

Evidence Project Final Report

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2. Project title
3. Contractor organisation(s)
4. Total Defra project costs (agreed fixed price)
5. Project: start date
end date

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Executive Summary

The highest prevalence of ESBL *E. coli* in cattle is in calves on most farms (Liebana and others 2006, Watson and others 2012, Schmid and others 2013) and it was hypothesized that feeding waste milk containing antibiotics, particularly the 3rd and 4th generation cephalosporins might be an important risk factor. The study was designed to assess this risk and also methods for risk mitigation. There was a lack of information of current practice on dairy farms regarding feeding of waste milk and this was addressed by a postal questionnaire.

In order to survey antibiotic usage and waste milk feeding practices on dairy farms in England and Wales a suitable sampling frame of dairy farmers was established and an adequate number of responses obtained. Of the 557 farms that responded to the survey, 514 (92%) kept dairy cows and reared calves to weaning and were therefore able to provide complete set of relevant information. An intramammary treatment containing cefquinome, a fourth generation cephalosporin, was the first choice on 29% of 514 farms surveyed for the treatment of mastitis during lactation. Eighty three per cent of respondents fed waste milk to calves and the most common reasons reported for this practice were to save money (44%), and because it was convenient (40%). Other reasons included it being a way of improving calf growth and immunity (18 of 50 other reasons given (36%)). Waste milk samples and further information on recent antimicrobial treatments were collected from 103 farms, randomly selected from the 404 respondents who fed waste milk and agreed to further participation. Waste milk samples were delivered to the respective laboratories for bacteriology and antibiotic residue analysis. When asked if they would consider stopping feeding waste milk to calves, 37 % of farmers said yes and 63 % no. When asked how waste milk would be disposed of if it was not fed to calves, 94 % said they would dispose of it in the wastewater or slurry system.

The 103 waste milk samples collected from these farms were initially screened for 14 beta-lactam antibiotics and subsequently analysed for 8 beta-lactam antibiotics detected in initial screening. Seventy-one waste milk samples were found to be 'positive', with residues > 4 µg kg⁻¹, for compounds with a β-lactam structure; of these 22 contained cefquinome, a 4th generation cephalosporin. The maximum cefquinome concentration detected was 4600 µg kg⁻¹ and the mean concentration of cefquinome in the 22 positive samples was 1470 µg kg⁻¹.

Investigations were carried out to assess whether cephalosporin residues in waste milk could be reduced. The stability of cefquinome in milk at different temperature and pH was investigated as well as the effect of fermentation and treatment with a beta-lactamase enzyme. The most effective method for degrading cefquinome in spiked milk was to increase the alkalinity to pH 10. The second most effective treatment for cefquinome degradation was to add β-lactamase. Heat treatment of unpasteurised milk at 37°C was a

simple and effective approach for degrading cefquinome within 24 hours, largely due to the fermentative effect of the endogenous flora, and it was considered likely that further heat treatment at higher temperatures would also be required to kill the endogenous flora present in unpasteurised milk. Such fermentation was more effective than heat treatment at 37°C of sterile UHT milk. Larger scale incubations (10 litres) of cefquinome spiked unpasteurised milk (containing a larger inoculum of endogenous ESBL *Enterobacteriaceae*), at 37°C for 8 hours, were sufficient to decrease cefquinome residues to negligible concentrations. However, at 60°C, heating for greater than 2 hours was required to kill the enriched bacterial population consistently. Cefquinome was not degraded by fermentation of waste milk containing endogenous ESBL *Enterobacteriaceae* at 10°C (i.e. close to the average UK outside air temperature), so using this method in UK would likely require heating.

The waste milk samples collected from 103 farms were also examined for Extended-spectrum beta-lactamase (ESBL)-producing bacteria, in particular the CTX-M ESBL that is most frequently detected in cattle (Hunter and others 2010, Snow and others 2012). Six samples were positive for CTX-M bacteria of which 4 were positive for CTX-M *E. coli*. The CTX-M types identified were 1, 14, 14b and 15, all previously detected in cattle in England and Wales (Hunter and others 2010, Snow and others 2012). Follow up sampling on 3 farms detected the same CTX-M types in cattle as were present in waste milk.

In subsequent work, ESBL *E. coli* free calves were colonised with a CTX-M-14 *E. coli* and then fed different concentrations of cefquinome in milk replacer. The concentrations used were based on the findings from the surveillance of waste milk collected from farms, with a maximum concentration of 15 µg/ml. There was no significant difference observed in the duration of shedding of CTX-M *E. coli* between groups of calves which received cefquinome in the milk and those which received un-spiked milk. Also the study did not demonstrate a significant increase in the numbers of CTX-M *E. coli* shed in groups of calves which received cefquinome in the milk, compared to controls. Possible reasons for this include the small sample size (each study group had 5 calves and there was large variation in shedding between calves), age of calves and strain of *E. coli* used for colonisation (which colonised very efficiently).

A larger on-farm field study was then completed comparing naturally ESBL *E. coli* colonised calves fed either waste milk (which might contain antimicrobial residues) or milk replacer (no antimicrobial residues) with 25 calves in each group. The two groups of calves were located on a farm known to be positive for ESBL *E. coli* and which also used third and fourth generation cephalosporins in dairy cows. Calves fed waste milk were heavier (gained 5.9kg per week compared with 4.0kg for control calves) and showed less disease (no disease reported in the group fed waste milk compared with ten animals treated in control group). The study indicated that feeding waste milk increased the prevalence of calves infected with CTX-M-positive *E. coli*, and increased the amount of resistant bacteria shed in the faeces. There was no significant difference between groups in the proportion of isolates that were resistant throughout the study. Shedding of CTX-M-positive *E. coli* persisted for longer in calves fed waste milk until after weaning.

A stochastic simulation model was developed to assess the risk of transfer of ESBL *E. coli* to calves via the feeding of waste milk from dairy cattle on the same farm. There is wide variability (and uncertainty) in the actual dairy cow prevalence of ESBL *E. coli* and sensitivity analysis of the model suggests that this is very important with regards to the predicted ESBL *E. coli* prevalence in calves. The scenario analyses indicated that completely stopping the practice of feeding waste milk to calves was the most effective measure, but the scenario assuming complete eradication of ESBL *E. coli* and residues from the waste milk was almost as effective. The model suggests that any potential intervention package targeted at the waste milk tanks would need to deliver a large reduction of both ESBL *E. coli* and residues in the waste milk tank to be considered effective. Contamination of waste milk with ESBL *E. coli* may be a variable which is highly dependent on (or related to) the degree of environmental contamination.

Finally, the results were reviewed and advice proposed for reducing selection for ESBL *E. coli* in calves through the feeding of waste milk. Completely stopping the feeding of waste milk was considered the most effective method in the scenario analyses above. Most farmers who do not feed waste milk feed milk replacer as an alternative, which would be an additional cost. However, although the calves fed waste milk in this study showed less disease, feeding milk replacer is likely to have health benefits, particularly in preventing Johne's disease but also in the control of other important diseases such as *Mycoplasma bovis* infection and salmonellosis. Other alternatives were also considered which would result in the elimination or reduction of cephalosporins in waste milk fed to calves. These could involve not using cephalosporins in the herd or managing waste milk so that milk containing cephalosporins or milk containing higher concentrations of cephalosporins is not fed to calves. A range of treatments of waste milk to reduce the concentration of cephalosporin were also reviewed and it was considered that a treatment that eliminated cephalosporin residues and ESBL *E. coli* would, if developed produce similar results to not feeding waste milk. These treatments of waste milk all require further work to establish if they would be practical solutions on farm. The possible environmental effects of disposing of unused waste milk containing antibiotics in slurry or wastewater require further investigation.

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
- the objectives as set out in the contract;
 - the extent to which the objectives set out in the contract have been met;
 - details of methods used and the results obtained, including statistical analysis (if appropriate);
 - a discussion of the results and their reliability;
 - the main implications of the findings;
 - possible future work; and
 - any action resulting from the research (e.g. IP, Knowledge Exchange).

Objective 01 - Survey of antibiotic usage and waste milk feeding on dairy farms in England and Wales

Objective 01/01 *Waste milk survey*

There was little current information on how widespread different waste milk feeding practices are in the UK dairy industry. The purpose of Objective 1 was to examine the current situation relating to antibiotic use in dairy cows, which might lead to the presence of antimicrobial residues in colostrum or waste milk and to investigate the practices associated with feeding these substances to calves.

Methods

A questionnaire was designed to collect information on farm practices relating to different therapeutic regimens and usage of veterinary antibiotic products. Questions about the occurrence of mastitis and lactating/dry cow treatments were included as well as questions relating to the feeding of waste milk to calves including any storage, fermentation or pasteurisation and the disposal of waste milk. Basic farm details were also collected such as the number of milking cows and husbandry practices.

A sampling frame of dairy farms in England and Wales, stratified by herd size, was constructed from the Cattle Tracing System (CTS) in which only farms with more than 50 animals of dairy breed were eligible for inclusion. The survey took place between September and November 2010. The questionnaire was sent to 2003 farms with a covering letter explaining the purpose and background to the study and a pre-paid envelope to return their response. Information on data protection was provided within the questionnaire and the results were analysed anonymously. A small inconvenience payment was offered for completion and return of the questionnaire. A MS Access database was constructed to hold the data obtained in the completed questionnaires and data were analysed using STATA (StataCorp. 2007: release 10) and MS Excel. Statistical testing was performed using the Wilcoxon rank-sum test and the Chi-squared test with Yates correction for two categories of data. Additional information on non-respondents was extracted from the CTS database and also analysed using STATA and MS Excel.

Results

At least 385 responses were required to estimate the proportion of farms carrying out any given practice with 5% accuracy and 95 % confidence. Of the 2003 farms surveyed, 557 (28%) successfully completed their questionnaire, 34 (1.7 %) did not wish to participate in the study, were no longer a dairy farm or had less than 50 milking cows, and 19 (0.95 %) responded after the deadline so were not included in the analysis. The remaining 1393 farmers did not respond. No significant difference was observed in herd size or farm location between respondents and non-respondents using the Wilcoxon rank-sum test.

Of the 557 farms that responded to the survey, 514 (92 %) kept dairy cows and reared calves to weaning and so could complete the questionnaire in full. The herd size (adult milking cows) reported by all respondents ranged from 1 to 1000 (median = 130). The mean number of cases of mastitis per herd in the previous year was 47.

Ninety-three per cent of respondents used antibiotic intra-mammary tubes to treat mastitis cases (95% CI = 91 %-95 %). The two most frequently used lactating cow antibiotic tubes contained dihydrostreptomycin, neomycin, novobiocin, and procaine penicillin (37 %) or the fourth generation cephalosporin cefquinome (29 %). Ninety-six per cent of respondents used antibiotic tubes at the cessation of lactation ("drying off"), and of these, 85 % treated all cows in their herd. The most frequently used dry cow antibiotic tube (43 %) contained cefalonium. Frequently used injectable antibiotics included tylosin (27 %), dihydrostreptomycin and procaine penicillin (20 %) and the third generation cephalosporin ceftiofur (13 %).

On the majority of farms surveyed, dairy heifer calves were left to suckle from their dams for up to 24 hours after birth (39 %), although around 15 % of farms did remove calves immediately. Eighty-three per cent of respondents (413) fed waste milk to calves. Most farms that fed waste milk to calves stated that their waste milk sometimes contained milk or colostrum from freshly calved cows and heifers that had received dry cow antibiotics or from lactating cows that had received antibiotic therapy (93 % and 90 %

respectively). Milk from high cell count cows was also reported to be sometimes included in waste milk fed to calves on over 70 % of farms. The majority of respondents that fed waste milk to calves fed it fresh (407/413; 99 %), while 6 % let it ferment prior to feeding. Very few respondents refrigerated, froze or pasteurised their waste milk (1 %, 1 % and 0.5 % respectively) (more than one answer per farm possible). Only 30 % (95% CI = 26 %-35 %) discarded the milk from the first milking after antibiotic treatment, and 87% fed milk to calves from cows with mastitis (95% CI = 83 %-90%).

The most common reasons reported for feeding waste milk to calves was to save money (44%), and because it was convenient (40%). Other reasons included it being a way of improving calf growth and immunity (18 of 50 other reasons given (36%%)).

Discussion

The results reported here are from the first survey in England and Wales on antibiotic treatment in dairy cows combined with the feeding of waste milk to calves. The response rate for this survey was 30 % which is low but not uncommon for mailed questionnaires. Monetary incentives were used in this study and are likely to have encouraged responses; although further methods such as follow-up contact could have been employed to improve the response rate. Despite the low response rate the sample size requirements were met. No significant difference was observed between the herd size of farmers who responded and those who did not. Additionally, the response rate did not appear to be associated with the location of the farm or the cattle holding density within the farms locality. However, since the practices within the underlying population could not be predetermined, it is not known whether antibiotic usage and waste milk feeding practices of respondents in this survey were truly representative of the general population.

Eighty-three per cent of responding dairy farmers who kept calves to weaning fed waste milk to calves. This practice has not been surveyed previously in the UK but the frequency of feeding waste milk to calves reported here is higher than reports from the United States Department of Agriculture's (USDA) National Animal Health Monitoring System where 30.6 % and 2.8 % of USA dairy farms were reported to have fed unpasteurised and pasteurised waste milk to dairy heifer calves respectively (USDA 2007).

