APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2006, p. 3872–3878 Vol. 72, No. 6
0099-2240/06/$08.00+0 doi:10.1128/AEM.02239-05

Field Trial Evaluating the Influence of Prophylactic and Therapeutic Antimicrobial Administration on Antimicrobial Resistance of Fecal *Escherichia coli* in Dairy Calves

Anna Catharina B. Berge,* Dale A. Moore, and William M. Sischo

University of California, Davis, Veterinary Medicine Teaching and Research Center, 18830 Road 112, Tulare, California 93274

Received 21 September 2005/Accepted 20 March 2006

The objective of this study was to describe the influence of in-feed and therapeutic antimicrobials on resistance in commensal fecal *Escherichia coli* isolated from preweaned calves. Four groups of 30, day-old calf-ranch calves were enrolled and raised until 4 weeks of age. Groups 1 to 3 were raised without antimicrobials in the feed. Group 1 was isolated from the other groups and received no antimicrobial therapy. Group 2 was housed on the calf ranch and did not receive antimicrobial therapy, whereas groups 3 and 4 could be treated with antimicrobials. Group 4 was fed neomycin and tetracycline HCl in the milk replacer. Fecal samples were collected from calves on days 1, 14, and 28. Three *E. coli* isolates per sample were evaluated for susceptibility to 12 antimicrobials. Cluster analysis was used to group isolates having similar susceptibility patterns. Cumulative logistic regression was used to evaluate factors associated with increasing levels of multiple antimicrobial resistance. In-feed antimicrobials were associated with higher levels of multiple antimicrobial resistance in fecal *E. coli*.f In calves not receiving in-feed antimicrobials, older calves had higher levels of resistance compared to day-old calves. Individual antimicrobial therapy increased resistance in these calves but appeared to be transient. There was no environmental influence on resistance in *E. coli* populations among study groups.

Although antimicrobials are used in calf rearing to treat and prevent disease, their use is increasingly viewed as a factor in the emergence of bacterial antimicrobial resistance in animal and human pathogens (13, 15, 16). Bull calves destined for beef are often raised on dedicated calf ranches in the United States. In these systems, calves are brought onto the ranch as day-olds and individually housed in hutches until they are weaned between 50 and 70 days of age. The challenge to management is that assembling large numbers of calves from multiple sources can create a highly susceptible population to infectious disease. To counteract this, antimicrobials such as tetracycline and neomycin are often incorporated into the milk replacer to prevent disease and raise healthy calves (8, 21, 24). The practice of adding antimicrobials into feed (milk or grain) creates a selection pressure for antimicrobial-resistant bacteria that could affect human health, but data associating antimicrobial resistance with prophylactic and metaphylactic antimicrobial use in animals are limited (23).

Multiple studies have shown that the young, preweaned calf harbors a multiply resistant commensal *Escherichia coli* enteric flora (17, 20, 25, 27). A recent study of commensal *E. coli* isolated from preweaned calves on calf ranches and dairies described the many factors associated with antimicrobial resistance in these organisms (4, 5). The central finding was that multiple-resistant fecal *E. coli* were predominant in preweaned calves regardless of antimicrobial exposure. Farm type (calf ranch versus dairy) and individual antimicrobial therapy were both associated with increasing levels of multiple antimicrobial resistance. The age of the calf (predominantly 2 to 4 weeks of age), a factor not directly associated with antimicrobial use, was also associated with increased levels of multiple antimicrobial resistance. These studies indicate that antimicrobial resistance is dynamic, and the effect of therapeutic and metaphylactic antimicrobial administration requires separate accounting from environmental and host-specific factors. The objective of this clinical trial was to assess the relative importance of antimicrobial and nonantimicrobial approaches to managing calf health as they relate to the development and persistence of antimicrobial resistance in commensal *E. coli* isolated from preweaned dairy calves. The null hypothesis tested was that the level of multiple-resistant commensal *E. coli* is independent of therapeutic or prophylactic administration of antimicrobials to preweaned calves.

MATERIALS AND METHODS

Study site. A clinical trial was conducted in California on a commercial calf ranch located in the southern San Joaquin Valley. The ranch raised calves for heifer replacements, veal, and dairy beef, and a daily inventory of approximately 2,000 calves on milk was maintained. Day-old calves from local dairies arrived at the ranch daily and were housed in wooden units that held three calves in individual hutches. Each unit had slatted floors and a plywood roof. The calves in each unit had the opportunity for nose-to-nose contact with their immediate neighbors. Each hutch was on cement blocks that raised them approximately 30 cm off the ground. Calves were bottle fed twice daily with 1.86 liters of milk replacer composed of a proprietary mix of milk powder and whey protein concentrate. Vitamins and minerals were added to each feeding according to farm protocols. The calves were introduced to nonmedicated starter grain in buckets on day 3, and this was replenished fresh daily thereafter. Fresh water was available ad libitum. On the day of arrival, all calves received 1.86 liters of a plasma-derived colostrum supplement (Lifeline; APC, Ames, Iowa) and were vaccinated as previously described (7).

Calf enrollment and processing. A total of 120, day-old, random source dairy bull calves were purchased from a commercial calf supplier. These calves were enrolled over a 2-day period, followed through 28 days of age, and then sold.

* Corresponding author. Mailing address: University of California, Davis, Veterinary Medicine Teaching and Research Center, 18830 Road 112, Tulare, CA 93274. Phone: (559) 688-1731, ext. 203. Fax: (559) 686-4231. E-mail: caberge@ucdavis.edu.

