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Full Length Research Paper

Sensitivity of ruminal bacterial isolates of sheep, cattle and buffalo to 13 therapeutic antibiotics

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Ruminal bacterial isolates, 59 from two sheep, five cows and nine buffaloes were used to evaluate sensitivity to the therapeutic antibiotics amikacin, cefadroxil, cefoperazone, cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, piperacillin, polymyxin, roxithromycin, streptomycin and vancomycin. Sensitivity of ruminal bacterial isolates to each was determined by the clearance zone (CZ) in the Kirby-Bauer disc diffusion susceptibility test. Bacterial isolates from sheep exhibited, in general, lower resistance (*P*=0.040) to antibiotics than buffalo. Irrespective of ruminant species, bacterial isolates had a higher tolerance (*P*<0.001) to cefadroxil (CZ=3.1 mm), whereas ciprofloxacin (CZ=24.4 mm) followed by erythromycin (CZ=20.9 mm) and amikacin (CZ=20.0 mm) were the most toxic antibiotics to all isolates. Inhibitory effects of other antibiotics to ruminal bacterial isolates were intermediate, with two groups of antibiotics according to CZ size, being those with a CZ of 12-19 mm (gentamicin > roxithromycin > cefotaxime = vancomycin > cefoperazone > piperacillin), and those with CZ size of 7-10 mm (streptomycin > chloramphenicol = polymyxin). Sub-therapeutic antibiotic use in ruminant feeding to optimize rumen fermentation may lead to residues in meat and milk, as well as increase their inhibition to ruminal bacterial populations.

Key words*:* Antibiotics, buffalo, cattle, sheep, clearance zone, ruminal bacteria isolates.

INTRODUCTION

Oral administration of therapeutic antibiotics in ruminants is limited by their potential adverse effects on the gastro-intestinal tract of microorganisms. However, in large herds of free ranging ruminants it may be the only practical way to administer them. Antibiotics are, nevertheless, widely used in feeds of housed food animals because of their positive effects on growth rates and lactation performance, as well as decreased incidence and severity of disease including a reduction in mortality (Goldberg, 1959). To avoid the danger of development of drug-resistant strains and transfer of resistance among bacterial species, it would be best if the antibiotics used in feed be different from those therapeutic antibiotics used in the treatment of human and animal diseases.

In the last years, there has been a debate concerning the causes of antibiotic resistance and steps that should be taken to prevent its occurrence (Lewis et al., 2002). This debate has been divided between physicians and

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Abbreviation: CZ, clearance zone.

veterinarians who use antibiotics therapeutically to treat acute disease and livestock producers who use antibiotics sub-therapeutically to enhance animal performance. Physicians/veterinarians argued that routine use of antibiotics in animal feed creates a selection pressure for resistances that eventually spread to man (Gustafson, 1991; Russell and Houlihan, 2003). However, others have argued that resistance is more likely to appear when physicians and veterinarians misdiagnose infections and improperly administer antibiotics (Fisher and Scott, 2008).

Beef cattle in feedlots worldwide are routinely fed a class of antibiotics known as ionophores which can increase feed efficiency by as much as 10% (Goodrich et al., 1984; Russell and Strobel, 1989), thereby reducing their environmental impact. Ruminal bacteria resistant to one antibiotic can also be resistant to another (Russell and Strobel, 1989) but, until recently, the mechanism of this resistance was not well defined (Callaway and Russell, 1999; Rychlik and Russell, 2002). In Europe, the use of antibiotics was banned as a precautionary measure to prevent potential development of human microbial resistances to these antibiotics (European Commission Directorate-General XXIV, 1999).

