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Impact of the Direct Application of Therapeutic Agents to the Terminal Recta of Experimentally Colonized Calves on *Escherichia coli* O157:H7 Shedding[∇]

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Enterohemorrhagic *Escherichia coli* O157:H7 is an important intestinal pathogen of humans with a main reservoir of domesticated ruminants, particularly cattle. It is anticipated that the risk of human infection can be reduced by controlling the organism within its reservoir hosts. Several options for the control of *E. coli* O157:H7 in cattle have been proposed, but none have been demonstrated to be successful in the field. Here we describe a novel experimental method, based on the terminal-rectum-restricted colonization described previously, to eliminate fecal carriage of *E. coli* O157:H7. In experimentally challenged calves, direct application to the rectal mucosa of either of two therapeutic agents, polymyxin B or chlorhexidine, greatly reduced bacterial shedding levels in the immediate posttreatment period. The most efficacious therapeutic agent, chlorhexidine, was compared in orally and rectally challenged calves. The treatment eliminated high-level shedding and reduced low-level shedding by killing bacteria at the terminal rectum. A rapid-detection system based on the ability to identify *E. coli* O157:H7 from swabs of the rectal mucosa was also assessed. This test was sufficiently sensitive to identify high-level bacterial carriage. Thus, a combination of the detection method and treatment regimens could be used in the field to eliminate high-level fecal excretion of *E. coli* O157:H7, so greatly reducing its prevalence within this host and the risk of human infection.

Enterohemorrhagic *Escherichia coli* (EHEC) has emerged in developed countries over the past 20 years as an important cause of human intestinal disease. In addition to bloody diarrhea, intestinal infection can lead to potentially fatal systemic sequelae resulting from the activity of Shiga toxins. The majority of these infections in the United States, Canada, United Kingdom, and Japan are caused by *E. coli* O157:H7 (23). This serotype has been frequently isolated from cattle feces, and most human *E. coli* O157:H7 infections originate, either directly or indirectly, from this source (2, 3). Control measures have sought to minimize the transmission of *E. coli* O157:H7 and other EHEC strains from cattle to humans via the food chain. Among the preslaughter control strategies, the most effective would be those that reduce fecal shedding from food-producing animals (18). For example, a reduction in the incidence of human infections in the United States coincided with improved screening and removal of *E. coli* O157:H7-positive meat in beef-processing plants following a Food Safety and Inspection Service notice in 2002 (9). However, routes of transmission other than the food chain, such as infection through drinking water (20) or direct contact with animal feces (10), are significant in certain parts of the world. Therefore, to reduce the risk of *E. coli* O157:H7 infection for humans requires control within the bovine population.

To our knowledge, there is no method currently being applied to control the colonization by this microorganism in food-producing animals. However, several methods have been tested experimentally and these have been thoroughly reviewed (8, 30). Vaccination is potentially the most effective and economically viable option, and several candidate vaccines have been examined (14, 19, 34). Though a vaccine based on secreted proteins showed experimental promise (32), at field scale this did not perform satisfactorily (25). Other vaccines are being developed, but the possibility exists that none will be sufficiently protective to substantially reduce prevalence levels. Several groups have observed an effect on fecal shedding (determined either by prevalence, duration of shedding, or fecal concentration) from various biological controls (4, 5, 29, 31, 33, 35). Dietary manipulations have also been reported to alter fecal shedding of *E. coli* O157:H7 in cattle, but data from different studies are conflicting (17, 27). Chemical compounds with antimicrobial activity, esculin (15) and sodium chlorate (7), have both been shown to reduce prevalence experimentally, but the latter has not been approved for use in food-producing animals due to potential mammalian toxicity. More-conventional antimicrobials, such as neomycin and oxytetracycline, had little effect on shedding levels in calves in one study (1), but in another study neomycin was shown to reduce shedding in calves (16). However, continuous use of antimicrobials to treat *E. coli* O157:H7 has been traditionally seen as unacceptable through fears of selecting for bacterial resistance.

Since the maintenance of *E. coli* O157:H7 within a cattle population can be dependent upon a small proportion of cattle that are shedding high levels of the organism in feces (22), a