This survey has shown that antibiotic intra-mammary tubes are commonly used in lactating cows in England and Wales, and that cefquinome, a fourth generation cephalosporin is the first choice treatment for mastitis on nearly a third of responding dairy farms (147/506). Cefquinome concentrations of between 10,000 and 27,000 ng ml⁻¹ have been detected in treated quarters, but this fell rapidly below the EU Maximum Residue Limit 2-3 days after the last intra-mammary dosing (Thal and others 2011). This indicates that discarding the first milking after treatment may reduce exposure of calves to this antibiotic in waste milk. However, this was done on only 30 % of dairy farms in this survey.

Antibiotic intra-mammary tubes are also commonly used at drying off, and often in the whole herd as a prophylactic measure. Therefore on farms where the whole herd is treated, all calves would normally receive colostrum containing antibiotic residues, unless they feed a powdered colostrum substitute which was rare in this study (approximately 3 %). All calves need to ingest colostrum during the first few hours of life to provide protection against infection, and it is beneficial to continue feeding colostrum, as the antibodies it contains have a local action within the gut to protect against enteric diseases. In this survey, the most commonly used dry cow intra-mammary antibiotic was cefalonium, a first generation cephalosporin.

Fermentation of colostrum and waste milk may degrade certain antibiotics, however the appropriate conditions for fermentation are difficult to achieve in the UK (Wray and others 1990). This survey has shown that only 5 % and 6 % of responding dairy farms in England and Wales ferment their colostrum or waste milk respectively. Pasteurisation of waste milk before feeding has been recommended to reduce microbial load. However according to this survey this practice is rarely carried out. This is similar to findings from studies in the USA and Canada (Kehoe and others 2007; Vasseur and others 2010). The low occurrence of this practice may well be due to the significant investment required to routinely pasteurise small volumes of liquid. Even when used, pasteurisation is unlikely to affect the antimicrobial concentration in milk and may reduce the immunological benefits of colostrum.

More than a third of the farms surveyed in England and Wales leave calves to suckle their own dam for up to 24 hours after birth (39 %), while 15 % remove calves from their dam straight away. An American study of dairy farms in Pennsylvania reported that it was common practice to remove dairy heifer calves from their dam straight away with 87 % of farms feeding colostrum via a bucket or bottle (Kehoe and others 2007).

The most common reason for farmers to feed waste milk to calves was to save money. If feeding of waste milk containing antibiotic residues is shown to be associated with the occurrence of ESBL *E. coli* in calves then there could be economic implications in advising not to feed it. This survey provides an overview of current practices on a large sample of English and Welsh dairy farms in relation to antimicrobial usage and the feeding of waste milk, information which has not been available previously.

The following paper describing the results of Objective 1 has been published in Veterinary Record and is provided in Appendix 1:

BRUNTON, L. A., DUNCAN, D., COLDHAM, N. G., SNOW, L. C. & JONES, J. R. (2012). A survey of antimicrobial usage on dairy farms and waste milk feeding practices in England and Wales. *Veterinary Record* 171, 296

Objective 01/02 – Collection of waste milk

As part of the survey above, farms were asked if they would be willing to provide a waste milk sample for analysis, and 404 farms responded positively to this request. Farms were stratified by milking herd size with the aim to achieve a distribution of herd size that represented the surveyed population. One hundred and twenty farms were randomly selected across the strata, with the number selected from each stratum proportionate as a percentage of 120 to the percentage of total farms in that stratum. Herd size, location and antibiotic use among the population of 120 farms was assessed and found to be similar to the underlying population of surveyed farms (Objective 01/01). Each of the 120 farms was contacted by telephone and 103 were recruited to provide a sample of waste milk being fed to calves. Sampling kits were sent to each farm so that the farmers could collect the samples themselves and information was collected on the treatments the contributing cows had received. Each farm was allocated to a former-VLA regional laboratory based on its location, and staff from the regional laboratories were responsible for collection of the samples from farmers on the day they were taken. The samples were aliquoted at the regional laboratories where part of the sample was sent chilled to AHVLA Weybridge for bacteriology (see Objective 4); while the remainder was frozen at -80°C and sent to FERA for analysis for antimicrobials (see Objective 2).

Not all farms completed the questionnaire accompanying the waste milk samples, but data was obtained from 99 farms, although not all of these farms answered every question. The average number of cows contributing to the total waste milk being fed when the samples were collected was 5 and ranged from 0 to 60. Considering the containers from which the samples were collected, on average 4 cows had contributed to each container (range: 0-60). The average number of calves that received waste milk at the feeding time at which the samples were collected was 15 and ranged from 0-140, while on average 12 calves would receive waste milk from the specific container sampled (range: 0-140). The most commonly used antibiotic in cows contributing to the waste milk sample was penicillin G. Cefquinome was recorded as being used as a treatment in cows contributing on 37 farms.

Questions about waste milk storage were also asked. The median time that waste milk was stored was 1 hour, although 17 % of farms stored waste milk for more than 5 hours. In cold conditions, 92 % of farms fed waste milk fresh while 3 % refrigerated, 1 % froze and 10 % fermented waste milk. In warm conditions, 89 % of farms fed waste milk fresh while 3 % refrigerated, 1 % froze and 11 % fermented waste milk. No farms pasteurised waste milk under cold conditions, but four farms did pasteurise under warm conditions.

On average, waste milk was fed to calves twice a day (93 % of farms) and the average amount of waste milk fed to each calf at each feed was 3 litres. Around 37 % of farms had waste milk left over at the end of the day. On these farms, 60 % disposed of the milk while 40 % fed it the next day with 91 % of those using it the next day storing it at room temperature.

When asked if they would consider stopping feeding waste milk to calves, 37 % of farmers said yes and 63 % no. When asked how waste milk would be disposed of if it was not fed to calves, 94 % said they would dispose of it in the waste or slurry system.

The data gathered with the waste milk samples provides more detailed information on the composition of waste milk on farms and feeding and storage practices. The potential for antibiotics to have a selective effect in the slurry/ waste water and subsequent environmental contamination needs to be considered since responses indicate that this would be the most common method of disposal of waste milk.

Objective 2 Antibiotic residues in waste milk

Objective 02/01 Waste milk sample receipt

Objective 02/02 Screening waste milk samples for antibiotic residues

Objective 02/03 Confirmation of antibiotic residues in waste milk

A total of 103 waste milk samples were received at Fera in March 2011 (objective 02/01). These samples were screened for 14 compounds with a β -lactam structure (amoxicillin, ampicillin, cloxacillin, dicloxacillin, nafcillin, oxacillin, penicillin G, penicillin V, cefalexin, cefalonium, cefapirin, cefazolin, cefoperazone and cefquinome); objective 02/02). Objective 02/02 was completed on 28 June 2011. It was subsequently agreed with the project leader that all 103 samples would be subjected to a confirmatory method based on Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) to provide quantitative data for eight β -lactam antibiotics that were detected during the initial screening exercise (i.e. amoxicillin, ampicillin, cloxacillin, penicillin G, cefalexin, cefalonium, cefapirin and cefquinome).

In the original project plan a selection of the 103 samples were also to be screened for other potential antibiotic residues by a 'generic method' based on LC-Time of Flight Mass spectrometry (LC-ToF-MS).

However, as the questionnaires received from the farmers indicated that aminoglycosides were being used on regular basis, it was agreed¹, that the ToF-MS work would be replaced by the analysis of approximately 60 waste milk samples for the target aminoglycosides of dihydrostreptomycin, streptomycin and neomycin (which includes the compound framycetin). These samples comprised 47 from farms with known treatment of cows with aminoglycosides and 13 selected randomly from the remaining farms out of the 103 waste milk sample set.

Approach

2.1 Cephalosporins and penicillins (compounds with a β -lactam structure)

Residues of cephalosporins and penicillins were quantified using an in-house Standard Operating Procedure (Method Code FSG377 issue v1) validated to the requirements of Commission Decision 2002/657/EC. The principle of this method is as follows:

Acetonitrile is added to the test sample. After homogenising, shaking and centrifugation, the supernatant is cleaned-up using C18 dispersive solid phase extraction (dSPE). After further shaking and centrifugation the supernatant is concentrated through evaporation under nitrogen before a final centrifugation and filtration. Analysis of cleaned-up sample extracts is carried out by LC-MS/MS. Method calibration was via the use of a six- or seven- point series of matrix extracted standards. Four deuterated (d) internal standards (d4-amoxicillin, d5-ampicillin, d7-penicillin G and d5-cefalexin) were added to both the waste milk samples and to the calibration samples to assist with the quantification. The sample data table (Appendix 1) indicates which internal (or external) standards were used in the quantification of each target analyte. Quality Assurance data for the analysis of replicate positive control samples, that were included in each analytical batch, is attached in Appendix 2a. All data has been corrected for analytical recovery.

Seventy-one out of 103 waste milk samples were found to be 'positive', with residues $> 4 \mu\text{g kg}^{-1}$, for compounds with a β -lactam structure, of which 22 related to cefquinome, a 4th generation cephalosporin.

2.2 Aminoglycosides

Residues of aminoglycosides were determined by using an in-house Standard Operating Procedure (Method Code FSG350 issue v4) that was validated to Commission Decision 2002/657/EC under a VMD funded R&D project (see VM02139). An outline of the method can be found on the Defra website at http://randd.defra.gov.uk/Document.aspx?Document=VM02139_7146_FRA.pdf. Method calibration was via the use of a five- point series of matrix extracted standards. Internal standards are not available for this method, so all quantification was via the external calibration series. Quality assurance data for positive control samples is attached in Appendix 2b. All data has been corrected for analytical recovery.

The analyses of the selected waste milk samples were performed between 02 September 2011 and 21 September 2011.

Fifty-two out of 60 waste milk samples were found to be positive for aminoglycosides, with a residue $> 100 \mu\text{g kg}^{-1}$ (dihydrostreptomycin/streptomycin) or $> 750 \mu\text{g kg}^{-1}$ (neomycin) respectively. One sample containing streptomycin and five samples containing dihydrostreptomycin had residue concentrations which were greater than the top calibration point for these two analytes of $2000 \mu\text{g kg}^{-1}$. The residue concentrations in these six samples are therefore 'indicative' only, and the true concentration may be much higher than the estimated concentration.

Detailed results are available at Appendix 2.

Quality assurance:

3.1 Controls

With each batch negative (blanks) and positive (spikes) controls were analysed. All blanks proved to be negative; while the data for the spiked samples are presented in Appendix 3a and 3b.

3.2 Repeatability

A small selection of waste milk samples were analysed repeatedly for residues of penicillin G and cefquinome. These repeatability data are presented in Appendix 3c.

The following paper describing the results of Objective 2 and 4 has been published:

RANDALL, L., K. HEINRICH, R. HORTON, L. BRUNTON, M. SHARMAN, V. BAILEY-HORNE, M. SHARMA, I. MCLAREN, N. COLDHAM, C. TEALE AND J. JONES (2014). Detection of antibiotic residues and association of cefquinome residues with the occurrence of Extended-Spectrum beta-Lactamase (ESBL)-producing bacteria in waste milk samples from dairy farms in England and Wales in 2011. *Res Vet Sci* **96**(1): 15-24.

Objective 03/ Stability of antibiotic residues in waste milk

The aim of this part of the project was to determine the effect of various practices including storage, fermentation and bioremediation upon the stability of antibiotic residues in waste milk. The practices considered were heating to different temperatures, adjustment of pH, fermentation and hydrolysis by β -

¹ Email of 1 July 2011 from Fera to AHVLA refers.

lactamase treatment of cefquinome spiked unpasteurised milk. For some experiments cefquinome-spiked UHT milk was also used as a control for natural fermentation of unpasteurised milk. Cefquinome was selected for these experiments because this drug was found to be most prevalent in waste milk from a survey of selected farms (mean cefquinome concentration in waste milk of positive samples was 1.47µg/ml).

Methods:

For each experiment, milk was spiked with cefquinome in bulk to a final concentration of 2µg/ml to model the mean concentrations of cefquinome found in waste milk from the surveyed farms. Aliquots of spiked milk (10g) were then placed in 50 ml centrifuge tubes in **duplicate** for each time point (n=10) for each experimental condition to be tested. At the appropriate time, each tube was rapidly frozen in a cardice/ethanol bath and stored frozen at -80°C prior to analysis by quantitative liquid chromatography tandem mass spectrometry undertaken at FERA, limit of detection (LOD) = 125 µg/kg. The concentrations of cefquinome were originally reported by FERA as µg/kg, but have been converted to µg/ml (by using the conversion factor 1.03/1000 and assuming the mean density of milk to be approximately 1.03) to be more readily comparable to MIC data. The pseudo first order rate constant (k), for the degradation of cefquinome by the different treatments examined, was obtained from the slope of the linear regression of the cefquinome concentration data (after transformation to natural logarithms) against time ($k = \text{slope} \times -1$). The half life ($t_{1/2}$) for the degradation of cefquinome was calculated from the pseudo first order rate constant where $t_{1/2} = \ln(2)/k$.

Thermal stability of antibiotic residues in milk (Obj. 03/01): Spiked unpasteurised milk was incubated at 4°C, 18°C, 37°C and 50°C for up to 240 hours. A second experiment was carried out in spiked UHT milk to try to distinguish between temperature effects alone and the effects of fermentation and pH changes caused by the growth of endogenous flora in unpasteurised milk. The spiked UHT milk was incubated under similar conditions for up to 144 hours, with the exception that the temperature conditions designed to mimic average UK outside air temperature was adjusted to 10°C for the UHT milk (cf 18° for the unpasteurised milk).

pH stability study of antibiotic residues in milk (Obj 03/02): Cefquinome-spiked UHT milk was incubated for up to 168 hours at pH 1, pH 4, pH ~6.7 (i.e. natural pH of milk) and at pH 10. Preliminary studies were undertaken to determine the minimum volumes of HCl and NaOH to add to 10g of milk to obtain the appropriate pH conditions (results not shown).