Several antibiotics have been examined at sub-therapeutic levels in ruminant production systems to optimize rumen fermentation patterns resulting in favorable metabolic changes in the rumen (McGuffey et al., 2001; Virkel et al., 2004). Alterations in ruminal fermentation are generally attributed to shifts in microbial populations (Chen and Wolin, 1979; Dennis and Nagaraja, 1981). *In vitro* studies with pure cultures of ruminal bacteria have suggested that hydrogen, formic acid, acetic acid, lactic acid, and butyric acid producing bacteria tend to be susceptible to antibiotics, whereas succinic acid producing and lactic acid fermenting bacteria tend to be resistant (Dennis and Nagaraja, 1981; Henderson et al., 1981). Ruminal metabolic changes induced by many antimicrobial compounds are similar to those induced by lasalocid and sodium monensin (Demeyer and VanNevel, 1985; Merchen and Berger, 1985). However, effects of these antimicrobial compounds on specific ruminal bacterial species of different ruminants have not been definitively determined.

This study was designed to determine the susceptibility of some ruminal bacterial species isolated from sheep, cattle and buffalo to 13 traditional therapeutic antibiotics.

MATERIALS AND METHODS

Animals and bacterial isolates

Rumen samples (~100 ml/sample of mixed solid and liquid ruminal contents) were collected immediately after the animals were slaughter in an abattoir. Rumen contents were sampled from 2 sheep, 5 cows and 9 buffaloes. Two samples were collected from each animal and homogenized to a single sample that was consequently used for inoculation of cultures previously prepared with thioglycollate agar medium (Merck, 1982).

Isolation of ruminal bacteria

Thioglycollate broth cultures containing (g/l): 0.5 L-cystine, 2.5 sodium chloride, 5.5 dextrose, 5 yeast extract (Oxoid L21), 15 pancreatic digest of casein (Oxoid) and 0.5 sodium thioglycollate, were used to cultivate and isolate ruminal bacteria in accordance with the recommendation of the National Institute of Health (1946).

From each homogenized fresh sample of rumen contents, one ml of fluid was used for inoculation of cultures, spreading the inoculum manually on the surface of a Petri dish containing thioglycollate agar medium. All plates were incubated anaerobically at 39°C for 72 h. Thereafter, colonies were picked up and streaked to confirm purity. All actions were under anaerobic conditions. Weekly transfers were necessary for survival of cultures and, for long term storage; cultures of each ruminal bacterial isolate were frozen in 200 g/l glycerol at –80°C in cryogenic plastic tubes.

Antibiotics sensitivity testing

Stock cultures of ruminal bacteria isolates were grown on fresh anaerobically sterilized media with cysteine hydrochloride as the reducing agent and sodium resazurin as the indicator to verify the absence of oxygen in the medium. Prior to sterilization, pH was adjusted to 6.8 and the medium was supplemented with 750 mg of agar-agar per liter of meidum. After sterilization at 121°C for 20 min, 7 to 8 ml aliquots of the medium were dispensed and spread into glass plates purged with oxygen-free CO_2. Plates were then inoculated and prepared for the assay to examine sensitivity of ruminal bacteria growing on the cultures to antibiotics. The numbers of rumen bacterial isolates used in the study were 9 from sheep, 16 from cattle and 34 from buffalo. Sensitivity of the isolated ruminal bacteria from the rumen of sheep, cattle and buffalo to antibiotics was determined by the Kirby-Bauer disc diffusion susceptibility test (Moolman and Wyk, 2004).

Filter paper discs (Whatman No. 1; 5 mm diameter) were impregnated with 10 µl of an aqueous solution containing 5 µg of the corresponding antibiotic. The antibiotics (Sigma-Aldrich Co. Ltd.,) used in the study were: amikacin, cefadroxil, cefoperazone, cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, piperacillin, polymyxin, roxithromycin, streptomycin and vancomycin.

Discs were applied to the surface of agar plates which had been previously inoculated with a standard amount of 48 h old cultures of ruminal bacteria isolates (1 ml of 10^5 colony forming units). Plates were incubated at 39°C and the diameter of clear inhibition zone (mm) was measured using a caliper after 72 h. Control discs were impregnated with 10 µl of dimethylsulfoxide solution. Each isolate was tested in duplicate.