control strategy that successfully targets this minority of positive animals should have a major impact on *E. coli* O157:H7 prevalence. Colonization of the terminal rectum (TR) is required for this high-level fecal shedding (21), and targeting of treatments to the TR (24) provides an excellent opportunity for a control method. The accessibility of the site means that antimicrobial agents can be applied directly to the TRs of food-producing animals in a single application. By the same principle, the effect of applying multiple doses of *E. coli* O157:H7 lytic bacteriophages to the TRs of colonized cattle has been recently assessed (29) and reduced but did not eliminate bacterial shedding. In this study, two compounds, polymyxin B and chlorhexidine, were assessed for the ability to reduce or eliminate *E. coli* O157:H7 from experimentally challenged calves by administration directly to the rectal mucosa. It has shown that a proportion of orally challenged calves have low levels of *E. coli* O157:H7 originating from regions other than the TR, as demonstrated by postmortem sampling of intestinal contents within this and previous studies (6, 11, 24). Our experimental design therefore involved a comparison of treatment efficacy in both rectally and orally challenged calves in order to assess the impact on fecal shedding of non-TR sites of colonization. An initial experiment (experiment 1) involved a total of 54 weaned calves in three batches of 18 that were challenged with a nalidixic acid-resistant, Shiga toxin-negative *E. coli* O157:H7 strain (ZAP 198) (24). The initial objective was to compare *E. coli* O157:H7 shedding following rectal challenge to the established oral challenge route (24), as it was important to demonstrate that the two challenge routes created qualitatively and quantitatively similar patterns of colonization at the TR. Rectal challenge would then permit an assessment of the efficacy of subsequent treatment methods in the absence of bacteria originating from nonrectal sites.

The objective of experiment 2 was to assess the impact of two agents applied to the TR mucosa on *E. coli* O157:H7 shedding. In experiment 2, a total of 53 calves in six batches were successfully colonized (defined by fecal *E. coli* O157:H7 concentrations of greater than 10^3 CFU/g immediately prior to treatment). Initially, both the chlorhexidine and polymyxin protocols were highly successful in rectally challenged calves but the chlorhexidine treatment was selected for more extensive assessment.

Detection of rectally colonized *E. coli* O157:H7 by rectal swabbing of colonized cattle is a highly sensitive means of identifying positive animals when combined with enrichment culture (13, 26). An additional objective within this study was to combine rectal swabbing with an immunochemical test of sufficient sensitivity to create a rapid field method to detect high-level bacterial shedding. Such a procedure could offer many advantages when used in conjunction with the aforementioned treatment regimen.

MATERIALS AND METHODS

Animals and experimental challenge. Experimental calf challenges were performed at Moredun Research Institute (MRI) in containment level 2 housing facilities authorized by Home Office license number 60/3179. Ethical consent was obtained from the MRI Animal Experiments Committee. Calves were reared conventionally on the same farm of origin until at least 2 weeks postweaning and transported to MRI, where they were acclimatized for 1 week prior to challenge. Calves were tethered in individual pens separated by solid walls. They were fed twice daily on QRD pellets (BOCM Pauls, Renfrew, United Kingdom) and had

ad libitum access to hay and water. Prior to challenge, fecal samples from each calf were confirmed to be negative for *E. coli* O157 by overnight enrichment culture in tryptic soya broth (Oxoid) containing 20 mg/liter novobiocin, followed by immunomagnetic separation with anti-O157 immunoglobulin-coated paramagnetic beads (IDG, United Kingdom) and culture on CT-SMAC plates (cefixime-tellurite sorbitol MacConkey; Oxoid). At the time of challenge, the mean calf ages were 114 days (experiment 1, orally challenged), 115 days (experiment 1, rectally challenged), 80 days (experiment 2, orally challenged), and 71 days (experiment 2, rectally challenged).

Bacterial challenge and enumeration. The challenge bacteria (nalidixic acid-resistant *E. coli* O157:H7 Walla 3) were cultured overnight in L broth (Invitrogen) at 37°C with aeration. Each oral inoculum was created by adding 1 ml of undiluted culture to 20 ml of phosphate-buffered saline (PBS), resulting in approximately 10^9 CFU per dose. The oral inocula were administered to the calves via a stomach tube and washed down with 500 ml of sterile PBS. Rectal challenge was performed by smearing a large cotton swab (150-mm Jumbo cotton swab; Fisher Scientific) saturated with approximately 1 ml of an undiluted overnight bacterial culture (10^9 CFU/ml) over the rectal mucosa, a method similar to that described by Sheng et al. (28).

Feces were sampled on a daily basis and separated into surface and core components as previously described (24). There were some missing data; one calf died of unrelated causes, and three calves in one group became colonized with a nalidixic acid-resistant, sorbitol-fermenting *E. coli* strain that obscured the selective media used for enumeration. Stools were graded according to the ease with which the surface and core components were separated (A, separation likely to be poor due to feces being dry and pelleted; B, separation likely to be good; C, separation likely to be poor due to feces being too soft; D, separation impossible due to feces being liquid). Samples too liquid to be separated were processed as "whole feces." Ten-gram quantities of feces were homogenized in 90 ml of sterile PBS and where appropriate were serially diluted in 10-fold steps in PBS. One hundred microliters of each dilution was spread onto sorbitol MacConkey agar plates containing 15 µg ml^{-1} nalidixic acid (N-SMAC; Oxoid). All inoculated media were incubated overnight at 37°C. Non-sorbitol-fermenting colonies were counted, and a colony from each sample was tested for O157 lipopolysaccharide (LPS) with a latex agglutination test kit (Oxoid). The concentration of *E. coli* O157:H7 in log$_{10}$ CFU per gram was calculated. When performed, enrichment cultures consisted of 1 ml of an undiluted fecal suspension added to 9 ml of L broth containing 15 µg ml^{-1} nalidixic acid. Following incubation at 37°C for 24 h, the enrichment cultures were streaked onto N-SMAC plates and these were incubated overnight at 37°C.

