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Detection of antibiotic residues and association of cefquinome residues with the occurrence of Extended-Spectrum β-Lactamase (ESBL)-producing bacteria in waste milk samples from dairy farms in England and Wales in 2011

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ABSTRACT

Waste milk samples from 103 farms in England and Wales were examined for the presence of β-lactam antibiotics and ESBL-producing *Enterobacteriaceae*. Approximately ten months after the initial sampling, further waste milk, environmental and faecal samples from farms shown to be positive for CTX-M *Escherichia coli* were investigated further. Isolates with an ESBL phenotype were tested by PCR for the presence of $bla_{\text{CTX-M}}$, bla_{OXA}, bla_{SHV} and bla_{TEM} genes. Isolates positive for $bla_{\text{CTX-M}}$ were sequenced to determine CTX-M type. Representative isolates were further examined by PFGE, plasmid replicon typing and serotyping. Of particular interest, 21.4% of waste milk samples contained residues of the cephalosporin cefquinome, which was significantly associated with CTX-M bacteria. Such bacteria occurred in 5.8% of the waste milk samples (including 3.9% CTX-M *Escherichia coli*). CTX-M types identified were 1, 14, 14b and 15, but none of the *E. coli* were serotype O25, the serotype of the human pandemic strain.

1. Introduction

Waste milk is milk unfit for human consumption and may be designated unfit for various reasons. It includes milk from cows immediately post-calving (colostrum), milk from cows with udder infections (mastitis) and milk from cows undergoing treatment with antibiotics or other medicines in which drug residues in excess of Maximum Residue Limits may be present. This waste milk cannot be sold for human consumption, but is widely used to feed calves.

In a recent survey of 557 dairy farms in England and Wales, 93% of the respondents to a postal questionnaire used antibiotic intra-mammary tubes to treat mastitis; this study reported that cefquinome, a fourth generation (extended spectrum) cephalosporin, was used by 29% of respondents as their current most frequent mastitis therapy (Brunton et al., 2012). Use of third and fourth generation cephalosporins in livestock is likely to provide a selective pressure for the maintenance of resistance or emergence of resistance to these compounds. In the above study (Brunton et al., 2012), 83% of 499 respondents or 413 individual farmers reported feeding waste milk to calves. If waste milk were found to contain third or fourth generation cephalosporin residues, then such milk could potentially exert a selective pressure influencing the occurrence of Extended Spectrum β-Lactamase (ESBL) resistant bacteria in calves fed waste milk.

Several studies have reported the presence of CTX-M bacteria in faecal samples from dairy cattle in the UK (Liebana et al., 2006; Snow et al., 2011; Teale et al., 2005; Watson et al., 2012). One study on a commercial farm in the United Kingdom in 2008 found that 30.3% of milking cows, 17% of the whole herd and 95.6% of two day old calves were positive for CTX-M ESBL *E. coli* (Watson et al., 2012). Recently a positive association was reported between the use of 3rd and 4th generation

cephalosporins on dairy farms and the presence of CTX-M ESBL *E. coli* in cattle on those farms, although no association was found with the use of first and second generation cephalosporin veterinary medicines (Snow et al., 2012).

Third and fourth generation cephalosporins are important antibiotics for the treatment of human bacterial diseases (Collignon et al., 2009; Livermore et al., 2007). The human pandemic O25:H4-ST131 *E. coli* clone, which is often amikacin resistant, and carries CTX-M-15 on an plasmids such as Inc FII, is of particular concern as this strain can cause significant morbidity and mortality in humans (Lau et al., 2008). To date, none of the CTX-M-positive *E. coli* isolated from cattle (Horton et al., 2011; Liebana et al., 2006; Snow et al., 2011; Teale et al., 2005; Watson et al., 2012), or chickens and turkeys (Randall et al., 2011) in the UK have been shown to belong to the main human pandemic clone. However, detailed molecular analysis of other *E. coli* serotypes, has revealed that a plasmid designated pCT bearing a CTX-M-14 ESBL initially isolated from a veterinary source, is present in some *E. coli* from humans and different species of food producing animals (Cottell et al., 2011; Stokes et al., 2012), suggesting that *E. coli* or other *Enterobacteriaceae* have access to a shared global pool of resistance elements.

The aim of this study was to examine waste milk samples from farms in England and Wales for antibiotic residues and ESBL-producing bacteria, in particular those bearing the CTX-M gene, and to characterise any ESBL-bacteria isolated. Faecal and environmental samples from calves and adult cattle were also examined from a small number (three) of these farms to further investigate possible clonal links between the waste milk fed to calves and the presence of CTX-M-positive *E. coli* in the animals and their environment.

2. Materials and methods

2.1 Control isolates

For presumptive phenotypic ESBL tests using MAST disks, *E. coli* NCTC 10418 was used as negative control and *E. coli* NCTC 13351 (TEM 3, broad spectrum), 13352 (TEM 10, ceftazidimase) and 13353 (CTX-M 15, CTX-M-1, cefotaximase) were used as ESBL-positive controls. PCRs for CTX-M, OXA, SHV and TEM genes (Fang et al., 2008) were also performed with suitable positive and negative control isolates for each gene tested.

2.2 Antimicrobials and chemicals

Antimicrobials and chemicals used in this study were obtained from Sigma-Aldrich (Poole, Dorset, UK). Antimicrobial disks for detection of putative AmpC or ESBL phenotype were obtained from MAST (MAST group Ltd, UK). Brilliance ESBL agar was obtained from Oxoid (Thermo Fisher Scientific, UK), whilst CHROMagar ECC and CHROMagar CTX supplement were obtained from CHROMagar (CHROMagar, France).

2.3 Waste milk samples

Waste milk samples were collected from farms in England and Wales in February and March 2011. A sampling frame of approximately 370 farms was stratified by herd size to reflect the distribution of herd size as reported elsewhere (Brunton et al., 2012). From this sampling frame, 120 farms were randomly selected in proportion to the percentage of farms in each stratum. The distribution of herd size and geographical location within the selection was examined and found to correlate well with the population described previously (Brunton et al., 2012). Farms were contacted

by telephone to request a sample of waste milk, and 103 were successfully recruited. This final selection of farms suitably reflected the dairy herd population that participated in the previous survey based on herd size and location (Brunton et al., 2012).

