

## Effect of Penicillin and Virginiamycin on Drug Resistance in Lactose-Fermenting Enteric Flora

STUART A. GAINES,\* LARRY D. ROLLINS, ROBERT D. WILLIAMS,† AND MURRAY SELWYN‡

*Division of Veterinary Medical Research, Food and Drug Administration, Beltsville, Maryland 20705*

Three groups of beagle dogs were fed either a control diet, a diet containing virginiamycin (55 µg/g of diet), or a diet containing penicillin (110 µg/g of diet). The proportions of lactose-fermenting organisms in their feces that were resistant to ampicillin, dihydrostreptomycin, tetracycline, or chloramphenicol were measured by a comparative plate-counting procedure. Both antibiotic-supplemented diets resulted in an increase ( $P < 0.001$ ) in the occurrence of ampicillin, dihydrostreptomycin, and tetracycline resistances during the time of their administration. The occurrence of these resistances was greater ( $P < 0.001$ ) in the group receiving penicillin than in the group receiving virginiamycin. In addition to the above resistances, a greater ( $P < 0.001$ ) occurrence of resistance to a sulfonamide (sulfamethoxypyridazine) due to treatment was found by susceptibility testing of isolates. Representative isolates were able to transfer their resistance to a strain of *Escherichia coli* K-12.

The general usage pattern for subtherapeutic levels of antibiotics, including penicillin (P) and virginiamycin (VM), is to feed them continuously to the appropriate food-producing animals on a herd basis throughout an extended period of their growing life. An increase in the proportion of drug-resistant gram-negative bacteria occurs when antimicrobial drugs that have a wide spectrum of activity against gram-negative bacteria are used in this type of management system (4, 12, 14). Although P is generally considered to be more active against gram-positive than gram-negative bacteria, Katz et al. (6) have reported an increase in the occurrence of drug-resistant gram-negative organisms in chickens that were receiving P-supplemented diets.

VM is also approved for subtherapeutic and therapeutic use in animal feed (2); this drug is a peptolide antibiotic whose main spectrum of activity is also against gram-positive organisms (13), but unlike P it has not been used as extensively as a feed additive. Therefore, it was of interest to verify the effect of P and to determine whether the feeding of VM, which has a spectrum of activity similar to that of P, would influence the antibiotic susceptibility of the gram-negative enteric flora. An animal model was used to determine the effect that feeds containing subtherapeutic levels of these two antibiotics have on the proportion of drug-resistant, lactose-fermenting (LF) organisms in feces.

### MATERIALS AND METHODS

**Experimental design.** In the first study, 21 adult male and female beagle dogs weighing 12.6 to 15.8 kg were divided equally into three experimental groups; they were housed in individual cages, with separate buildings for each group. Exposure of the animals was limited to study personnel. The dog was previously shown to be a suitable animal for testing the effect that antibiotics have on the proportion of drug-resistant gram-negative organisms (9).

Each experimental animal was fed 350 g of a ground meal ration (Ralston Purina Co., St. Louis, Mo.) per day. One group received meal to which P had been added at a calculated concentration of 110 µg/g of feed, and another group received meal supplemented with VM at 55 µg/g of feed; the diets were continued for 62 days. Feed was mixed by study technicians 2 weeks before being used in the experiment. When the supplemented feed was assayed by an AOAC method (5), 108 to 130 µg of P per g was detected in six batches of the supplemented feed, but only 19 µg/g was found in one batch. The amount of VM in supplemented feed was not verified by assay in this study; however, the labeled potency of the animal feed grade VM used was confirmed by a disk-plate method submitted by the SmithKline Corp. (private communication, 1973). Assays (5) on the treatment feed before the addition of drug and on the unsupplemented feed that was used for the control group did not detect P, dihydrostreptomycin (DSM), chlortetracycline, or neomycin.

Since the amount of VM in the supplemented diet was not confirmed, a second study with five treated and five control dogs was conducted to verify the increased occurrence of drug resistance that was observed in the VM group during the first study. VM-supplemented feed was assayed by the disk method and was found to contain 65 µg of VM per g of feed.

Fresh fecal samples were obtained from a collection

\* Present address: IMC Chemical Group, Inc., Terre Haute, IN 47808.

† Present address: Ciba-Geigy Corp., Summit, NJ 07901.

pan located under the cage floor, and sample processing was initiated approximately 1 h after the feces were collected. Collections were made four times before the diets were started, with the last sample being collected 2 days before the diets were started. During antibiotic administration, samples were collected on days 5, 12, 19, 26, 35, 43, 50, and 57. In an attempt to determine whether the increase in the prevalence of drug-resistant organisms decreased when antibiotic-supplemented feed was withdrawn, the dogs were returned to an antibiotic-free diet for 64 days. Fecal samples for the second study were collected four times before the VM diet was started, on days 7, 15, 21, 29, 35, and 42 of the VM administration period, and on days 19 and 33 after the diets had been discontinued.