Postmortem sampling. Postmortem sampling was performed on calves shedding *E. coli* O157:H7 beyond day 14 postchallenge in experiment 1 to confirm the TR-restricted colonization of rectally challenged calves and provide information on the possible degree of non-TR colonization of orally challenged calves. A full description of the methods and results of the postmortem sampling will be submitted for publication elsewhere. Essentially, 10-g samples of gut contents were obtained and *E. coli* O157:H7 bacteria were enumerated as described for feces above. For this paper, results from the large intestinal contents have been pooled. The proximal colon concentration was the mean of contents from three sites: the apex of the cecum, the ileocecocolic valve, and the site just proximal to the narrowing where the proximal colon becomes the spiral colon. The mid/distal-colon concentration was the mean of the contents from the spiral colon (point of inflection) and the distal colon (midway between the spiral colon and rectum).

Application of therapeutic agents to the TR. In experiment 2, application of therapeutic agents was performed at 7 days postchallenge. This timing band was selected, on the basis of results of experiment 1 and previous experience with this model, to allow sufficient time for establishment of colonization and before the point at which levels of *E. coli* O157:H7 shedding were expected to fall. Two treatment preparations were developed. One preparation included polymyxin B sulfate at a concentration of 2 mg/ml with sodium alginate at 10 mg/ml. This was applied via a syringe to the rectal mucosa in a volume of 100 ml. The second preparation contained chlorhexidine gluconate at a concentration of 2% (wt/vol) in a total volume of 500 ml. The choices of therapeutic agents were based on minimal gut absorption, low host toxicity, bactericidal efficacy, and a low risk of generating bacterial resistance. Alginate was used in the polymyxin preparation to increase the time of contact with the mucosal surface. This was not used for the chlorhexidine preparation, as a larger volume was applied. Therapeutic concentrations were based upon in vitro determination of the concentration and time required to achieve 100% killing of the challenge strain (data not shown).

Swab recovery and *E. coli* O157 detection. The rectal mucosas of calves to be treated with therapeutic agents in experiment 2 were swabbed with large cotton swabs that were then vortexed in 5 ml of PBS. The presence of *E. coli* O157 was
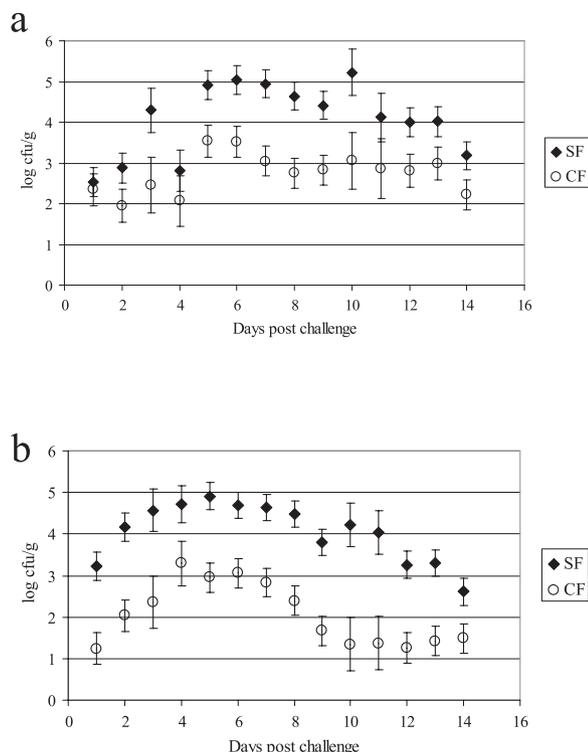
FIG. 1. Mean daily fecal *E. coli* O157:H7 concentrations ($\log_{10}$ CFU per gram) for orally (a) and rectally (b) challenged calves within experiment 1. Surface (SF) and core (CF) fecal compartments were calculated separately, allowing comparison on the $\log_{10}$ scale. Data include all samples where separation of fecal compartments was possible within the first 14 days postchallenge. Error bars indicate the standard errors of the means.

detected by immersing an O157 Coli-Strip (Coris BioConcept, Belgium) in 0.25 ml of swab suspension and reading the result 15 min later. The strength of the band, if positive, was subjectively categorized as weak, moderate, or strong. The concentration of *E. coli* O157:H7 in the swab suspension was determined by plating a serial dilution onto N-SMAC plates as described for fecal bacterial enumeration. Samples obtained in the latter part of the experiment were additionally processed if the initial test result was negative; the swab suspension was filtered through a 3-μm-pore-size glass fiber filter (type A/D; Pall) and centrifuged (1,500 × *g* for 10 min), and the deposit was resuspended in 100 μl of sample diluent. A second O157 Coli-Strip was then immersed in the suspension and read 15 min later.