Approximately 100 ml of waste milk was collected from each farm using a sterile sampler. Farmers collected samples that were representative of waste milk being fed to calves, stirring the waste milk in the container before collection. Pots of waste milk were refrigerated before collection.

The farmer also completed a short questionnaire concerning which antibiotics had been administered to the cows contributing to the waste milk as well as farm management practices related to feeding waste milk to calves. The waste milk samples were kept chilled and on the day of receipt at the laboratory approximately 40 ml were frozen at -80° C in two aliquots of ~ 20 ml for antibiotic residue analysis. Approximately 20 ml were kept chilled for bacteriology which was initiated the following day. After bacteriological examination of all waste milk samples, 10% sterile glycerol was added to each sample as a cryo-protectant and these waste milk samples were also frozen at -80° C in case further bacteriological tests were required.

2.4 Farm studies

Three of the farms that were positive for CTX-M-positive *E. coli* in the first part of the study were re-visited (approximately 10 months after analysis of the original waste milk sample) for further sampling. During this second part of the study, waste milk samples were collected twice in the two weeks prior to a herd visit and on the day of the herd visit. Additionally, on the day of the herd visit, 90 faecal samples and

10 environmental samples were collected. Environmental samples included samples from calf pens, collecting yards, pen rails, water troughs and waste milk feeders; puddles, tractor foot well, cubicle house scraper, roadway, seepage from animal pens, waste milk store and tractor scraper. On all farms samples were collected from water troughs, animal pens, tractor foot wells and scraper. Some of the other samples were unique to one or two of the farms only, but all environmental samples were from areas of the farm contaminated or potentially contaminated with calf or adult cattle faeces.

Approximately 30% to 50% of faecal samples were taken from high yielding cows and the remaining faecal samples were taken from other cattle on the farm including, for example, weaned and unweaned calves, low yielding cows and dry (i.e. non-lactating) cows.

Faecal, environmental and waste milk samples were examined for the presence of ESBL-producing bacteria, in particular CTX-M bacteria.

2.5 Analysis of antibiotics in waste milk

A total of 103 waste milk samples were screened for 14 compounds with a β-lactam structure; these comprised eight penicillins (amoxicillin, ampicillin, oxacillin, cloxacillin, dicloxacillin, nafcillin, penicillin G and V) and six cephalosporins (cefalexin, cefalonium, cefapirin, cefazolin, cefoperazone and cefquinome).

Confirmatory analyses were performed for seven β-lactam antibiotics that were detected during the initial screening exercise (amoxicillin, cloxacillin, penicillin G, cefalexin, cefalonium, cefapirin and cefquinome). An IS0/IEC 17025:2005 accredited procedure, validated to Commission Decision 2002/657/EC (Anonymous, 2002), was employed. Screening and confirmatory residue analyses were performed quantitatively by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

The follow-up visits to three of the farms that were positive for CTX-M-positive *E. coli* in waste milk yielded further waste milk samples for the second part of the study that were also examined for β-lactam antibiotics.

2.6 Isolation of bacteria from waste milk samples

Waste milk samples (n = 103) were diluted in sterile PBS and 100µl of suitable dilutions (Miles et al., 1938) were plated onto four different agars both to provide a bacterial counts on that media and to provide isolates for further study.

The agars were blood agar (BA) as a non-selective medium for aerobic bacteria, CHROMagar ECC (CA-ECC) for mainly *Enterobacteriaceae*, CA-ECC + 16 mg/L cefoxitin (CA-FOX) to select bacteria with an AmpC resistance phenotype and CHROMagar CTX (CA-CTX) for presumptive cefotaximase-producing bacteria. Plates were incubated overnight at 37° C with the exception of CHROMagar CTX plates which were incubated for ~ 48 hours at 37° C. Waste milk samples yielding less than 10 colonies on the above agars before dilution were plated on the same agar after 18-24 hours enrichment in buffered peptone water (BPW). The latter was achieved by adding 1 ml of waste milk per 9 ml BPW before overnight incubation at 37° C for 18-24 hours.

Stored waste milk samples (after thawing) were also plated onto Oxoid Brilliance ESBL agar (BRILL), before and after enrichment. Counts were not performed on this agar as the total bacterial count may have dropped during storage at -80° C with 10% v/v glycerol for between 5 days and ~ 8 weeks, depending on when the waste milk sample originally arrived.

194 *2.7 Isolation of bacteria from follow-up farms study*

195 Isolation of bacteria from faecal / environmental samples in the follow-up farm visits
196 used the same procedures as for waste milk samples, except that ~ 1 gram of cattle
197 faeces or environmental sample was added to 9 ml of BPW and plated directly to CA-
198 ECC, CA-FOX and CA-CTX agar plates. Samples yielding less than 10 cfu/ml *E. coli*
199 were plated to the same agar after enrichment at 37° C for 18-24 hours in BPW. For
200 waste milk samples from these follow-up visits, bacterial counts were performed
201 (Miles et al., 1938) and samples were also plated to BRILL agar.

202

203 *2.8 Phenotypic ESBL tests*

204 The ESBL phenotype was determined for presumptive *Enterobacteriaceae* identified
205 from the initial 103 waste milk samples using disc diffusion tests for clavulanate
206 synergy. Disk combinations used included cefotaxime and ceftazidime with and
207 without clavulanic acid as well as ESBL/AmpC detection discs.

208

209 *MICs.* MICs of cefotaxime, cefquinome, ceftazidime and cephalexin were determined
210 against representative ESBL isolates by the method of the British Society for
211 Antimicrobial Chemotherapy (BSAC, 2011).

212

213 *2.9 PCR and sequencing*

214 For isolates from the initial 103 waste milk samples, all isolates with an ESBL
215 phenotype were tested for the presence of CTX-M, OXA, SHV and TEM genes using
216 a multiplex PCR (Fang et al., 2008). The CTX-M genes from all CTX-M positive
217 isolates from these initial 103 waste milk samples were also sequenced to determine

the CTX-M type using primers as previously described for group 1 and group 9 CTX-M sequence types (Carattoli et al., 2008; Sabate et al., 2002).