Statistical analyses

Differences between the sensitivity, based upon inhibition zone diameter of the ruminal bacteria isolates of the ruminant species to the antibiotics were statistically analyzed according to a factorial design (Steel and Torrie, 1980) with ruminant species and antibiotics as fixed effects and isolate (considered as the experimental unit) as the random effect, using mixed-design analysis of variance of SAS (1999). As interactions of animal species × antibiotics occurred, differences among ruminal species isolates were tested for each antibiotic using MIXED of SAS with ruminant species as the fixed effect and isolate as the random effect.

RESULTS

Profiles of inhibition of the ruminal bacteria isolates by

various antibiotics (*P<0.001*) are illustrated in Figure 1. Based on average clearance zone (CZ) among all isolates, ciprofloxacin (CZ=24.4 mm) followed by erythromycin (CZ=20.9 mm) and amikacin (CZ=20.0 mm) had the most pronounced inhibitory effect on ruminal bacterial growth. In contrast, cefadroxil (CZ=3.1 mm) had the lowest effect. For other antibiotics, inhibitory effects on ruminal bacterial isolates were intermediate with two groups of antibiotics according to the average size of CZ, being those with a CZ of 12 to 19 mm (gentamicin > roxithromycin > cefotaxime = vancomycin > cefoperazone > piperacillin), and those with average CZ of 7 to 10 mm (streptomycin > chloramphenicol = polymyxin).

Inhibition effects of the antibiotics on ruminal bacterial isolates from sheep, cattle and buffalo are shown in Table 1. Among all the antibiotics studied, isolates from buffalo ruminal contents were the most tolerant (P=0.040) to the antibiotics, having the smallest CZ (12.7 mm), whereas CZ were higher for sheep (CZ=15.6 mm) and cattle (CZ=14.5 mm) rumen bacterial isolates, without differences between buffalo and cattle (Figure 2). As interactions between ruminant species and antibiotics occurred (*P<0.001*), comparisons among ruminant species from isolates were examined (Tables 1 and 2). Differences among buffalo *versus* cattle and sheep in their tolerance to antibiotics only occurred for gentamicin (*P<0.01*) with a higher tolerance for buffalo *versus* sheep and cattle isolates (Table 1), and a tendency (P=0.059) to be higher for buffalo isolates to erythromycin and roxithromycin.

All bacteria isolates from buffalo, sheep and cattle were sensitive (CZ>0) to amikacin and ciprofloxacin (Table 2), while bacterial isolates from sheep and cattle were sensitive (CZ>0) to erythromycin, gentamicin, piperacillin, roxithromycin and vancomycin. In contrast, there were buffalo isolates tolerant (CZ=0) to most antibiotics, except for amikacin and ciprofloxacin for which all isolates examined were affected to some extent (CZ>0). Fifteen to 26% of bacteria isolates from sheep, cattle and buffalo were not affected (CZ=0) by cefotaxime, chloramphenicol, polymyxin and streptomycin; and over 70% of the isolates had tolerance (CZ=0) against cefadroxil.

DISCUSSION

Antibiotics in our study may inhibit bacterial growth and replication by altering processes such as peptidoglycan synthesis, ribosome activity, DNA replication, mRNA transcription, nucleotide synthesis and/or membrane stability (Levinson and Jawetz, 2000). Antibiotics also affect microbial cell membranes, but they have a distinctly different mechanism of action from polymixin (Pressman, 1985). Vancomycin may inhibit ruminal bacteria isolates by constraining peptidoglycan synthesis, whereas chloramphenicol and erythromycin may affect cell ribosome activity.

The antibiotics used had highly lipophilic polyethers that accumulate in cell membranes and catalyze rapid ion movement through bacterial cells (Pressman, 1985). The antibiotics examined inhibited ruminal bacterial isolates to differing extents resulting in the rank: ciprofloxacin (most toxic) > erythromycin > amikacin > gentamicin > roxithromycin > vancomycin = cefotaxime > cefoperazone > piperacillin > streptomycin > polymyxin = chloramphenicol > cefadroxil (least toxic). These differences in inhibitory activity may be due to variable effects of the antibiotics on the direction of metal and proton movement across the bacterial cell membrane, which is ultimately dictated by the magnitude of ion gradients across the membrane (Russell and Strobel, 1989). Most living organisms maintain a higher concentration of K inside their cells, and they expel Na and protons (Harold, 1986). The rumen is rich in Na, and ruminal Na concentrations are higher than K (Durand and Kawashima, 1980). For example, when glycolyzing *Streptococcus bovis* cells were treated with monensin (Russell, 1987; Russell and Strobel, 1989), there was a rapid efflux of K from the bacterial cell, and a concomitant influx of Na and protons. Cells attempt to counteract this futile ion flux by activating membrane ATPases and transporters, becoming eventually de-energized. Other antibiotics can also translocate ions across the cell membranes of mammals, and this limits their therapeutic use (Pressman, 1985).