Effect of fermentation on the degradation of antibiotic residues in milk (Obj. 03/03): Cefquinome spiked UHT milk was fermented for up to 144 hours at either 10°C, to mimic the average annual UK outside air temperature, or at 37°C which is the optimum temperature for fermentation. The fermentation process was started by adding a small volume of inoculum (0.5 ml) of the probiotic suspension (2g/18 ml of 0.1M sterile PBS pH 7.2) or fermented milk culture to 10g of cefquinome spiked milk. Four different inocula were studied: 1) a suspension of Provita W.D. Powder Ruminant which is marketed by Provita Eurotech Ltd, Omagh as a feed additive containing *Lactobacillus acidophilus* and *Enterococcus faecium*, 2) a suspension of Protexin Pro-Soluble which is marketed by Centaur Services Ltd as a feed additive that contains *Enterococcus faecium*, 3) with an inoculum of unpasteurised milk that had been allowed to ferment naturally at 37°C for 18 hours, and 4) 0.5 ml of 0.1M PBS pH 7.2 alone i.e. no probiotic or fermenting bacteria added, to control for temperature and pH effects.

Larger scale incubation: 10 litres of cefquinome-spiked unpasteurised milk, containing a starter culture of approximately 500 ml of pre-fermented unpasteurised milk, was incubated at 37°C for 22 hours and at 60° for a further 2 hours. Aliquots (6x 10g) were taken at 0, 8, 22 and 24 hours for determination of cefquinome concentrations and bacterial counts on selective and non-selective agar.

Bioremediation of waste milk, degradation of cephalosporins by beta lactamase (Obj. 03/04): Preliminary studies indicated that conditioned Luria-Bertani medium without glucose (LB-G) from 24 hour growth of an *E. coli* strain expressing *bla*_{CTX-M-15} (LREC 99 from the AHVLA bacterial strain collection) possessed significant degradative activity against cefquinome at 10°C when incubated for up to 18 hours (results not shown). This enzymic activity was selected for the main study since conditioned media from other strains (LREC 90 expressing *bla*_{CTX-M-14}, LREC 98 expressing *bla*_{CTX-M-1}) and a commercial source of β-lactamase (M02075, FluoroChem, Derbyshire, UK) were less effective at degrading cefquinome at 10°C. The main study was conducted on both unpasteurised milk and UHT milk by adding 0.5 ml of the LB-G conditioned media from LREC99 to 9.5g of cefquinome spiked milk (approx. 5% v/v final) or adding 0.5 ml of unconditioned LB-G media alone as a control. These samples were incubated at either 10°C to mimic annual UK average outside air temperatures as before or at 37°C (for optimal enzymic activity) for up to 96 hours.

Results:

Thermal stability study (Obj. 03/01): In unpasteurised milk the optimal temperature for cefquinome degradation was 37°C (Fig. 1a), whereas in UHT milk the optimal temperature was 50 °C (Fig. 1b). This difference is attributed to the additional effects of fermentation of endogenous flora at 37°C in unpasteurised spiked milk.

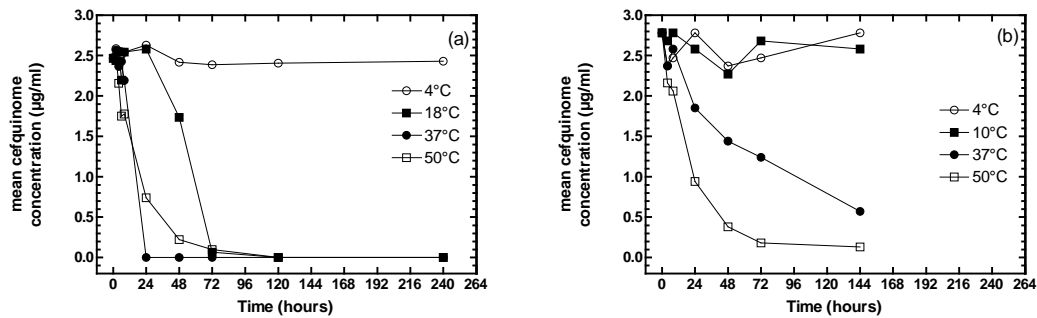


Fig 1. Temperature dependant degradation of cefquinome in spiked milk, (a) unpasteurised milk, (b) UHT milk

Table 1 Kinetic parameters for degradation of cefquinome in unpasteurised and UHT milk at different temperatures

Milk type	Temperature (°C)	Pseudo first order rate constant k (1/hours) ^a	t _{1/2} (hours) ^b
Unpasteurised	4	~ 0	~ ∞
	18	0.04045	17.1
	37	0.1322	5.2
	50	0.04679	14.8
UHT	4	~ 0	~ ∞
	10	~ 0	~ ∞
	37	0.01065	65.1
	50	0.03830	18.1 ^c

^a ~0 implies that the slope was not significantly different from 0.

^b ~ ∞ implies that the half life was infinitely long.

^c up to 72 hours.

pH stability study (Obj 03/02): Cefquinome was relatively stable in UHT milk at pH 1, 4 and ~6.7 for the whole of the experimental time period (168 hours). However cefquinome was degraded rapidly at pH 10 with concentrations falling to below the limit of detection after 8 hours (Fig.2).

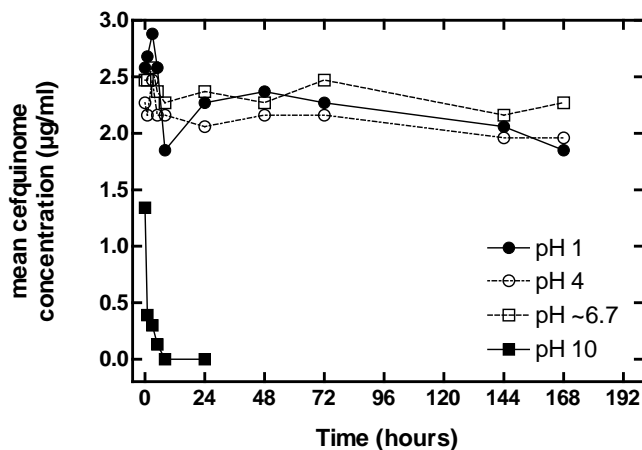


Fig. 2 pH dependant degradation of cefquinome in spiked UHT milk at 10 °C over time.

Table 2 Kinetic parameters for degradation of cefquinome in UHT milk at different pH's

pH	Pseudo first order rate constant k (1/hours)	t _{1/2} (hours)
1	0.001603	432.4
4	0.0008147	850.8
~6.7	0.0005502	1259.8
10	0.4064	1.7

Fermentation study (Obj. 03/03): At 10°C, cefquinome concentrations remained stable in the spiked UHT milk over the 6 day incubation period when incubated in the presence of the probiotics or fermented milk culture (Fig. 3a). The concentrations of cefquinome declined steadily when the milk was incubated at 37°C in the presence of the probiotic or starter culture (Fig. 3b). However the cefquinome concentrations also declined in the control tubes, in the absence of fermentative bacteria, suggesting that the higher temperature made a significant contribution to the cefquinome degradation observed.

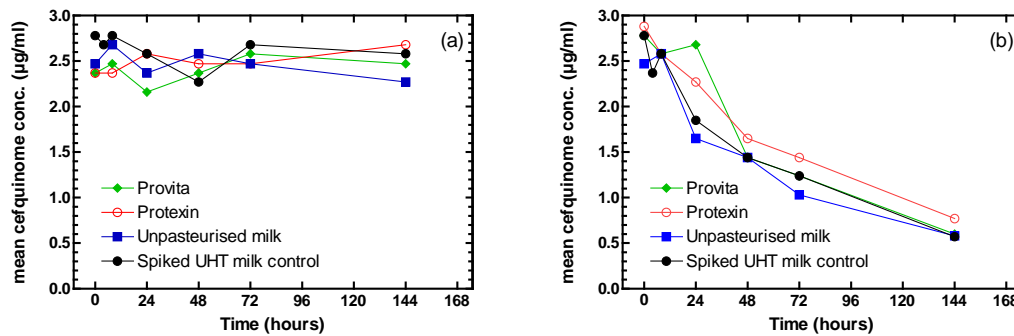


Fig. 3 Degradation of cefquinome in UHT milk by fermentation: (a) at 10°C and (b) at 37°C

Table 3 Kinetic parameters for degradation of cefquinome in UHT milk at with different fermentation and temperature conditions

Temperature (°C)	Inoculum	Pseudo first order rate constant (1/hours)	t _{1/2} (hours)
10	Provita	~ 0	~ ∞
	Protexin	~ 0	~ ∞
	Fermented raw milk	0.0007277	952.5
	PBS control	0.0004263	1626.0
37	Provita	0.01112	62.3
	Protexin	0.009065	76.5
	Fermented raw milk	0.01037	66.8
	PBS control	0.01065	65.1

Larger scale incubation: Cefquinome concentrations decreased rapidly at 37°C, to below the LOD after 8 hours. Total bacterial counts increased from 10⁶ to 10⁸ CFU/ml during this time period. The majority of these were ESBL *Enterobacteriaceae* bacteria. Heat treatment at 60°C for 2 hours significantly reduced the count of viable bacteria in the milk.

Bioremediation study (Obj. 03/04):

The concentrations of cefquinome in both unpasteurised and UHT spiked milk declined steadily over the first 8 hours and declined to below detection levels at 24 hours (Fig 4a and 4b respectively, p11). The results clearly demonstrate that the β-lactamase activity in the LB-G conditioned media was able to degrade cefquinome at 10°C. In contrast cefquinome concentrations were stable in the absence of β-lactamase activity (LB-G control). Similar responses were obtained for incubation conducted at 37°C (results not shown).

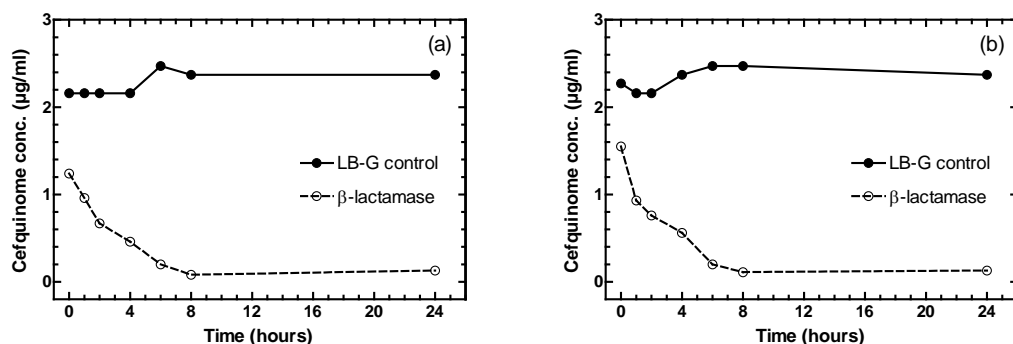


Fig. 4 Bioremediation of cefquinome spiked milk at 10°C using β -lactamase activity from LB-G conditioned media. (a) unpasteurised milk, (b) UHT milk.

Table 4 Summary of kinetic parameters for degradation of cefquinome in unpasteurised and UHT milk by β -lactamase activity at 10°C

Milk type	β -lactamase	Pseudo first order rate constant (1/hours)	$t_{1/2}$ (hours)
Unpasteurised milk	+	0.3332	2.1
	-	~ 0	$\sim \infty$
UHT milk	+	0.3197	2.2
	-	~ 0	$\sim \infty$

Conclusions

1. The most effective method for degrading cefquinome in spiked milk was to increase the pH to pH 10. This approach was able to completely eliminate cefquinome within 8 hours ($t_{1/2} = 1.7$ hours). However further work is required to assess whether milk treated in this way would be palatable for calves. Cefquinome was stable at pH 1, pH 4 and pH ~ 6.7 .
2. The second most effective treatment for cefquinome degradation was to add β -lactamase ($t_{1/2} \sim 2$ hours). However, this treatment is also problematic since it is likely that some method for inactivating the enzyme would be required before feeding the treated waste milk to calves. Further work is therefore required to make this a practical solution.
3. Heat treatment of unpasteurised milk at 37°C is a simple and effective approach for degrading cefquinome within 24 hours ($t_{1/2} \sim 5.2$ hours). However this temperature would also encourage the growth of endogenous flora and it seems likely that β -lactamase production from endogenous flora in unpasteurised milk played a significant part in the degradation observed since the half life was much longer ($t_{1/2} \sim 65.1$ hours) when the experiment was repeated in UHT milk in the absence of bacteria. If this approach is to be used then a further heat treatment at higher temperatures would also be required to kill endogenous flora from unpasteurised milk.
4. The effectiveness of fermentation is dependant upon the flora present in the milk. The endogenous flora of the unpasteurised milk used in this study was able to degrade cefquinome within 24 hours at 37°C. However this was not reproduced in UHT milk, the half life at optimal fermentation temperatures (37°C) was >60 hours in UHT milk with the probiotic bacteria or starter culture of the fermented unpasteurised milk. The difference between the rates of degradation between unpasteurised milk at 37°C and UHT milk inoculated with a small volume of fermented unpasteurised milk at 37°C could be attributed to either to a slower rate of growth of the bacteria in the UHT milk, or by the size of inoculum used (numbers of ESBL bacteria present in the inoculum). The effectiveness of fermentation was also highly dependant upon the incubation temperature. Cefquinome was not degraded by fermentation at 10°C (i.e. close to the average UK outside air temperature), the half life for fermentation by all treatments was >900 hours. Therefore it seems likely that any practical solutions for treatment of waste-milk on farm by fermentation would require the waste milk to be heated. Larger scale incubations (10 litres) of cefquinome spiked unpasteurised milk (containing a larger inoculum of endogenous ESBL

Enterobacteriaceae), at 37°C for 8 hours, were sufficient to decrease cefquinome residues to negligible concentrations. At 60°C, heating for greater than 2 hours is required to kill the enriched bacterial population consistently.