As a large number of waste milk, faecal and environmental samples yielded isolates on CHROMagar CTX from the three farms that were revisited, only a proportion (~ 35%) were tested directly for CTX-M group 1 (Carattoli et al., 2008; Fang et al., 2008) and CTX-M group 9 (Sabate et al., 2002) using sequencing primers and the resulting amplicons were sequenced to determine the CTX-M sequence type. The ~ 35% of isolates that were selected for sequencing from the three farms that were revisited were chosen to be representative of all of the different CTX-M positive isolates with respect to animal groups or environmental sites.

2.10 Identification of bacteria

Approximately 120 isolates representative from the waste milk samples from the first part of the study only were tested by MALDI-ToF, as previously described (Toszeghy et al., 2012) to elucidate bacterial identity.

2.11 Serotyping ('O' antigen testing) of E. coli

A total of 17 *E. coli* from the 103 waste samples in the first part of the study were selected for 'O' antigen identification (serotyping) (Randall et al., 2011) and these included all the CTX-M-positive *E. coli* as well as other representative *E. coli* that had a putative ESBL phenotype by at least one test. From the second part of the study (follow-up visits to three farms positive for CTX-M bacteria in waste milk) a total of 68 representative CTX-M-positive *E. coli* were serotyped to include isolates from waste milk samples (n = 6) and faecal samples (n = 53) from all three farms, and isolates from environmental samples (n = 9) from one of the farms.

2.12 PFGE

PFGE of selected CTX-M-positive *E. coli* isolates was performed as previously described (Ribot et al., 2006). From the initial 103 waste milk samples, six CTX-M-positive *E. coli* from three different farms were tested by PFGE. From the second part of the study (follow-up visits to three farms positive for CTX-M bacteria in waste milk), a total of 58 representative CTX-M-positive *E. coli* were tested by PFGE. This included isolates from waste milk samples (n = 3) and faecal samples (n = 45) from all three farms, and isolates from environmental samples (n = 10) from one of the farms.

2.13 Replicon typing

Replicon typing to determine the plasmid type responsible for ESBL carriage was performed for selected isolates (n = 7) from the follow-up farms study only, as previously described (Carattoli et al., 2005) and also using the PBRT replicon typing kit (Diatheva) according to the manufacturer's instructions. Replicon typing was performed on transconjugants selected on agar containing cefotaxime as previously described (Toszeghy et al., 2012).

2.14 Analysis of association of on-farm antibiotic use and other herd factors and the presence of ESBL bacteria in milk

Pearson's correlation coefficient was used to determine if there was a relationship between (i) the numbers of waste milks positive for different antibiotics to the numbers that would be theoretically positive for antibiotics, based on the on-farm questionnaire data of cows treated with specific antibiotic and then contributing to

267 waste milk; (ii) the relationship between number of cows contributing to waste milk
268 that were treated with cefquinome and the concentration of cefquinome in waste milk.
269 Data collected in the questionnaire accompanying the waste milk samples were
270 processed using MS Access and analysed using Stata 12 (Stata Corporation, College
271 Station, TX, USA). Any variables with more than 20 % of records missing were
272 excluded from the analysis. Odds ratios were calculated to measure the association
273 between each variable and the presence of CTX-M-positive bacteria in the samples.
274 Associations were tested at the univariable level using logistic regression for
275 continuous variables, or Pearson's X^2 statistic for dichotomous variables. Variables
276 significant at the 0.2 α-error level were considered for inclusion in the multivariable
277 analysis with consideration given to the biological plausibility of associations.
278 Because of the low number of positive outcomes in the dataset, a forward stepwise
279 approach was used to build a logistic regression model in order to minimise the risk of
280 the model not converging. Variables were included or excluded based on the
281 likelihood ratio test. Herd size was treated as an *a priori* confounder, and was
282 transformed to a dichotomous variable with all values less than or equal to the median
283 of 142 coded as "0" and all values greater than the median coded as "1". The
284 likelihood ratio test was used to ascertain whether herd size should be included in the
285 final model as a dichotomous or continuous variable. The fit of the model was
286 assessed using the Hosmer & Lemeshow goodness of fit test (Hosmer and Lemeshow,
287 2000).
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3. Results

3.1 Antibiotics in waste milk from 103 farms

The mean, median, maximum, minimum concentration and number of waste milks positive for β-lactam antibiotics in waste milk samples are shown in Table 1 with other relevant details.

In all, 66 / 103 or 64.1% of waste milks examined for the β-lactam antibiotics were positive for at least one of the antibiotics. Of the 103 waste milk samples, 6.8%, 0%, 3.9% and 32.0% contained detectable concentrations of the β-lactam antibiotics amoxicillin, ampicillin, cloxacillin and penicillin G respectively and 5.8%, 7.8%, 2.9% and 21.4% were positive for the cephalosporin antibiotics cefalexin, cefalonium, cefapirin and cefquinome respectively (Table 1). The most commonly detected antibiotic in the samples was penicillin G, followed by the fourth generation cephalosporin cefquinome.

The concentrations detected for each antibiotic ranged from < 4 to 4600 μg/kg (~ < 0.004 to 4.6 mg/L). For cefquinome in particular, the antibiotic range for the 22 / 103 initial waste milk samples that contained detectable concentrations of cefquinome was 6 to 4600 μg/kg (~ 0.006 to 4.6 mg/L) with a mean (for positives only) of approximately 1.4 mg/L (Table 1).

There was a strong correlation (Pearson's $r = 0.983$, $p < 0.001$) between actual antibiotics found in waste milk and the reported antibiotics used to treat cows that contributed to specific batches of waste milk. However, for most antibiotics, the theoretical number of waste milks that could have contained antibiotic, based on treatment history, was higher than the number of waste milks positive for specific antibiotics. Relatively few waste milks gave unexpected results of antibiotics being detected that were not reported in the treatment history of contributing cows.