Ciprofloxacin was the most toxic antibiotic to the bacterial isolates, possibly by inhibition of cell DNA gyrase and, although this antibiotic has some activity against gram-positive bacteria, it is against gram-negative organisms that it proved to be more potent than other fluoroquinolones antibiotics (Lebel, 1988). Erythromycin, a specific inhibitor of protein biosynthesis, inhibited incorporation of phenylalanine by a cell-free ribosomal system of bacterial cell (Wolfe and Hahn, 1964). Amikacin, gentamicin and streptomycin are aminoglycosides which may inhibit ruminal bacterial isolates by interfering with the proof-reading process, causing an increased error rate in synthesis with premature termination by inhibiting ribosomal translocation where the peptidyl-tRNA moves from the A-site to the P-site by disrupting the integrity of the bacterial cell membrane (Shakil et al., 2008), and by binding to the bacterial 30S or 50S ribosomal subunits (Champney, 2001).

Some antibiotics used in our study only affected the flow of a single ion, whereas others act as antiporters (Russell and Strobel, 1989) by binding protons or metal ions (for example, Na and K), so that only uncharged molecules containing either a proton or metal ion can move freely through the cell membrane. Metal ion binding is facilitated by loss of solvation water and the ability of the linear molecule to shield this charge (Riddell, 2002). Because the carboxyl group of antibiotics remains near the surface, its ionization is a pH-dependent function(Chow and Russell, 1990).

