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Verotoxins in commensal *Escherichia coli* in cattle: the effect of injectable subcutaneous oxytetracycline in addition to in-feed chlortetracycline on prevalence

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SUMMARY

Using a self-paired observational study, the association between therapeutic oxytetracycline use and the prevalence of virulence genes in commensal *Escherichia coli* (*E. coli*) from cattle was examined. Faeces were collected from 39 yearling bulls prior to and after treatment with oxytetracycline and from 44 untreated animals. Between samplings all animals received in-feed chlortetracycline for 16 days. Five *E. coli* were isolated from each sample and tested by a polymerase chain reaction (PCR) capable of detecting all verotoxin (*vt*) genes. Positive isolates were further tested with a multiplex PCR to detect *vt1*, *vt2*, *eaeA* and *hlyA*. For *vt*, 23 animals were positive at both samplings, 26 negative at both samplings, 22 negative animals became positive and 12 positive animals became negative. Sixty-eight per cent of the discordant pairs changed from *vt*-negative to *vt*-positive (95% CI 48–80) suggesting pressure toward becoming *vt*-positive perhaps due to the transfer of genes due to mixing of cattle in the months between samplings or an effect of chlortetracycline.

INTRODUCTION

The United States Food and Drug Administration's Center for Veterinary Medicine has included in its guidelines for the use of antimicrobials in food-producing animals a need to assess the effect of antimicrobial agents on the pathogen load in animals [1]. This recommendation arises from the concern that the use of antimicrobials in food animals may increase the number of enteric bacteria in the intestinal tract of animals that are capable of causing human illness. Increased public scrutiny has lead to questions about the use of in-feed and therapeutic antimicrobials by livestock industries, however at present most of the literature focuses on in-feed use and little is known about the effect of the therapeutic use of antibiotics on microbes in livestock. The present study examines the effect of treatment with injectable oxytetracycline in animals which subsequently received in-feed antimicrobials, a common occurrence in feedlot cattle production in North America. The aim, therefore, was to determine if additional therapeutic antibiotic use was associated with changes in virulence gene prevalence. The outcomes of interest examined were changes in the prevalence of the *vt* (verotoxin) genes, and if positive, in the prevalence of *vt1* (verotoxin 1 or VT1), *vt2* (verotoxin 2 or VT2), *eaeA* (intimin, a protein involved in attachment) and *hlyA* (enterohaemolysin) genes in *E. coli* isolated from bulls treated with in-feed antimicrobials only as compared to those treated additionally with injectable oxytetracycline. Information about the prevalence of virulence genes in commensal enteric bacteria is of interest as they are potential indicators of selection pressure on enteric bacteria and because they represent a reservoir of virulence genes for potentially pathogenic bacteria.

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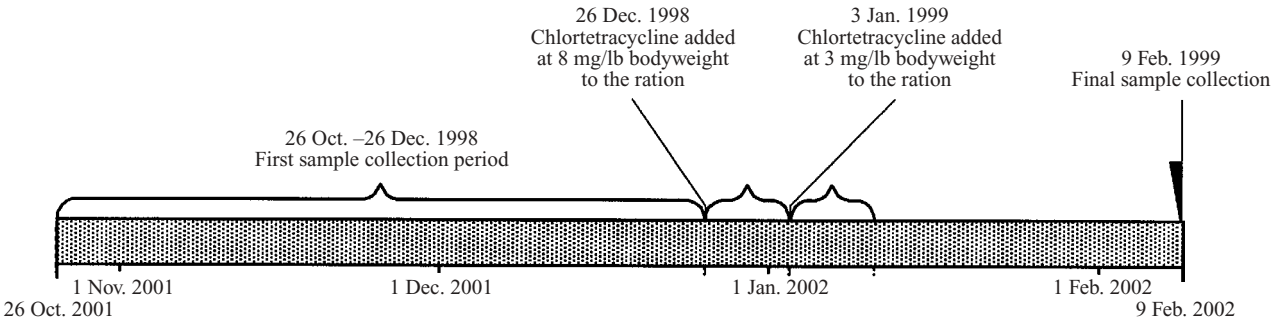

Fig. 1. Time line of study sampling and timing of administration and dosage of in-feed chlortetracycline to cattle.