TABLE 1. Criteria for clinical diagnosis and therapeutic decisions used in the clinical trial[a]

Health condition	Clinical sign(s)	Score	Therapeutic choice(s)	
			Nonantimicrobial	Antimicrobial[b]
Diarrhea	Formed	0		
	Semiformed	1		
	Watery	2	Bismuth, Kaolin-Pectin	Ceftiofur[c]
	Watery with mucus	3	Kaolin-Pectin	Ceftiofur[c]
	Blood in feces	4	Kaolin-Pectin, UAA	Ceftiofur[c]
Respiratory signs	Normal	0		
	Rhinitis	1		
	Coughing	2		Ceftiofur[c]
	Heavy thoracic breathing	3		Ceftiofur
	Abdominal breathing	4		Ceftiofur
Dehydration	Normal appearance	0		
	Sunken eyes	1	Electrolytes[b]	
	Skin tented 5–10 s	2	Electrolytes	
	Skin tented >10 s	3	Electrolytes	
Attitude	Alert	0		
	Depressed	1	Flunixine meglumine	
	Nonresponsive	2	Flunixine meglumine	
Appetite	Normal	0		
	Consuming <3/4 bottle	1		
Eye appearance	Normal	0		
	Swelling/redness/discharge	1		Penicillin G, subconjunctival
Umbilicus/joints	Normal	0		
	Swelling/heat/pain/lameness	1	Iodine, topical	Penicillin G
	Normal	0		
Otitis	Head tilt, hanging head	1		Tilmicosin

[a] The clinical trial assessed the influence of antimicrobial use in milk replacer and as disease therapy in 120 preweaned calves on antimicrobial susceptibility in fecal commensal *Escherichia coli*.
[b] Treatment was initiated if clinical sign was combined with appetite loss, depressed attitude, or multiple diagnosis.
[c] Ceftiofur dose, 2.2 mg/kg/day for 3 to 5 days; penicillin G, 300,000 IU/ml, 2.2 ml/100 kg/day for 1 to 3 days; tilmicosin, 10 mg/kg/day for 3 days.

Calves originated from several dairy farms, were comingled on the transportation truck, and were representative of dairy bull calves destined for beef. No information on dairy source was provided with the calves, though no more than three calves could have come from a single farm. The calves were randomly off-loaded from the trailer by the calf dealer. No further randomization was attempted, and calves were assigned to treatment groups in the order they were removed from the calf trailer. Calves were placed in newly constructed wooden hutch units. The feeding strategy and vaccination program were identical to those of the ranch's commercially raised calves for beef or heifer replacement. The veterinarian in charge of the trial and two technicians were responsible for overseeing all aspects of the trial. Separate equipment was used for feeding and treating the study calves.

Health and performance monitoring. Calves were monitored for feed intake at all feedings and received a visual health appraisal twice daily that was recorded by both a veterinarian and the calf ranch manager responsible for health management on the ranch. Both were blinded to study group allocation and not involved with feeding or treatment administration. The health assessment was objectively based on appetite, fecal consistency, hydration status, respiratory effort, and attitude criteria (Table 1). Based on these criteria, a calf received therapeutic treatment by the veterinarian in charge of the study according to the antimicrobial treatment protocol described in Table 1.

Experimental groups. The trial was designed with four study groups consisting of 30 calves each. In group 1, calves were housed separately from the other experimental groups in an area that had not previously housed calves. Hutches were elevated 30 cm above a concrete floor that had been scraped clean and disinfected with a 0.52% sodium hypochlorite solution prior to the trial. Environmental samples taken from the concrete area prior to the start of the trial were culture negative for *E. coli* and *Salmonella*. The area was enclosed by a steel and plastic mesh fence. A biosafety area was provided for caretakers and consisted of a plastic tarp where protective clothes, boots, and gloves were used. Prior to entry into the calf area, boots were disinfected with 0.52% hypochlorite solution and 0.15% iodine solution baths. Separate, new feeding equipment was used for this group. Milk bottles and grain were transported to the calves without coming into contact with any other calf ranch equipment or soil. The study veterinarian ensured compliance with the biosafety measures and carried out all calf care, including feeding and treatments. Calves in this group were managed without using antimicrobials in the milk replacer. Therapeutic treatments consisted only of nonantimicrobial alternatives, such as bismuth salts, kaolin-pectin, flunixin meglumine, and electrolytes (Table 1). Calves in group 2 received no antimicrobials in the milk replacer and only nonantimicrobial treatments, as described for group 1. These calves were housed in a row separate from the rest of the calf ranch facility. No specific measures were taken to isolate these calves from the calf ranch environment. In group 3, calves were housed adjacent to and within 1 meter of group 2. Group 3 calves received no antimicrobials in the milk replacer but received individual antimicrobial therapy for clinical disease (Table 1). The antimicrobials used to treat disease were primarily ceftiofur hydrochloride (Excenel; Pfizer, Inc., New York) and occasionally penicillin G procaine (Aquacilin; Vedco, St. Joseph, MO) and tilmicosin (Micotil 300; Elanco Animal Health, Indiana). Nonantimicrobial alternatives as described above were used concurrently with antimicrobial treatments. In group 4, calves were housed adjacent to and within 1 meter of group 3. At each feeding, they received a medicated milk replacer containing tetracycline hydrochloride (22 mg/kg of body weight/day; TET-324; Agripharm, Grapevine, TX) and neomycin sulfate (22 mg/kg/day; Neomix AG 325; Pfizer, Inc., New York). The antimicrobials were diluted in water and added directly to each bottle before the milk replacer was added in the milk-mixing stage. Therapeutic treatment options were the same as for group 3.