**Media and reagents.** Nutrient agar, MacConkey agar (Mc), triple sugar iron agar, Simmons citrate agar, and antimicrobial susceptibility disks were obtained from BBL Microbiology Systems, Cockeysville, Md. Ampicillin (AM) and dicloxacillin were obtained from Bristol Laboratories, Syracuse, N.Y.; DSM was from Hamilton Pharmaceutical Co., Hamilton, N.Y.; tetracycline (TE) was from ICN Pharmaceuticals, Inc., Covina, Calif.; nalidixic acid (NA) from Sterling Winthrop Research Institute, New York, N.Y.; VM was from SmithKline Corp., Philadelphia, Pa.; and P was from Pfizer Inc., New York, N.Y.

**Microbiological procedures.** In the first study, a comparative plate-counting procedure was used to determine the incidences of AM, DSM, TE, and chloramphenicol resistances in LF enteric organisms. One gram of fresh feces was mixed with 99 ml of sterile saline, and the suspension was serially diluted. Duplicate 0.1-ml aliquots were plated on Mc, Mc-10  $\mu\text{g}$  of AM per ml of agar, Mc-25  $\mu\text{g}$  of DSM per ml, Mc-4  $\mu\text{g}$  of TE per ml, and Mc-15  $\mu\text{g}$  of chloramphenicol per ml. Total number of LF enteric organisms per gram of feces were determined from drug-free Mc plates. The proportion of the total LF flora resistant to AM, DSM, TE, or chloramphenicol was converted to a percentage value and expressed as mean percentage of resistant LF bacteria. Samples from the second VM group were plated only on drug-free Mc plates.

Well-isolated colonies that produced biochemical reactions typical of *Escherichia coli* on drug-free Mc plates were further tested on triple sugar iron agar and Simmons citrate agar. Cultures giving reactions typical of *E. coli* were tested further for antimicrobial susceptibility by the method of Bauer et al. (1). Five isolates from each sample collected before antibiotic administration and three isolates from each sample collected during and after administration were tested with paper disks for their susceptibility to 10  $\mu\text{g}$  of AM, 10  $\mu\text{g}$  of streptomycin (SM), 30  $\mu\text{g}$  of cephalothin, 250  $\mu\text{g}$  of sulfamethoxypyridazine (SU), 10  $\mu\text{g}$  of colistin, 30  $\mu\text{g}$  of chloramphenicol, 100  $\mu\text{g}$  of furazolidone, 30  $\mu\text{g}$  of neomycin, 300 U of polymyxin B, 30  $\mu\text{g}$  of TE, and 30  $\mu\text{g}$  of NA.

Ninety-six resistant isolates were tested for their ability to transfer drug resistance to an NA-resistant *E. coli* recipient. A modification (10) of the method of Schroeder et al. (11) was used. Trypticase soy broth (BBL Microbiology Systems) cultures containing the drug-resistant donor and the recipient strain were

mated for 2 h at 37°C, and 0.1-ml aliquots of the mating mixture were plated on Mc containing 25  $\mu\text{g}$  of NA and 4  $\mu\text{g}$  of TE per ml of agar; Mc containing 10  $\mu\text{g}$  of AM, 10  $\mu\text{g}$  of dicloxacillin, and 25  $\mu\text{g}$  of NA per ml; Mc containing 25  $\mu\text{g}$  of NA per ml; and antibiotic-free Mc plates. The antimicrobial susceptibility of transconjugant strains was confirmed by the method of Bauer et al. (1).

**Statistical analysis.** Colony count data were subjected to analysis of variance procedures after the counts were transformed by taking common logarithms. When significant *F* values were encountered, the Student *t* test was conducted to determine which experimental groups were significantly different. Results obtained by susceptibility testing of individual isolates were analyzed by chi-square tests (7).

## RESULTS

The proportions (percent) of drug resistances as determined by a comparative plate-counting procedure are given in Table 1. Results are reported as the mean percentages of AM-, DSM-, and TE-resistant LF bacteria in the total (susceptible and resistant) LF fecal population. The mean counts of total LF and drug-resistant LF bacteria are also presented. The proportions of drug-resistant organisms were low for all groups before administration of the supplemented diets (zero time), ranging from 3 to 22%. Since the control group had a marginally significant ( $P < 0.1$ ) lower range of TE resistance than did the P or VM groups, values for percentage of resistance during and after antibiotic administration were adjusted for the pretreatment levels in the statistical analysis.

During antibiotic administration, the percentages of AM, DSM, and TE resistance were greater ( $P < 0.001$ ) in the VM and P groups than in the control group. The occurrence of resistance in the P group ranged from 80 to 98% for AM, 56 to 89% for DSM, and 69 to 86% for TE. The percentages of resistant organisms in the VM group were lower ( $P < 0.001$ ) than those in the P group; values reached peaks at 47% (AM), 53% (DSM), and 45% (TE).

In contrast to the higher values in the groups given P and VM, the proportion of AM-, DSM-, or TE-resistant organisms in the control group ranged from 2 to 23% during antibiotic administration. Chloramphenicol-resistant organisms were not detected.