Quantification of serum anti-O157 LPS IgG. Serum samples of experiment 1 calves were collected prior to challenge and at postmortem. Purified O157 (List Biological Laboratories) was conjugated to polymyxin B as previously described (12) and used as a coating antigen for an indirect enzyme-linked immunosorbent assay. Microtiter plates (Immulon 2 HB; Thermo Electron) were coated at the optimum coating concentration, determined to be 1 μg per well in 100 μl of carbonate buffer, and incubated overnight at 4°C. Twofold dilution series of test and standard sera from 1 in 12.5 to 12,800 were applied to the coated plates, which were then incubated for 1 h. Pre- and postchallenge serum samples from

the same calves were always tested on the same plate. After washing, the secondary antibody (sheep anti-bovine immunoglobulin G [IgG] conjugated with horseradish peroxidase; Serotec) diluted 1 in 800 was added for 1 h of incubation and then the plates were washed and developed with substrate (Sigma-Fast OPD; Sigma-Aldrich). Plates were read in a Dynex Revelation 3.04 enzyme-linked immunosorbent assay plate reader at 492 nm. Endpoint titers were determined, and the fold increase in the postcolonization samples relative to the prechallenge serum titer was calculated for each calf.

Data analysis. Unless otherwise stated, bacterial counts are expressed as the $\log_{10}$ (count plus one) to permit samples with zero plate counts to be expressed as zero. However, it is possible that a sample expressed as zero may have contained bacteria at levels below the limit of detection, in theory, $\log_{10}$ 1.5. A repeated-measures model was fitted to the log counts for the surface samples for the first 14 days postchallenge; after day 14, high-shedding calves were selectively culled. Calf group and route of challenge were included as factors in the model. Because the data set contained some missing observations, the correlation between observations on the same animal was modeled with a power model. Additional statistics were used to summarize the colonization ability and shedding patterns of the challenge strain administered by the rectal and oral routes. These statistics were calculated from *E. coli* O157:H7 counts from surface feces (or whole feces when separation was not possible). The area under the curve (AUC) for shedding for the first 14 days postchallenge was calculated by the trapezoidal method. Uptake was the proportion of challenged calves that became colonized initially (AUC, >10). The peak level was the highest *E. coli* O157:H7 concentration observed within the shedding period. Persistence was the proportion of calves that were initially colonized with *E. coli* O157:H7 still detectable by direct plating at day 14 postchallenge. The distribution of anti-O157 LPS IgG serum titers was positively skewed, and the results are expressed as $\log_2$ relative titers. Pre- and postcolonization titers were compared with a one-sample *t* test. The graphs in Fig. 1 feature mean surface and core fecal *E. coli* O157:H7 concentrations calculated from day 1 postchallenge by using all samples where separation was possible (grades A, B, and C) in all animals with confirmed uptake (AUC, >10).

In experiment 2, calves with surface fecal concentrations of at least 10^3 CFU/g on the day of treatment were included in the analyses of the treatment effect. In order to estimate the changes in fecal concentration in response to treatment, a general linear mixed model was fitted to the change in the number of CFU per gram between the immediate pretreatment and 1 day posttreatment (PT) samples. The type of intervention and route of challenge were included as fixed effects, and calf batch was included as a random effect. Approximate 95% confidence intervals for the mean change were calculated as the mean plus or minus two times the appropriate standard error. Further analysis was performed on the bacterial counts for several time points before and after intervention for the chlorhexidine and control groups by a repeated-measures model fitted to the data. The lack of independence between counts made on the same individual were modeled with a power model. Type of intervention (chlorhexidine or control) and route (oral or rectal) were fitted as fixed effects.

All statistical calculations were performed in Genstat 8th edition; the parameters of the repeated-measures and general linear mixed models were estimated with the restricted maximum-likelihood directive.