The primary hypothesis tested was that treatment with injectable oxytetracycline in the individual animal was not associated with any additional change in the prevalence of virulence genes in commensal *E. coli* isolates in cattle which subsequently receive chlortetracycline in feed. To examine the hypothesis a matched (self paired) analysis was conducted on a group of cattle being studied for the prevalence of antimicrobial resistance in intestinal commensal *E. coli* upon routine in-feed and selected individual treatment with tetracyclines.

METHODS

Animal enrolment and eligibility criteria

The study design is discussed in detail in another publication [2] and shown in Figure 1. The study consisted of two periods during which faecal samples were collected from cattle: (1) October to December 1998 and (2) February 1999. All cattle in the study received in-feed chlortetracycline for a period of 16 days from 26 December 1998 to 8 January 1999 (Fig. 1). The initial dose (8 mg/lb bodyweight per day) was used as the facility veterinarian diagnosed undifferentiated bovine respiratory disease in the cattle. Chlortetracycline is registered for use in the United States for treatment of bacterial pneumonia caused by *Pasteurella multocida* organisms at 10 mg/lb bodyweight per day. At the time the study was conducted this was an off-label drug use in Canada. The dose was reduced to 3 mg/lb bodyweight per day to allow adjustment of the ruminal flora. There is little evidence to support this practice, although it is commonplace. Prior to the addition of chlortetracycline to the diet, faecal samples were collected from animals immediately prior to treatment with injectable oxytetracycline by the subcutaneous route for either respiratory disease or infectious digital dermatitis. At that time, faecal samples were also collected from two untreated animals selected from a pool of untreated animals (Fig. 2). At the outset of the study a randomly generated list of animals in each pen was compiled. Each time an animal was treated for undifferentiated fever/depression or digital dermatitis, the next two animals from that pen on the untreated list were selected as controls. Treated animals were removed from the list of potential untreated animals. As the untreated animals were selected from the pen, some pens were not sampled as no animals became sick. Due to the longitudinal nature of an incidence density study, untreated animals could subsequently be treated and would then no longer be classified as untreated (Fig. 2). Several times both untreated animals were subsequently treated. On 9 February 1999, 30 days after the cessation of chlortetracycline treatment, faecal samples were collected from all animals in the facility. There were no individual animal therapeutic treatments with injectable oxytetracycline after 26 December.

Faecal sample preparation

After collection all samples were transported with ice packs in styrofoam containers to the laboratory for culture, isolation, identification and determination of antimicrobial resistance of *E. coli*, and to examine the isolates for the presence of *vt1*, *vt2*, *eaeA* and *hlyA* genes.

Escherichia coli isolation

Ten grams of faeces were diluted with 90 ml of buffered peptone water, homogenized in a stomacher and incubated at 37 °C for 18–24 h. Following incubation a loopful of faecal material was streaked onto two MacConkey agar plates. The MacConkey plates were incubated at 37 °C for 18–24 h. Five colonies per sample that were morphologically consistent with *E. coli* were selected for further identification. Isolates