The following paper describing the results of Objective 3 has been published: HORTON, R. A., L. P. RANDALL, V. BAILEY-HORNE, K. HEINRICH, M. SHARMAN, L. A. BRUNTON, R. M. LA RAGIONE AND J. R. JONES (2015). Degradation of cefquinome in spiked milk as a model for bioremediation of dairy farm waste milk containing cephalosporin residues. *J Appl Microbiol* 118(4): 901-910.

Objective 04 - Waste milk ESBL microbiology

Objective 04/01 Detection and identification of ESBL bacteria in waste milk

Objective 04/02 Molecular typing of ESBL bacteria

Objective 04/03 Investigation of farms with ESBLs in waste milk

Waste milk samples from 103 farms in England and Wales were examined for the presence of ESBL-producing *Enterobacteriaceae*. Approximately ten months after the initial sampling, further waste milk, environmental and faecal samples from three of the farms found positive for ESBL *E. coli* in waste milk were examined.

Methods

Waste milk samples – Objective 04/01. Waste milk samples from 103 farms in England and Wales were examined for the presence of ESBL-producing *Enterobacteriaceae*. Approximately 100 ml of waste milk was collected from each farm using a sterile sampler. Approximately 20 ml was kept chilled for bacteriology which was initiated the following day.

Isolation of bacteria from waste milk samples Objective 04/01. Waste milk samples (n = 103) were diluted in sterile PBS and 100µl of suitable dilutions (Miles and others, 1938) were plated onto the six different agars both to provide a count of bacteria on that media, and to provide isolates for further study. The six agars used were Blood agar (BA) for total aerobic bacteria, CHROMagar ECC (CA-ECC) for mainly *Enterobacteriaceae* (an agar on which white colonies may be non-fermenters or rarely glucuronidase-negative *E. coli*), CHROMagar ECC + 1mg/L ceftazidime (CA-CAZ) to isolate ceftazidimase-producers, CA-CAZ + 64 mg/L BZ (benzo[b]thien-2-ylboronic acid, CAS 98437) compound (CA-CAZ+ BZ) to isolate ceftazidimase-producers but with BZ compound to inhibit some AmpC strains, CA-ECC + 16 mg/L cefoxitin (CA-FOX) to isolate mainly 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. 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 milk per 9 ml BPW before overnight incubation at 37° C for 18-24 hours. After bacteriological examination of all waste milk samples, 10% of sterile glycerol was added to each sample for cryo-protection and these milk samples were also frozen at -80°C. Stored milk samples (after thawing) were also plated onto Oxoid Brilliance ESBL agar (BRILL), before and after enrichment, to determine if this agar might detect ESBL-producers not detected with the other agars.

Farm study – Objective 04/03. During the first part of the study, waste milk samples from 3 out of 103 farms were positive for CTX-M *E. coli*. These farms were re-visited (approximately 10 months after analysis of the original waste milk sample) for further sampling. Waste milk samples were collected one and two weeks prior to the sampling visits. From each farm on a single visit, approximately 100 samples were collected to include 90 faecal samples, 10 environmental samples and one waste milk sample (thus giving a total of 3 waste milk samples from each farm at three time points). Environmental samples included samples from animal calf pens, collecting yard, pen rails, water troughs, waste milk feeder, 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 such as 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.

Isolation of bacteria from follow-up farm study - Objective 04/03. The same procedures as for milk samples were used, except that ~ 1 gram of cattle faeces or environmental sample was added to 9 ml of

BPW and plated directly to CA-ECC, CA-FOX and CA-CTX agar plates. Samples yielding less than 10 cfu/ml *E. coli* were plated to the same agar after enrichment at 37°C for 18-24 hours in BPW. For milk samples only from these follow-up visits, bacterial counts were performed and samples were also plated to BRILL agar.

Phenotypic ESBL tests - Objectives 04/01 and 04/03. MAST ESBL and AmpC disk tests were used as a preliminary screen to identify isolates for further study and to give an indication of resistance phenotypes. The chromogenic CHROMagar media yielded colonies of different colours which were classified as follows: white colonies, non-fermenters and rarely glucuronidase-negative *E. coli*; purple colonies, *Enterobacteriaceae* other than *E. coli* including *Klebsiella*, *Citrobacter*, *Enterobacter*; blue colonies, presumptive *E. coli*. MAST ESBL disks are generally intended for use with *Enterobacteriaceae* and therefore results for non-fermenters could not be taken as indicative of a particular resistance phenotype, unless the isolate was subsequently identified as *E. coli* or another member of the *Enterobacteriaceae* family. To test for an ESBL or AmpC phenotype in isolates from the initial 103 milk samples only (not applied to isolates from follow on farm visits), MAST disks (ceftazidime [D62C] and cefotaxime [D64C] +/- clavulanic acid and ESBL / AmpC [D68C] detection sets) were used. MAST cefepime disks [D63C] +/- clavulanic acid were also used on isolates that were putative ESBL positive by any of the other disk tests in an attempt to give more reliable results for any isolates that may also have been AmpC producers.

MICs - Objective 04/02. MICs of amoxicillin, cefquinome, cefotaxime, ceftazidime, cefalexin, neomycin, oxytetracycline, streptomycin and sulfadiazine were determined by the method of the British Society for Antimicrobial Chemotherapy (BSAC) as previously described (Andrews, 2001). MICs were determined against a panel of ~ 130 isolates from waste milk samples in the first part of the project only (not from follow on farm visits). Isolates were chosen to be representative of various resistance phenotypes identified and included isolates from each of the different agars used.

Growth curves – Objective 04/02. Growth curves for isolates of interest with and without various concentrations of cefquinome, were determined in Luria-Bertanii broth for 24 hours at 37°C. Growth was recorded every 30 minutes by measuring optical density using a Fluorostar plate reader.

PCR and sequencing. For isolates from the initial 103 milk samples, all isolates with an ESBL phenotype were tested for the presence of CTX-M, OXA, SHV and TEM genes using a multiplex PCR (Fang and others, 2008). All CTX-M positive isolates from the initial 103 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 and others 2008, Sabate and others, 2002). As a large number of milk, faecal and environmental samples yielded isolates on CHROMagar CTX from the three farms that were revisited, a proportion (~ 35%) were tested directly for CTX-M group 1 and CTX-M group 9 using sequencing primers and the resulting amplicons were sequenced to determine CTX-M sequence type.

Identification of bacteria - Objectives 04/01. Approximately 120 isolates representative from the waste milk samples from the first part of the study only were identified by MALDI-ToF (Toszeghy and others 2012).

Determination of mecA and nuc genes in Staphylococcus isolates - Objective 04/02. All isolates found to be *Staphylococcus* by MALDI-ToF were tested by PCR, as previously described, for the presence of the *mecA* and *nuc* genes (Denmark NF10, 2008).

Serotyping ('O' antigen testing) of E. coli - Objectives 04/01 and 04/03. Serotyping (somatic antigen testing) of selected, mainly CTX-M, *E. coli* isolates was determined as previously described (Sojka, 1965; Guinee and others, 1972). A total of 17 *E. coli* from the 103 waste samples in the first part of the study were selected for 'O' antigen identification or serotype, and these included all the CTX-M *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), 68 representative CTX-M *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.

PFGE - Objectives 04/02. PFGE of selected CTX-M *E. coli* isolates was performed as previously described (Ribot and others 2006). Six CTX-M *E. coli* (in some instances more than one isolate from one milk sample was tested), from the 103 waste samples in the first part of the study, were selected for PFGE. From the second part of the study (follow-up visits to three farms positive for CTX-M bacteria in waste milk), 79 representative CTX-M *E. coli* were tested by PFGE to include isolates from waste milk samples (n = 5) and faecal samples (n = 58) from all three farms, and isolates from environmental samples (n = 16) from one of the farms.

Replicon typing - Objectives 04/02. Replicon typing to determine the plasmid type was performed for selected isolates (n = 7) from the follow on farm studies only, as previously described (Carattoli and

others, 2005) and also using the PBRT replicon typing kit (Diatheva, Cat No.MBK 0038) according to the manufacturer's instructions.

Statistical methods –To determine whether the detection of cefquinome residues in milk samples (from objective 2) was associated with the presence of CTX-M bacteria in the milk samples, the null hypothesis of no association between the detection of cefquinome in a milk sample and the presence of CTX-M bacteria was tested using Fisher's Exact Test. The Fisher's Exact test was also used to determine if there were significant differences in number of faecal samples positive for CTX-M *E. coli* from calves fed different types of milk from one of the farms that were re-visited.

Results

ESBL phenotype isolates and confirmed CTX-M isolates - Objectives 04/01. Almost all milks yielded growth on Blood agar (BA) and CHROMagar ECC (CA-ECC). Over 60% of milks yielded growth on CA-FOX. About 20% of milks yielded bacterial growth on CHROMagar CTX (CA-CTX), but only 4 milk samples yielded CTX-M *E. coli*. The mean bacterial count [~ maximum] without enrichment in the waste milk samples on BA was ~ 10,000,000 [1,000,000,000] cfu/ml and for *Enterobacteriaceae* on CA-ECC was ~1,000,000 [100,000,000], 100,000 [10,000,000], 10,000 [100,000] cfu/ml for white, purple and blue colonies (presumptive *E. coli*) respectively. On CA-CTX the mean total count [~ maximum] in waste milks was ~ 1000 [100,000], 10,000 [1000,000], 10 [1000] for white, purple and blue colonies (presumptive *E. coli*) respectively.

If only blue (presumptive *E. coli*) and purple (non *E. coli* but *Enterobacteriaceae*) colonies are considered then 7 /103 milk samples yielded 28 isolates that were positive for an ESBL phenotype by at least one test, but also negative for an AmpC phenotype (Appendix 4). Of these seven milk samples yielding isolates with a putative ESBL phenotype based on the above criteria, six of the milk samples were positive for CTX-M bacteria of sequence types 1, 14, 14b and 15 (Appendix 4). The seventh milk sample that was positive for an *E. coli* with a putative ESBL phenotype, was positive for the *bla*_{TEM} gene, but the sequence type of this strain was not determined. Four of the six CTX-M positive farms were positive for CTX-M *E. coli* in waste milk, whilst two of the farms were positive for the *bla*_{CTX-M} gene in *Citrobacter* or *Enterobacter*. In all, the *bla*_{CTX-M} gene was detected in *Citrobacter*, *Enterobacter*, *Escherichia coli*, *Kluyvera* and *Raoultella* (Appendix 4). Appendix 4 also shows the concentrations of cephalosporin in the waste milk samples (objective 2). There was a significant association ($p=0.002$) between the occurrence of CTX-M bacteria (present in 5.8% of the waste milk samples; 3.9% contained CTX-M *Escherichia coli*) and the occurrence of cefquinome residues, present in 21.4% of waste milk samples. None of the *E. coli* serotyped were O25 (the serotype of the human epidemic strain).

MICs - Objective 04/02. Isolates of *Citrobacter*, *Enterobacter*, *Escherichia coli*, *Kluyvera* and *Raoultella* that were positive for *bla*_{CTX-M} had MICs of ≥ 1 mg/L for all of the four cephalosporins tested (results not shown). The non β -lactam antibiotics (neomycin, oxytetracycline, streptomycin and sulfadiazine) showed varying levels of activity for the organisms tested (results not shown). In most cases, for each organism type there were examples of isolates that were sensitive and those that had reduced susceptibility.

Growth curves - Objective 04/02. Growth curves were derived for three different CTX-M sequence type strains of *E. coli* isolated from bovine waste milk and three *E. coli* strains isolated from bovine faeces. The growth of these strains was investigated in the presence of cefquinome at minimum inhibitory concentrations (MIC), sub-minimum inhibitory concentrations and in control broth. As expected, the time lag to the start of growth increased for all strains when they were incubated in the presence of increasing concentrations of cefquinome and the maximal growth obtained decreased in the presence of increasing cefquinome concentrations. The MICs of cefquinome against all isolates were well above clinical breakpoints of cephalosporin antibiotics which tend to be 0.5 to 2 μ g/ml (BSAC). Cefquinome was also able to partially inhibit growth of the strains from waste milk at concentrations below the MIC (~ 1/8 MIC) suggesting that this antibiotic in waste milk could select for ESBLs and in particular CTX-M ESBLs at sub-MIC levels.

Farms re-sampled – antibiotic levels – The results for the three follow-up farms found to be positive for CTX-M *E. coli* in waste milk from the first part of the study and re-visited ~ 10 months after the initial samples were examined are shown in Appendix 5.