Fecal sample collection and processing. Using two sterile cotton-tipped swabs, rectal fecal samples were taken from all calves on days 1, 14, and 28. Each fecal sample was streaked for colony isolation directly onto MacConkey agar and incubated for 24 h at 37°C. Three lactose-positive colonies of different morphologies were selected and restreaked onto MacConkey agar and incubated for 18 to 24 h at 37°C. Biochemical confirmation of the strains was performed on all isolates using triple sugar iron, sulfide indole motility, urea, Simmon's citrate, and oxidase tests. *E. coli* was defined as oxidase negative, indole positive, Simmons citrate negative, urease negative, and hydrogen sulfide negative (12). At least one isolate per calf and sampling occasion was stored in tryptic soy broth with 20% glycerol at −80°C.

Antibiograms. Antimicrobial susceptibility to 12 antimicrobials for each biochemically confirmed *E. coli* isolates was determined using a disk diffusion assay following CLSI (formerly NCCLS) standards and as previously described (2, 4, 22). The antimicrobial disks used were the following: ampicillin (AMP), 10 μg;

TABLE 2. Antimicrobial susceptibility clusters of fecal *E. coli* from pre-weaned calves[a]

Cluster	No. of isolates	Mean inhibition zone size (mm)											
		Beta-lactams				Aminoglycosides			Sulfonamides		TET	CHL	NAL
		AMC	AMP	CEF	XNL	AMK	GEN	STR	SULF	SXT			
A	357	22	20	18	27	22	21	16	24	28	21	23	23
B	55	21	19	18	27	22	20	15	22	27	**7**	21	22
C	3	**9**	**6**	**6**	21	23	21	17	26	28	21	21	19
D	7	18	**10**	15	25	22	21	**11**	**6**	20	20	24	24
E	67	21	19	18	26	22	21	**9**	**6**	20	**6**	23	23
F	16	19	**6**	17	25	21	19	**11**	20	27	**6**	21	22
G	13	24	23	22	28	22	19	**6**	**6**	**6**	**6**	24	24
H	4	**10**	**6**	**6**	**19**	23	21	**11**	25	27	**6**	24	22
I	35	19	**6**	17	27	22	21	**7**	**6**	19	**6**	23	23
J	8	22	20	19	25	21	16	**6**	**6**	16	**6**	**6**	22
K	6	19	**6**	19	27	23	21	**7**	**6**	**6**	**6**	23	23
L	26	**10**	**6**	**6**	**20**	22	21	**8**	**6**	20	**6**	22	23
M	35	19	**6**	17	27	22	**7**	**7**	**6**	**6**	**6**	24	23
N	8	19	**6**	18	26	22	20	**6**	**6**	**6**	**6**	**6**	25
O	14	**10**	**6**	**6**	**17**	22	21	**6**	**6**	**6**	**6**	23	22
P	12	18	**6**	13	26	22	**6**	**7**	**6**	**6**	**6**	**6**	25
Q	16	**10**	**6**	**6**	**16**	21	19	**6**	**6**	19	**6**	**6**	22
R	7	19	**10**	17	26	22	**10**	**6**	**6**	**6**	**6**	**6**	**6**
S	35	**9**	**6**	**6**	**18**	21	**7**	**8**	**6**	**6**	**6**	23	24
T	25	**10**	**6**	**6**	**18**	22	21	**7**	**6**	**6**	**6**	**6**	25
U	10	**9**	**6**	**6**	**18**	22	**7**	**6**	**6**	21	**7**	**6**	23
V	42	**9**	**6**	**6**	**17**	22	**9**	**6**	**6**	**6**	**6**	**6**	23
W	105	**10**	**6**	**6**	**15**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	21
X	3	**9**	**6**	**6**	**17**	16	**9**	**6**	**6**	**6**	**6**	**6**	**6**
Sum	909												

[a] This clinical trial evaluated the influence of antimicrobials in the milk replacer and as therapy on antimicrobial susceptibility in fecal commensal *E. coli*. The clusters are described by their mean zone sizes (in mm) in the disk diffusion assay to the 12 antimicrobials and ordered according to decreasing sum of mean zone sizes to the 12 antimicrobials. Boldface numbers indicate clusters where the mean antibiotic zone size was described as resistant.

amoxicillin-clavulanic acid (AMC), 20/10 μg; cephalothin (CEF), 30 μg; ceftiofur (XNL), 30 μg; amikacin (AMK), 30 μg; gentamicin (GEN), 10 μg; streptomycin (STR), 10 μg; sulfisoxazole (SULF), 250 μg; sulfamethoxazole-trimethoprim (SXT), 23.75–1.25 μg; tetracycline (TET), 30 μg; chloramphenicol (CHL), 30 μg; nalidixic acid (NAL), 30 μg. For each batch of isolates tested, quality control strain *E. coli* ATCC 25922 (ATCC, Manassas, Va.) was included in the assay set. Zone sizes (in mm) were measured with digital calipers to two decimal points, and these measurements were used for all quantitative analyses. The distributions of the zone sizes were assessed and graphed.