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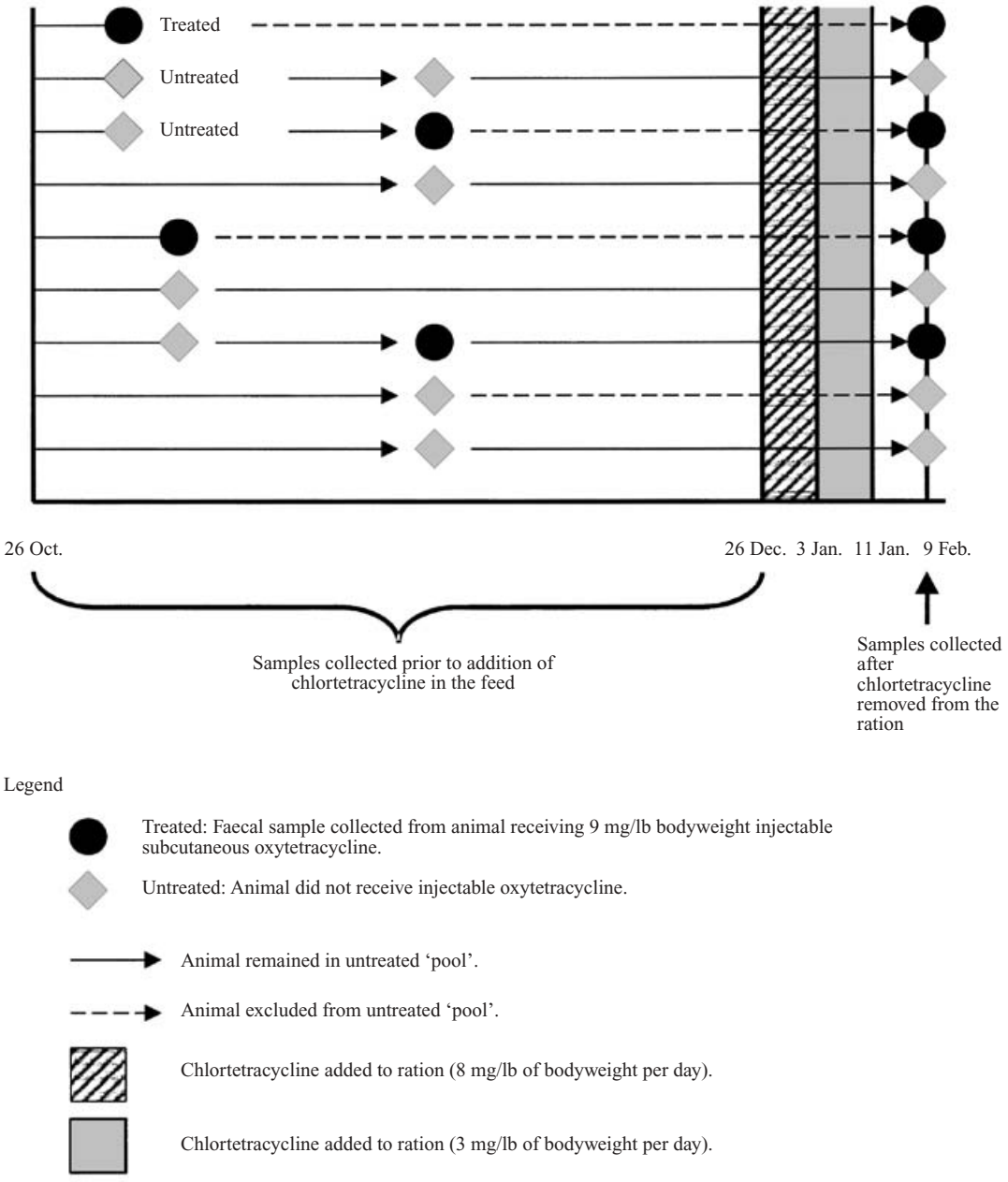

Fig. 2. An incidence density study design in which faecal samples were collected from animals that received injectable oxytetracycline for treatment of undifferentiated respiratory disease or digital dermatitis and from untreated animals.

that were positive for indole production and did not utilize citrate were identified as *E. coli*.

Detection of verotoxin genes in faecal samples

Faecal samples were tested by a polymerase chain reaction (PCR) capable of detecting all *vt* genes [3]. Lysates for PCR reactions were prepared according to the method of Read and co-workers [3]. Briefly, faecal samples were inoculated into 1 ml of Brain Heart Infusion broth (Difco, MI, USA) and incubated for 18 h at 37 °C. The culture was centrifuged at 12 000 ***g*** for 1 min, the supernatant was decanted, the pellet was suspended in 1·0 ml of FA buffer (Difco) and centrifuged again. The supernatant was discarded and the pellet was suspended in 500 μl of sterile distilled water. The samples were placed in a boiling water bath for 10 min and then placed on ice. Prior to use in a PCR reaction, the samples were centrifuged at 12 000 ***g*** for 1 min. The PCR protocol and cycling