The prevalence of resistant organisms in the VM group after the antibiotic-supplemented diets were replaced by an antibiotic-free diet decreased to values of 3 to 11% for AM, 4 to 38% for DSM, and 3 to 24% for TE, approximating those in the control group (Table 2). Although the occurrence of resistant organisms decreased in the P group, the lowest percentage of resistant

TABLE 1. Effect of a P- or VM-supplemented diet on the numbers and proportions of drug-resistant LF organisms in dogs

Day	Group <sup>a</sup>	Total LF organisms/g of feces <sup>b</sup>			Resistant organisms <sup>c</sup>						Proportion of resistant organisms					
		No.	SD <sup>d</sup>	AM	AM		DSM		TE		AM		DSM		TE	
					No.	SD	No.	SD	No.	SD	%	SD	%	SD	%	SD
0 (Before diet)	C	4.8 × 10 <sup>7</sup>	1.3 × 10 <sup>7</sup>	5.0 × 10 <sup>5</sup>	1.0 × 10 <sup>6</sup>	1.4 × 10 <sup>6</sup>	4.6 × 10 <sup>5</sup>	6.2 × 10 <sup>5</sup>	1.9 × 10 <sup>6</sup>	7.8	13.0	16.4	14.5	7.5	9.6	
	P	6.2 × 10 <sup>7</sup>	1.7 × 10 <sup>7</sup>	7.2 × 10 <sup>6</sup>	2.2 × 10 <sup>6</sup>	1.2 × 10 <sup>6</sup>	2.8 × 10 <sup>6</sup>	9.2 × 10 <sup>6</sup>	2.2 × 10 <sup>6</sup>	5.8	5.9	21.8	15.6	17.2	17.8	
5	VM	2.8 × 10 <sup>7</sup>	2.8 × 10 <sup>7</sup>	1.1 × 10 <sup>6</sup>	2.4 × 10 <sup>6</sup>	1.0 × 10 <sup>6</sup>	2.2 × 10 <sup>6</sup>	9.7 × 10 <sup>6</sup>	2.7 × 10 <sup>6</sup>	3.1	4.2	11.4	11.2	15.1	22.5	
	C	3.2 × 10 <sup>6</sup>	3.9 × 10 <sup>6</sup>	4.2 × 10 <sup>6</sup>	2.7 × 10 <sup>5</sup>	5.5 × 10 <sup>6</sup>	3.0 × 10 <sup>6</sup>	8.5 × 10 <sup>4</sup>	6.7 × 10 <sup>4</sup>	17.7	14.3	5.4	5.5	3.3	3.4	
12	P	4.0 × 10 <sup>7</sup>	4.1 × 10 <sup>7</sup>	3.2 × 10 <sup>7</sup>	3.0 × 10 <sup>7</sup>	3.8 × 10 <sup>7</sup>	4.6 × 10 <sup>7</sup>	3.6 × 10 <sup>7</sup>	3.5 × 10 <sup>7</sup>	80.3	15.2	78.1	25.1	80.6	11.4	
	VM	1.0 × 10 <sup>8</sup>	6.0 × 10 <sup>7</sup>	4.7 × 10 <sup>7</sup>	5.4 × 10 <sup>7</sup>	2.1 × 10 <sup>7</sup>	2.0 × 10 <sup>7</sup>	3.4 × 10 <sup>7</sup>	3.0 × 10 <sup>7</sup>	41.0	30.2	23.2	16.1	36.2	24.2	
19	C	1.4 × 10 <sup>6</sup>	1.7 × 10 <sup>6</sup>	8.0 × 10 <sup>4</sup>	1.1 × 10 <sup>5</sup>	1.0 × 10 <sup>5</sup>	1.3 × 10 <sup>5</sup>	5.2 × 10 <sup>4</sup>	7.7 × 10 <sup>4</sup>	6.6	11	9.1	16.9	1.8	1.9	
	P	7.7 × 10 <sup>7</sup>	7.3 × 10 <sup>7</sup>	8.3 × 10 <sup>6</sup>	8.8 × 10 <sup>6</sup>	6.7 × 10 <sup>6</sup>	7.4 × 10 <sup>6</sup>	7.3 × 10 <sup>6</sup>	7.9 × 10 <sup>6</sup>	87.3	16	89.3	13.6	86.1	21.9	
26	VM	1.7 × 10 <sup>8</sup>	3.7 × 10 <sup>7</sup>	2.9 × 10 <sup>7</sup>	4.9 × 10 <sup>7</sup>	6.5 × 10 <sup>7</sup>	6.8 × 10 <sup>7</sup>	5.8 × 10 <sup>7</sup>	5.2 × 10 <sup>7</sup>	15.8	22.8	37.7	31.1	29.0	29.0	
	C	1.4 × 10 <sup>7</sup>	3.6 × 10 <sup>7</sup>	1.3 × 10 <sup>4</sup>	2.2 × 10 <sup>4</sup>	1.6 × 10 <sup>4</sup>	2.1 × 10 <sup>4</sup>	5.8 × 10 <sup>5</sup>	1.5 × 10 <sup>5</sup>	3.6	4.0	15.8	25.2	2.3	2.4	
35	P	3.8 × 10 <sup>7</sup>	3.8 × 10 <sup>7</sup>	4.0 × 10 <sup>7</sup>	3.5 × 10 <sup>7</sup>	2.0 × 10 <sup>7</sup>	1.9 × 10 <sup>7</sup>	2.7 × 10 <sup>7</sup>	2.6 × 10 <sup>7</sup>	92.4	17.9	64.8	22.0	76.6	21.4	
	VM	1.1 × 10 <sup>7</sup>	8.9 × 10 <sup>6</sup>	1.6 × 10 <sup>6</sup>	2.5 × 10 <sup>6</sup>	4.7 × 10 <sup>6</sup>	6.6 × 10 <sup>6</sup>	1.3 × 10 <sup>7</sup>	1.5 × 10 <sup>7</sup>	11.3	9.4	28.0	25.0	17.7	6.2	