In addition to the antibiotics investigated above, waste milks (number) also included milk from cows treated with dihydrostreptomycin (36), framycetin / neomycin (43), kanamycin (7), lincomycin (1), marbofloxacin (2), novobiocin (32), oxytetracycline (4), streptomycin (5), sulphadiazine (3), trimethoprim (3) and tylosin (10).

3.2 Antibiotics from waste milk from the three re-visited farms

In the follow-up study, for the three re-visited farms (re-visited ~ 10 months after the initial sampling), cefquinome was found in the waste milk samples from all three farms, over the range < 4 to 27,000 µg/kg (~ < 0.004 to 27 mg/L) as shown in Table 2. The mean cefquinome concentration for all of the nine waste milk samples collected as part of the follow-up study was 3,763 µg/kg . These waste milk samples were also tested for the other antibiotics (as in Table 1), but were negative for these.

3.3 Cows contributing cefquinome to waste milk or injected with cefquinome

Twenty two of the 103 waste milk samples were positive for cefquinome. According to the farmer's records, for 21 of these waste milk samples, at least some of the cows contributing to the waste milk had been treated with a cefquinome product. No relationship was observed between the number of treated cows contributing to the waste milk sample and the concentration of cefquinome detected in the sample (Pearson correlation coefficient 0.177, not significant at the 5% level with n-2 degrees of freedom).

3.4 Isolation of bacteria from different agars

Before enrichment, 99%, 85%, 62.1% and 21.4% of samples yielded bacterial isolates on blood agar (total aerobic bacteria), CHROMagar ECC (*Enterobacteriaceae* and

some non-fermentative Gram-negative organisms such as *Pseudomonas*), CA-FOX and CA-CTX respectively respectively.

The mean total count on blood agar was 1.4 x 10^7 cfu/ml. Mean counts for presumptive *Enterobacteriaceae* [presumptive *E. coli*] on CA-ECC, CA-FOX and CA-CTX were ~ 4.0 x 10^4 [~ 8 x 10^3], ~ 6.0 x 10^3 [~ 20], ~ 6.0 x 10^3 [~ 10] respectively.

Although it was not possible to compare CHROMagar CTX and BRILL (the two agars for isolation of putative ESBL) directly, as samples were plated to BRILL after a period of storage, a similar number of waste milks were positive for CTX-M bacteria on both these agars, and these two agars were the most effective for isolation of CTX-M bacteria.

3.5 Identification of isolates in waste milk.

One hundred and twenty isolates from 65 of the farms were identified by MALDI-ToF to give an idea of the main aerobic bacterial species present in waste milk. These isolates were identified as (number identified): *Escherichia coli* (25), *Hafnei alvei* (15), *Kluyvera intermedia* (9), *Enterobacter cloacae* (8), *Hafnia* species (6), *Kluyvera* species (5), *Staphylococcus* species (3), including one *aureus* (methicillin sensitive) and one *equorum* with good IDs but also one *sciuri* with confidence level to genera only, *Serratia* species (4), *Citrobacter braakii* (3), *Citrobacter* species (3), *Raoultella terrigena* (3), *Aerococcus viridans* and species (2), *Aeromonas* spp. (2), *Streptococcus uberis* (2), *Yersinia* species (2), other *Citrobacter* (2), *Enterobacter* species (1), *Delftia* species (1), *Enterococcus faecalis* (1), *Enterococcus italicus* (1), *Pseudomonas tolaasii* (1), *Raoultella* species (1), *Yersinia enterocolitica* (1), not identified (18).

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368 *3.6 Putative AmpC phenotype of isolates*

369 Considering only blue (presumptive *E. coli*) and purple (non *E. coli* but
370 *Enterobacteriaceae*) colonies, then 48 waste milk samples (farms) yielded isolates
371 (blue 14.5%, purple 85.5%) that had an AmpC phenotype, including those with an
372 ESBL and AmpC phenotype. About 50% of these blue and purple individual colonies
373 from the 48 milk samples as above were identified by MALDI-ToF and were
374 *Citrobacter, Enterobacter, Escherichia, Hafnia, Kluyvera* and *Yersinia* spp.

375

376 *3.7 Putative ESBL phenotype isolates and confirmed CTX-M isolates*

377 If only blue (presumptive *E. coli*) and purple (presumptive *Enterobacteriaceae* other
378 then *E. coli*) colonies are considered, then 7 /103 waste milk samples from the initial
379 survey yielded isolates that were positive for an ESBL phenotype by at least one test
380 (Table 3), but also negative for an AmpC phenotype (results not shown). Of these
381 seven waste milk samples yielding isolates with a putative ESBL phenotype based on
382 the above criteria, six of the waste milk samples were positive for CTX-M bacteria of
383 sequence types 1, 14, 14b and 15 (Table 3). The seventh waste milk sample that was
384 positive for an *E. coli* with a putative ESBL phenotype, was positive for the bla_{TEM}
385 family of genes, but was not examined further. Four of the six CTX-M positive farms
386 were positive for CTX-M-positive *E. coli* in waste milk, whilst two of the farms were
387 positive for the $bla_{\text{CTX-M}}$ gene in *Citrobacter* or *Enterobacter*. In all, the $bla_{\text{CTX-M}}$ gene
388 was detected in *Citrobacter, Enterobacter, Escherichia coli, Kluyvera* and *Raoultella*
389 (Table 3). The MICs of the cephalosporin antibiotics tested against these

representative ESBL isolates were in the range 1 to > 128 μg/ml for the CTX-M positive isolates (Table 3)

3.8 Statistical analysis

Factors explored for associations with the presence of CTX-M-positive bacteria in waste milk are listed in Table 4. Significant associations at the univariable level were only identified for the detection of cefquinome in the milk (OR = 23.53; $p < 0.01$) and the reported use of cefquinome by the farmer (OR = 13.65; $p < 0.01$). A weak association was observed with increasing herd size (OR = 5.54; $p = 0.08$) and with increasing quantity (litres) of milk produced per year (OR = 1.00; $p = 0.1$). The reported use of cefquinome and litres of milk produced per year were excluded from the regression model based on comparison of the model with and without these variables using the likelihood ratio test. There was no statistical evidence that herd size should rather be included in the final model as a continuous variable rather than a dichotomous variable (the p value for the likelihood ratio test was < 0.01 for both models). The odds ratios for the variables included in the final model are listed in Table 5. The model showed no evidence of lack of fit based on the Hosmer-Lemeshow test statistic ($p = 0.11$). The large odds ratio observed for the detection of cefquinome in the milk suggested that CTX-M-positive bacteria were 20 times more likely to be present in waste milk when cefquinome was also present. A significant association was not observed between herd size and the presence of CTX-M-positive bacteria in waste milk in the final model, but herd size was retained in this model since it modified the effect of presence of cefquinome from 23.53 to 20.35 and improved the fit of the model based on the likelihood ratio test ($p = 0.0016$).