Serum inhibition bioassay. Blood samples (5 ml) were collected from all calves on the day of arrival. The samples were transported chilled directly to the laboratory for serum separation, and a serum inhibition bioassay was performed directly. The aim of this assay was to detect the presence of inhibitory substances in the blood. The method has been previously described (3, 5). Briefly, Mueller-Hinton agar containing *Bacillus subtilis* ATCC 6633 (1,600 CFU/ml in initial inoculation) was poured over a 20- by 20-cm plate, and 64 wells for 90-μl samples were made in the agar. Standard concentrations of penicillin were added in eight serial dilutions to the wells, and serum samples were added in triplicate to the remaining wells. The plates were incubated for 20 h at 37°C. The diameters of the zones of inhibition surrounding the wells were measured. Based on the penicillin standards, a standard curve was calculated and the inhibition zones of the calf serum were transformed into serum μg penicillin/ml. The *Bacillus subtilis* strain used in the assay had been tested in our laboratory and found sensitive to a set of 20 antimicrobials commonly used in bovine animals.

Data analyses. The statistical software program SAS (version 8.2; SAS Institute, Cary, NC) and StatXact-4 (Cytel Software Corporation, Cambridge, MA) were used for data analyses. The statistical unit of analysis was the *E. coli* isolate. Each isolate had a profile consisting of the measured inhibition zone size to the described 12 antimicrobials. All antimicrobials were used in the cluster analysis to group isolates having similar resistance patterns together. The cluster analysis methodology has been described elsewhere (4). Clusters were obtained using the squared Euclidean distance as a dissimilarity measure and Ward's minimum variance method (Proc Cluster method = wards). For each cluster, the mean zone sizes to the 12 antimicrobials were calculated. The clusters were ranked in order of decreasing sum of the mean zone size to the 12 antimicrobials. Susceptible clusters had large sums, while multiple-resistant clusters had relatively small sums. The order of the clusters therefore corresponded to increasing levels of resistance. Stratified analysis (Proc freq) was first used to evaluate shifts in antimicrobial resistance clusters between the calf groups and between sampling occasions (1, 14, and 28 days). The trends in the distributions were assessed using the chi-square statistic or the asymptotic nonparametric Jonckheere-Terpstra test (JT test). Cumulative logistic regression models utilizing a generalized estimating equation (GEE; Proc Genmod) were used to model trends in increasing levels of resistance by using ranked resistance cluster as the outcome variable (5). A repeated measure on each calf sampling time with an independent covariance matrix to account for evaluating three *E. coli* isolates per fecal sample was incorporated (1, 18). The models predicted the odds of an *E. coli* isolate belonging to a more resistant cluster compared to all less resistant clusters in the cluster hierarchy (5). For each isolate, experimental group affiliation and individual antimicrobial treatments received by the calf within 5 days of sampling were evaluated as covariates for shifts in antimicrobial resistance (5). The principle covariates, second-, and third-order interactions were tested for inclusion in the model, with a P value for entry set at 0.3 and a P value for retention in the model of 0.15.

RESULTS

No evidence of inhibition was revealed by the serum inhibition bioassays for detection of antimicrobials in calf serum, suggesting that none of the calves had received antimicrobial treatment prior to arrival on the farm. The mortality was high in the trial cohorts; 22 calves died, and 11 calves that were not eligible for antimicrobial treatment were censored from the study for animal welfare reasons (7). Five of the 22 calves died within the first 4 days of arrival and belonged to groups without

TABLE 3. Distribution of antimicrobial susceptibility clusters of fecal commensal *E. coli* isolates from calves[a]

Cluster	No. of isolates in calves at age:											
	1 day				14 days				28 days			
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
A	56	63	59	78	21	16	5	0	14	25	20	0
B	3	5	6	3	0	5	15	0	0	0	18	0
C	0	1	0	0	1	1	0	0	0	0	0	0
D	1	1	0	0	1	2	1	0	1	0	0	0
E	11	3	0	4	14	6	6	0	16	1	4	2
F	2	0	0	1	1	2	3	0	0	3	4	0
G	0	4	2	0	0	1	0	0	5	1	0	0
H	0	0	0	0	1	0	0	0	3	0	0	0
I	3	1	5	0	1	7	2	0	3	5	5	3
J	0	0	0	0	0	0	0	0	3	3	1	1
K	0	0	0	0	0	3	0	1	0	2	0	0
L	2	2	1	0	3	6	4	0	1	6	1	0
M	1	0	0	2	8	7	5	0	4	8	0	0
N	0	0	0	0	0	8	0	0	0	0	0	0
O	0	1	2	0	0	0	3	3	1	0	0	4
P	0	0	1	0	0	0	0	6	0	3	2	0
Q	0	0	0	0	0	0	11	5	0	0	0	0
R	0	0	0	0	1	0	0	0	0	0	1	5
S	0	0	0	4	3	6	13	1	0	2	3	3
T	0	1	3	0	0	2	7	3	0	0	1	8
U	0	0	0	0	6	0	4	0	0	0	0	0
V	0	0	1	0	5	0	1	21	0	0	10	4
W	0	0	0	0	0	0	4	50	0	0	3	48
X	0	0	0	0	0	0	0	0	0	0	0	3
Sum	79	82	80	92	66	72	84	90	51	59	73	81

[a] The influence of antimicrobials in the milk replacer and as therapy on the antimicrobial susceptibility in *E. coli* was evaluated. Group 1, no antimicrobial therapy and housed isolated from other study groups; group 2, no antimicrobial therapy and housed within the calf ranch; group 3, antimicrobial therapy available and housed within the calf ranch; group 4, antimicrobial therapy available, housed within the calf ranch, and received milk replacer medicated with antibiotics.

in-feed antibiotics. The isolates from these calves were excluded from the study. From the fecal samples obtained at days 1, 14, and 28, a total of 909 bacterial isolates were collected and biochemically confirmed as *E. coli*. Fifteen calves had been treated with ceftiofur within 5 days of the day 14 sampling, and one of these had also received tilmicosin. Eight calves had been treated with ceftiofur within 5 days of the day 28 sampling, and one of these had also received penicillin. A total of 69 *E. coli* isolates were therefore from calves that had been treated within 5 days of collection of the fecal sample.