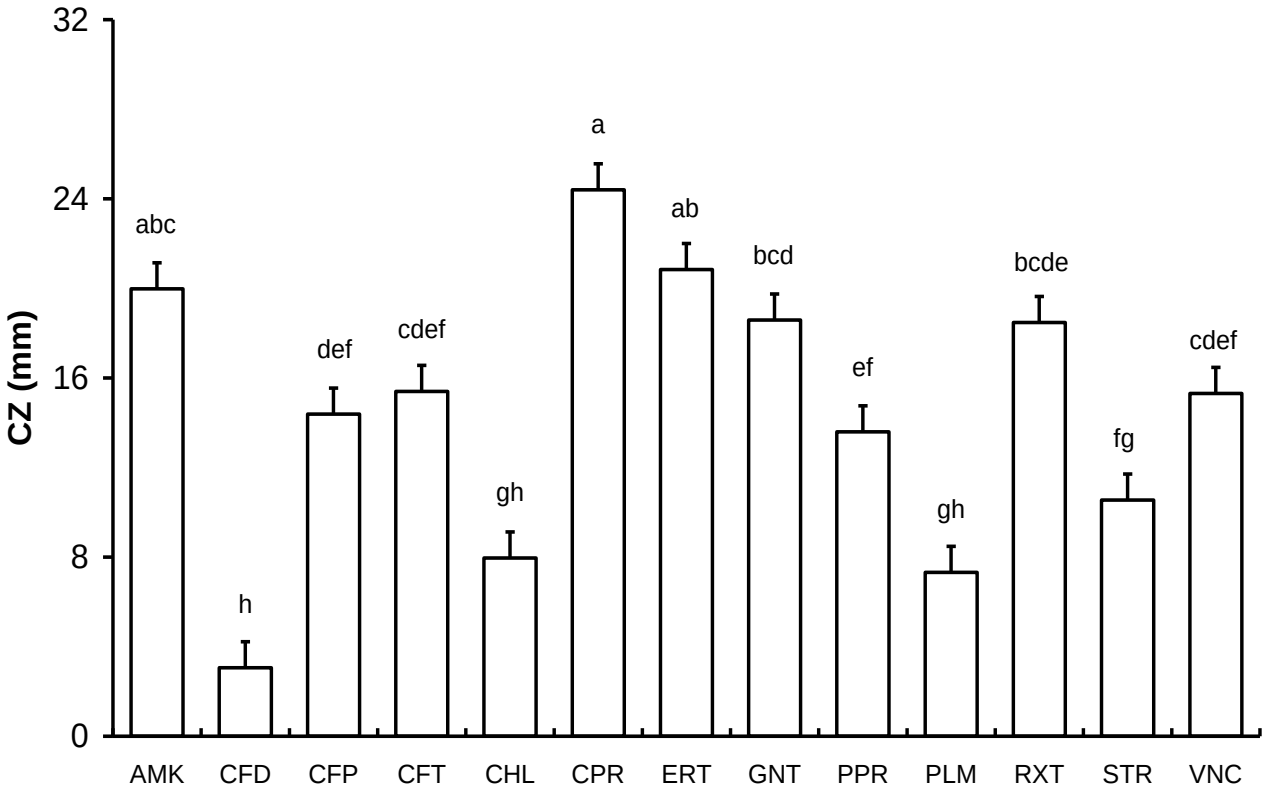

Figure 1. Average size (mm) of clearance inhibition zone (CZ) for the different antibiotics ($P < 0.001$). [a, b, c, d, e, f, g, h: bars with different superscripts differ for their clearance inhibition zone value ($P<0.05$). AMK; amikacin, CFD; cefadroxil, CFP; cefoperazone, CFT; cefotaxime, CHL, chloramphenicol; CPR, ciprofloxacin; ERT, erythromycin; GNT, gentamicin; PPR, piperacilli; PLM, polymyxin; RXT, roxithromycin; STR, streptomycin; VNC, vancomycin].

Table 1. Differences among animal species from which ruminal bacteria were isolated in their responses (based on the clearance inhibition zone in mm) to each antibiotic.

Antibiotic	Buffalo	Cattle	Sheep	SEM	*P*
Amikacin	19.2	18.7	22.0	2.13	0.436
Cefadroxil	2.4	3.8	3.0	1.97	0.757
Cefoperazone	11.0	15.0	17.1	2.73	0.097
Cefotaxime	15.2	15.1	15.9	3.32	0.979
Chloramphenicol	7.8	7.4	8.7	2.36	0.911
Ciprofloxacin	23.1	24.2	25.9	1.95	0.460
Erythromycin	16.4	21.5	24.7	3.30	0.059
Gentamicin	12.8 a	19.9 b	23.1 b	2.41	<0.001
Piperacillin	11.8	15.3	13.7	2.48	0.314
Polymyxin	7.0	5.9	9.0	2.21	0.538
Roxithromycin	13.9	19.8	21.7	3.36	0.059
Streptomycin	10.4	11.9	9.3	1.94	0.535
Vancomycin	14.2	15.2	16.4	2.63	0.751

a, b. Within antibiotic, values in the same row with different superscripts differ ($P<0.05$).

The use of antibiotics in ruminant feeding for optimization of rumen fermentation patterns to meet various objectives, such as increased animal growth or reduced environmental impact, could increase the concerns about antibiotic residues, such as fumaric acid, as it could to be one of the most useful because of its potential to reduce methanogenesis by diverting H_2 to propionate (Newbold and Rode, 2006). Increased H_2