Waste milk samples from all three repeat farm visits were also positive for cefquinome (although for one farm only 1/3 samples were positive), and this was the only antibiotic found in the milk samples from the re-sampling. Cefquinome concentrations in waste milk samples over the three samples taken for each farm were in the ranges 970 to 27,000, 360 to 1,700 and < 4 to 740 μ g/kg respectively.

PFGE types - Objective 04/02. 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 (results not shown). This demonstrates that the CTX-M plasmids were present in a diverse array of *E. coli* isolates as well as in some non *E. coli* isolates. Of particular interest was the observation that one PFGE type associated with serotype O128 was present in a CTX-M-15 isolate from the waste milk from the first part of the study from farm 5687, 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 5438, where one PFGE strain type associated with 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.

A table showing Farms (milk samples) positive for representative *Enterobacteriaceae* that were AmpC negative by MAST disks, but ESBL positive by at least one MAST disk is presented at *Appendix 4*.

Farms re-sampled – CTX-M E. coli - Objective 04/03. All farms that were re-sampled (farm numbers 5438, 5687 and 11585) were still positive for CTX-M *E. coli* in the waste milk and also had CTX-M *E. coli* in 33.3% to 74.4% of faecal samples (*Appendix 5*). All samples from calves and adult cattle harboured CTX-M *E. coli*, although only one of the farms (5687) was also positive for CTX-M *E. coli* in most of the environmental samples taken (*Appendix 5*). For farm 5438, percentages of faecal samples from calves positive for CTX-M *E. coli* were higher than for faecal samples from older animals (*Appendix 5*).

Two of the re-sampled farms (5438 and 5687) 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 (*Appendix 5*). None of the serotypes were the O25 serotype of the human pandemic strain (Lau and others, 2008). The remaining farm (11585) was positive for CTX-M sequence types 14 and 15 in waste milk and faecal samples whilst on the previous visit the milk sample was only positive for CTX-M sequence type 14. One CTX-M-14 isolate from this farm had an N replicon type.

On farm 5438 there were four groups of calves which either received colostrum or non-waste milk from a machine or milk powder or waste milk *Appendix 5*. Although these four groups of calves received different types of milk, the percentages of samples from these groups of calves that were positive for CTX-M *E. coli* were not significantly different (Fisher's Exact test for differences between the four groups was $p = 0.466$ or when comparing waste milk with the other milks combined was $p = 0.29$). Pens for calves on the milk machine and for calves on milk powder were adjacent to each other but the pens for calves on colostrum and waste milk were distant and separated from each other. However, cross-contamination of groups was likely as the calves could be moved between accommodation as they grew in size and there was no thorough cleaning of pens between occupancy.

A table showing 'Presence of CTX-M *E. coli* on three farms previously found to have CTX-M *E. coli* in waste milk' is presented at *Appendix 5*.

Conclusion

In waste milk the presence of antimicrobials may be associated with the presence of resistant organisms. Cefquinome, was significantly associated ($p = 0.002$) with CTX-M bacteria present in 5.8% of the milk samples (3.9% for CTX-M *E. coli*). Feeding waste milk to calves can therefore lead to exposure of those animals to ESBL-producing bacteria. Subsequent visits to three of the farms positive for both cefquinome and CTX-M *E. coli* in waste milk showed evidence of CTX-M *E. coli* in all of the different groups of animals tested and on one farm, also in the environment. 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).

The following paper describing the results of Objective 2 and 4 has been published:

RANDALL, L., K. HEINRICH, R. HORTON, L. BRUNTON, M. SHARMAN, V. BAILEY-HORNE, M. SHARMA, I. MCLAREN, N. COLDHAM, C. TEALE AND J. JONES (2014). "Detection of antibiotic residues and association of cefquinome residues with the occurrence of Extended-Spectrum beta-Lactamase (ESBL)-producing bacteria in waste milk samples from dairy farms in England and Wales in 2011." *Res Vet Sci* **96**(1): 15-24.

Objective 05 Selective pressure of a 3rd/4th generation cephalosporin in milk fed to calves.

05/01 *Selective effect of cephalosporin fortified milk on ESBL E. coli in calves*

05/02 *Qualitative and quantitative assessment of selective pressure on ESBLs*

The aim of objective 5 was to determine whether the feeding of milk supplemented with a 3rd/4th generation cephalosporin could exert a selective pressure and thus lead to increased shedding of ESBL *E. coli*.

Methods

05/01 Selective effect of cephalosporin fortified milk on ESBL *E. coli* in calves

Forty cross breed calves (8 groups; n=5 per group and each group housed separately) were recruited to the study at approximately 10-15 days of age. After one week of acclimatisation, five groups (Groups 1-5) of calves were dosed with 10⁹ CFU of CTX-M-14 *E. coli* O33 as previously described (Reeves *and others.*, 2013) and allowed to stabilise for 4 days. The remaining 3 groups (Groups 6-8) were then dosed with a phosphate buffered saline (PBS) placebo. The feeding of calf milk replacer mixed according to the manufacturer's instructions and fortified with cefquinome at concentrations of 0.15 µg/ ml (Group 2), 1.5 µg/ ml (Groups 3 and 7), 4.5 µg/ ml (Group 4) and 15 µg/ ml (Groups 5 and 8) commenced at day 11 and continued for 4 weeks. Groups 1 and 6 received no antibiotics in the milk; instead an aliquot of sterile water was added to the milk substitute. The concentrations of cefquinome added were reflective of the concentrations found in waste milk samples on farms where 1.5 µg/ ml was the approximate mean of positive samples tested (see Appendix 2). The calves also had access to coarse mix, hay and mains water. Rectal faecal samples were taken three days prior to challenge and then daily post challenge to monitor the shedding of total and CTX-M *E. coli*. Three animals from each group were randomly selected and euthanased at week 5 for evaluation of challenge strain tissue tropism; all remaining animals were euthanased at the end of the study.

The numbers of total and presumptive CTX-M *E. coli* in the faecal samples were enumerated by culture on CHROMagar ECC and CHROMagar CTX, respectively. Faecal samples (1 g) were suspended in 9 ml buffered peptone water (BPW) and ten-fold serial dilutions in sterile PBS were plated directly onto the respective agars. If growth was not observed from the direct plating of the faecal samples in PBS, then cultures were enriched at 37°C for 16-18 hours in the initial buffered peptone water (BPW) prior to plating as above. Therefore, in the latter case, numbers of total and presumptive CTX-M *E. coli* were calculated for the faecal sample after enrichment.

Data were initially analysed using GraphPad Prism software version 5.0 (San Diego, CA, USA) at group level using ANOVA or Chi squared tests to examine differences between the groups. Following the initial analysis three sets of regression analyses were performed using the CTX-M *E. coli* count data, the CTX-M non-*E. coli* data and the total *E. coli* data. All analyses were carried out in STATA 12 (Stata Corporation, College Station, TX, USA) and p <=0.05 was used as the significance level in all analyses. A population-averaged approach based on generalised estimating equations (GEE) was used to account for repeated measurements on individual calves over time.

05/02 Qualitative and quantitative assessment of selective pressure on ESBLs

The selective pressure of antibiotic in milk was also recorded by assessing transfer of the CTX-M gene to other *E. coli* strains and serotypes, and to other species of bacteria. The strain, serotype and species of CTX-M bearing bacteria recovered from faeces was assessed by standard serological and molecular techniques. The *E. coli* isolates were analysed by Pulse Field Gel Electrophoresis (PFGE) (Ribot *and others.*, 2006). The serotype (somatic O antigen) of CTX-M bearing *E. coli* was assessed by standard serological techniques (Sojka, 1965; Guinee *and others.*, 1972). A selection of presumptive *E. coli* colonies (n=38) from Chromagar CTX plates (blue) and other (white) colonies (n= 90) were speciated by MALDI-ToF to determine if the CTX-M bearing plasmid has transferred to other bacterial species. A selection of presumptive CTX-M *E. coli* colonies from CHROMagar CTX and presumptive CTX non- *E. coli* colonies from CHROMagar ECC were tested for the presence of the CTX-M group 9 ESBL genes by PCR with sequencing primers as described previously (Sabate *and others.*, 2002; Carattoli *and others.*, 2008)

Results:

05/01 Selective effect of cephalosporin fortified milk on ESBL *E. coli* in calves

All calves were clinically normal at the onset of the study. All calves screened negative for faecal CTX-M *E. coli* prior to oral dosing with the challenge strain. Interestingly, a wide variation in the magnitude and duration of faecal shedding of CTX-M *E. coli* was observed among individual calves. Calves receiving cefquinome at the lowest concentration (0.15 µg/ ml) showed, on average, increased shedding of CTX-M *E. coli* in the first week of the feeding compared to calves which were receiving un-spiked milk (Figure 1, p18). After 15 days there was significantly less shedding of CTX-M *E. coli* (p= 0.032) in groups receiving the highest concentrations of antibiotics. The levels of CTX-M *E. coli* shed gradually decreased in all groups until there were no significant differences between calves being fed un-spiked and cefquinome spiked milk. At least one calf from each of the infected groups continued to shed CTX-M *E. coli* throughout the course of the study. Regression models through the CTX-M *E. coli* data showed no difference between the treatment groups and the control group in terms of initial shedding levels and the rates at which shedding declined when group 1 (control) was used as the baseline group. The binomial model suggested that there was no difference between the groups in the percentage of animals shedding CTX-M *E. coli* over the course of the study. The univariate analysis found no significant difference in the proportion of total *E. coli* that were resistant between groups which were infected with CTX-M *E. coli* O33 (the challenge strain). There were significant differences in the shedding rate of total *E. coli* between the control and groups 4 (p = 0.048) and 5 (p = 0.001) where shedding in those groups decreased at a slower

rate compared with the control group. Calves which were receiving cefquinome in the milk in the non-colonised groups (7 and 8) showed a significantly quicker decrease ($p < 0.05$) in levels of total *E. coli* shed in the faeces compared to calves in group 6 (non-infected control) which were receiving no cefquinome.

Prior to the start of the study all calves tested negative for CTX-M positive *Enterobacteriaceae*. None of the animals in the group 1 (control) shed any CTX-M non-*E. coli*. This made the group an unsuitable baseline for the model so group 2 was used as the baseline. At the start of the study, only group 4 was significantly different to group 2 ($p = 0.001$). The model suggested that the shedding of CTX-M non-*E. coli* increased significantly faster in group 3 ($p = 0.000$) and group 5 ($p = 0.001$) compared with group 2. This coincided with a decrease in levels of CTX-M *E. coli* in the same groups.

05/02 Qualitative and quantitative assessment of selective pressure on ESBLs

A number ($n=90$) of non-*E. coli* isolates were identified using MALDI- ToF analysis. There were five different *Enterobacteriaceae* identified, as detailed in Table 1 (p 18), with 54 % of isolates identified as *Citrobacter* spp. A number of suspected CTX-M-14 *E. coli* isolates ($n=35$) recovered from faecal samples were selected for O serotyping to assess plasmid transfer. Of the 35 isolates selected, 89 % were O33 CTX-M *E. coli* and four isolates belonged to other serotypes. PFGE analysis of *E. coli* isolates showed four distinct clonal groups (A-D) (Figure 5). All of the CTX-M isolates recovered from faecal samples that were O33 by serotyping and the O33 CTX-M *E. coli* challenge strain (EC768/06) belonged to PFGE clonal group B. The CTX-M group PCR revealed that 65 % of *E. coli* isolates belonged to CTX-M group 9 and the presence of the *bla*_{CTX-M-14} gene was confirmed by ABI sequencing. Sixty per cent of non-*E. coli* isolates were shown to contain the *bla*_{CTX-M-14} gene whilst 40 % were shown to be CTX-M-1 (Table 1, p 18).

Discussion

The data obtained from this calf-feeding trial revealed some statistically significant associations but the detailed picture is complicated. There was no significant difference observed in the duration of shedding of CTX-M *E. coli* between groups of calves which received cefquinome in the milk and those which received un-spiked milk. This is comparable with findings of the study reported by Alali and others (2004) where there was no significant difference in faecal shedding of neomycin and oxytetracycline resistant *E. coli* O157 in calves fed milk replacer containing neomycin and oxytetracycline compared with controls.

Interestingly, there was an observed increase in the mean levels of CTX-M *E. coli* shed in the faeces of calves in group 2 (0.15 µg/ ml cefquinome in the milk) at day 6 post inoculation, however when the GEE model was run using data from individual calves and the control group as the baseline, no significant difference was observed. The small sample size is likely to be an important factor affecting these results as there were only five calves per group and there was a high degree of variation in the levels of shedding between individual calves.

Interestingly, at day 15 post inoculation, there was a significant decrease in the numbers of CTX-M *E. coli* shed in the faeces of calves which received 1.5 µg/ ml and 15 µg/ ml cefquinome in the milk which coincided with a significant increase in CTX-M non-*E. coli* being shed in the same groups.

Based upon counts where the number of resistant *E. coli* exceeded the number of total *E. coli*, we have observed a margin of error around our counts up to a maximum of 2.8 logs and a median error by which one count can exceed another of 1.3 logs. Therefore, we can only be confident that we have detected true differences between the counts on CHROMagar ECC and CHROMagar CTX when these differences are greater than 2.8 logs (maximum error).