***E. coli* antimicrobial resistance clusters.** The inhibition zone size distributions of the isolates revealed bimodal distributions to all 12 antibiotics tested. For descriptive and illustrative purposes only, the isolates were defined as resistant or susceptible to an antibiotic based on the trough in the bimodal distribution. The cut points (troughs, in mm) were as follows: AMK, 14; AMC, 14; AMP, 15; CEF, 12; XNL, 21; CHL, 14; GEN, 16; NAL, 16; STR, 13; SULF, 12; TET, 17; SXT, 12. Isolates with zone sizes equal to or larger than the cut point were defined as sensitive, and those with zones smaller than the cut point were defined as resistant. Multiple resistance was defined as exhibiting resistance to two or more antimicrobials.

The antimicrobial resistance patterns of the *E. coli* isolates to the 12 antimicrobials in the panel were grouped into 24 clusters, ordered by increasing level of resistance, and labeled A to X (Table 2). The number of antimicrobials to which the *E. coli* in the clusters exhibited resistance varied from 0 to 11 antimicrobials. Thirty-nine percent (357/909) of the *E. coli* isolates were sensitive to all antimicrobials tested; 6% (55/909) were resistant to a single antimicrobial, while 55% (412/909) of the isolates were multiply resistant. The quality control performed within limits during the study, and the standard deviation of the *E. coli* ATCC 25922 tests was between 1.2 and 2.3 mm for all antimicrobials.

Stratified analysis of antimicrobial cluster membership. Regardless of experimental group, isolates from day-old calves were predominantly susceptible to the 12 antimicrobials tested, with 71 to 85% of isolates belonging to cluster A (Table 3). The *E. coli* populations in all groups shifted to more resistant clusters at 14 and 28 days of age (JT test, $P < 0.0001$ for all four groups). The patterns of the shifts were not uniform across the experimental groups. Isolates from calves receiving no antimicrobials in the milk replacer or antimicrobial treatments (experimental groups 1 and 2) exhibited similar cluster distributions (JT test, $P = 0.31$) and generally belonged to less resistant clusters than the calves exposed to antimicrobials (groups 3 and 4) (JT test, $P < 0.0001$). At 14 days of age, calves with an opportunity for antimicrobial exposure (group 3) had more isolates in clusters containing ceftiofur resistance (H, L, O, Q, S, T, U, V, and W) than groups 1 and 2 (chi-square test, $P < 0.0001$). This effect was primarily associated with isolates from calves that received ceftiofur antimicrobial therapy in the 5 days prior to sampling (Table 4) (chi-square test, $P = 0.0001$). More *E. coli* isolates (48% of isolates) belonging to the most resistant clusters (V, W, and X) were isolated from calves receiving antimicrobials in the milk replacer (group 4)

TABLE 4. Antimicrobial susceptibility cluster distribution of fecal *E. coli* isolates from calves treated with ceftiofur within 5 days of sampling compared to calves not treated[a]

Cluster	No. (%) of isolates in cluster			
	Group 3[b]		Group 4[c]	
	No treatment	Treatment	No treatment	Treatment
A	18 (17.5)	7 (13.0)	0	0
B	21 (20.4)	12 (22.2)	0	0
C	0	0	0	0
D	1 (1.0)	0	0	0
E	9 (8.7)	1 (1.9)	2 (1.3)	0
F	7 (6.8)	0	0	0
G	0	0	0	0
H	0	0	0	0
I	7 (6.8)	0	3 (1.9)	0
J	1 (1.0)	0	1 (0.6)	0
K	0	0	1 (0.6)	0
L[d]	**4 (3.9)**	**1 (1.9)**	**0**	**0**
M	5 (4.9)	0	0	0
N	**0**	**0**	**0**	**0**
O	**0**	**3 (5.6)**	**7 (4.5)**	**0**
P	2 (1.9)	0	5 (3.2)	1 (6.7)
Q	**7 (6.8)**	**4 (7.4)**	**3 (1.9)**	**2 (13.3)**
R	1 (1.0)	0	5 (3.2)	0
S	**9 (8.7)**	**7 (13.0)**	**4 (2.6)**	**0**
T	**4 (3.9)**	**4 (7.4)**	**11 (7.1)**	**0**
U	**0**	**4 (7.4)**	**0**	**0**
V	**4 (3.9)**	**7 (13.0)**	**17 (10.9)**	**8 (53.3)**
W	**3 (2.9)**	**4 (7.4)**	**94 (60.3)**	**4 (26.7)**
X	**0**	**0**	**3 (1.9)**	**0**
Sum	103	54	156	15

[a] Comparison includes 14- and 28-day samples from calves not supplemented with antimicrobials in their milk replacer (group 3) and calves supplemented with antimicrobials in the milk replacer (group 4) on a commercial calf ranch.
[b] Calves fed no antimicrobials in milk replacer.
[c] Calves fed neomycin sulfate and tetracycline HCl in milk replacer.
[d] Boldface indicates clusters with ceftiofur resistance.

than in other groups (4% of isolates) (chi-square test, $P < 0.0001$). Cluster W was the dominant resistance pattern in group 4, containing 56 to 59% of the isolates at 14 and 28 days of age, respectively. Less than 5% of the *E. coli* isolates from calves in group 3 were found in cluster W, whereas no isolates from groups 1 and 2 belonged to this cluster. Few isolates from calves in group 4 belonged to the more susceptible clusters (A to J) at 14 and 28 days of age. There were no detectable shifts in clusters in calves in group 4 due to individual antibiotic treatment (Table 4).