3.9 Farms re-sampled – CTX-M-positive E. coli

All farms that were re-sampled were still positive for CTX-M-positive *E. coli* in the waste milk and also had CTX-M-positive *E. coli* in 33.3% to 74.4% of all faecal samples (Table 2).

Each of the different calf and adult cattle groups on farms which were re-sampled harboured CTX-M-positive *E. coli,* and one of the farms (farm B), was also positive for CTX-M-positive *E. coli* in most of the environmental samples taken (Table 2). For farm A, a greater proportion of faecal samples from calves were positive for CTX-M-positive *E. coli* compared with faecal samples from older animals (Table 2).

Two of the re-sampled farms (A and B) were positive for CTX-M sequence type 15 only (same CTX-M type as first sampling) and this was associated with a range of serotypes and plasmid type I1-γ (Table 2). None of the serotypes were the 'O' 25 serotype of the human pandemic strain (Lau et al., 2008). The remaining farm (C) was positive for *E. coli* with CTX-M sequence types 14 and 15 in waste milk and faecal samples, whilst the initial waste milk sample had been positive for *E. coli* with CTX-M sequence type 14 only. One CTX-M 14 isolate tested for replicon type from this farm had an N replicon type.

3.10 PFGE types

PFGE of 64 CTX-M positive *E. coli* obtained from both the first part of the study (103 collected waste milks) and the second part of the study (three of the farms positive for ESBL *E. coli* sampled) showed 17 different PFGE profiles that corresponded to some

extent with the different serotypes seen when an 80% cut-off was used (Figure 1). This demonstrates that the CTX-M plasmids were present in a diverse array of *E. coli* isolates as well as in some of the non *E. coli* isolates that were not subjected to PFGE. Of particular interest was the observation that one PFGE type associated with *E. coli* serotype O128 was present in a CTX-M 15 *E. coli* isolate from the waste milk from the first part of the study from farm B, and also from seven faecal samples (from weaned calves, low yielding cows and lame cattle) and three environmental samples from the same farm in the second part of the study. This shows the presence of this particular CTX-M type, serotype and PFGE strain type in waste milk, animals and their environment over approximately a 10 month period. A similar scenario was also observed for farm A, where one PFGE strain type associated with *E. coli* serotype O45 was present in CTX-M-15 *E. coli* from waste milk in the first part of the study, and then from one faecal sample (from an in-calf heifer) from the same farm ~ 10 months later.

4. Discussion

Cefquinome was found in 21.4% of the waste milk samples and this can be compared with the proportion of farmers (29%) who stated in a recent survey (Brunton et al., 2012) that they used cefquinome intra-mammaries as first choice treatment for mastitis in lactating cows. Cefalonium was present in 7.8% of waste milk samples and has been reported to be used as a first choice treatment by 43% of farmers in treating cows at the end of lactation in what is referred to as “dry cow therapy” (Brunton et al., 2012). Since dry cow therapy is administered approximately once annually, and waste milk sampling was done on a single occasion during the year, the apparent difference with the previous study (Brunton et al., 2012) is easily reconciled. There was a strong

correlation (Pearson's r = 0.983, p <0.001) between antibiotics reportedly used by farmers and those found in waste milk.

High bacterial counts (Wray et al., 1990) and antibiotic resistant bacteria have previously been shown to be present in waste milk (Selim and Cullor, 1997). In the study of Selim and Cullor (Selim and Cullor, 1997), *Streptococcus* species (84/165 samples) and *Enterobacteriaceae* (83/165 samples) were the predominant bacterial species identified, followed by *Staphylococci* (68/165 samples). *Escherichia coli* was the Gram-negative species most commonly isolated (52/165 samples; 32%). This corresponds to some extent with the findings of this study, in that ~ 70% and ~ 40% of waste milks without enrichment were positive for presumptive *Enterobacteriaceae* or presumptive *E. coli* respectively on CHROMagar ECC.

In this study only a low number of waste milk samples contained bacteria with CTX-M genes of sequence types 1, 14, 14b or 15. Cefquinome residues in waste milk were the only antibiotic residues significantly associated with the presence of CTX-M bacteria in waste milk, and 5 / 6 milks that contained CTX-M bacteria also contained detectable cefquinome residues. Although the odds of a sample containing CTX-M bacteria were increased when cefquinome was present in the waste milk, it is important to note the large confidence interval obtained indicating that this odds ratio should be interpreted with caution. This wide confidence interval could be due to the relative rarity of finding a positive sample. An increase in sample size would have increased the power of the study to detect weaker associations with other variables. Although herd size was not found to be significantly associated with the presence of CTX-M-positive bacteria in waste milk, it was found to modify the effect of the detection of cefquinome in the samples. Snow et al., (2012) similarly found herd size to be weakly associated with the presence of ESBLs on dairy farms at the univariable

level but not at the multivariable level. It is plausible that farms with more adult dairy cattle will use greater amounts of cefquinome making it more likely to be detected in waste milk.

Sequencing, PFGE and serotyping clearly showed in some cases the same CTX-M sequence type and similar / identical clones of *E. coli* in both waste milk samples and in animal faecal samples and their environment. Persistence seems likely, though repeated re-introduction from external sources remains a possibility.