Whilst this study did not demonstrate a significant increase in the levels of CTX-M *E. coli* shed in groups of calves which received cefquinome in the milk, the outputs suggest that further investigation is necessary in a field rather than laboratory situation. The sample size used will have limited the power of the study to detect an association between cefquinome concentration and the level of CTX-M *E. coli* and there was high variation in shedding levels between individual calves. A larger sample size in a farm situation would overcome this. The challenge strain used was a field strain which has been previously shown to colonise very well in the bovine gut (Horton and others, 2011, Reeves, 2013) and the inherent ability to colonise bovine intestine efficiently may have out-weighed any effect of the selective pressure of the antibiotics in the milk. Another factor that may have affected the shedding result is the age of the calves when feeding of antibiotic fortified milk commenced as usually calves begin receiving waste milk at in the first week of life, however due to rules regarding transport of animals, we were not able to begin feeding the cefquinome spiked milk until approximately three weeks of age. It is possible that in the presence of challenge strains which would ordinarily colonise to a lower level, a greater association would be observed between exposure to cefquinome and the occurrence of CTX-M *E. coli* levels in calves. The detection of CTX-M-1 in bacteria other than *E. coli* in some calves during the experiment was unexpected; some calves may have been carrying such organisms prior to the start of the study but not detected by our screening processes.

Table 1: Non-*E. coli* bacteria recovered from the faeces of calves orally dosed with CTX-M *E. coli* O33

Bacterial Species	% Isolates (n=90) identified	% Isolates CTX-M positive	CTX-M Type
<i>Citrobacter</i> Spp.	54.4	89.8	14
<i>Pseudomonas</i> Spp.	23.3	28.6	1
<i>Enterobacter</i> Spp.	15.6	64.3	14
<i>Acinetobacter</i> Spp.	12.2	0	NA
<i>Raoultella</i> Spp.	5.6	100	1

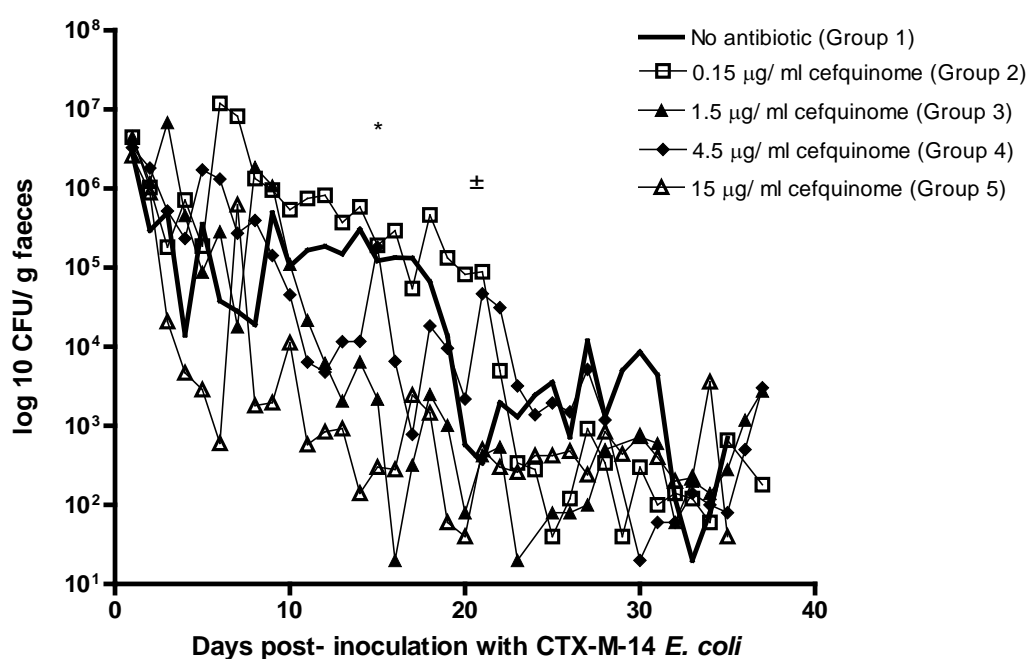


Figure 1: Daily faecal shedding of CTX-M *E. coli* as group averages

*The group average shedding for calves receiving 0.15 µg/ ml cefquinome in the milk was higher than the control group during the first week post-inoculation

±Groups receiving 1.5 µg/ ml cefquinome and 15 µg/ ml cefquinome in the milk shed significantly lower levels of ($p < 0.01$) CTX-M *E. coli* at day 15 post- inoculation.

Objective 06 - On farm waste milk exposure study to assess the impact of feeding waste milk with antibiotic residues on the prevalence and incidence of ESBLs in calves

Objective 06/01 Feeding of waste milk with antibiotic residues affects the proportion of cefotaximase (CTX-M)-positive *E. coli* in the gut of calves and that this will vary with calf age

Objective 06/02 Feeding of waste milk with antibiotic residues affects the prevalence of calves infected with CTX-M-positive *E. coli* Under Objective 2, 69 % of waste milk samples were found to contain residues $> 4 \mu\text{g kg}^{-1}$ for compounds with a β -lactam structure, and a third of these were cefquinome, a 4th generation cephalosporin. The aim of this objective was to assess on farm the impact of feeding waste milk containing antibiotic residues on the prevalence of ESBL-producing *E. coli* in the intestinal flora of calves in order to test the following hypotheses: that the feeding of waste milk with antibiotic residues affects the proportion of cefotaximase (CTX-M)-positive *E. coli* in the gut of calves and that this will vary with calf age (objective 06/02); that the feeding of waste milk with antibiotic residues affects the prevalence of calves infected with CTX-M-positive *E. coli* (objective 06/01).

Methods

A longitudinal case-control study was carried out on a farm known to harbour CTX-M-positive *E. coli*. Fifty calves were recruited to the study at 1-2 days of age and allocated sequentially to either a milk replacer-

fed group (control) or waste milk-fed group (treatment). Calves were housed on straw, and water was available *ad libitum*. They were weaned at 7 weeks of age and then fed for a further 6 weeks. A statistical power analysis based on previous prevalence estimates indicated that 25 animals per group would be an appropriate sample size to detect significant differences with 95 % confidence and 90 % power, assuming a mean CTX-M-positive *E. coli* prevalence of 50 % in the control group and 90 % in the treatment group. Animals were weighed at 4 time points: on entry to the study and at 4, 7 and 13 weeks. Any treatments given to the calves or to the group were recorded, as were any health events or changes to management practices. Faecal samples were collected from all calves daily for the first week after enrolment, then twice weekly until weaning, then weekly for a further four weeks. Environmental samples from the calf housing were collected weekly. Total *E. coli* and CTX-M-positive *E. coli* in faecal samples were enumerated using CHROMagar ECC and CHROMagar CTX supplement respectively using the methods described for Objective 5. A subset of 45 waste milk and 3 milk replacer samples were examined for CTX-M-positive *E. coli* (see Objective 4), and for antibiotic residues using the screening method described for Objective 2. Data were explored initially using STATA 12 at group level using ANOVA or Chi squared tests to examine differences in the proportion of total *E. coli* that were resistant, the number of infected calves over time, and the mean number of CTX-M bacteria shed over time in each group. These variables were subjected to regression analysis if significant differences were observed between groups in the initial analysis ($p < 0.05$). Survival analysis was used to compare the rate of colonisation of calves with CTX-M-positive *E. coli* between the groups. Regression analyses were performed on the bacterial count data using a population-averaged approach based on generalised estimating equations (GEE) to account for repeated measurements on individual calves over time. For all models, the QIC command in STATA was used to confirm which correlation structure was most appropriate. An interaction term between the calf treatment group and sample number (1-25) was included in the models to allow for changes in shedding over time to be examined and compared between the groups.

Results

At entry to the study there was no difference in the average calf weight between groups. But at each of the subsequent time points the average weight of calves in the treatment group was significantly higher than those in the control group. The average rate of weight gain was estimated at 4.0 kg per week for the control group and 5.9 kg per week for the treatment group ($p = 0.000$). Only calves in the control group experienced diarrhoea which indicated a significant difference between groups (6 v 0, $p = 0.018$). Cefquinome, a fourth generation cephalosporin, was the most commonly used intramammary and injectable antibiotic in cows that contributed to the waste milk. Cefalonium, a first generation cephalosporin was also frequently used. In line with the treatment history data, cefquinome residues were detected in 39/45 waste milk samples, with a concentration range of 390 to 1,700 $\mu\text{g kg}^{-1}$ and a mean concentration of 746 $\mu\text{g kg}^{-1}$. Penicillin G could be detected in 12/45 waste milk samples, but only quantified for one of those at 360 $\mu\text{g kg}^{-1}$. Milk replacer fed to the control group was also examined and found to be free of any detectable β -lactam residues (minimum detection limit of 400 $\mu\text{g kg}^{-1}$). Bacteriological testing failed to isolate CTX-M-positive *E. coli* from any of the milk samples. However, 25 of the waste milk samples yielded colonies presumptively identified as CTX-M-positive *Klebsiella* spp. Two hundred and sixty environmental samples were collected throughout the study (130 per treatment group) from eight different locations within the pens. The same numbers of samples were taken from each location per treatment group. Overall no significant difference was observed in the number of samples positive for *E. coli* ($p = 0.705$) or CTX-M-positive *E. coli* ($p = 0.457$) between the treatment groups. All sampling locations yielded resistant isolates, but a significant difference in positive samples between treatment groups was only found for samples taken from the pen floor using boot swabs ($p = 0.016$). Consistently high numbers of *E. coli* were isolated from calf faecal samples throughout the study, with a slight decline in numbers as the animals increased in age. The numbers of CTX-M-positive *E. coli* isolated from calves were consistently higher in the treatment group (statistically significant for sample 8, mean value of 4.8×10^6 in the treatment group versus 7.06×10^5 in the control group, $p = 0.0005$), sample 11 (mean value of 5.53×10^5 in the treatment group versus 6.22×10^4 in the control group, $p = 0.0091$) and sample 12 (mean value of 8.44×10^5 in the treatment group versus 6.96×10^4 in the control group, $p = 0.0265$) and tended to follow a similar pattern of decline in number over time compared with the control group. Representative isolates from each group were confirmed as CTX-M-positive by PCR (25 isolates per group). In the treatment group, 18 isolates were CTX-M group 1 and seven were group 9. In the control group 20 isolates were CTX-M group 1 and five were group 9. Initial examination of the proportion of total *E. coli* that were CTX-M-positive indicated there was an overall difference between the two groups ($p < 0.001$) (Figure 6.1a, p20). However, the regression analysis found no significant difference in the proportion of isolates that were resistant at the start of the study between the two groups, or in the proportion of isolates that were resistant over time (Figure 6.1a, p20). A higher proportion of samples from calves in the treatment group were positive for CTX-M-positive *E. coli* compared with the control group. A chi squared test was used to examine differences in the percentage of calves shedding between the two groups at different time points. This indicated that there was no difference in the proportion of calves shedding CTX-M-positive *E. coli* at the start of the study, but that a significant difference emerged at around four weeks of age. This was confirmed by the regression analysis

which found no significant difference between the proportion of animals shedding in the control and treatment groups at the start of the study, but showed that the proportion shedding in the treatment group declined significantly slower than the control group (0.055% less of a decrease per day, $p < 0.001$) indicating that more animals continued to shed in the treatment group over time. The survival analysis indicated that there was no difference in the incidence rate of colonisation of calves with CTX-M-positive *E. coli* between the two groups ($p = 0.736$).

A great deal of variability was observed in the shedding within groups, and overall the counts of CFU/g faeces ranged from < 100 to 6×10^7 . ANOVA revealed a significant difference in the counts of CTX-M-positive *E. coli* between the two groups at around four weeks of age (samples 11 & 12), but not at the start or end of the study. The regression analyses identified a significant difference between the control and treatment groups throughout the study (treatment group shed 1.555 log CFU per gram more per day, $p < 0.001$) and a significant difference in the rate at which counts decreased over time (counts in the control group decreased by 0.157 log CFU per gram more per day, $p < 0.001$) (Figure 6.1b, p20)..

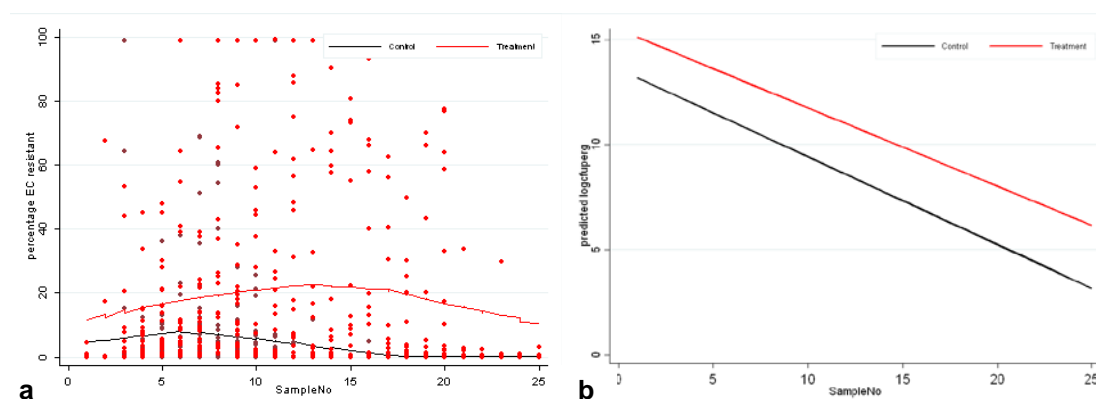


Figure 6.1 – a) Proportion of total *E. coli* that were CTX-M-positive at each time point based on mean counts. Significant differences between groups as determined by ANOVA are illustrated as * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$; b) Fitted lines from linear GEE model comparing the degree of shedding over time for animals in the control and treatment groups.