43	C	9.8 × 10 <sup>5</sup>	1.1 × 10 <sup>6</sup>	2.8 × 10 <sup>4</sup>	5.7 × 10 <sup>4</sup>	1.2 × 10 <sup>5</sup>	2.2 × 10 <sup>5</sup>	8.3 × 10 <sup>4</sup>	1.6 × 10 <sup>5</sup>	6.0	13.8	6.3	9.7	11.3	12.5	
	P	5.6 × 10 <sup>7</sup>	7.1 × 10 <sup>7</sup>	4.8 × 10 <sup>7</sup>	6.4 × 10 <sup>7</sup>	4.0 × 10 <sup>7</sup>	6.1 × 10 <sup>7</sup>	4.7 × 10 <sup>7</sup>	6.1 × 10 <sup>7</sup>	97.6	4.0	72.7	24.5	82.2	18.7	
50	VM	6.9 × 10 <sup>7</sup>	7.2 × 10 <sup>7</sup>	4.6 × 10 <sup>7</sup>	5.7 × 10 <sup>7</sup>	4.5 × 10 <sup>7</sup>	5.0 × 10 <sup>7</sup>	4.5 × 10 <sup>7</sup>	5.9 × 10 <sup>7</sup>	43.0	35.0	43.3	35.5	42.4	33.3	
	C	2.3 × 10 <sup>7</sup>	3.0 × 10 <sup>7</sup>	2.1 × 10 <sup>5</sup>	3.6 × 10 <sup>5</sup>	5.5 × 10 <sup>6</sup>	9.5 × 10 <sup>6</sup>	2.3 × 10 <sup>5</sup>	3.7 × 10 <sup>5</sup>	4.3	6.1	18.1	36.6	6.6	7.0	
57	P	9.6 × 10 <sup>7</sup>	8.4 × 10 <sup>7</sup>	9.8 × 10 <sup>7</sup>	8.7 × 10 <sup>7</sup>	8.2 × 10 <sup>7</sup>	7.4 × 10 <sup>7</sup>	9.6 × 10 <sup>7</sup>	9.7 × 10 <sup>7</sup>	98.2	2.5	80.8	14.3	75.8	31.2	
	VM	1.2 × 10 <sup>8</sup>	9.6 × 10 <sup>7</sup>	4.9 × 10 <sup>7</sup>	6.3 × 10 <sup>7</sup>	6.3 × 10 <sup>7</sup>	3.8 × 10 <sup>7</sup>	6.1 × 10 <sup>7</sup>	4.1 × 10 <sup>7</sup>	47.2	33.8	53.3	24.5	36.8	23.9	
57	C	4.3 × 10 <sup>5</sup>	4.4 × 10 <sup>5</sup>	4.3 × 10 <sup>4</sup>	1.0 × 10 <sup>5</sup>	6.3 × 10 <sup>4</sup>	8.4 × 10 <sup>4</sup>	2.3 × 10 <sup>4</sup>	4.8 × 10 <sup>4</sup>	22.5	27.8	18.1	23.4	3.1	4.1	
	P	1.4 × 10 <sup>8</sup>	1.3 × 10 <sup>8</sup>	7.8 × 10 <sup>7</sup>	6.7 × 10 <sup>7</sup>	7.8 × 10 <sup>7</sup>	1.2 × 10 <sup>8</sup>	9.0 × 10 <sup>7</sup>	7.7 × 10 <sup>7</sup>	74.7	26.4	55.7	33.9	72.4	24.6	
57	VM	1.5 × 10 <sup>8</sup>	2.3 × 10 <sup>8</sup>	5.8 × 10 <sup>7</sup>	7.4 × 10 <sup>7</sup>	6.5 × 10 <sup>7</sup>	7.4 × 10 <sup>7</sup>	5.7 × 10 <sup>7</sup>	8.6 × 10 <sup>7</sup>	31.3	31.3	36.0	32.2	35.2	20.0	
	C	4.6 × 10 <sup>6</sup>	6.5 × 10 <sup>6</sup>	2.8 × 10 <sup>5</sup>	4.2 × 10 <sup>5</sup>	6.1 × 10 <sup>5</sup>	1.0 × 10 <sup>6</sup>	5.9 × 10 <sup>5</sup>	1.1 × 10 <sup>6</sup>	12.4	17.1	16.9	22.6	17.0	24.3	
57	P	1.0 × 10 <sup>7</sup>	7.0 × 10 <sup>6</sup>	1.2 × 10 <sup>7</sup>	1.1 × 10 <sup>7</sup>	8.8 × 10 <sup>6</sup>	6.8 × 10 <sup>6</sup>	7.6 × 10 <sup>6</sup>	5.3 × 10 <sup>6</sup>	96.0	6.7	81.0	14.0	76.8	19.5	
	VM	2.1 × 10 <sup>7</sup>	2.1 × 10 <sup>7</sup>	5.8 × 10 <sup>6</sup>	8.5 × 10 <sup>6</sup>	1.2 × 10 <sup>7</sup>	1.6 × 10 <sup>7</sup>	1.6 × 10 <sup>7</sup>	2.0 × 10 <sup>7</sup>	21.1	14.8	27.7	15.4	37.0	24.0	
57	C	9.9 × 10 <sup>6</sup>	2.1 × 10 <sup>7</sup>	5.5 × 10 <sup>5</sup>	1.2 × 10 <sup>6</sup>	8.3 × 10 <sup>5</sup>	1.4 × 10 <sup>6</sup>	1.6 × 10 <sup>6</sup>	3.5 × 10 <sup>6</sup>	2.2	2.3	12.2	13.6	10.1	8.6	
	P	1.3 × 10 <sup>8</sup>	2.2 × 10 <sup>8</sup>	3.8 × 10 <sup>7</sup>	4.8 × 10 <sup>7</sup>	9.1 × 10 <sup>7</sup>	1.6 × 10 <sup>8</sup>	6.7 × 10 <sup>7</sup>	1.1 × 10 <sup>8</sup>	81.0	12.4	69.4	15.5	69.4	26.1	
57	VM	5.8 × 10 <sup>7</sup>	5.9 × 10 <sup>7</sup>	2.5 × 10 <sup>7</sup>	3.4 × 10 <sup>7</sup>	1.2 × 10 <sup>7</sup>	1.0 × 10 <sup>7</sup>	4.6 × 10 <sup>7</sup>	7.1 × 10 <sup>7</sup>	31.4	22.1	23.5	12.5	44.8	29.5	