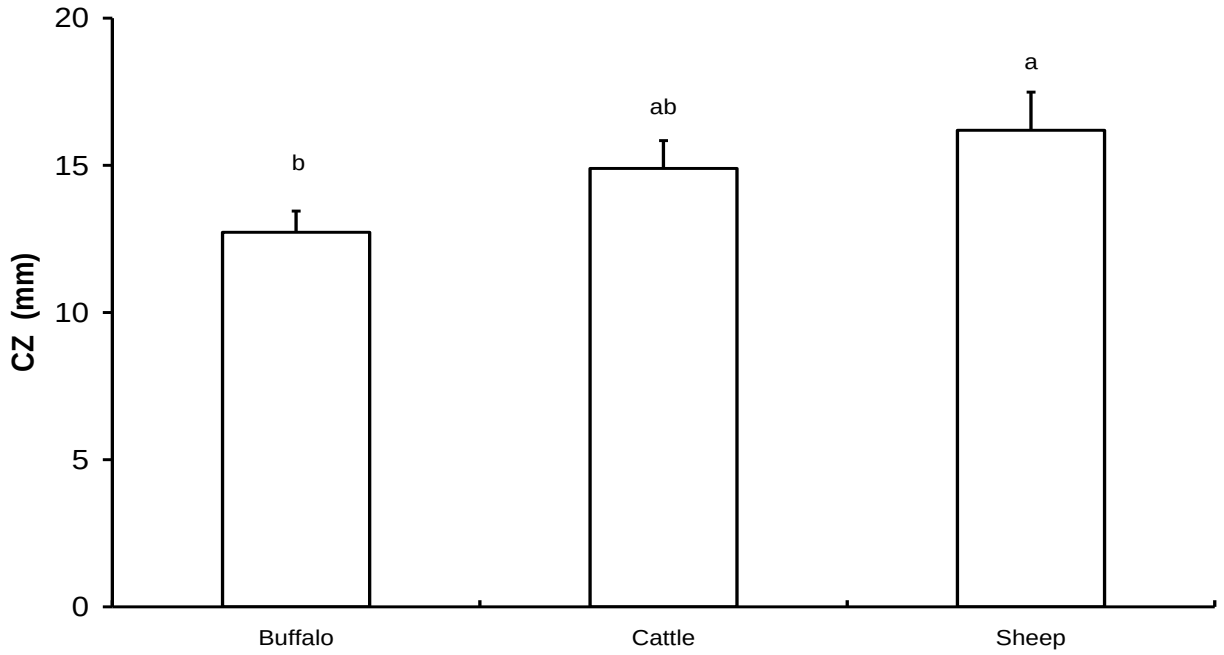

Figure 2. Average size (mm) of clearance inhibition zone (CZ) for the different ruminant species from which bacterial isolates were obtained across all antibiotics (P = 0.040). [a, b: bars with different superscripts differ in their clearance inhibition zone value (P < 0.05)].

Table 2. Percentage of ruminal bacteria isolates from different ruminant species that show tolerance (clearance zone =0) against the antibiotics.

Antibiotic	Buffalo	Cattle	Sheep	Mean	Median
Amikacin	0.0	0.0	0.0	0.0	0.0
Cefadroxil	82.8	58.8	66.7	69.4	66.7
Cefoperazone	20.7	05.9	0.0	8.9	5.9
Cefotaxime	10.3	11.8	22.2	14.8	11.8
Chloramphenicol	37.9	29.4	11.1	26.2	29.4
Ciprofloxacin	0.0	0.0	0.0	0.0	0.0
Erythromycin	17.2	0.0	0.0	5.7	0.0
Gentamicin	3.4	0.0	0.0	1.1	0.0
Piperacillin	13.8	0.0	0.0	4.6	0.0
Polymyxin	27.6	29.4	11.1	22.7	27.6
Roxithromycin	37.9	0.0	0.0	12.6	0.0
Streptomycin	13.8	5.9	22.2	14.0	13.8
Vancomycin	24.1	5.9	0.0	10.0	5.9
Mean	22.3	11.3	10.3		
Median	17.2	5.9	0.0		

utilization by fumarate reducing bacteria could also stimulate cellulolytic bacteria and enhance cellulose digestion (Wallace et al., 2006). However, inconsistent effects of fumaric acid on animal performance (Newbold and Rode, 2006), have limited its use in practice. One of the major constraints to induction of the effects fumaric acid is that the affinity of fumarate reducing bacteria to H_2 is lower than the affinity of methanogens and, as a result, the maximum potential of fumarate to divert H_2 from CH_4 is limited because methanogens utilize H_2 more rapidly than fumarate utilizing bacteria. Asanuma et al. (1999) suggested that fumarate utilizing bacteria have a disadvantage in utilization of H_2 compared with methanogens, especially when the partial pressure of H_2 is low. In this regard, ciliate protozoa facilitate methanogenesis by consuming O_2 and establishing a high redox potential (Newbold et al., 1995). Defaunating agents were found to strongly inhibit methanogenesis

and direct H_2 to propionate production (Santra et al., 1996).

