of *bla*$_{CTX-M}$ *E. coli* in human populations have shown mixed results. Luvsansharav et al. (2012) (29) found a prevalence of 65.7% amongst commensal isolates in Thailand. In the UK, a study across four regions reported commensal faecal carriage of *bla*$_{CTX-M}$ *E. coli* to be approximately 7% (30). A recent analysis of human urinary samples from the same region as the farms surveyed in this study gave a sample-level prevalence of *bla*$_{CTX-M}$ *E. coli* of approximately 5% (8). It should be noted that all farm samples in the present study were from faecally contaminated sites, not individual animals, and so it is possible that the number of animals carrying *bla*$_{CTX-M}$ *E. coli* was much lower than the

reported sample-level prevalence. Direct comparison with human and other farm animal carriage studies should therefore be made with caution.

AB contamination of colostrum as a possible driver of *bla*$_{CTX-M}$ *E. coli* positivity in dairy calves - evidence of direct and co-selection

It has been shown experimentally that waste (AB-contaminated) milk feeding to calves increases faecal excretion of ABR bacteria (31). This practice is reducing on UK dairy farms and, in the analysis presented here, waste milk feeding was not associated with an increased risk of finding *bla*$_{CTX-M}$ *E. coli*. In contrast, the choice of dry cow therapy (an antibacterial preparation inserted into a cow's udder between lactations to help treat or prevent mastitis) was associated with *bla*$_{CTX-M}$ *E. coli* positivity in Calf samples. Colostrum management is a hugely important part of early life for most farmed mammals and is universally encouraged in dairy farming. In this study, cefquinome (a 4GC) dry cow therapy was most significantly associated with *bla*$_{CTX-M}$ *E. coli* in Calf samples (**Table 2**), and it has previously been shown that colostrum from cows given cefquinome dry cow therapy is heavily contaminated with cefquinome (32). There was also a clear positive association between the usage of framycetin as part of a dry cow therapy combination and the risk of finding *bla*$_{CTX-M}$ *E. coli* in Calf samples (**Table 2**). Whilst no work has been published on the contamination of colostrum with framycetin, its use as a mastitis therapy for milking cows results in identifiable residues in milk (33), so it is highly likely to also contaminate colostrum. It is possible, therefore, that feeding of colostrum, which can be contaminated with AB used for dry cow therapy, is a driver of *bla*$_{CTX-M}$ *E. coli* in calves. An alternative (or indeed an additional) explanation for this observed association is that *E. coli* (a species known to be found in the udders of dairy cows

(34) that carry *bla*$_{CTX-M}$ are selected within the udder during AB dry cow therapy and contaminate colostrum alongside the AB used.

WGS showed that *bla*$_{CTX-M}$ was co-located with framycetin resistance genes in 77.4% of 115 fully sequenced *E. coli* isolates across study farms. Accordingly, it is hypothesised that framycetin dry cow therapy drives *bla*$_{CTX-M}$ *E. coli* positivity in calves because of co-selection of bacteria carrying *bla*$_{CTX-M}$ and a framycetin resistance gene, just as cefquinome use drives *bla*$_{CTX-M}$ *E. coli* positivity by direct selection of *bla*$_{CTX-M}$. Whilst regional differences in the ecology of circulating resistance may mean that the specific observation made here is not universally applicable, this is still an important “real-world” example of the frequently identified laboratory phenomenon of co-selection and illustrates why policy changes to reduce the usage of HP-CIAs such as cefquinome may not remove all selective pressure for presence of HP-CIA resistance on farms. Snow et al. (2012) (19) also identified overall use of 3/4GCs as a risk factor for *bla*$_{CTX-M}$ *E. coli* presence on dairy farms. Other studies have not made a link between the usage of framycetin and prevalence of *bla*$_{CTX-M}$ *E. coli*. However, it is not always clear whether other studies have separated out different dry cow therapies since they have tended to focus on systemic AB use.