<sup>a</sup> C, Control group.  
<sup>b</sup> Plate counts on Mc agar without antibiotic.  
<sup>c</sup> Plate counts on Mc agar containing AM (10 µg/ml), DSM (25 µg/ml), or TE (4 µg/ml).  
<sup>d</sup> SD, Standard deviation.

TABLE 2. Numbers and proportions of drug-resistant LF organisms in dogs after withdrawal from a P- or VM-supplemented diet<sup>a</sup>

Withdrawal day	Group <sup>b</sup>	Total LF organisms/g of feces <sup>c</sup>			Proportion of resistant organisms												
		Resistant organisms <sup>d</sup>			AM				TE				DSM				
		No.	SD	%	No.	SD	%	No.	SD	%	No.	SD	%	No.	SD	%	TE
8	C	4.6 × 10 <sup>6</sup>	4.9 × 10 <sup>6</sup>	1.7 × 10 <sup>5</sup>	1.5 × 10 <sup>6</sup>	1.8 × 10 <sup>6</sup>	3.2 × 10 <sup>5</sup>	7.4 × 10 <sup>5</sup>	0.9	1.8	9.7	13.3	4.6	7.5			
	P	1.2 × 10 <sup>7</sup>	2.8 × 10 <sup>7</sup>	2.9 × 10 <sup>7</sup>	1.1 × 10 <sup>7</sup>	2.8 × 10 <sup>7</sup>	4.6 × 10 <sup>6</sup>	9.8 × 10 <sup>6</sup>	90.1	17.3	83.3	20.2	62.7	21.4			
	VM	5.7 × 10 <sup>6</sup>	5.3 × 10 <sup>6</sup>	5.7 × 10 <sup>6</sup>	9.8 × 10 <sup>6</sup>	7.9 × 10 <sup>6</sup>	1.4 × 10 <sup>6</sup>	1.8 × 10 <sup>6</sup>	11.0	6.3	38.0	31.0	24.2	26.0			
15	C	1.5 × 10 <sup>7</sup>	2.0 × 10 <sup>7</sup>	2.9 × 10 <sup>5</sup>	1.6 × 10 <sup>6</sup>	1.9 × 10 <sup>6</sup>	8.4 × 10 <sup>5</sup>	1.1 × 10 <sup>6</sup>	4.4	6.9	11.3	11.6	8.0	11.2			
	P	3.0 × 10 <sup>7</sup>	5.8 × 10 <sup>7</sup>	7.7 × 10 <sup>7</sup>	2.0 × 10 <sup>7</sup>	4.0 × 10 <sup>7</sup>	1.8 × 10 <sup>7</sup>	4.0 × 10 <sup>7</sup>	72.7	26.3	52.1	22.7	51.8	23.2			
	VM	2.4 × 10 <sup>7</sup>	6.0 × 10 <sup>7</sup>	1.8 × 10 <sup>6</sup>	2.3 × 10 <sup>7</sup>	6.0 × 10 <sup>7</sup>	3.9 × 10 <sup>6</sup>	3.5 × 10 <sup>6</sup>	12.0	9.5	38.0	27.0	8.9	14.0			
22	C	3.3 × 10 <sup>6</sup>	5.8 × 10 <sup>6</sup>	2.4 × 10 <sup>5</sup>	6.1 × 10 <sup>6</sup>	9.4 × 10 <sup>6</sup>	5.5 × 10 <sup>5</sup>	8.9 × 10 <sup>5</sup>	10.2	6.5	13.0	7.4	12.8	11.3			
	P	4.2 × 10 <sup>6</sup>	6.4 × 10 <sup>6</sup>	4.6 × 10 <sup>6</sup>	2.1 × 10 <sup>6</sup>	3.0 × 10 <sup>6</sup>	1.9 × 10 <sup>6</sup>	3.4 × 10 <sup>6</sup>	87.1	16.6	60.0	35.5	64.0	32.9			
	VM	1.0 × 10 <sup>7</sup>	1.3 × 10 <sup>7</sup>	6.2 × 10 <sup>5</sup>	1.6 × 10 <sup>6</sup>	2.1 × 10 <sup>6</sup>	3.0 × 10 <sup>5</sup>	5.4 × 10 <sup>5</sup>	2.8	2.2	24.6	38.1	3.5	5.3			
29	C	5.1 × 10 <sup>5</sup>	5.3 × 10 <sup>5</sup>	2.7 × 10 <sup>4</sup>	1.3 × 10 <sup>5</sup>	1.5 × 10 <sup>5</sup>	4.7 × 10 <sup>4</sup>	8.5 × 10 <sup>4</sup>	7.2	12.0	24.4	18.8	9.5	8.9			