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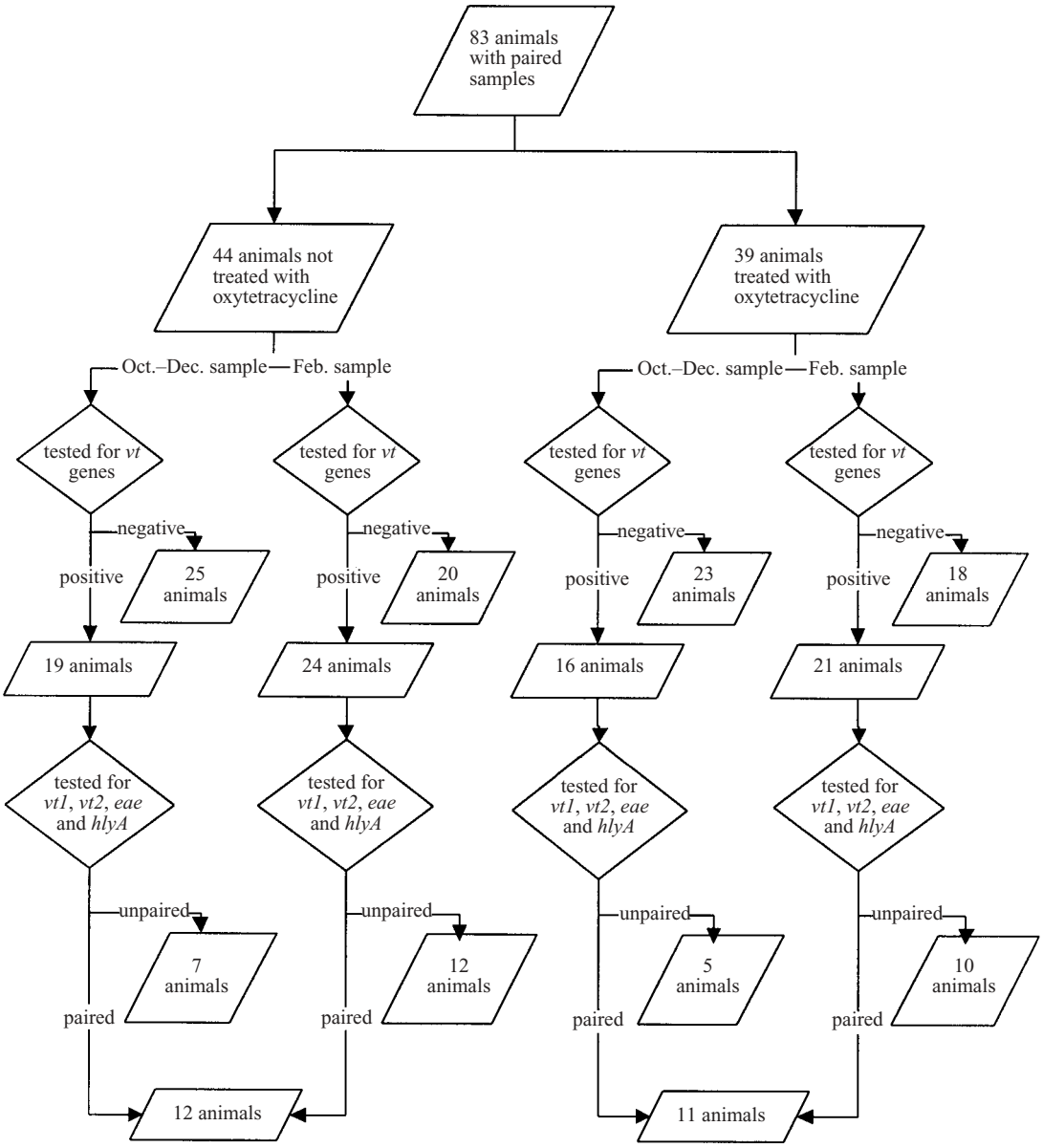

Fig. 3. Flowchart describing the number of animals enrolled in the study and the results of testing during the study.

conditions used have been published [3]. If the faecal samples were positive for *vt* genes by PCR, the *E. coli* strains isolated from the individual faecal samples (see section *Escherichia coli* isolation) were tested using a multiplex PCR capable of differentiating *vt1*, *vt2* and detecting *eaeA* and *hlyA* [4]. The lysate was prepared as previously described [3].

Serotyping of *E. coli*

Escherichia coli isolates that possessed any of the *vt1*, *vt2*, *eaeA* or *hlyA* genes were serotyped by standard serological methods [5].

Statistical analyses

All statistical procedures were performed using SAS version 8.1 (SAS Institute Inc., Cary, NC, USA). Data in the analyses are only for the matched pairs as described below and in Figure 3. For each animal only the first sample collected between October and December 1998 was used to represent this period. This is because, as the analysis was based on the individual animal, animals with greater than one collection would have a greater opportunity of being declared positive and therefore may have biased the study findings. Given that the purpose of the October–December period of sample collection and examination of the

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Table 1. *Distribution of the paired results among 83 bulls housed at a bull test station that had faecal samples collected at both the October–December and the February sampling times*

	February sampling			
October–December sampling	Verotoxin positive†	Verotoxin negative	Total	*P* value*
Verotoxin positive†	23	12	35	
Verotoxin negative	22	26	48	
Total	45	38	83	0·12

* *P* value for the binomial tests $H_0 = p = 0{\cdot}5$ for the discordant pairs.
† Positive for verotoxin based on a PCR capable of detecting all *vt* genes.

isolated *E. coli* was to establish the baseline prevalence prior to treatment, the first sample collected from each animal is an untreated sample. Matched animals had samples collected at the October–December and the February sampling. Among these animals a second level of matching was conducted when both samples tested positive for generic verotoxin. Eighty-three animals met the first criterion. Among those 83 animals, 23 animals met the second criteria (Fig. 3).