RESULTS

Comparison of challenge routes. Several parameters were used to compare the oral and rectal challenge routes in experiment 1 (Table 1). These included uptake, mean peak count, mean AUC, persistence, and mean serum IgG anti-O157 LPS responses to colonization. No statistically significant differences were demonstrated for these parameters between the

TABLE 1. Comparison of the two challenge routes in experiment 1 calves

Challenge route	Uptake (no. of calves colonized/total)	Persistence (no. of calves shedding/total)	Mean peak count ($\log_{10}$ CFU/g) ± SD (CI[a])	Mean serum IgG LPS ($\log_2$ relative titer) ± SD (CI)
Rectal	22/24	16/22	5.8 ± 0.6 (5.5–6.0)	2.5 ± 1.9 (1.5–3.5)
Oral	24/30	15/20[b]	5.5 ± 0.7 (5.1–5.8)	2.3 ± 1.9 (1.3–3.3)

[a] CI, 95% confidence interval for the mean.
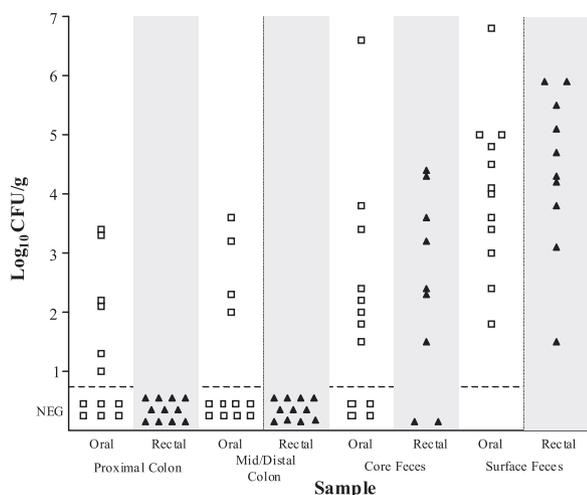[b] The records for four calves were incomplete.

FIG. 2. Individual *E. coli* O157:H7 concentrations ($\log_{10}$ CFU per gram) in postmortem-derived gut contents and antemortem fecal compartments in experiment 1 calves. Rectally and orally challenged calves are indicated in separate columns. Samples negative by direct plating are in the region below the broken line labeled NEG.

two challenge routes. The daily mean levels of bacterial shedding generated by the two challenge routes throughout the 14-day observation period are compared in Fig. 1a and b by using mean fecal surface and core *E. coli* O157:H7 concentrations. Typically, an establishment period of a few days elapsed in which the fecal concentrations gradually increased. In the orally challenged calves, this increase coincided with the appearance of a large surface-to-core ratio. In contrast, in the rectally challenged calves the surface-to-core ratio was immediately high, suggesting that rectal colonization occurred at challenge. This colonization was delayed in the orally challenged calves, and the fecal *E. coli* O157:H7 bacteria detected prior to this point were most likely a result of the transient passage of the initial challenge bacteria. The mean surface-to-core ratios beyond day 5 postchallenge were similar for both challenge routes when calculated for grade B samples, approximately $\log_{10}$ 2. Anti-O157 LPS IgG serum titers increased significantly in both challenge groups between the pre- and postchallenge sampling points ($P < 0.001$), but this increase did not differ significantly between challenge groups (Table 1).

It was demonstrated previously (24) that although the majority of fecal *E. coli* O157:H7 bacteria originate at the TR, a

proportion of individuals carry low levels at other sites. These levels typically arise in the proximal colon, though numbers of *E. coli* O157:H7 bacteria in gut contents do not increase significantly until the TR, where those originating from higher intestinal colonization sites are obscured by much higher concentrations at the rectal mucosa. To confirm the TR-restricted colonization of rectally challenged calves and provide information on the possible degree of non-TR colonization of orally challenged calves, postmortem sampling was performed on calves in experiment 1 still shedding beyond day 14 postchallenge. The large-intestinal-content concentrations of *E. coli* O157:H7 at postmortem are shown in Fig. 2. The proximal-colon contents were all negative for rectally challenged calves. *E. coli* O157:H7 within the core samples of these calves therefore originated from the surface feces rather than non-TR sites. *E. coli* O157:H7 concentrations were highest in the surface feces, the difference being at least 10-fold and often considerably higher where reliable separation of the surface and core was achieved. For the orally challenged calves, a third (4 of 12) of the mid- and distal-colon contents contained detectable *E. coli* O157:H7 but target bacterial numbers in surface fecal samples were always much higher and the core fecal samples therefore contained a mixture of *E. coli* O157:H7 from non-TR and TR sources.

Treatment effects in the immediate PT period. A total of 22 rectally challenged calves were colonized. Of these, 4 were left as untreated controls, 6 were treated with polymyxin, and 12 were treated with chlorhexidine. Thirty-one orally challenged calves were successfully colonized, and of these, 6 were untreated controls, 2 were treated with polymyxin, and 23 were treated with chlorhexidine. Treatments were applied at 7 days after challenge, and daily fecal samples were taken up to this point and for 7 further days for enumeration of *E. coli* O157:H7 bacteria. On day 7 PT, the fecal samples, if negative by direct plating, were subjected to enrichment culture to confirm the absence of the challenge strain. The 11 calves in the final group were maintained for 3 weeks after treatment to monitor for reacquisition of the challenge strain.

