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Detection of sensitive and mutant ruminal bacteria isolates from sheep, cattle, and buffalo using 14 therapeutic antibiotics

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Abstract: In the present study, sensitive and mutant colonies of some ruminal bacterial species isolated from sheep, cattle, and buffalo were detected. We counted and considered "mutant colonies" the bacterial colonies grown in the clear inhibition zone in the Kirby–Bauer disk diffusion susceptibility test. Detected mutant colonies were higher in buffalo than in cattle and sheep. Duricef and metronidazole caused no mutations in any species. The others formed mutant colonies, where roxithromycin = polymyxin = chloramphenicol = gentamicin < erythromycin < vancomycin < piperacillin = cefotaxime < streptomycin < cefoperazone < ciprofloxacin < amikacin. Sheep had the highest number of sensitive isolates, and the number of sensitive isolates was dramatically lower in cattle and buffalo. There were no sensitive isolates with the antibiotic metronidazole, and there was a low number of sensitive isolates with duricef. The other antibiotics had more sensitive isolates (gentamicin = ciprofloxacin = amikacin > streptomycin = piperacillin > erythromycin > vancomycin = cefoperazone = cefotaxime > roxithromycin > polymyxin > chloramphenicol). The number of sensitive isolates of the different ruminant species for all the antibiotics was highest in buffalo, followed by cattle and then sheep ($P < 0.05$). We could conclude that subtherapeutic antibiotic use in ruminant feeding may lead to the formation of antibiotic-resistant mutant colonies, making their subtherapeutic effect nonexistent.

Key words: Antibiotics, mutant isolates, resistance, ruminal bacteria

1. Introduction

The use of antibiotics as antimicrobial feed additives in farm animal production has contributed to treating clinical disease, to preventing and controlling common disease events, and to enhancing animal growth (1). Recently, Landers et al. (2) showed that feeding antibiotics to livestock might lead to changes in the commensal bacteria in the gastrointestinal tract of animals fed such antibiotics, which in turn could lead to an increase in antibiotic resistance genes and pathogen transfer. Guidelines were therefore developed for the prudent use of antibiotics. These give criteria for selecting the most appropriate antibiotic preparation and determining dosage and duration of therapy for necessary treatment. At the same time, new and more efficient types of antibiotics should be developed in order to introduce new alternative antibiotic preparations if any bacteria species have developed resistance to the existing treatments (1).

In large herds of free-ranging ruminants, therapeutic oral antibiotic administration may be the only practical way to administer antibiotics. In cattle, 75.3% of drugs were applied orally, followed by 17.3% parenteral and 7.4% local applications (1). Oral administration can cause microbial resistance to administered antibiotics; however, it has been suggested that resistance is more likely to appear when physicians and veterinarians misdiagnose infections and improperly administer antibiotics (3). Ruminal bacteria resistant to one antibiotic can also be resistant to another (4), but the mechanism of this resistance was not well defined until recently (5).

Subtherapeutic antibiotic use in ruminant feeding to optimize rumen fermentation may lead to residues in meat and milk (6), as well as an increase in the inhibition of ruminal bacterial populations (7). For this reason, in 2006, the European Union banned the use of antibiotics as growth promoters in livestock feeding due to potential toxicities to host animals, potential rumen microbial adaptation, and risk of the presence of residues of these compounds in milk and meat, with potential effects on human health (8).

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The inhibitory effects of antibiotics in bacterial growth and replication could be due to their effects on processes such as peptidoglycan synthesis, ribosome activity, DNA replication, mRNA transcription, nucleotide synthesis, and/or membrane stability (9,10). There were differences in antibiotic resistance among animal species. Sheep exhibited, in general, lower resistance than buffalo to antibiotics (7). Irrespective of ruminant species, bacterial isolates had different levels of sensitivity to different antibiotics. A higher tolerance was noticed for cefadroxil, whereas ciprofloxacin, erythromycin, and amikacin were the most toxic antibiotics (7).

This study aimed to detect sensitive and mutant colonies of some ruminal bacterial species isolated from sheep, cattle, and buffalo for the 14 traditional therapeutic antibiotics.

2. Materials and methods

2.1. Animals and bacterial isolates

Samples that were a mix of solid and liquid ruminal contents (~100 mL) were collected from a local animal slaughterhouse immediately after animal slaughter. Rumen content samples were sampled from 2 sheep, 5 cows, and 9 buffaloes, with 2 samples collected from each animal. Samples were homogenized to a single sample, which was subsequently used for inoculation of cultures previously prepared with thioglycolate agar medium (11).

2.2. Isolation of ruminal bacteria

Thioglycolate broth cultures were used to cultivate and isolate ruminal bacteria in accordance to the recommendations of the National Institutes of Health (12). The culture contained (g/L): 500 sodium thioglycolate, 500 L-cystine, 5000 yeast extract (Oxoid L21), 15,000 pancreatic digest of casein (Oxoid), and 2500 sodium chloride dextrose.