Low risk of finding *bla*$_{CTX-M}$ *E. coli* on publicly accessible farm sites and little evidence of sharing with locally resident people

This study identified a lower chance of detecting *bla*$_{CTX-M}$ *E. coli* in samples collected on pastureland than elsewhere on the farm. Because pastureland may be more affected by the elements, this finding may be partly linked with the finding that lower average monthly temperature was associated with decreased risk of detecting *bla*$_{CTX-M}$ *E. coli*. Importantly, 395/630 (62.7%) of samples from pastureland were from

publicly accessible sites so the finding of a low positivity for *bla*$_{CTX-M}$ *E. coli* on pastureland led to the hypothesis that dairy farms are unlikely to be sources of transmission of *bla*$_{CTX-M}$ *E. coli* into the local human population. Analysis of fully sequenced genomes of 296 *bla*$_{CTX-M}$ *E. coli* from study farms and from urine samples provided by people living in the same geographical range as the farms over the same time period did not suggest any evidence of direct sharing of *E. coli* between farms and the local human population (i.e. none were the same ST, carrying the same plasmid with the same *bla*$_{CTX-M}$ variant). However, two farm *E. coli* isolates carried a *bla*$_{CTX-M}$ plasmid almost identical to a plasmid circulating in the local human population. One plasmid (pCT) is already known to be spread widely amongst *E. coli* from humans and animals on three continents (Cottell 2011). Whilst unpublished, the other plasmid (pTC_N40607) has been identified in *E. coli* in the USA so is likely to be similarly widespread. Accordingly, whilst there is some evidence of shared circulating plasmids, as reported in a number of recent studies (35-37), the overall level of overlap between farm and human *bla*$_{CTX-M}$ *E. coli* identified in this study was very small and not suggestive of any novel or recent transmission events.

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Transparency declarations

D.C.B. was president of the British Cattle Veterinary Association 2018-19. Otherwise, the authors declare no competing 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.

Author Contributions

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Tables

Table 1. Prevalence of *E. coli* carrying *bla*$_{CTX-M}$ at farm and sample levels

Sample type		Farm level	Sample level
Overall	**Total sample size**	53	4145
	Total (%) positive for CTX-M-carrying *E. coli*	42 (79.2%)	224 (5.4%)
Adult	**Total sample size**	52	1835
	Total (%) positive for CTX-M-carrying *E. coli*	25 (48.1%)	76 (4.1%)
Dry Cow	**Total sample size**	46	282
	Total (%) positive for CTX-M-carrying *E. coli*	7 (15.2%)	8 (2.8%)
Calf	**Total sample size**	51	631
	Total (%) positive for CTX-M-carrying *E. coli*	33 (64.7%)	98 (15.5%)
Heifer	**Total sample size**	41	1235
	Total (%) positive for CTX-M-carrying *E. coli*	18 (44%)	40 (3.2%)
Pastureland	**Total sample size**	47	630
	Total (%) positive for CTX-M-carrying *E. coli*	8 (17%)	12 (1.9%)
Pastureland that is publicly accessible (Footpath)	**Total sample size**	41	395
	Total (%) positive for CTX-M-carrying *E. coli*	8 (20.0%)	11 (2.8%)

Table 2. Fixed effects from the multilevel, multivariable logistic regression model performed on Calf samples

Risk factor	**Odds ratio [95% confidence interval]**	**p**
Use of cefquinome dry cow therapy in the last six months	4.12 [2.11, 8.25]	0.00003
Daily water trough cleaning	0.44 [0.29, 0.69]	0.0002
Average monthly temperature	1.57 [1.20, 2.06]	0.0008
Use of framycetin dry cow therapy in the last six months	1.91 [1.01, 3.61]	0.04

Table 3. Fixed effects from the multilevel, multivariable logistic regression model performed on the full dataset

Risk factor	**Odds ratio [95% confidence interval]**	**p**
Sample taken from the environment of pre-weaned heifers	4.51 [3.25, 6.27]	0
Average monthly temperature	1.60 [1.34, 1.89]	0.00000001
Sample taken from pastureland	0.32 [0.17,0.61]	0.0000001
Feeding of maize silage	3.28 [1.50, 7.18]	0.002

Figures

Figure 1A. Average monthly temperature vs. presence (1) or absence (0) of CTX-M in samples from pre-weaned calves. A multilevel, multivariable logistic regression model revealed a positive association with increased temperature (p=0.0008).