Since cefquinome was the only antibiotic associated with ESBL-producing bacteria, it is pertinent to consider the concentrations at which it was found in waste milk. The concentrations were 6 to 4,600 μg/kg for positive samples detected in the initial 103 waste milk samples or ~ 0.006 to 4.6 mg/L with a mean of ~ 1.4 mg/L. The mean concentration of cefquinome observed should be sufficient to kill most *Enterobacteriaceae* that lacked some form of acquired resistance such as an ESBL gene and as such should provide a selective pressure for ESBL-producing bacteria. Even the highest cefquinome concentration of approximately 27 mg/L (in one of the waste milks from re-sampled farm A) represents a sub-MIC concentration for some of the ESBL isolates from waste milk in this study, and as such confers a potential to select, rather than kill, some ESBL bacteria. In a work reported elsewhere (Orden et al., 1999), the MIC_{90} of cefquinome against 195 *E. coli* from calves was 0.125 mg/L, whilst for CTX-M isolates from this study, the cefquinome MICs were 2 to 64 mg/L. Based on these values, if one considers concentrations of 1 mg/L and above as concentrations of cefquinome that are likely to select for ESBLs if they are present on a particular farm, then about 10% of the waste milks examined contained these concentrations. Once waste milk is ingested by the calf, the situation is obviously

514 complex in relation to the intestinal concentration achieved and likely effect on the
515 intestinal bacterial flora.
516 In a study, where five lactating cows (suffering from clinical mastitis) were treated
517 with cefquinome by simultaneous intramammary and intramuscular injection,
518 maximum cefquinome residues of 10 to 27 mg/L were found in the waste milk from
519 treated udders (Thal et al., 2011). This correlates well with the maximum cefquinome
520 concentrations which were detected in the waste milk samples from this study. In
521 view of the dilution of waste milk from treated cows with that from untreated cows
522 and that waste milk must be discarded for 84 hours (7 milkings if cows are milked
523 twice daily) after the last cefquinome treatment, there is the potential to produce the
524 range of concentrations of cefquinome observed in waste milk in this study.
525 One solution to prevent calves being exposed to antibiotic resistant bacteria in waste
526 milk would be to pasteurise all waste milk fed to calves. This is recommended
527 practice in some publications (Godden et al., 2005; Jamaluddin et al., 1996), since
528 waste milk may contain pathogenic bacteria such as *Mycobacterium avium*
529 *paratuberculosis*, the bacterium causing Johne's disease, *Salmonella* spp.,
530 *Mycoplasma* spp., and *Escherichia coli* (AFIA, 2008). However pasteurisation is not
531 likely to alter the activity of many antibiotics in waste milk, which may then exert a
532 selective pressure on the intestinal flora (AFIA, 2008), and this study shows that
533 ESBL *E. coli* are generally widespread in the farm environment on farms where such
534 bacteria occur.
535 To conclude, cefquinome, a fourth generation cephalosporin antibiotic, was detected
536 in some waste milk samples tested and was significantly associated with the presence
537 of CTX-M-positive bacteria. Subsequent visits to three of the farms positive for both
538 cefquinome and CTX-M-positive *E. coli* in waste milk showed evidence of CTX-M-

positive *E. coli* in all of the different groups of animals tested and for one farm, also in the environment. The relative importance of waste milk versus other potential sources (such as the environment or other animals) in exposing calves to colonising CTX-M-positive *E. coli* is not known. Similarly, the relative importance of antimicrobial residues in waste milk in exerting a selective pressure influencing the colonization of calves by ESBL *E. coli* remains to be determined.

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Transparency Declarations

CHROMagar CTX was developed at the AHVLA as reported previously.(Randall et al., 2009) The transparency statement used in the previous publication is as below:-

The details of the specific formula to make CHROMagar EEC selective for the isolating of CTX-M bacteria have been sold to CHROMagar (France) by the Veterinary Laboratories Agency, UK. CHROMagar (France) have filed a patent application relating to the formula used in CHROMagar CTX. L. P. R and F. C.-H. received a fixed sum 'inventors' reward for their part in the development of the agar. All other authors: none to declare.

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682 **Table 1.** β-lactam antibiotics in waste milk samples from 103 farms in England and

683 Wales

Waste milk results type for antibiotics detected	Antibiotic detected [μg/kg]							
	AMX	AMP	CLX	PEN-G	LEX	CFL	HAP	CFQ
Number waste milks positive	7	0	4	33	6	8	3	22
Mean conc. waste milks > LOD	258	NA	80	371	132	98	882	1433
Median conc. waste milks > LOD	18	NA	43	120	25	26	140	985
Mean conc. all waste milks	18	NA	3	119	8	8	26	306
Median conc. all waste milks	< LOD	NA	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Maximum conc.	1300	NA	230	2500	660	580	2500	4600
Minimum conc. > LOD	5	NA	4	5	4	6	5	6
95% of samples at/below conc.	6	4	4	700	4	18	4	2200
Detection limit	4	4	4	4	4	4	4	4
No. added [a]	14	3	8	45	13	8	3	34
Unexpected positive [b]	2	0	1	5	1	3	0	1
MRL μg/kg [c]	4	4	30	4	100	20	60	20

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686 AMX - amoxicillin; AMP - ampicillin; CLX - cloxacillin; PEN-G - penicillin G; LEX
687 – cefalexin (first generation cephalosporin); CFL – cefalonium (first generation
688 cephalosporin); HAP – cefapirin (first generation cephalosporin); CFQ – cefquinome
689 (fourth generation cephalosporin); conc. – concentration; LOD – limit of detection;
690 NA – not applicable.
691 *a* – No. added refers to the number of farms that reported adding stated antibiotic to
692 the waste milk tank via milk from treated cows;
693 *b* – Unexpected positives are where the sample was positive for stated antibiotic but
694 the farmer had not reported in a voluntary survey the recent use of the stated antibiotic
695 for cows contributing to waste milk. This does not relate to non-compliance with farm
696 recording obligations.
697 *c* – MRL - Maximum residue limit in milk (Commission regulation [EU] No
698 37/2010).

Table 2. Presence of CTX-M-positive *E. coli* on three farms previously found to have CTX-M-positive *E. coli* in waste milk.