Multivariate statistical models of predictive factors for increasing levels of resistance. The multivariate cumulative logistic regression models assessed the odds of fecal *E. coli* isolates belonging to a more resistant cluster compared to less resistant clusters at all levels in the cluster hierarchy. There were significant second- and third-order interactions between experimental groups, time, and therapeutic treatment covariates in the saturated logistic regression model. For ease of interpretation of the models, the analysis was split, and the data from groups 1 to 3 were analyzed separately from group 4 data. Both models were tested for interactions between the covariates.

The antimicrobial resistance patterns of fecal *E. coli* from calves that received no antimicrobials in the milk (study groups 1 to 3) were influenced by calf age at sampling and individual antimicrobial treatment within 5 days of sampling, but not by study group affiliation (Table 5). Compared to day-old calves, 14- and 28-day-old calves were more likely to shed increasingly multiple-resistant *E. coli*, with 14-day-old calves having the greatest odds of shedding increasingly resistant bacteria. The *E. coli* from calves that received individual antimicrobial treatment within 5 days of sampling were more resistant than the *E.*

TABLE 5. Two separate cumulative logistic regression GEE models assessing the influence of prior antimicrobial treatment, sampling time, and trial group affiliation on increasing multiple resistance of fecal *E. coli*

Variable	*N*	Estimate	*P* value	Odds ratio[b]	95% C.I.[a]
No antibiotic in milk replacer (groups 1 to 3; $N = 646$)					
Antimicrobial therapy within 5 days of sampling					
None	592	Reference			
Treated	54	1.11	0.02	3.03	1.15–7.98
Study group					
1	196	Reference			
2	213	−0.05	0.87	0.95	0.56–1.65
3	237	0.23	0.43	1.26	0.71–2.25
Calf age at sampling					
1 day	241	Reference			
14 days	222	2.32	<0.001	10.18	5.69–18.3
28 days	183	1.64	<0.001	5.16	2.82–9.41
Antibiotic in milk replacer (group 4; $N = 263$)					
Antimicrobial therapy within 5 days of sampling					
None	248	Reference			
Treated	15	−0.67	0.13	0.51	0.21–1.24
Calf age at sampling					
1 day	92	Reference			
14 days	90	6.41	<0.001	608.5	186.27–1,989.03
28 days	81	6.47	<0.001	645.87	196.25–2,125.79

[a] C.I., confidence interval.
[b] Cumulative odds ratio for increasing levels of resistance.

coli from untreated calves. The level of resistance observed in the *E. coli* isolates was not affected by being adjacent to or isolated from the other calf groups.

In the group fed antimicrobials in the milk replacer, *E. coli* isolates from 14- and 28-day-old calves were more likely to be increasingly multiple resistant compared to day-old calves (Table 5). There was no observed difference in antimicrobial resistance patterns of isolates collected at 14 days compared with 28 days of age. In addition, antimicrobial resistance patterns of *E. coli* isolated from these calves were not significantly affected by antimicrobial therapy.

DISCUSSION

This study demonstrated several risk factors associated with increasing multiple antimicrobial resistance in fecal *E. coli* isolated from preweaned calves: in-milk neomycin sulfate and tetracycline HCl fed as a prophylactic, ceftiofur administered as individual antimicrobial therapy for clinical disease, and calf age (14- and 28-day-old calves were at higher risk compared to day-old calves). The latter two risk factors were conditional on whether the calf received in-milk antimicrobials.

By themselves, antimicrobials in the calf milk replacer selected for a highly resistant *E. coli* population. In these calves, the influence on the *E. coli* population of other risk factors (antibiotic treatment or calf age) was not observed. The milk replacer containing neomycin sulfate and tetracycline HCl selected for bacteria with resistance to antimicrobials not used at the ranch, such as the aminoglycosides (amikacin, streptomycin, and gentamicin), chloramphenicol (florfenicol; the chloramphenicol-related veterinary drug was not used in the study calves), and sulfonamides (sulfisoxazole and sulfadimethoxazole-trimethoprim). We did not evaluate the persistence of these highly resistant *E. coli* isolates in calves receiving medicated milk replacer after 4 weeks. This would be of interest to further evaluate potential public and animal health risks associated with prophylactic antimicrobial therapy as these animals enter the food chain. It should be noted that the dosage of antibiotics added to the milk replacer in this study, while typical for many large calf ranches, exceeded the level used for prophylactic purposes in medicated feeds (14). We have observed similar shifts in *E. coli* antibiotic resistance patterns on other farms with prophylactic dose medicated feed (A. C. B. Berge, unpublished data).

Calves not receiving in-milk antimicrobials but being treated for clinical disease with individual antimicrobial therapy transiently shed a more resistant *E. coli* population than untreated calves. The *E. coli* isolates from treated calves belonged to clusters containing ceftiofur resistance, the antimicrobial used for the majority of treatments. The resistance pattern for these isolates included not only ceftiofur but other antimicrobials as well, i.e., the isolates were multiply resistant. This apparent selection effect was observed at both 14 and 28 days of age.