The first hypothesis tested, unconditioned on individual treatment, if the time of measurement was associated with a change in the prevalence of any *vt* genes. Matched samples from 83 animals were used in this analysis. An exact test for the binomial distribution of the discordant pairs was conducted and the null hypothesis was that the proportion = 0·5. Fisher's exact *P* value was calculated. The discordant pairs refer to those animals that were either found initially positive for any *vt* genes (October–December) and then found negative at the second testing (February) or vice versa. This method of analysis was based on the concept that if time of sampling was unrelated to the probability of becoming positive, then an equal number of animals should change from positive to negative for *vt* genes as change from negative to positive. Therefore the discordant pairs should have a binomial distribution with *p* (proportion) = 0·5. As this test was conducted on the faecal sample, the unit of analysis and observation for this test was the individual animal.

The second hypothesis tested the effect of individual animal treatment on the prevalence of specific virulence genes. However only 23 animals met the entry criteria for evaluation of the effect of individual animal treatment on verotoxin prevalence. Therefore, due to insufficient power for discrimination, only descriptive data are presented.

RESULTS

The number of animals, that is the number of faecal samples that tested positive by PCR for any *vt* genes among the 83 matched pairs is shown in Figure 3. The distribution of the 83 matched pairs is presented in Table 1. In our study, 22 of the 34 discordant pairs (64%) changed from *vt*-gene negative to *vt*-gene positive. The exact confidence interval for the binomial distribution of the discordant matched pairs tested by PCR for any *vt* genes was 0·48–0·80. This can be interpreted as saying that the 95% confidence interval for the proportion (*p*) of animals that changed from *vt*-gene negative at the first sampling to *vt*-gene positive at the second sample is 48–80%. Fisher's exact two-sided *P* value for the proportion of the discordant pairs, i.e. that $p = 0{\cdot}5$ was 0·12.

Table 2 presents the number of animals that tested positive for the presence of *vt1*, *vt2*, *eaeA* and *hlyA*, the number of positive *E. coli* within each animal, and the serotype of the positive *E. coli*. The *E. coli* isolates possessing any of the *vt1*, *vt2*, *eaeA* and *hlyA* genes or combinations thereof belonged to 31 different *E. coli* serovars. Note these tests for *vt1*, *vt2*, *eaeA* and *hlyA* genes were only performed on *E. coli* isolated from the 57 animals that tested positive by PCR. Among these 57 animals, 23 animals were paired, and among these animals the effect of being treated with oxytetracycline was not significantly associated with the distribution of the matched pairs.

DISCUSSION

A recent literature review from the US Center for Veterinary Medicine suggests that there is very little literature available that discusses the effect of antimicrobials on pathogen load in livestock [6]. Among

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Table 2. *The serotypes and distribution of specific virulence genes within verotoxin-gene positive commensal* E. coli *isolated from cattle*