A summary of the bacterial shedding by calves before and after treatment with chlorhexidine and the same values obtained for untreated controls are shown in Table 2. The most direct means of assessing the effect of treatment is to compare the *E. coli* O157:H7 concentrations in feces immediately before and 1 day after treatment application. Table 2 shows the mean change in $\log_{10}$ CFU per gram for the chlorhexidine

TABLE 2. Comparison of chlorhexidine-treated and untreated groups

Challenge route and treatment	Pretreatment peak concn	Immediate pretreatment concn	Day 1 PT concn	Change in concn	Peak/day 7 PT concn	No. of calves
Oral						
Chlorhexidine	4.8[a]	4.3	1.5	−2.8 (−3.3, −2.3)[b]	2.1/0.7	23
None	4.6	3.8	2.9	−0.9 (−2.0, 0.1)	3.4/1.5	6
Rectal						
Chlorhexidine	4.9	4.5	0.5	−4.0 (−4.7, −3.3)	1.4/0.4	12
None	4.9	4.7	4.0	−0.7 (−2.0, 0.6)	4.5/1.9	4

[a] The values shown are $\log_{10}$ CFU/g.
[b] The values in parentheses are approximate 95% confidence intervals for the mean changes in concentration.

TABLE 3. Day 1 PT surface and core fecal *E. coli* O157:H7 levels of orally challenged, chlorhexidine-treated calves[a]

Challenge route, TR/non-TR colonization category, and calf ID[b] no.	SF[c]	CF[d]	S/C ratio[e]
Oral			
Likely/unlikely			
659	3.8	0.0	
663	2.4	0.0	
732	1.5	0.0	
666	2.4	0.0	
686	1.8	0.0	
Unlikely/likely			
602	1.5	1.5	1.0
641	2.4	2.3	1.3
651	2.6	2.8	0.5
679	4.6	4.3	1.8
689	2.8	2.7	1.3
604	2.7	2.5	1.4
Likely/possible			
635	3.6	1.8	53.7
726	2.5	1.8	4.4
Rectal			
Likely/unlikely			
661	3.9	0.0	
684	2.2	0.0	

[a] Excludes calves with no detectable *E. coli* O157:H7.
[b] ID, identification.
[c] SF, surface fecal concentration.
[d] CF, core fecal concentration.
[e] S/C ratio, ratio of surface to core fecal concentrations (not $\log_{10}$ transformed).

treatment groups together with approximate 95% confidence intervals estimated from the general linear mixed model. A general linear model was used to estimate significant differences between the change in the mean *E. coli* O157:H7 concentration for treatment type and challenge route. The reduction in mean bacterial concentration of the treated groups was significantly greater than that of the untreated groups ($P < 0.001$). Both polymyxin and chlorhexidine produced a significant reduction in $\log_{10}$ CFU per gram (mean changes of -3.4 and -3.2, respectively) relative to the untreated groups (mean change of -0.8), but there was no evidence that one was more effective than the other. There was evidence of a difference in treatment effects for calves challenged by different routes. The rectal route had a significantly greater ($P = 0.016$) reduction in $\log_{10}$ CFU per gram than the oral route (mean changes of -4.0 and -2.8, respectively, for the chlorhexidine group).

Factors confounding treatment success. Within the rectally challenged group, the treatment was highly successful, with the majority (10/12) being cleared of any detectable fecal *E. coli* O157:H7. This gives an indication of the direct effect of the treatment at eliminating the target organism at the TR. The success of treatment of the orally challenged group was inferior to that of the rectally challenged group, but this difference is only partially accounted for by *E. coli* O157:H7 originating from nonrectal sites. Examination of the surface-to-core ratios 1 day after treatment (Table 3) suggests that the failure to kill bacteria at the TR occurred in a greater proportion of the

orally challenged group (7/23) than in the rectally challenged group (2/12). However, a Fisher's exact test of these proportions yielded a mid-*P* value of 0.209, indicating that the difference was insignificant. Of the 13 orally challenged calves with $>10^{1.5}$ CFU/g in the day 1 PT feces, 7 exhibited evidence of TR colonization, with 5 having *E. coli* O157:H7 only in the surface feces and 2 having surface-to-core ratios of 4 or greater. Eight of the orally challenged calves had *E. coli* O157:H7 in the core feces at day 1 PT, six of which had surface-to-core ratios of less than 2, indicating likely nonrectal colonization. The fecal *E. coli* O157:H7 concentrations in all but one of these were less than 10^3 CFU/g.