	P	2.0 × 10 <sup>7</sup>	4.4 × 10 <sup>7</sup>	2.1 × 10 <sup>7</sup>	1.2 × 10 <sup>7</sup>	2.9 × 10 <sup>7</sup>	3.3 × 10 <sup>6</sup>	6.9 × 10 <sup>6</sup>	65.4	23.4	50.1	29.3	23.2	24.0			
	VM	6.1 × 10 <sup>6</sup>	1.1 × 10 <sup>7</sup>	2.6 × 10 <sup>6</sup>	4.9 × 10 <sup>6</sup>	5.2 × 10 <sup>6</sup>	1.0 × 10 <sup>6</sup>	1.4 × 10 <sup>6</sup>	8.3	9.5	30.3	38.1	10.7	17.9			
36	C	4.0 × 10 <sup>7</sup>	9.7 × 10 <sup>7</sup>	4.4 × 10 <sup>5</sup>	3.1 × 10 <sup>6</sup>	5.1 × 10 <sup>6</sup>	1.8 × 10 <sup>5</sup>	3.0 × 10 <sup>5</sup>	11.1	26.1	5.6	6.8	3.6	3.3			
	P	3.7 × 10 <sup>7</sup>	8.9 × 10 <sup>7</sup>	7.8 × 10 <sup>7</sup>	2.9 × 10 <sup>7</sup>	7.1 × 10 <sup>7</sup>	3.4 × 10 <sup>7</sup>	9.5 × 10 <sup>7</sup>	63.7	31.7	48.7	36.6	56.3	46.3			
	VM	8.7 × 10 <sup>7</sup>	1.5 × 10 <sup>8</sup>	1.4 × 10 <sup>7</sup>	9.8 × 10 <sup>6</sup>	1.2 × 10 <sup>7</sup>	1.9 × 10 <sup>7</sup>	4.0 × 10 <sup>7</sup>	4.8	4.8	29.0	37.6	23.4	36.1			
43	C	3.5 × 10 <sup>6</sup>	3.7 × 10 <sup>6</sup>	4.5 × 10 <sup>5</sup>	7.3 × 10 <sup>6</sup>	1.1 × 10 <sup>6</sup>	1.9 × 10 <sup>6</sup>	3.4 × 10 <sup>6</sup>	3.3	5.4	24.4	25.2	10.6	20.8			
	P	3.9 × 10 <sup>6</sup>	6.7 × 10 <sup>6</sup>	8.2 × 10 <sup>6</sup>	6.6 × 10 <sup>6</sup>	7.5 × 10 <sup>6</sup>	5.6 × 10 <sup>6</sup>	5.7 × 10 <sup>6</sup>	38.8	35.9	36.1	29.2	33.9	37.2			
	VM	4.5 × 10 <sup>6</sup>	3.3 × 10 <sup>6</sup>	2.3 × 10 <sup>5</sup>	3.0 × 10 <sup>6</sup>	3.2 × 10 <sup>6</sup>	3.9 × 10 <sup>6</sup>	4.6 × 10 <sup>6</sup>	3.6	7.7	6.9	7.9	7.6	10.7			
50	C	3.4 × 10 <sup>6</sup>	2.2 × 10 <sup>6</sup>	1.2 × 10 <sup>4</sup>	2.2 × 10 <sup>4</sup>	1.4 × 10 <sup>4</sup>	3.1 × 10 <sup>4</sup>	4.1 × 10 <sup>4</sup>	5.6	9.6	8.6	5.5	13.3	17.7			
	P	1.9 × 10 <sup>7</sup>	4.5 × 10 <sup>7</sup>	2.7 × 10 <sup>6</sup>	2.3 × 10 <sup>6</sup>	2.2 × 10 <sup>6</sup>	4.1 × 10 <sup>6</sup>	4.7 × 10 <sup>6</sup>	48.1	27.1	43.9	33.4	16.1	15.1			
	VM	3.1 × 10 <sup>7</sup>	6.2 × 10 <sup>7</sup>	1.5 × 10 <sup>6</sup>	2.2 × 10 <sup>7</sup>	5.6 × 10 <sup>7</sup>	2.2 × 10 <sup>7</sup>	5.6 × 10 <sup>7</sup>	1.5	2.7	21.1	29.3	17.1	30.5			
64	C	2.3 × 10 <sup>6</sup>	1.9 × 10 <sup>6</sup>	4.6 × 10 <sup>4</sup>	7.3 × 10 <sup>4</sup>	1.3 × 10 <sup>5</sup>	5.4 × 10 <sup>4</sup>	7.0 × 10 <sup>4</sup>	0.7	0.7	2.0	3.0	2.0	2.0			
	P	3.2 × 10 <sup>6</sup>	6.0 × 10 <sup>6</sup>	2.7 × 10 <sup>5</sup>	3.5 × 10 <sup>4</sup>	3.5 × 10 <sup>4</sup>	2.4 × 10 <sup>6</sup>	3.6 × 10 <sup>6</sup>	31.3	35.5	32.6	39.9	22.6	32.6			
	VM	4.6 × 10 <sup>6</sup>	6.3 × 10 <sup>6</sup>	1.5 × 10 <sup>6</sup>	1.2 × 10 <sup>6</sup>	2.0 × 10 <sup>6</sup>	1.4 × 10 <sup>6</sup>	3.6 × 10 <sup>6</sup>	2.3	1.1	4.1	2.6	24.2	17.7			