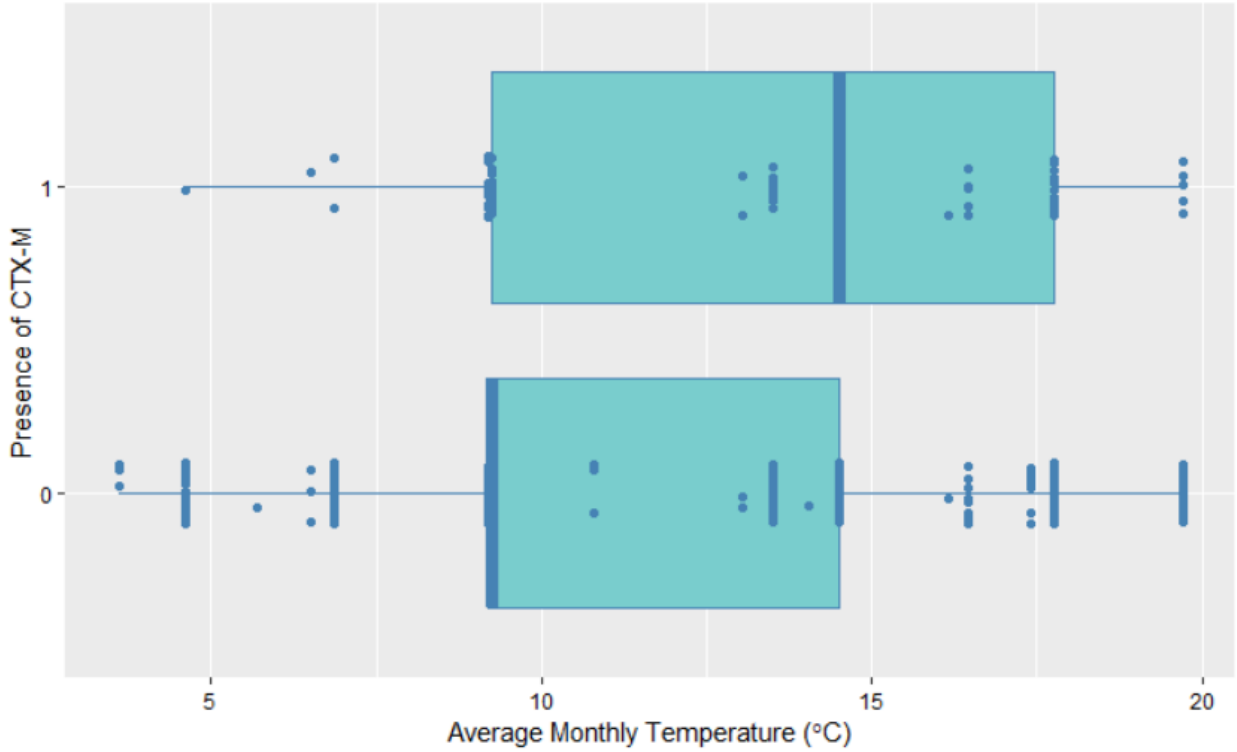


Figure 1B. Average monthly temperature vs. presence (1) or absence (0) of CTX-M in all samples of faecally contaminated dairy farm environments. A multilevel, multivariable logistic regression model revealed a positive association with increased temperature (p=0.00000001).