Farm ID	Sample type	No. of samples	% + CTX *E. coli* [b]	CTX-M type [c]	Sero-types of presumptive CTX-M isolates [c] (No. tested) [cefquinome concentrations in waste milk in µg/kg]	Plasmid type (No. tested)
A	Environmental	10	0			
	All faecal [a]	90	33.3	15	8,11,12,22,45,73,75,77,101,RO (23)	
	Waste milk	3	33.3	15	UT (1) [970; 2,000; 27,000]	
	Calves on MA	2	100	15	12,77 (2)	I1- γ (1)
	Calves on MP	6	66.6	15	77,101 (3)	
	Calves on CL	2	100	15	77, RO (2)	
	Calves on WM	16	75.0	ND	12, 22,75 (5)	
	HY cows	45	17.8	15	73,75,77 (5)	I1- γ (1)
	LY cows	9	22.2	15	75 (1)	
	Other cows	10	50	15	8,11,45,75,RO (5)	I1- γ (1)
B	Environmental	10	90.0	15	2, 20,77,128, 147, UT (9)	I1- γ (1)
	All faecal [a]	90	74.4	15	2,71,77,101,128,147, UT (16)	
	Waste milk	3	66.7	15	71,UT (2) [360; 1,100; 1,700]	I1- γ (1)
	Calves UW	17	100	15	77,101, UT (3)	
	Calves WE	12	100	15	2,128 (3)	
	HY cows	38	47.4	15	147,UT (3)	I1- γ (1)
	LY cows	17	88.2	15	77,128 (3)	
	Other cows	5	100	15	71,128 (4)	
C	Environmental	10	0			
	All faecal [a]	90	55.5	14+15	6,8,64,101,RO,UT (14)	
	Waste milk	3	100	14+15	8,68,UT (3) [<4; <4; 740]	N (1)
	Calves UW	22	50.0	14	8, RO,UT (3)	
	HY cows	30	56.7	14	8,101,UT (3)	
	LY cows	20	60.0	14	101,UT (3)	
	Cows on AB	8	75.0	14+15	64, 101 (3)	
	Cows dry	10	40.0	14	6,8 (2)	

ND - not determined; UT - un-typable; RO - rough; AB - antibiotic; CL - colostrum; HY - high yielding; LY - low yielding; MA - milk machine; MP - milk powder; UW - unweaned; WE - weaned; WM - waste milk.

a - Faecal samples were from a representative spectra of the cattle on the farm as detailed. *b* - Isolates that grew as blue colonies on CHROMagar CTX were presumed to be CTX-M-positive *E. coli. c* - Approximately 35% of presumptive CTX-M bacteria were tested for CTX-M sequence type.

Table 3. Farms (waste milk samples) positive for representative[a] *Enterobacteriaceae* that were AmpC negative by MAST disks, but ESBL positive by at least one MAST disk.

Farm ID	Identification by MALDI-ToF		PCR and sequencing		MICs of antibiotics against isolates (µg/ml)				Antibiotic concentration in waste milk [µg/kg]	
	Genus	species	COST PCR	CTX Type	CTX	CEQ	CAZ	LEX	Anti-biotic	Conc.
D	ESCHERICHIA	coli	T		0.06	0.03	0.25	4	CLX	45
A[b]	ESCHERICHIA	coli	C	15	128	32	16	>128	CFQ	3500
B[b]	ESCHERICHIA	coli	CT	15	128	32	4	>128	CFQ	2500
E	ESCHERICHIA	coli	CT	1	128	32	4	>128	CFQ	1400
E	ESCHERICHIA	coli	C	1	8	4	1	64	CFQ	1400
E	KLUYVERA[b]	intermedia	C	1	16	8	2	64	CFQ	1400
E	RAOULTELLA	terrigena	C	1	8	4	2	64	CFQ	1400
F[c]	ENTEROBACTER	cloacae	C	14b	128	64	8	128	CFL/CFQ	18/1100
C	ENTEROBACTER	cloacae	C	14	8	4	1	64	CFQ	590
C	ENTEROBACTER	cloacae	ST		16	2	128	128	CFQ	590
C	ESCHERICHIA	coli	C	14	16	4	1	64	CFQ	590
C	ESCHERICHIA	coli	ST		1	0.25	16	8	CFQ	590
G	CITROBACTER	species	CT	1	16	2	4	64	PEN-G	27

Grey highlight denotes farms that were chosen up for follow-up sampling ~ 10 months after initial sampling of waste milks – see Table 2.

CTX – cefotaxime, CEQ – cefquinome, CAZ – ceftazidime, LEX – cefalexin.

a - For each farm, results are shown for only one isolates of a particular bacteria species for a particular phenotype and genotype.
b - *Kluyvera* species are the natural and ancestral host of $bla_{CTX\text{-}M}$ genes.
c – On these three farms there were records of cattle contributing to waste milk receiving cefquinome by injection
See also table 1 for abbreviations, also CFP - cefepime. COST PCR, PCR for CTX-M (C), OXA (O), SHV (S) and TEM (T) genes.

Table 4. Univariable analysis of the association between antibiotics in waste milk and farm management factors and the presence of CTX-M bacteria in waste milk