<sup>a</sup> Values are given as means ± standard deviations (SD) based on results from seven animals per group.

<sup>b</sup> C, Control group.

<sup>c</sup> Plate counts on Mc agar without antibiotic.

<sup>d</sup> Plate counts on Mc agar containing AM (10 µg/ml), DSM (25 µg/ml), or TE (4 µg/ml).

organisms remained higher (AM, 31%; DSM, 32%; and TE, 16%) than values for the control of VM group ( $P < 0.001$ ). The total counts of LF organisms (susceptible and resistant) on Mc agar were higher in the P and VM groups than in the control group at all but one sampling day.

From 123 to 216 LF organisms were isolated from each group before, during, and after administration of the supplemented diets. A higher prevalence ( $P < 0.001$ ) of drug-resistant isolates occurred during administration in the P group (95% AM, 75% SM, 78% SU, 77% TE) and the VM group (22% AM, 27% SM, 34% SU, 33% TE); values for the control group were not higher than 13%. After the diets were discontinued, the occurrences of AM-, SM-, SU-, or TE-resistant isolates in the P group remained higher ( $P < 0.01$ ) than those in the control group. These results are similar to those for occurrence and persistence of drug-resistant organisms detected with the comparative plate count procedure (Table 1). Thirty-five percent of the isolates tested were able to transfer resistance.

In the second study, the prevalence of isolates that were resistant to at least 1 of the 11 antimicrobial drugs that were tested was higher in the VM group than in the control group ( $P < 0.001$ ). Before the VM-supplemented diet was started, the proportion of drug-resistant isolates from the VM or the control group was not greater than 25%, but during VM administration 44% of isolates from the VM group were drug-resistant compared with 6% of those from the control group. The percentages of VM isolates that were resistant to AM (7%), SM (24%), SU (14%), and TE (26%) were again lower than the proportion of AM-, SM-, SU-, or TE-resistant isolates from the P group of the first study. However, the values from the VM group during antibiotic administration were higher ( $P < 0.01$ ) than the occurrence (<4%) of resistance to the same drugs in the isolates from the control group.

## DISCUSSION

In these studies, the occurrences of antimicrobial drug-resistant isolates were higher in dogs that were receiving VM- or P-supplemented feed than in the control group; both procedures used, a comparative plate count method and the disk diffusion test of Bauer et al. (1), detected a higher proportion of resistant organisms. These results are similar to those of Katz et al. (6), who observed a higher percentage of SM- and TE-resistant organisms in chickens that were receiving a P-supplemented diet.