The majority of isolates (54%) from the calves in group 3 that had not received antimicrobial therapy within 5 days of sampling (though they could have been treated prior to this time) belonged to susceptibility clusters A to F. The majority of these isolates were either susceptible to all tested antibiotics or were resistant to only the β-lactams (not ceftiofur). These results were comparable to isolates from calves in groups 1 and 2, which received no antimicrobial treatments. A study in beef calf steers that assessed the effect of a single dose of florfenicol on antimicrobial resistance patterns of fecal *E. coli* detected similar transiently increased levels of multiple-resistant bacteria (6).

The age-related shift in resistance patterns in fecal *E. coli* in calves observed in this study has been described previously (5, 19, 20). Shifts towards higher levels of resistance in the nontreated calves indicate that there is a selection for more resistant bacteria that is not due to antimicrobial pressure.

Our study indicated there was little or no environmental transfer of resistant traits or bacteria between calves. Very few isolates with resistance to the study's primary therapeutic antimicrobial, ceftiofur, were isolated from calves that did not receive antimicrobial therapy within 5 days of sampling or had not been receiving in-milk antimicrobials. In a previous study, investigators were unable to detect increasing resistance in the nontreated control animals housed in the same pens as the treated animals (6).

We used cluster analyses based on disk diffusion zone sizes to group the bacteria into antimicrobial susceptibility profiles (4). The clustering methodology allows for the grouping of a large number of bacterial isolates on a large number of antimicrobial susceptibility phenotypes. Because this approach does not rely on characterizing isolates as resistant or susceptible based on clinical breakpoints, it is more appropriate for ecological studies (26).

For our analyses, we created a resistance cluster hierarchy based on the sum of the inhibition zones for the 12 antimicrobials tested. While this hierarchy may not reflect the underlying relationships of the genes governing the observed antimicrobial resistance, it also does not judge which type of antimicrobial resistance is "worse." As alternative analytical approaches, we analyzed the data using the number of specific antimicrobial resistances present based on the clinical cut points for human-source *E. coli* and also classified the isolates into four groups based upon the number of antimicrobials to which they were resistant. These models resulted in only minor changes in the coefficient estimates and confirmed the trends observed in the models assigning our hierarchical cluster as the dependent variable. The multinomial logistic regression model revealed factors associated with increasing trends in antimicrobial resistance. These models incorporated all patterns of resistance described in the rank-order as described. The objective to describe trends in antimicrobial resistance was therefore well met by the present modeling approach. Further studies of individual unique or minor resistance patterns would be of interest but were not addressed in this paper.

While it is clear from our study and others that the occurrence of antimicrobial resistance in commensal *E. coli* from calves has multiple causes, the use of antimicrobials is a dominant selective influence. This is particularly true for the use of antimicrobials in milk replacer, which selected for a highly resistant population of *E. coli* in our study. The use of antimicrobials in animal feed is controversial, with a consistent argument from public health practitioners that is a threat to the public health (16). Others have argued that continuing the use of antimicrobials in food animal production is important and not an important source of antibiotic resistance for humans (23). The most important question is whether there is any

support for their use for food animal health. Although antimicrobials have been routinely added to milk replacer for preweaned calves for decades, few studies exist that document their efficacy for reducing morbidity and mortality. A review paper by Constable cited only a few studies to support the efficacy of antimicrobials in milk replacer (9). More recent data suggested that calves receiving antimicrobials in milk had less mortality, had better weight gain, and had better overall health than calves not receiving in-milk antimicrobials (6). It is significant to point out that in the same study, failure of passive transfer of immunity, due to a management failure to provide neonatal calves with colostrum, was significantly associated with increased mortality, poorer calf health, and increased need for therapeutic treatments. It is reasonable to speculate that the use of in-milk antimicrobials to improve calf health is due to failure to support the calf's immune system and provide optimum rearing environments. Decreasing the use of in-milk antimicrobials would decrease the prevalence and likely duration of multiple antimicrobial-resistant commensal *E. coli* isolates observed in calves, but it could come at a cost to animal health and possibly safety of the food system, given the current status of calf health. In order to achieve a reduction of the use of in-feed antimicrobials, more effort needs to be made to optimize the components of the disease triad: host, pathogen, and environment. This requires that all calves receive adequate colostrum, be reared in clean, ventilated environments, and receive adequate nutritional support and that measures be taken to minimize spread of calf pathogens. It also requires that we continue to investigate management strategies that support the calf's systemic and local immunity, such as nutritional and immunologic supplements. The industry also needs to closely monitor their use of antimicrobials to ensure they are being appropriately applied through the use of treatment protocols and susceptibility testing of pathogens.

ACKNOWLEDGMENTS

This work was supported by the California Dairy Research Foundation, Center for Food Animal Health-SVM University of California, and NIFSI-CSREES (grant no. 00-51110-9721). Study protocol for animal use and care no. 9658 was approved by the International Animal Care and Use Committee (date of review, 3 July 2002).

We extend our gratitude to the calf ranch and ranch personnel participating in this study for providing facilities, equipment, and advice on calf rearing. For technical assistance, appreciation and thanks go to Staci Barnett, Paul Lindeque, Tina Lindeque, Mary Mecca, Katrin Newman, Bethann Palermo, Sonya Vasquez, and Chengling Xiao.