Exposure Variable	Level	CTX status		OR [a]	95 % confidence interval	Chi *P* value [b]
		Positive	Negative			
Antibiotic treatments						
Cefquinome detected in milk	Yes	5	17	23.53	2.32-1125.89	0.0001
	No	1	80	*1.00*		
Amoxicillin detected in milk	Yes	0	6	0.00	0.00-10.96	0.5302
	No	6	91	*1.00*		
Penicillin G detected in milk	Yes	1	32	0.41	0.01-3.87	0.4057
	No	2	65	*1.00*		
Cefalexin detected in milk	Yes	0	6	0.00	0.00-10.96	0.5302
	No	6	91	*1.00*		
Cefalonium detected in milk	Yes	1	7	2.57	0.05-28.06	0.4013
	No	5	90	*1.00*		
Cefapirin detected in milk	Yes	0	3	0.00	0.00-23.44	0.6620
	No	6	94	*1.00*		
Cloxacillin detected in milk	Yes	0	4	0.00	0.00-17.22	0.6119
	No	6	93	*1.00*		
Cefquinome use reported by farmer	Yes	5	26	13.65	1.39-654.47	0.0034
	No	1	71	*1.00*		
Farm management						
Herd size (adult dairy cattle)	≤ 142	1	51	*1.00*		0.0878
	>142	5	46	5.54	0.58-267.18	
Number of cows contributing milk to the sampled container	≤ 2	2	53	*1.00*		0.3100
	> 2	4	44	2.41	0.32-27.58	
Number of hours waste milk is stored before feeding	≤ 2 hours	3	69	*1.00*		0.2734
	> 2 hours	3	28	2.46	0.31-19.35	
Volume of sampled container	≤ 20 litres	3	54	*1.00*		0.7863
	> 20 litres	3	43	1.26	0.16-9.83	
All waste milk used by the end of the day?	Yes	4	60	*1.00*		0.8136
	No	2	37	0.81	0.07-5.99	
Continuous variables				**OR**	**95 % CI**	P_{wald}
Time since last treatment of cows contributing to waste milk: cefquinome				1.07	0.96-1.19	0.248
Time since last treatment of cows contributing to waste milk: any antibiotic				0.99	0.93-1.04	0.658
Litres of milk produced per year				1.00	0.99-1.00	0.102

[a] Odds ratios are rounded to two decimal places. The baseline level is indicated by an OR of 1.00 in italics.

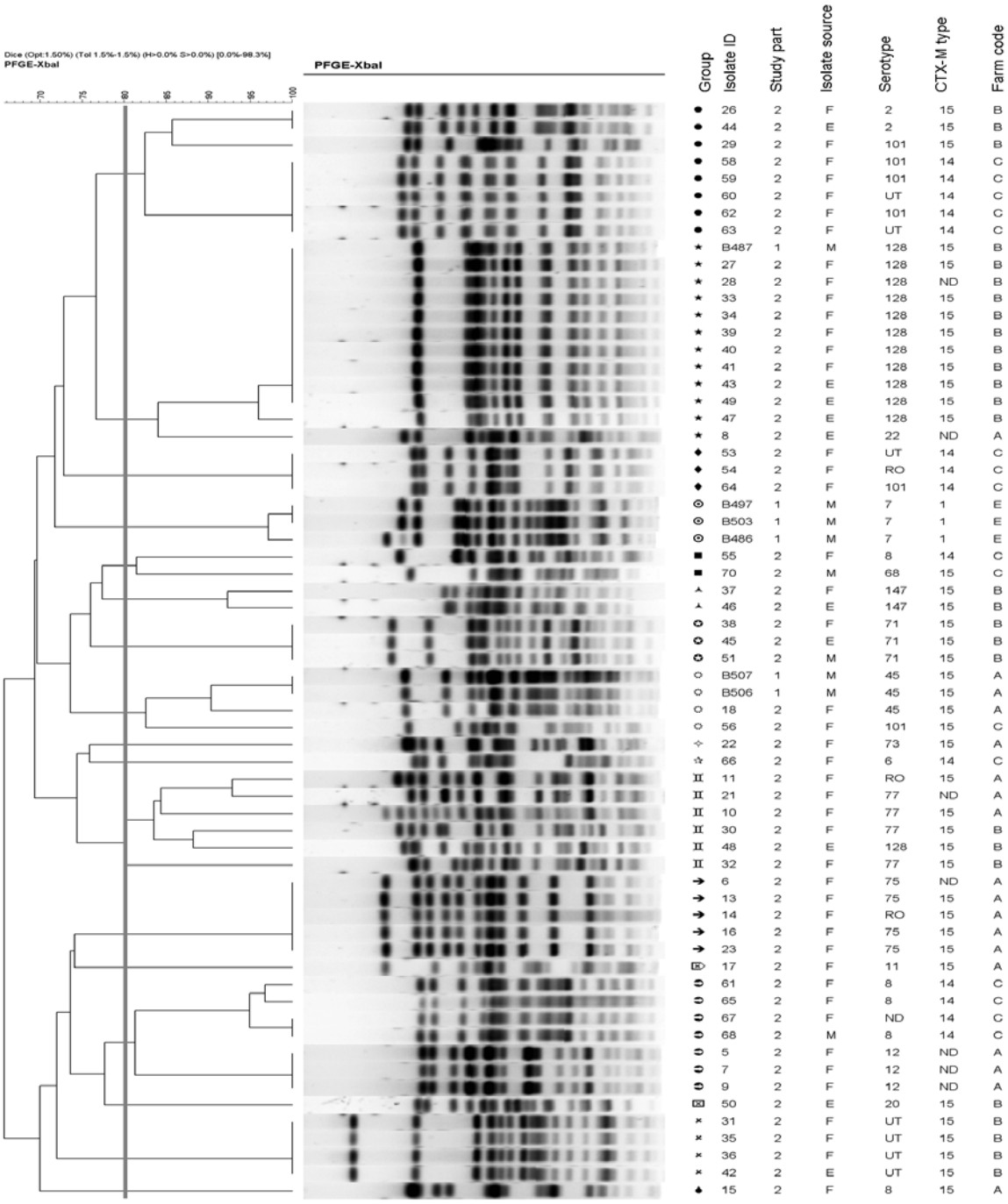
[b] Underlined P values indicate variables considered as significant or approaching significance that were considered for the multivariable analysis.

Table 5. Multivariable model results

Variable	Level	Odds ratio	95 % confidence interval	P_{wald}
Cefquinome detected in milk	No			
	Yes	20.35	2.19 – 189.14	0.008
Herd size (adult dairy cattle)	≤ 142			
	>142	4.06	0.42 – 39.72	0.228

Hosmer-Lemeshow goodness of fit $p = 0.11$

Figure 1. PFGE profiles with serotypes for CTX-M-positive *E. coli* from waste milk, faeces and the farm environment.



Grey line and groups denote 80% cut off. Survey part – 1, initial 103 waste milk samples; 2 Milk (M), faeces (F) and environmental (E) samples from the three follow-up farms. UT - untypable; RO - rough.