Serotype	Animal ID	Sampling time	*vt1*	*vt2*	*eaeA*	*hlyA*	No. of isolates
O153:NM	48	Oct./Dec.	+	+	−	+	2
O26:NM	28	Oct./Dec.	−	−	+	−	1
O121:H7	31	Oct./Dec.	+	−	−	−	1
O121:H7	34	Oct./Dec.	+	−	−	−	1
O113:H21	103	Oct./Dec.	−	+	−	+	3
O126:H11	103	Oct./Dec.	+	−	+	+	1
O175:H16	119	Oct./Dec.	−	−	−	−	1
O46:H2	131	Oct./Dec.	−	+	−	−	1
O119:H?	131	Oct./Dec.	−	−	+	+	1
O?:H12	136	Oct./Dec.	−	−	−	−	1
O26:NM	145	Oct./Dec.	−	−	+	−	1
O116:NM	140	Oct./Dec.	−	+	−	+	1
O113:H21	104	Oct./Dec.	+	−	−	+	1
O113:H21	104	Oct./Dec.	−	+	−	+	1
O46:H2	1	Feb.	−	+	−	−	1
O116:NM	3	Feb.	−	+	−	+	3
O?:NM	3	Feb.	−	+	−	−	1
O116:NM	4	Feb.	−	+	−	+	2
O28:NM	8	Feb.	−	+	−	+	5
O156:H8	18	Feb.	−	−	+	−	5
O26:NM	22	Feb.	+	−	+	+	1
O26:NM	28	Feb.	+	−	+	+	1
O121:H7	31	Feb.	+	−	−	−	1
O26:NM	33	Feb.	+	−	+	+	3
O26:H11	34	Feb.	+	−	+	+	3
O26:NM	34	Feb.	+	−	+	+	1
O156:H8	41	Feb.	−	−	+	−	5
O156:H8	42	Feb.	−	−	+	−	3
O171:H2	46	Feb.	−	+	−	−	1
O153:NM	48	Feb.	+	+	−	+	1
O156:H8	60	Feb.	−	−	+	−	3
O113:H21	64	Feb.	−	+	−	+	1
O?:H31	65	Feb.	−	−	−	−	1
O10:NM	109	Feb.	−	−	−	−	1
O115:NM	110	Feb.	−	−	−	−	1
O10:NM	121	Feb.	−	−	+	+	1
O130:H38	121	Feb.	+	+	−	+	1
O10:NM	121	Feb.	+	+	−	+	2
O?:H8	125	Feb.	−	+	−	−	1
O107:NM	133	Feb.	−	−	+	+	1
O4:H37	136	Feb.	−	−	−	+	1
O2:H25	148	Feb.	−	−	−	+	1
O136:H12	150	Feb.	−	+	−	+	1
O2:H27	150	Feb.	−	−	−	+	1
O153:H21	150	Feb.	−	−	+	−	1
O64:H38	151	Feb.	−	−	+	+	1
O115:NM	151	Feb.	−	−	−	+	2
O2:H27	153	Feb.	−	−	−	+	2
O153:H21	153	Feb.	−	−	+	−	1
O136:H12	153	Feb.	−	+	−	+	1
O2:H25	153	Feb.	−	+	−	+	1
O171:H2	178	Feb.	−	+	−	−	3
O113:NM	179	Feb.	+	+	−	−	1
O35:H19	183	Feb.	−	+	−	+	4
O15:H16	185	Feb.	−	+	−	−	4

Example: In Animal ID 103 (row 5) three isolates were serotype O126:H11. These three isolates were *vt1*, *vt2* and *hlyA* positive. In animal 104 (row 13 and 14) two isolates were serotype O113:H21. However these two serotypes had different patterns therefore they are a different row in the table.

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29 studies available, only two studies on calves appear to be available in the peer-reviewed literature [6]. All studies examined *Salmonella* and the conclusion drawn by the review suggests that in swine and poultry, the only species studied to any degree, there is little to suggest that use of antimicrobials increases the pathogen load. This study, however, did not examine changes in the actual number of pathogenic bacteria in food-producing animals; rather it focused on the prevalence of virulence genes within the population. The rationale behind this approach was two-fold: first, the previously cited review suggests that to date there is little evidence to suggest that antimicrobials do increase pathogen load, secondly, it seems feasible that rather than inducing changes in the overall population size of particular bacteria, antibiotics may cause a change in the population demographics so that within species some genes become more prevalent, in a similar manner in which resistance genes become more prevalent under selection pressure from antimicrobial use.

Our intent was to examine the association between therapeutic use and the prevalence of virulence genes and we did not find an association between individual treatment with oxytetracycline and the prevalence of virulence genes in the matched pairs. The use of the paired analysis in a study of this type significantly reduces the potential number of data-points that are available for analysis as only the discordant pairs are used. The study design utilized self-pairing to control for potential differences in baseline levels of prevalence of *vt* genes in the bacteria. This approach was required because allocation to the treatment groups, i.e. receiving injectable oxytetracycline (yes/no) was non-random. Normally, in experiments or field trials, we expect randomization to control for differences in confounding variables between study treatment groups. However, in this observation study non-random allocation of oxytetracycline to animals occurred, that is only sick animals received oxytetracycline and we had no control over which animal became sick, as a result we could not be sure that the baseline levels of *vt* prevalence between the well and sick animals were equal. We also had reasonable justification to believe that being 'sick' could cause changes in the gut flora, as these animals were likely to have reduced feed intake. Technically, this should have increased the power to detect differences, as it should reduce the variability of the outcome, however, as with all rare outcomes this can still lead to small data sets. A study of similar size using independent populations will probably have less power due to initial differences in the baseline prevalence of the outcome and within and between animal variation.