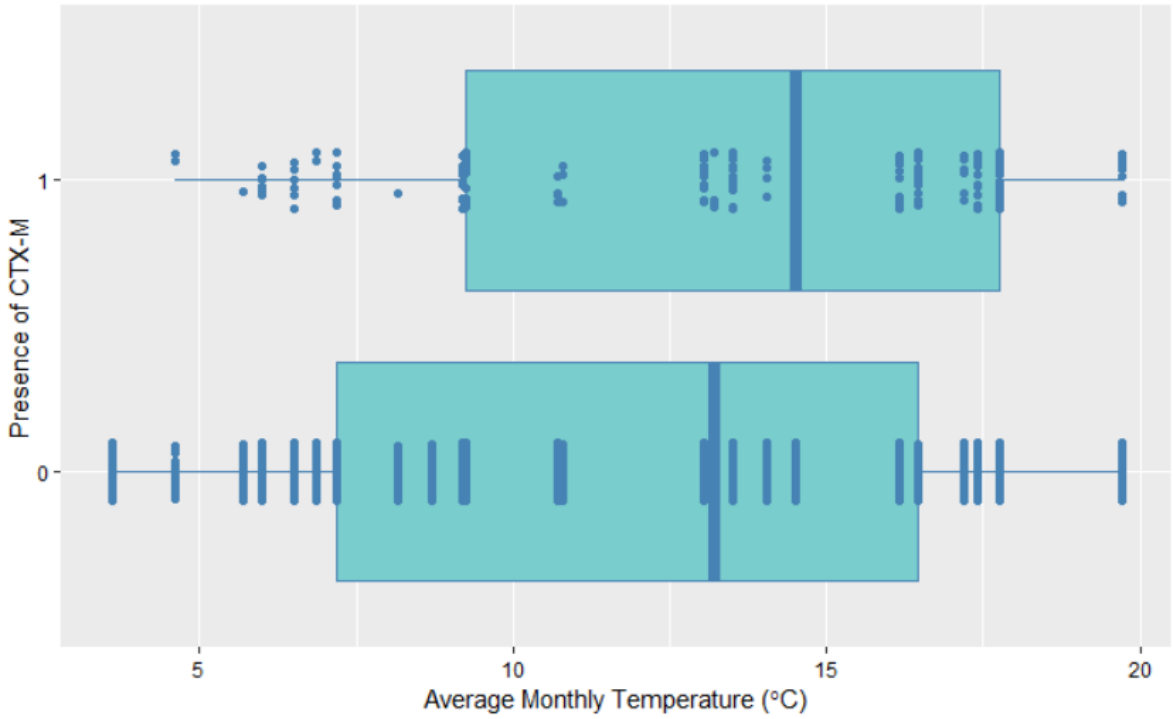
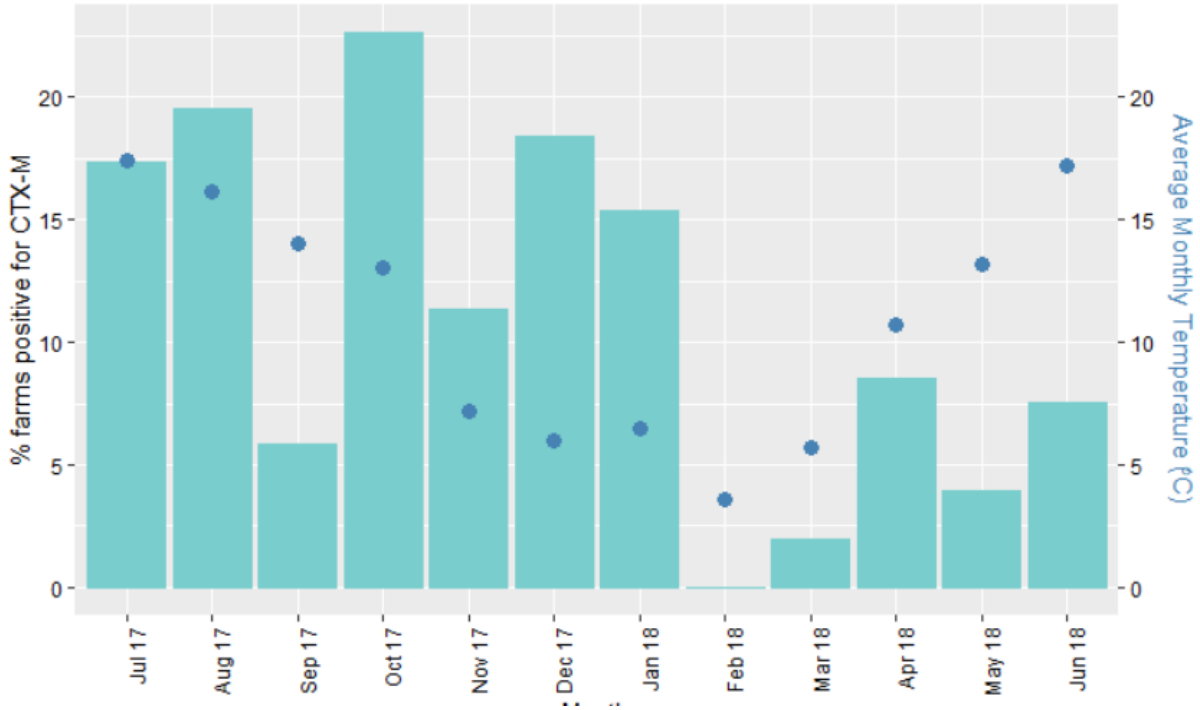


Figure 2. Percentage of farms positive for CTX-M by month and by average monthly temperature representing a year during the middle period of this study. Samples from calves have been excluded.



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