From each homogenized fresh sample of rumen contents, 1 mL of fluid was used to inoculate cultures by spreading it manually on the surface of a petri dish (9 cm in diameter) containing thioglycolate medium. Plates were poured to a depth of 5 mm (about 15 mL of medium) and dried for 30 min. All plates were incubated anaerobically at 39 °C for 72 h. Thereafter, colonies were picked up and streaked to confirm purity. All actions were done under anaerobic conditions. Weekly transfers were necessary for culture survival; for long-term storage, cultures of each ruminal bacterial isolate were frozen in 200 g/L glycerol at –80 °C in cryogenic plastic tubes.

2.3. Mutants and sensitive isolates to antibiotics

On a freshly anaerobically sterilized medium with cysteine hydrochloride as a reducing agent and sodium resazurin as the indicator of oxygen absence in the medium, stock cultures of ruminal bacteria isolates were grown. Medium pH was adjusted to 6.8 and then the medium was supplemented with 750 mg of agar-agar. The medium was sterilized at 121 °C for 20 min, and then about 7–8 mL aliquots of the medium were dispensed and spread onto glass plates purged with oxygen-free CO_2. After medium spreading, plates were inoculated and prepared for assay to examine the sensitivity of ruminal bacteria to the different antibiotics. Nine rumen bacterial isolates from sheep, 16 from cattle, and 34 from buffalo were used in the study. The number of antibiotic-sensitive isolates was determined by the Kirby–Bauer disk diffusion susceptibility test (13). Filter paper disks (Whatman No. 1; 5 mm in diameter) were impregnated with 10 µL of an aqueous solution of water containing 5 µg of roxithromycin, piperacillin, streptomycin, cefotaxime, cefoperazone, vancomycin, polymyxin, amikacin, chloramphenicol, duricef, ciprofloxacin, erythromycin, gentamicin, or metronidazole. The used antibiotics were manufactured by Sigma-Aldrich. Control disks were impregnated with 10 µL of dimethyl sulfoxide solution.

The impregnated disks were applied to the surface of agar plates previously inoculated with a standard amount of 48-h-old cultures of ruminal bacteria isolates (1 mL of 10^5 colony-forming units) and incubated at 39 °C for 72 h. After incubation, the diameter of the formed clear inhibition zone (mm) was measured using a caliper. All the bacterial colonies grown in the clear inhibition zone were counted and considered as mutant colonies. Each isolate was tested in duplicate.

2.4. Statistical analyses

We analyzed differences among ruminant species for the number of sensitive isolates and percentage of mutant colonies for tested antibiotics according to a completely randomized design (14). Ruminant species and antibiotics were considered as fixed effects and isolates (considered the experimental unit) as the random effect, using mixed-design analysis of variance (15).

3. Results

Based on the mutant colonies detected among animal species, buffalo had more mutant colonies than cattle or sheep, the former of which had the lowest. The number of mutant colonies detected as a result of antibiotic use varied. Neither duricef nor metronidazole caused any mutants. Another 4 antibiotics (roxithromycin, polymyxin, chloramphenicol, and gentamicin) caused one mutant colony each, but none of those occurred in sheep. Two antibiotics (amikacin > ciprofloxacin) increased the numbers of mutant colonies detected, most of those for buffalo. The other 6 antibiotics had moderate numbers of mutant colonies (erythromycin < vancomycin < piperacillin = cefotaxime < streptomycin < ciprofloxacin) (Table 1).

Table 1. Number of mutant colonies detected in response to each individual antibiotic in the 3 different ruminant species.

Antibiotic	Number of mutant isolates			Total mutants
	Sheep	Cattle	Buffalo	
Roxithromycin	0	1	0	1
Piperacillin	1	1	2	4
Streptomycin	0	1	4	5
Cefotaxime	1	2	1	4
Cefoperazone	3	3	0	6
Vancomycin	2	1	0	3
Polymyxin	0	0	1	1
Amikacin	4	4	8	16
Chloramphenicol	0	1	0	1
Duricef	0	0	0	0
Ciprofloxacin	2	2	5	9
Erythromycin	0	1	1	2
Gentamicin	0	0	1	1
Metronidazole	0	0	0	0
Mean	0.9	1.2	1.6	3.8

Table 2. Number of sensitive isolates detected in response to each individual antibiotic in the 3 different ruminant species.