Discussion

This study was designed to be representative of the normal calf husbandry practices on a commercial dairy farm. In order to investigate the effect of feeding waste milk on calves, all other management and environmental factors were kept the same for both groups. The sample size enabled the study to be conducted in a natural situation, whilst still providing the study with enough power to detect significant associations.

Aust *et al.* (2012) examined the effect of feeding untreated and pasteurised waste milk or bulk milk on calf performance and health. They found no significant difference in average daily gain between groups. In this study, calves were weighed on four occasions and a significant difference observed between groups over the course of the study. One of the reasons why farmers in England and Wales chose to feed waste milk to calves was because it improved calf growth (3.5% of respondents to survey in Objective 1 - Brunton *et al.* 2012); the findings of this study would appear to support this.

Cefquinome was detected in 87 % of the waste milk samples at a mean concentration of $746 \mu\text{g kg}^{-1}$ (0.746 mg/l). Orden *et al.* (1999) calculated the MIC_{90} of cefquinome against 195 isolates of *E. coli* from calves to be 0.125 mg/l, while previous work at AHVLA has found the MIC_{90} of cefquinome against CTX-M-positive *E. coli* from calves to be at least 2 mg/l (R. Horton - unpublished observations). Based on this, the concentrations detected in waste milk in our study should be high enough to kill susceptible isolates, but low enough to provide a selective pressure in favour of *E. coli* with acquired ESBL resistance. However, it is not known from this study what concentration of cefquinome would reach the *E. coli* population in the gut. There is likely to be degradation of cefquinome in the stomach, plus a dilution effect with the consumption of water and saliva.

The first hypothesis of this study was that feeding waste milk with antibiotic residues to calves would affect the proportion of CTX-M-positive *E. coli* in the gut of calves and that this would vary with calf age. Aust *et al.* (2012) found the proportion of *E. coli* resistant to some antibiotics including cefotaxime to be higher in calves fed waste milk compared with those fed bulk milk. The results of our analysis have shown that there was no difference between calves fed waste milk or calves fed milk replacer in the proportion of *E. coli* isolates that were CTX-M-positive. This was observed at the start of the study and throughout. However, although no difference was observed in the proportion of *E. coli* that were resistant, calves in the waste milk group shed greater numbers of CTX-M-positive *E. coli* than calves in the control group throughout the study, although shedding decreased at an equal rate in both groups.

The second hypothesis was that feeding waste milk with antibiotic residues would affect the prevalence of calves infected with CTX-M-positive *E. coli*. The results of the analysis showed that at the start of the study there was no significant difference in the prevalence of infected calves between the groups, but over

time, a significant difference between groups did emerge. This suggests that infection with CTX-M-positive *E. coli* persisted in a larger number of calves fed waste milk compared with calves fed a milk replacer where the prevalence declined faster. Wray *et al.* (1990) found that resistant *E. coli* were only able to persist in the calf gut for a short duration without selective pressure. Our results show that although the prevalence of calves infected with CTX-M-positive *E. coli* does decline post-weaning (i.e. once the selective pressure of antibiotics in waste milk has been removed), there was a difference in persistence between the control and treatment groups.

The time variable used in the regression analyses was sample number (1-25). As samples were collected at different time points throughout the study (i.e. daily, then twice weekly, then weekly) the amount of time between two adjacent sampling occasions could vary. This variation was not considered in the models. However, the results of the survival analysis indicated that there was no difference in the rate of colonisation with CTX-M-positive *E. coli* between treatment groups. This would suggest that the choice of time variable was unlikely to have an effect on the associations observed between waste milk feeding and the increased calf prevalence and level of shedding.

Around half of the environmental samples collected in this study contained CTX-M-positive *E. coli* and all sampling locations yielded resistant isolates. Although no overall difference was observed in the proportion of positive samples between the two groups, significantly more pen floor samples were positive in the treatment group. This is likely to be as a result of the higher level of shedding of CTX-M-positive *E. coli* in the treatment, but could also have contributed to maintaining infection within that group.

The findings of this study indicate that feeding waste milk on this farm increases the prevalence of calves infected with CTX-M-positive *E. coli*, and increases the amount of resistant bacteria shed in the faeces. Shedding of CTX-M-positive *E. coli* persists after weaning in both treatment and control calves, but persists for longer in calves fed waste milk. These findings are applicable to the situation observed on this farm, but may differ on other farms where the contents of the waste milk may vary. However, similarity was observed between the most commonly used antimicrobials on this farm, and those commonly used on farms surveyed in Objective 1, indicating that this study is likely to provide some representation of the situation on other dairy farms in England and Wales.

The following paper describing the results of Objective 6 has been published

Brunton, L. A., H. E. Reeves, L. C. Snow and J. R. Jones (2014). "A longitudinal field trial assessing the impact of feeding waste milk containing antibiotic residues on the prevalence of ESBL-producing *Escherichia coli* in calves." *Prev Vet Med* **117**(2): 403-412.

Objective 7: Quantitative risk assessment for antibiotic residues in waste milk.

Objective 07/01 Development of Risk pathway for ESBL selection by waste milk.

Objective 07/02 Risk model data collection and collation.

Objective 07/03 Development of model to assess the risk of antibiotic residues in waste milk on ESBL selection.

Aims: The aim of this objective was to develop a stochastic simulation model to assess the risk of transfer of antimicrobial resistant *Escherichia Coli* (ESBL *E. coli*) to calves via the feeding of waste milk from dairy cattle on the same farm. The model was designed to consider the effect of the presence of cephalosporin residues (hereafter referred to as 'residues') in the waste milk, due to the intramammary usage of 3rd and 4th generation cephalosporins, and enable investigation of the impact of practical control measures aimed to minimise the development/selection of ESBL *E. coli* from feeding calves waste milk.

Materials & Methods: The risk pathway is described in Figure 1(p 22), which shows the potential routes by which calves are fed milk from cattle on-farm (the model does not consider milk from outside sources or other potential transmission routes, as the focus is on whether infection occurs within farm and possible prevention measures).

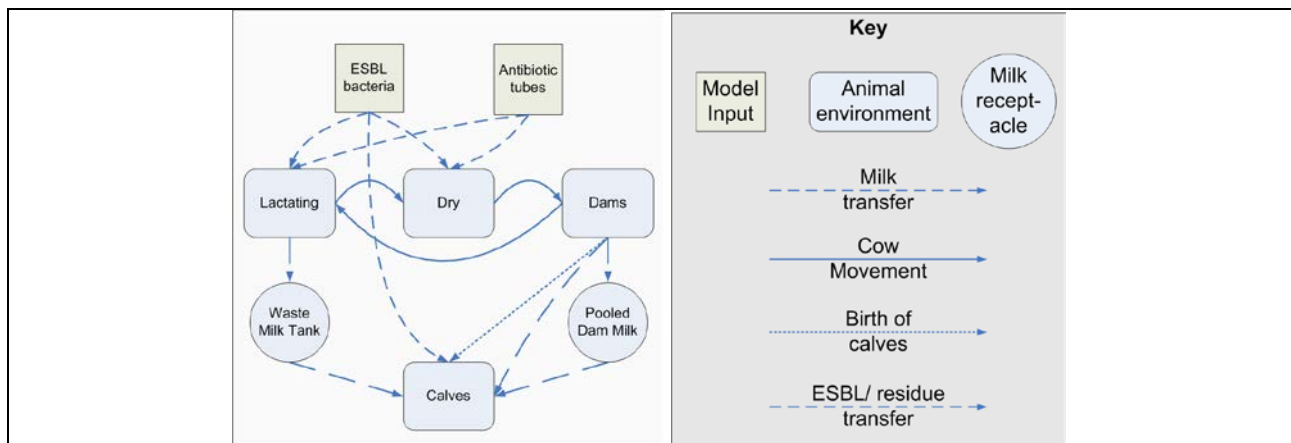


Figure 1: Top level model framework, showing the route of the waste milk from cows to calves.

The model was developed in Matlab and is a quantitative stochastic transmission model, using Monte Carlo simulation techniques to incorporate variability in parameter values within and between farms. Data obtained as part of OD2031, in particular from the waste milk survey (Objective 1) and the waste milk sampling study (Objective 2), were used to help parameterise the model. The model is run for 5,000 iterations, representing 5,000 different farms. On each farm the model simulates individual dairy cows, calves and waste milk tanks. The time step of the model is primarily in days, but there can be multiple milking and feeding events within the day. From day 1 of each iteration animals on the farm may acquire ESBL *E. coli* and residues; the model assumes no effects from exposure prior to this time. The numbers of ESBL bacteria with and without the presence of residues is approximated by a model developed as part of a previous project (see Appendix A of the Defra project report for OD2028 for full description) to simulate the levels of bacteria in the bovine gut and subsequently in the faeces.

Intervention analyses: Table 1(p22) shows a list of intervention scenarios simulated by the model. For each intervention measure we specify what effect it will have on ESBL *E. Coli* and the residues, with regards to an increase or decrease in numbers/ concentration at a particular stage. For example, a study conducted as part of OD2031 (objective 3) suggests that addition of β lactamase is likely to almost completely remove residues in a short space of time, but unlikely to affect the level of ESBL *E. Coli*. Conversely pasteurisation is likely to kill most ESBL *E. Coli* but may not be as effective against residues. There will, in reality, be large variation in the actual observed effect. However, for the purposes of this report we implemented a 'best case' effect (e.g. addition of β lactamase will completely remove residues).

Table 1: List of intervention measures and their effect on ESBL *E. coli* and residues

Intervention/ Scenario	Description	Stage implemented	Effect on ESBL <i>E. coli</i>	Effect on residues
<i>Baseline</i>	No intervention	N/A	N/A	N/A
<i>Pasteur</i>	Based on classic sterilization (120 degrees for 20 minutes)	Waste milk tanks	Complete removal of <i>E. coli</i>	1 log10 decrease*
<i>BetaLactam</i>	Addition of β lactamase to waste milk tanks	Waste milk tanks	No Effect	Complete removal of residues
<i>pH10to2</i>	pH is first raised to pH10 and then reduced to pH2	Waste milk tanks	Complete removal of <i>E. coli</i>	Complete removal of residues
<i>NoCephOnFarm</i>	Stop using 3rd or 4th generation cephalosporins on farm	All adult cows	No Effect	Complete removal of residues on farm.
<i>NoCowsWM</i>	Waste milk from cows being given 3rd or 4th generation cephalosporins not fed to calves	Lactating Cows	No Effect	Dairy cows will not contribute residues to waste milk
<i>NoCalvesWM</i>	Don't feed waste milk at all	Calves	Removal of waste milk source	Removal of waste milk source

*1 log 10 decrease = 90% reduction.

Results: The results of the baseline model run predict an average farm level prevalence (over 5,000 simulated farms) of calves with ESBL *E. coli* in their faeces of ~60% (5th and 95th percentiles 0% and 100%), while the average prevalence of calves with residues in their gut was ~70% (5th and 95th percentiles 0% and 100%). This was much higher than the prevalence of adult cows with residues in their milk (<1%). This is due to the fact that when a cow that has been treated with cephalosporins contributes to the waste milk, residues will be present in the waste milk and can then potentially be fed to a large number of calves, all of whom, the model assumes, will consume some amount of residue. The baseline model predicted an average concentration of ESBL *E. coli* in calf faeces of ~5.5 log₁₀ cfu/g (5th and 95th percentiles ~1.75 log₁₀ cfu/g and 8 log₁₀ cfu/g), while the average concentration in scenarios with no residues present was only ~2 log₁₀ cfu/g.

Figure 2 (p 23) shows the effect of the intervention measures on ESBL *E. coli* prevalence (top), residue prevalence (middle) and average ESBL *E. coli* numbers (bottom) in calves. The graphs show that scenarios which greatly reduce the level of residues present in the calves (e.g. *BetaLactam*, *NoCephOnFarm*) reduces the average concentration in calves, but those interventions that greatly reduce the level of both the residues and the ESBL *E. coli* (e.g. *pH10to2*, *NoCowsWM*) will reduce the average concentration by even more. Interestingly the pasteurisation scenario, *Pasteur*, (which kills all ESBL *E. coli* in the waste milk tank but only reduces the residues by 1 log₁₀ cfu) initially reduces the average ESBL *E. coli* concentration in calves, but by 60 days the concentration has increased again. This is because the calves can still be infected with ESBL *E. coli* via the dams at a very young age. When they are a bit older, these infected calves can then become exposed to cephalosporin residues via the waste milk, which will react with the already present ESBL *E. coli*, increasing the concentration. Fewer calves are likely to be infected with both ESBL *E. coli* and residues during the first 30-40 days, hence the initial decline in the pasteurisation scenario.