The proportion of drug-resistant organisms in this study ranged from 56 to 98% in the P group

and is similar to the occurrence of drug resistance when oxytetracycline, TE, or racephenicol was fed to pigs, chickens, and calves (4, 6, 8, 10, 12). The level of resistance in the VM group is considered intermediate since the proportion of drug-resistant organisms did not exceed 53%. In addition, high levels of resistant organisms persisted for longer periods of time in the P group than in the VM group. These characteristics of VM suggest that its use as a feed additive may result in a lower proportion of drug-resistant gram-negative organisms than other drugs.

VM is currently approved for use in swine feed at 10, 25, 50, and 100 g/ton (ca. 907 kg) of feed (2). Approved use of 100 g/ton is limited to 2 weeks, but the lower levels can be used for longer periods of time. The dog was used as the target animal in this study because of the difficulty in obtaining swine with a low proportion of drug-resistant organisms. If VM affects the susceptibility of the gram-negative enteric flora in swine as it did in the dog, then the use of VM at 50 to 100 g/ton may result in at least a similar intermediate response. The effect of VM at 10 or 25 g/ton on the occurrence of drug resistance requires additional studies.

Hypotheses about the mechanism by which the increase in drug resistance occurred are beyond the scope and intent of these studies. However, Decuypere et al. (3) have reported that when pigs received a VM-supplemented diet, the enterococcal and coliform populations increased and the number of lactobacilli decreased. Although we did not monitor the number of lactobacilli or enterococci in the present study, our results show that a small increase in the number of LF organisms occurred in both groups given the antibiotic-supplemented diets.

Our experiments show that feeding two drugs that are primarily active against gram-positive organisms caused an increase in the proportion of drug-resistant gram-negative bacteria that are ordinarily considered susceptible. Therefore, the evaluation of the effects that antibiotic-supplemented diets have on the occurrence of gram-negative resistant organisms should not be limited to drugs that are primarily active against gram-negative bacteria.

## ACKNOWLEDGMENTS

We thank Kay Langenbach, Beverly Leming, and Elaine Bennett for their technical assistance. We also thank Henry H. Bryant and Gordon C. Carter, National Center for Antibiotics Analysis, Food and Drug Administration, for the virginiamycin assays.

## LITERATURE CITED

1. Bauer, A. W., W. M. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a stan-

- standardized single disk method. *Am. J. Clin. Pathol.* **45**: 493-496.
2. **Code of Federal Regulations.** 1977. New animal drugs for use in animal feeds. Title 21, Part 558. Government Printing Office, Washington, D.C.
  3. **Decuypere, J., H. Henderick, and I. Vervaeke.** 1973. Influence of nutritional doses of virginiamycin and spiramycin on the quantitative and topographical composition of the gastrointestinal flora of artificially reared piglets. *Zentrabl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A* **223**:348-355.
  4. **Guinee, P.** 1971. Bacterial drug resistance in animals. *Ann. N.Y. Acad. Sci.* **182**:40-51.
  5. **Horwitz, W. (ed.)** 1975. Official methods of analysis of the Association of Official Analytical Chemists, 12th ed. Association of Official Analytical Chemists, Washington, D.C.
  6. **Katz, S. E., C. A. Fassbender, P. S. Dinnerstein, and J. J. Dowling, Jr.** 1974. Effects of feeding penicillin to chickens. *J. Assoc. Off. Anal. Chem.* **57**:522-526.
  7. **Kullback, S.** 1959. Contingency tables, p. 155-188a. *In* Information, theory and statistics. Dover Publications, New York.
  8. **Loken, K. I., L. W. Wagner, and C. L. Henke.** 1971. Transmissible drug resistance in *Enterobacteriaceae* isolated from calves given antibiotics. *Am. J. Vet. Res.* **32**:1207-1212.
  9. **Rollins, L. D., S. A. Gaines, D. Pocurull, and H. D. Mercer.** 1975. Animal model for determining the no-effect level of an antimicrobial drug on drug resistance in the lactose-fermenting enteric flora. *Antimicrob. Agents Chemother.* **7**:661-665.
  10. **Rollins, L. D., D. W. Pocurull, and S. Reynolds.** 1973. Effects of racephenicol on antibiotic resistance of lactose-fermenting enteric bacteria in chickens. *Antimicrob. Agents Chemother.* **4**:277-280.
  11. **Schroeder, S. A., P. M. Terry, and J. V. Bennett.** 1968. Antibiotic resistance transfer factor in *Salmonella* in the United States, 1967. *J. Am. Med. Assoc.* **205**:903-906.
  12. **Smith, H. W.** 1971. The effect of the use of antibacterial drugs on the emergence of drug resistant bacteria in animals. *Adv. Vet. Sci. Comp. Med.* **15**:67-100.
  13. **VanDijck, P. J.** 1969. Further evaluation of virginiamycin. *Chemotherapy (Basel)* **14**:322-332.
  14. **VanHouweling, C. D.** 1972. Report of the Commissioner of the Food and Drug Administration (Task Force on the Use of Antibiotics in Animal Feeds). FDA Publication 72-6008. Food and Drug Administration, Rockville, Md.