Further analysis was performed on the bacterial counts for several time points before and after intervention for the chlorhexidine and control groups with a repeated-measures model fitted to the data. Group means and standard errors estimated from this model are displayed in Fig. 3. There was very strong evidence of a difference in shedding pattern over time between the two groups (time × intervention interaction, $P < 0.001$). In the chlorhexidine-treated groups, there was a marked decrease in the mean number of CFU per gram after intervention. There was also evidence of an effect of the route on the pattern of responses (route × intervention interaction, $P = 0.019$). In the oral control group, the mean counts decreased fairly steadily after the time of intervention (7 days postchallenge), whereas the decrease appeared to be delayed in the rectal group; however, these observations are based on small numbers of animals and the means have high estimated standard errors. For animals treated with chlorhexidine, the mean decrease in counts was more pronounced for the rectally challenged group than for the orally challenged group.

For rectally challenged calves, an increase in shedding beyond day 1 PT was substantial (an increase of $>\log_{10} 1$, with a peak PT concentration of $>\log_{10} 2$) in only 1 out of 12 individuals. Five of the 23 orally challenged calves had substantially increased shedding beyond day 1 PT. Analysis of the day 1 PT surface-to-core ratios of these individuals (Table 3) indicates examples in which recovery was likely to have originated from TR surviving bacteria (calf 663) and non-TR colonization
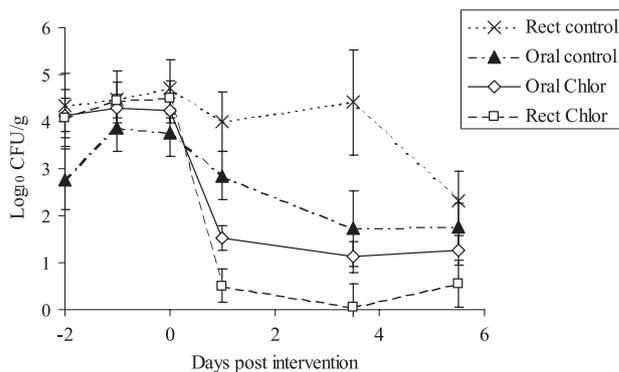

FIG. 3. Analysis of the mean daily fecal *E. coli* O157:H7 concentrations ($\log_{10}$ CFU per gram) around the time of intervention (day 0) for the chlorhexidine (Chlor) and control groups. A repeated-measures model was fitted to the data. Plots show the means, and error bars indicate the standard errors estimated from the model. Rect, rectal.

(calf 604), but the source of recovery was unclear for the other three calves.

On the basis of this analysis and the colonization parameters in Table 2, neither of the chlorhexidine-treated groups exhibited marked recovery of *E. coli* O157:H7. In order to assess recovery over a longer time, the final group of 11 calves was maintained for 3 to 4 weeks PT. Although occasional samples were enrichment culture positive for the challenge strain, there was no evidence of recolonization once the levels became negative by direct plating (data not shown).

Rapid detection based on direct recovery of *E. coli* O157 from the TR. The system used to identify cattle shedding high levels of *E. coli* O157 was based on the Coris BioConcept O157 Coli-Strip immunochemical detection kit. Although designed for use with broth enrichment cultures, the test strips can detect 10^4 *E. coli* O157 CFU/ml. Initial work suggested that this level could be obtained by swabbing the rectal mucosa of colonized calves. The concentrations of *E. coli* O157:H7 within rectal swab suspensions were compared to the corresponding fecal concentrations, collected up to an hour before the swab (data not shown). A positive linear relationship between the bacterial surface fecal levels and the swab recovery levels was demonstrated. The detection test positively identified all samples with *E. coli* O157:H7 concentrations in excess of 10^4 CFU/ml and the majority with concentrations at this detection limit. However, fecal concentration is used to determine the force of *E. coli* O157 contamination in field studies and transmission models and the detection limit of the procedure may be unacceptably high. For example, it did not identify all calves shedding *E. coli* O157:H7 above 10^4 CFU/g as positive. To lower the detection limit, swab suspensions negative by the immediate test were filtered and concentrated by centrifugation and the subsequent resuspended filtrate was retested. This procedure yielded a positive result for samples with 10^2 CFU/ml or greater within the preprocessed swab suspension (data not shown). The lowest corresponding fecal concentration of those testing positive after processing was 3.3×10^1 CFU/g, while the highest fecal *E. coli* O157:H7 concentration of those that were negative by both tests was 3.5×10^3 CFU/g.

DISCUSSION

An initial objective of this study was to assess different challenge routes in establishing persistent colonization of calves (experiment 1). In addition to the conventional oral challenge route, a rectal challenge protocol with a methodology and resultant colonization similar to those reported previously (28) was used to assess the efficacy of the treatment methods in the absence of bacteria originating from nonrectal sites. Importantly, the two challenge routes created qualitatively and quantitatively similar patterns of *E. coli* O157:H7 colonization, confirming the previous findings of Sheng et al. (28). Both challenge routes resulted in similar levels of uptake, *E. coli* O157:H7 shedding levels, persistence, and surface-to-core ratios that indicated colonization primarily occurred at the TR. Differences in the early stages after challenge reflect the physical differences between the two routes, as a rectal challenge deposits bacteria immediately onto the TR mucosa. We therefore conclude that, beyond the initial period of establishment of colonization, the rectal challenge method effectively reproduced the TR-restricted colonization pattern observed after an oral challenge.