We did, however, find an increase in the prevalence of virulence genes between the two sampling times. Despite observing a Fisher's exact P value of 0·12 in the present study, we would argue that the data suggest an increase in the prevalence of the PCR for *vt* genes being positive at the second sampling, because the confidence interval of the proportion of animals that changed from negative to positive (from 48 to 80%) is skewed towards the right and this suggests that the prevalence of *vt* genes increased between sampling, i.e. many more point estimates greater than 50% than those less than 50% are consistent with the study data. If there was no external pressure for animals to become *vt*-gene positive, then the binomial distribution of the discordant pairs should approach $p=0{\cdot}5$ in large samples. In our small sample, these requirements are met suggesting no association. However, ignoring the distribution of the confidence interval and its accompanying P value function and relying solely on P value and hypothesis testing to interpret the data relies too heavily on significance testing [7–9]. Furthermore, Fisher's exact P value for the binomial distribution is a two-sided test, i.e. testing that $p=0{\cdot}50$, not that $p>0{\cdot}50$ or $p<0{\cdot}50$. A two-sided test was conducted, as theoretically a one-sided test should only be used when the proportion can only move in one direction, which was not the case in this study. In our opinion, the data are suggestive of an association.

This study was inappropriate to address the question of whether additional in-feed antibiotics are associated with an increase in the prevalence of verotoxin genes, however the data arising from it is suggestive of a need for further study in this area. The study design is inappropriate because it cannot differentiate between the many factors that may have changed between the two sampling times. Two obvious factors that may have influenced the prevalence of *vt* genes are (1) changes due to transfer of bacteria and/or bacterial genes between animals during the study period and (2) the addition of chlortetracycline to the diet. As the animals in the study had only entered the facility several weeks prior to testing, ample opportunity for mixing of animal and faecal flora might not have occurred by the time the animals were sampled for the first time. However, at the second sampling the animals had

been in the facility for about 6 months. During that time, faecal bacteria or the *vt* genes may have been transferred between cattle. Alternatively, the addition of chlortetracycline may have been associated with the change in the prevalence of the virulence genes.

The mechanism by which antimicrobial use, in particular, chlortetracycline use, might select for *E. coli* containing the virulence genes or result in the propagation and release of bacteriophages encoding the *vt* genes, thereby increasing the prevalence of virulence genes in faecal *E. coli* is not clear. The *vt* genes are located downstream from a phage-encoded regulatory gene (Q gene) that regulates expression of the late phage genes including the *vt* operon [10]. It has been demonstrated that the toxin-converting bacteriophage may be induced to express phage functions and lytic growth by exposure to mitomycon C. Increased replication of the phages results in an increase in toxin production that is released by phage-mediated lysis of the bacteria [11]. Other reports have also indicated the probability of induction of the prophages by different antibiotics [12–14]. This may be one mechanism by which increased prevalence of *vt* genes in faecal *E. coli* might occur. However, we are not aware of studies that have examined the effect of the administration of tetracyclines on induction of prophages encoding verotoxins and the transfer of *vt* prophages from one *E. coli* to another in the intestinal tract. In a study of a large outbreak of enterohaemorrhagic *E. coli* O157:H7 infection in Japan, 14·8% of the O157:H7 isolates from patients were resistant to tetracycline and 10·6% were resistant to tetracycline and streptomycin; in contrast, none of the O157 isolates from potato salad implicated as the source of the outbreak were resistant to antimicrobials [15]. Tetracycline resistance was encoded on a conjugative plasmid that showed a high rate of transfer by conjugation to the *E. coli* C600 recipient strain. This would suggest that administration of tetracyclines to animals or humans may select for transfer of tetracycline resistance genes encoded on a conjugative plasmid and increase the likelihood of colonization with tetracycline-resistant verotoxigenic *E. coli* strains [13, 16].

We noted that the *E. coli* strains possessing any of the *vt1*, *vt2*, *eaeA* and *hlyA* genes or combinations thereof belonged to more than 30 different *E. coli* serovars. Thus the prevalence of verotoxigenic *E. coli* was not due to the spread of one or a few serovars only.

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