The ability to tolerate antibiotics at doses which inhibit sensitive bacteria is highly species-specific. The higher antibiotic resistance of bacteria in buffalo and cattle, *versus* sheep, was probably due to differences in ruminal bacterial species among ruminant species. Limited information is available on differences among ruminant species in their ruminal microbial communities and, in particular, on the sensitivity of ruminal bacteria from different animal species to antibiotics. Hassanain et al. (2011) collected a total of 310 samples of faeces and digesta, including 50 fecal droppings of broiler chickens and 260 intestinal content of 105 broiler chickens, 50 cattle, 55 buffalo and 50 sheep, and 48 human fecal samples, and examined antibiotic susceptibility of the isolated Campylobacter strains to antibiotics. They found that poultry Campylobacter strains displayed a resistance of 64.7% to ampicillin, streptomycin and chloramphenicol and 58.8% to erythromycin and tetracycline. In contrast, human strain resistance patterns were 87.5% to ampicillin, 75.0% to streptomycin and tetracycline, 62.5% to erythromycin and 50.0% to chloramphenicol. Consistent with our results, sensitivity to antibiotics of ruminal bacteria differed in the different host animals.

Resistance of bacterial isolates to some antibiotics, such cefadroxil, chloramphenicol, polymyxin and streptomycin appears to be mediated by extracellular polysaccharides (that is, glycocalyx) that repel antibiotics from the cell membrane. Genes responsible for antibiotic resistance in bacterial cells have not been identified, and there is no clear evidence that antibiotics resistance can be spread from one bacterium to another. Given these observations, use of antibiotics in animal feed at sub-therapeutic levels is not likely to have an important impact on transfer of antibiotic resistance from animals to man. Sengupta et al. (2011) concluded that Gram-negative bacteria in the anaerobic bacterial populations are the major reservoir of integrons and transposons screened, but they do not seem to be responsible for spread of multi-resistance phenotype among Gram-positive bacteria.

Some reports indicate that extracellular polysaccharide plays a key role in ionophore resistance of some ruminal bacterial species. When *Prevotella bryantii* B14 (Callaway and Russell, 1999) and *Clostridium aminophilum* F (Rychlik and Russell, 2002) cultures were selected with monensin, the monensin-resistant cells were more easily dispersed, had an increased amount of anthrone-reactive material, and were no longer agglutinated by lysozyme (a positively charged protein). Because the resistant cells did not persist after the ionophore was withdrawn, there was little indication that resistance was mediated by a traditional mechanism (for example, a degradative enzyme or a pump that expelled antibiotics). Little is known about the genetics of extracellular polysaccharide production in ruminal bacterial species, but studies with non-ruminal bacterial species indicate that it is encoded by a large number of inducible genes (Roberts, 1996).

Conclusion

Sub-therapeutic antibiotic used in ruminant feeding for optimization of rumen fermentation patterns to increase animal performance and/or reduce environmental impacts may be led to increasing concerns that their residues in animal products might give rise to resistance to antibiotics used therapeutically in humans. The inhibition response of the antibiotics examined in this study to the isolated bacterial populations of sheep, cattle and buffalo differed with higher inhibition in sheep versus buffalo and cattle. Antibiotic inhibitory effects ranked in the order: ciprofloxacin (most toxic) > erythromycin > amikacin > gentamicin > roxithromycin > vancomycin = cefotaxime > cefoperazone > piperacillin > streptomycin > polymyxin = chloramphenicol > cefadroxil (least toxic). Further research will be needed to investigate the residues of antibiotics in animal products.

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