It was anticipated from previous data (24) that low *E. coli* O157:H7 levels could originate from nonrectal regions after an oral challenge, and this was confirmed by postmortem sampling. The challenge strain was not recovered from the gut contents of rectally challenged calves, but relatively low concentrations were demonstrated in the core feces, indicating that separation of the two fecal components was rarely absolute. Incomplete separation of the two components when the surface concentration was high would therefore obscure any low concentrations of *E. coli* O157:H7 present in the core feces originating from nonrectal regions of orally challenged calves.

As an oral challenge may present the bacteria to sites such as the Peyer's patch, the colonization of host tissues may result in different humoral immune responses between challenge routes and potentially influence the clearance of the challenge bacteria. Serum anti-O157 LPS antibody responses were therefore used to compare the possible degree of exposure and responses for the two challenge routes. Although the serum anti-O157 LPS IgG titers increased significantly in response to colonization, the mean responses did not differ significantly between challenge groups.

The objective of the second part of this study (experiment 2) was the testing of two treatments applied to the TR in experimentally challenged calves. Our model generates groups of calves composed primarily of high shedders, and only those shedding $>10^3$ CFU/g at the time of intervention were used in the analyses. In orally challenged individuals, high numbers of inoculated bacteria increase the potential for non-TR colonization. Thus, this experiment represents a more stringent test of the treatment than may be expected for treatment of naturally colonized cattle, in which the majority of positive individuals shed very low levels (22). It is likely that high-level-shedding individuals would be the most resistant to a single treatment and are also the most important to target (18, 22).

Overall, the direct treatments were successful in reducing *E. coli* O157:H7 carriage and bacterial numbers were brought below the thresholds suggested by Matthews et al. (22) as necessary to eliminate effective transmission of the organism. Initially, two treatments were compared in rectally challenged calves and shedding parameters were determined before and after treatment. Though the difference in change between treatment types was small, the chlorhexidine treatment was chosen for further study. This choice was justified by the fact that an antiseptic such as chlorhexidine does not have the perceived risk of generating bacterial resistance associated with an antibiotic. In addition, the lower cost of chlorhexidine allows larger volumes to be applied, maximizing mucosal distribution, and is especially important when treating large numbers of large animals.

Various reasons for the failure of a treatment directly applied to the TR were anticipated. These included (i) failure of the treatment to kill bacteria at the TR, (ii) bacteria present in the digesta originating from nonrectal colonization sites, (iii) recolonization of the TR by bacteria surviving the treatment at the TR, and (iv) recolonization of the TR by bacteria originating from nonrectal sites. The difference in treatment effect between challenge routes was significant, and detailed analysis of the surface-to-core ratios in the PT feces of the chlorhexi-

dine-treated calves suggests that this was partially a result of bacteria originating from nonrectal regions in orally challenged calves (6 out of 23 calves) with the remaining difference being due to less efficacious bacterial killing at the TR in the orally challenged, relative to the rectally challenged, calves. The latter difference could be accounted for by random variability, as it was statistically insignificant and postmortem sampling (experiment 1) did not demonstrate any obvious difference between challenge routes in the pattern of colonization within the rectum (data not shown). However, the possibility that a hypothetical physiological effect, such as a stress response or a change in gene expression following passage through the gastrointestinal tract, reduced the bactericidal efficacy of the treatment in orally challenged calves cannot be ruled out.

The ability to identify high shedders with a rapid field test offers many advantages for use in conjunction with the treatment methods. As the direct test can be performed on the farm and results would be available in a very short time, it could identify individuals shedding high levels of *E. coli* O157 and facilitate the control of this microorganism within cattle in conjunction with the direct treatment method described in this paper.

It is beyond the scope of this paper to determine exactly how these methods would be best applied in the field, but treatment would have the greatest effect when applied at certain critical control points—for example, maintenance of clean herds by screening of introduced animals, treatment and removal of high shedders within an infected establishment or to limit transmission to other farms, markets, or slaughterhouses by implementation prior to transport. Since a single treatment was successful in the majority of individuals and reduced shedding levels below the threshold suggested by Matthews et al. (22), a large-scale experiment or field trial should be conducted in which the maintenance of *E. coli* O157 is dependent upon natural routes of transmission, to allow an accurate assessment of the efficacy of these experimental detection and treatment regimens. The applicability of this approach to the control of other EHEC strains originating from cattle would depend upon these strains also exhibiting a TR tropism. There is no evidence from published experimental challenges that this is the case, although the control of *E. coli* O157:H7 would lead to a considerable reduction in EHEC-mediated human disease in the majority of developed countries.

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