REFERENCES

1. **Agresti, A.** 2002. Categorical data analysis, p. 267–302. John Wiley and Sons, New York, N.Y.
2. **Bauer, A. W., W. M. Kirby, J. C. Sherris, and M. Turck.** 1966. Antimicrobial susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. **45:**493–496.
3. **Bennett, J. V., J. L. Brodie, E. J. Benner, and W. M. Kirby.** 1966. Simplified, accurate method for antimicrobial assay of clinical specimens. Appl. Microbiol. **14:**170–177.
4. **Berge, A. C. B., E. R. Atwill, and W. M. Sischo.** 2003. Assessing antimicrobial resistance in fecal *Escherichia coli* in young calves using cluster analysis techniques. Prev. Vet. Med. **61:**91–102.
5. **Berge, A. C. B., E. R. Atwill, and W. M. Sischo.** 2005. Animal and farm influences on the dynamics of antimicrobial resistance in faecal *Escherichia coli* in young dairy calves. Prev. Vet. Med. **69:**25–38.
6. **Berge, A. C. B., W. B. Epperson, and R. H. Pritchard.** 2005. Assessing the effect of a single dose florfenicol treatment in feedlot cattle on the antimicrobial resistance patterns in fecal *Escherichia coli*. J. Vet. Res. **36:**723–734.
7. **Berge, A. C. B., P. Lindeque, D. A. Moore, and W. M. Sischo.** 2005. A clinical trial evaluating prophylactic and therapeutic antimicrobial use on health and performance of calves. J. Dairy Sci. **88:**2166–2177.
8. **Braidwood, J. C., and N. W. Henry.** 1990. Clinical efficacy of chlortetracycline hydrochloride administered in milk replacer to calves. Vet. Rec. **127:** 297–301.
9. **Constable, P. D.** 2003. Use of antimicrobials to prevent calf diarrhea and septicemia. Bov. Pract. **37:**137–142.
10. Reference deleted.
11. Reference deleted.
12. **Farmer, J. J., III.** 1999. *Enterobacteriaceae*: introduction and identification, p. 442–459. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology. ASM Press, Washington, D.C.
13. **Fey, P. D., T. J. Safranek, M. E. Rupp, E. F. Dunne, E. Ribot, P. C. Iwen, P. A. Bradford, F. J. Angulo, and S. H. Hinrichs.** 2000. Ceftriaxone-resistant salmonella infection acquired by a child from cattle. N. Engl. J. Med. **342:** 1242–1249.
14. **Food and Drug Administration.** 2005. Approved animal drug products. 21 CFR 558. [Online.] http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&sid=a5d0bbd066eaa2639fc.
15. **Goldsmith, R. S., and P. M. Schur.** 2002. Use of subtherapeutic antimicrobials in livestock (as supplements or feed additives) with the induction of resistance in human bacterial pathogens, the magnitude and complexity of the problem become increasingly clear. J. Environ. Health **65:**7–21.
16. **Gorbach, S. L.** 2001. Antimicrobial use in animal feed—time to stop. N. Engl. J. Med. **345:**1202–1203.
17. **Hinton, M., Z. A. Al Chalaby, V. Allen, and A. H. Linton.** 1982. The persistence of drug resistant *Escherichia coli* in the intestinal flora of healthy broiler chicks. J. Hyg. (London) **89:**269–278.
18. **Hosmer, D. W., and S. Lemeshow.** 2000. Applied logistic regression, p. 308–330. John Wiley & Sons, Inc., New York, N.Y.
19. **Hoyle, D. V., H. I. Knight, D. J. Shaw, K. Hillman, M. C. Pearce, J. C. Low, G. J. Gunn, and M. E. Woolhouse.** 2004. Acquisition and epidemiology of antimicrobial-resistant *Escherichia coli* in a cohort of newborn calves. J. Antimicrob. Chemother. **53:**867–871.
20. **Khachatryan, A. R., D. D. Hancock, T. E. Besser, and D. R. Call.** 2004. Role of calf-adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. Appl. Environ. Microbiol. **70:**752–757.
21. **Morrill, J. L., A. D. Dayton, and R. Mickelsen.** 1977. Cultured milk and antimicrobials for young calves. J. Dairy Sci. **60:**1105–1108.
22. **National Committee for Clinical Laboratory Standards.** 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard, 2nd ed. National Committee for Clinical Laboratory Standards, Wayne, Pa.
23. **Phillips, I., M. Casewell, T. Cox, B. De Groot, C. Friis, R. Jones, C. Nightingale, R. Preston, and J. Waddell.** 2004. Does the use of antimicrobials in food animals pose a risk to human health? A critical review of published data. J. Antimicrob. Chemother. **53:**28–52.
24. **Quigley, J. D., J. J. Drewry, L. M. Murray, and S. J. Ivey.** 1997. Body weight gain, feed efficiency, and fecal scores of dairy calves in response to galactosyllactose or antimicrobials in milk replacers. J. Dairy Sci. **80:**1751–1754.
25. **Smith, H. W.** 1966. The incidence of infective drug resistance in strains of *Escherichia coli* isolated from diseased human beings and domestic animals. J. Hyg. (London) **64:**465–474.
26. **Wagner, B. A., D. A. Dargatz, P. S. Morley, T. J. Keefe, and M. D. Salman.** 2003. Analysis methods for evaluating bacterial antimicrobial resistance outcomes. Am. J. Vet. Res. **64:**1570–1579.
27. **Wierup, M.** 1975. Antimicrobial resistance and transferable antimicrobial resistance of Escherichia coli isolated from Swedish calves 5 and 30 days old. Nord. Vet. Med. **27:**77–84.