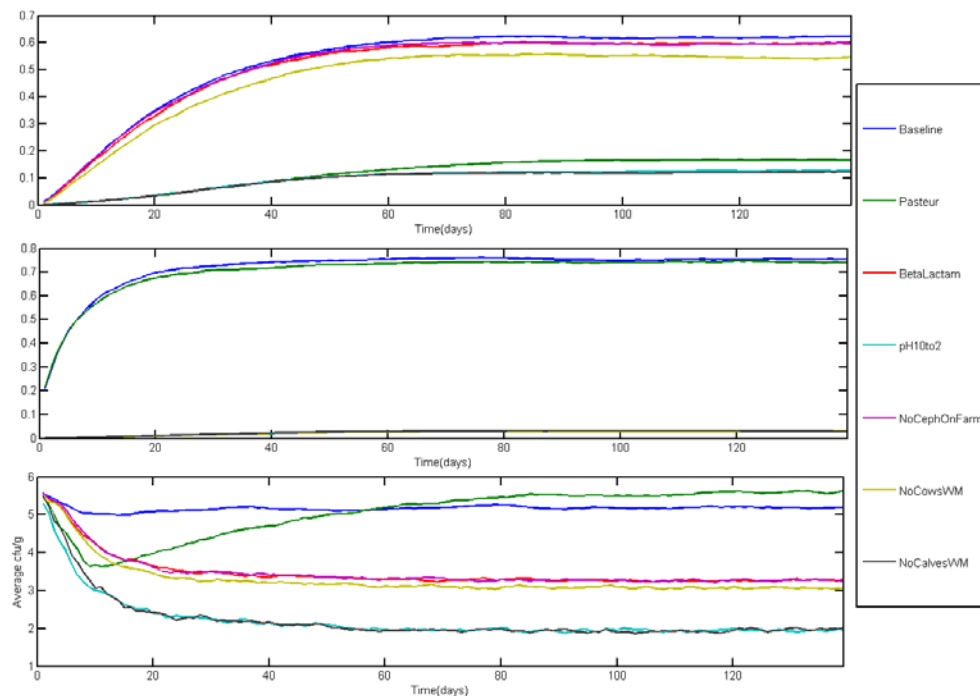


Figure 2: Results of baseline and intervention scenarios: average ESBL *E. coli* prevalence in calves (top), average residue prevalence in calves (middle) and average log₁₀ concentration of farm level mean ESBL *E. coli* in calves (bottom).

Discussion: The baseline model suggests that colonisation of calves with ESBL *E. coli* and exposure to cephalosporin residues via waste milk is likely, but with wide variation between farms. There is wide variability (and uncertainty) in the actual dairy cow prevalence of ESBL *E. coli* and sensitivity analysis of the model suggests that this is very important with regards to the model predicted ESBL *E. coli* prevalence in calves. The baseline model assumes a relatively low prevalence of ESBL *E. coli* infection among dairy cows (on average about 10% on a given day).

There are many other potential routes by which calves may be exposed to ESBL *E. coli* (e.g. via the environment, direct contact with other infected calves, importation of infected animals), that were outside the scope of this project. Some of these (e.g. environmental contamination) may be strongly linked to the presence of ESBL *E. coli* in waste milk. However, they may have an impact on the effectiveness of the waste milk interventions. For example, interventions that remove ESBL *E. coli* from the waste milk would likely be less effective if the calves are rapidly re-infected from an environment heavily contaminated with ESBL *E. coli*.

The scenario analyses indicated that completely stopping the practice of feeding waste milk to calves

(NoCalvesWM) was the most effective measure, but the scenario assuming complete eradication of ESBL *E. coli* and residues from the waste milk (pH10to2) was almost as effective. A simulation of hypothetical control measures that had a medium effect on both ESBL *E. coli* and residues in waste milk (e.g. 2 or 3 log₁₀ decrease) did not appear to be as effective as control measures that had a large effect on one or the other (e.g. complete elimination of residues but no effect on ESBL *E. coli* in waste milk). Thus, the model suggests that any potential intervention package targeted at the waste milk tanks would need to deliver a large reduction of both ESBL *E. coli* and residues in the waste milk tank to be considered effective.

Objective 8. Recommendations to minimise the effect of antibiotic residues in waste milk.

Introduction

In objective 6, carried out on a farm where ESBL *E. coli* were present and 3rd and 4th generation cephalosporins were used in dairy cows, the prevalence of ESBL *E. coli* was increased in calves fed waste milk and that shedding of ESBL *E. coli* persisted for longer and persisted after weaning. Also, recently evidence that feeding waste milk to calves does select for antibiotic resistance in *E. coli* in calf faeces has been published by Aust and others (2012). Their study showed significantly higher proportion of faecal *E. coli* isolates resistant to cefotaxime (3rd generation cephalosporin), cephalothin (1st generation cephalosporin), nalidixic acid (quinolone) and sulfamethazole/ trimethoprim (potentiated sulphonamide) in calves fed either waste milk and /or pasteurised waste milk compared to calves fed bulk (saleable) milk. The resistance mechanisms were not determined, but resistance in *E. coli* to cefotaxime may be associated with ESBLs, in particular CTX-M ESBLs (Aust and others, 2012). Schmid and others (2013) found that the calves fed waste milk were more likely to shed ESBL *E. coli* positive faeces samples but this was not statistically significant.

Options for control of potential risk

1. Do not feed waste milk and feed milk replacer instead.

The scenario analyses (objective 7) indicated that completely stopping the practice of feeding waste milk to calves was the most effective measure. The strategy used on most dairy farms that do not feed waste milk is to feed milk replacer although some farmers feed whole milk (from bulk tank) (Appendix 1).

Costs: Waste milk is considered to have no value, as the alternative to feeding it to animals on farm is normally disposal. The cost of feeding milk replacer will primarily be the milk replacer cost and additional costs will vary greatly depending on the system used. An estimate of consumption until weaning is 168 litres, although this will vary depending on weaning age and whether ad lib or restricted feeding. In 2012 the cost of milk replacer was given as 15-23p (figure from 2012) but cost may have changed since then. If a consumption of 168 litres is assumed until weaning and 2012 figures used (15-23p/litre) the additional cost of milk replacer per calf would have been in the range of £25.20-£38.64. There are many methods of feeding milk replacer and the potential additional or reduced labour costs, and equipment costs will vary depending on the system used. Labour costs for feeding milk replacer were estimated as between £2.40 and £13.80 per calf until weaning in 2012 depending on the system (Mole Valley Farmers, 2012).

Calf growth rates/ performance: The weight gains of calves will undoubtedly be influenced by the amount of waste milk or milk replacer fed and the constituents and therefore results are likely to be variable between trials. Around 3 % of farmers that responded to the postal survey (objective 1) stated that improvement in calf health and/or performance was a reason for feeding waste milk was fed. Godden and others (2005) found that calves fed pasteurised waste milk had a higher growth rate than calves fed milk replacer. In objective 6, less disease was observed in calves fed waste milk and calves fed waste milk were heavier than those fed milk replacer. However Hill and others (2009) found that calves fed milk replacer had a higher growth rate than calves fed saleable milk and commented that this was a more consistent product. There are other trials published in the farming press that suggest increased growth rates with named milk replacers.

Transmission of infectious disease by feeding waste milk: It has been shown that in herds feeding waste milk, this practice is associated with an increased occurrence of Johne's disease (Ridge and others, 2005) and that feeding pooled milk increases the risk of a cow testing antibody positive for *Mycobacterium avium paratuberculosis* (map) (Nielsen and others, 2008). *Mycoplasma bovis* infection in calves has been attributed to feeding waste milk (Walz and others, 1997) and *Salmonella* infections have been associated with feeding waste milk (Edrington and others, 2012). *Mycobacterium bovis* may also be transmitted to calves by milk feeding (Evangelista and De Anda, 1996; White and Minett, 1941). It is suggested that feeding calves waste (mastitic) milk may also result in a higher risk of mastitis in heifers but McDougal and others (2009) considered the effect unclear.

The risks of transmission of infectious disease via waste milk may be reduced or eliminated by

pasteurisation of waste milk, and control of infectious disease is likely the reason why fewer farmers feed waste milk to their dairy replacement heifers than other calves. Pasteurisation of waste milk is rare in England and Wales (objective 1).

Environmental impact: If waste milk is disposed of in slurry or wastewater systems, the environmental impact of this needs to be considered. The β -lactam antibiotics will degrade rapidly in the environment. In a study of dairy farms in Northern Germany, no analysed antibiotics were detected in slurry (liquid manure) or leachate from grassland or maize field. The authors suggested this was due to the lower amounts of antibiotic used in dairy farming compared to pig and poultry farming, degradation during storage of manure and degradation or absorption in soil when applied to grassland. However, there is no mention of whether waste milk was added to the slurry tested. The authors conclude that 'the pathway of excretion of antibiotics into the environment via cattle manure can be neglected' (Kemper and others, 2008). Also previous Defra funded studies (OD2008) conclude that slurry is not a good medium for transfer of genes by conjugation, an important method of transfer of ESBL genes, partly due to low winter temperatures (Snary and others, 2003), although again the addition of waste milk was not considered.

2. Control the use of waste milk containing extended spectrum cephalosporins.

These measures will reduce or eliminate the residues of cephalosporins fed to calves. According to the model (objective 7), interventions such as this which greatly reduce the level of residues fed to calves reduce the average concentration of ESBL *E. coli* in calves, but not as much as those that eliminate residues and ESBL *E. coli* in waste milk fed or stopping feeding waste milk. These interventions have not been costed.

Do not feed waste milk from cows treated with a third or fourth generation cephalosporin that is likely to have resulted in residues in milk (this would exclude ceftiofur injectable products): This is a potential mechanism of preventing the residues of 3rd and 4th generation cephalosporins being present in waste milk fed to calves. The additional cost would only fall to those farmers using the specified 3rd and 4th generation cephalosporins and it may be possible to implement by amending the product instructions.

Discard the milk during treatment and after first milk after end of extended spectrum cephalosporin treatment: This will reduce costs for the farmer compared to discarding all the milk from treated cows and will reduce the concentration of antibiotic residues in milk fed to calves. Cefquinome residues in milk following milking cow treatments have been shown to rapidly decline after the first milking after the treatment is completed (Knappstein and others, 2003; Thal and others, 2011).

Stop using 3rd and 4th generation cephalosporins in dairy herds: Stopping using extended spectrum cephalosporins will prevent these antibiotics being present in waste milk and may have an additional benefit (not modelled) in reducing the prevalence of ESBL *E. coli* in faeces of adult cattle. Sensitivity analysis of the model (objective 7) suggests the prevalence of ESBL *E. coli* in dairy cows is very important with regards to the model predicted ESBL *E. coli* prevalence in calves. Selection of faecal ESBL *E. coli* by injectable ceftiofur and cefquinome has been demonstrated in pigs (Cavaco and others, 2008).

3. Treat waste milk to remove cephalosporins

According to the scenario analyses (objective 7), complete eradication of ESBL *E. coli* and residues from the waste milk will produce an effect similar to stopping feeding waste milk. Removal of residues only will reduce the average concentration of ESBL *E. coli* in calves, but not as much as those that eliminate residues and ESBL *E. coli*. A number of techniques have been used effectively to destroy the cefquinome in waste milk, but on a laboratory scale only at this stage.

Adjusting pH of waste milk to pH 10 is very effective and cost would be low but there would be concerns about the safety of adding strong alkalis on farm and palatability has not been investigated.

Adding a beta-lactamase has been trialled and again is very effective against cefquinome but the beta-lactamase used was expensive. However, there is a commercial beta-lactamase available for this purpose in Finland which appears to have a more limited spectrum of activity against beta-lactam antibiotics (Antipen;

http://www.kissa.fi/user_files/files/Pakkauselosteet/Antipen%20PL%20hyv.%203.1.07.pdf) which might suggest this approach has some commercial potential. Currently it is difficult to see any economic advantage for farmers to use such a product. Further investigation of the potential of a commercial beta-lactamase effective against a wide range of beta-lactam antibiotics may be worthwhile.

Fermentation in the UK does not appear to be particularly effective without additional heat as the average temperature of 10°C is not high enough.

Heating has perhaps the greatest practical potential, especially if it destruction of cephalosporins such as cefquinome in the milk and with killing most bacteria including some pathogenic bacteria of concern (similar to the large scale incubation in obj. 3). On farm studies would be required to establish the

feasibility of such methods and the cost would be significant including a suitable machine to heat the milk, energy, maintenance and time.

Discussion.

If it is considered necessary to control the risk of selection for ESBL *E. coli* by feeding waste milk, the most practical method is likely to be to stop feeding waste milk and to feed milk replacer instead. This will also have some health advantages particularly in the control of Johne's disease. The effect on calf performance (growth rates) appears to be variable and dependent on the precise feeding regime used. The environmental effects of disposing of unused waste milk containing antibiotics in slurry or wastewater may be negligible but would require further investigation. Feeding milk replacer will incur an additional cost for farmers.

According to the model (objective 7), not using extended-spectrum cephalosporins in the adult herd is likely to result in a reduced ESBL *E. coli* prevalence in calves due to calves not drinking milk containing cephalosporin. Additionally this may also result in reduced prevalence in the adult herd which sensitivity analysis suggests is very important with regards to the model predicted ESBL *E. coli* prevalence in calves, but this possible additional effect is not included in the model. The effect on prevalence and shedding of ESBL *E. coli* in dairy cattle of using or not using extended-spectrum cephalosporins is an important knowledge gap. Other alternatives to reduce the risk of ESBL *E. coli* in calves would be to not feed waste milk likely to contain extended-spectrum cephalosporins and not feed the milk from cows during treatment and the first milk after cephalosporin treatment ceases.

None of the methods of reducing antibiotic (cefquinome) concentrations in waste milk prior to feeding are at a stage where they could be used on farm, although some show potential. A heating process to degrade cefquinome and kill ESBL *E. coli*, (plus some pathogens) may hold the greatest potential in this area. However, all would require significant development costs and there is currently no financial incentive for farmers to adopt such measures.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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