Antibiotic	Number of sensitive isolates			Totalsensitive isolates
	Sheep	Cattle	Buffalo	
Roxithromycin	17	8	5	30
Piperacillin	17	8	5	35
Streptomycin	23	7	5	35
Cefotaxime	23	6	3	32
Cefoperazone	20	7	5	32
Vancomycin	19	8	5	32
Polymyxin	18	6	4	28
Amikacin	26	8	5	39
Chloramphenicol	16	6	4	26
Duricef	3	1	0	4
Ciprofloxacin	26	8	5	39
Erythromycin	21	8	5	34
Gentamicin	26	8	5	39
Metronidazole	0	0	0	0
Mean	18.2	6.4	4.0	28.9

The number of sensitive isolates was extremely variable. Sheep had the highest number, which was dramatically greater than in cattle and buffalo. The sensitivity of isolates to the antibiotics was also extremely variable. No sensitive isolates were detected with metronidazole, and low numbers were detected with duricef. However, the other antibiotics had more sensitive isolates (gentamicin = ciprofloxacin = amikacin > streptomycin = piperacillin > erythromycin > vancomycin = cefoperazone = cefotaxime > roxithromycin > polymyxin > chloramphenicol) (Table 2).

Table 3 illustrates the percentage of the detected mutant colonies of the sensitive isolates for each ruminant species among the antibiotics used. Because buffalo had the highest number of mutant colonies, with the lowest number of sensitive isolates, that group had the highest mutant percentage of sensitive isolates. The opposite was true in sheep, which had the lowest mutant percentage of sensitive isolates. Duricef and metronidazole had no mutant colonies, while amikacin had the highest number.

Overall, the number of sensitive isolates in the ruminant species among the different antibiotics tested was highest in buffalo, followed by cattle and then sheep ($P < 0.05$), as shown in Figure 1. Figure 2 shows that the mean of the detected mutant colonies as a percentage of the sensitive isolates of the different ruminant species among antibiotics was highest in sheep followed by cattle, and lowest in buffalo ($P < 0.05$).

4. Discussion

The use of antibiotics in animal feeding at subtherapeutic levels is unlikely to have an important impact on transferring antibiotic resistance from animals to humans. This is because the genes responsible for antibiotic resistance in bacterial cells have not been identified, and there is no clear evidence that antibiotic resistance can transfer from one bacterium to another. Recently available information has indicated that the rumen has the ability to be a site of gene transfer among microorganisms (16). Rumen protozoa are active predators of bacteria that can harbor antibiotic resistance genes. There is therefore an opportunity for exchange at the genetic level. Gene transfer in the rumen is relevant given the mounting spread of antibiotic-resistant bacteria and the public health implications for veterinary antimicrobial therapies. Defaunation may prove to be an important method to reduce the spread of antibiotics resistance genes (17). Sengupta et al. (18) concluded that gram-negative bacteria in anaerobic bacteria populations are the major reservoir of screened integrons and transposons, but they do not seem to be responsible for the spread of multiresistance phenotypes among gram-positive bacteria.

There is limited information available on the differences between ruminant species in their ruminal microbial communities and, in particular, on the sensitivity of ruminal bacteria from different animal species to antibiotics. In our study, buffalo and cattle had a higher

Table 3. Detected mutant colonies as a percentage of sensitive isolates in response to each individual antibiotic for the 3 different ruminant species.

Antibiotic	Mutant % of the sensitive isolates			Mutants %
	Sheep	Cattle	Buffalo	
Roxithromycin	0.0	12.5	0.0	3.3
Piperacillin	11.8	12.5	20.0	11.4
Streptomycin	17.4	14.3	0.0	14.3
Cefotaxime	4.4	33.3	33.3	12.5
Cefoperazone	0.0	42.9	60.0	18.8
Vancomycin	0.0	12.5	40.0	9.4
Polymyxin	5.6	0.0	0.0	3.6
Amikacin	30.8	50.0	80.0	41.0
Chloramphenicol	0.0	16.7	0.0	3.9
Duricef	0.0	0.0	0.0	0.0
Ciprofloxacin	19.2	25.0	40.0	23.1
Erythromycin	4.8	12.5	0.0	5.9
Gentamicin	3.9	0.0	0.0	2.7
Metronidazole	0.0	0.0	0.0	0.0
Mean	7.0	16.6	19.5	10.7

number of bacterial mutants than did sheep. This may be due to differences in ruminal bacterial species among our ruminant species (7).

In our study, the number of mutant colonies observed with different antibiotics can be summarized as follows: amikacin > ciprofloxacin (least toxic, i.e. higher numbers of mutant colonies) > ciprofloxacin > cefoperazone > streptomycin > cefotaxime = piperacillin > vancomycin > erythromycin > gentamicin = chloramphenicol = polymyxin = roxithromycin > metronidazole = duricef (most toxic, i.e. no mutant colonies). These differences in the formation of mutant colonies may be due to different 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 (19). There is variability in response to antibiotics, especially concerning mutant colony formation. The presence of live mutant colonies in the presence of antibiotics suggests that these colonies were not killed, but were rather merely inhibited by the concentration of antibiotics in the clear zone (20). Bacteria can use different mechanisms to resist antibiotics, including degradation or modification of the antibiotic, alteration of the bacterial target of the antibiotic, and targeted protection and reduction of the intracellular concentration of the antibiotic, either by a decreased

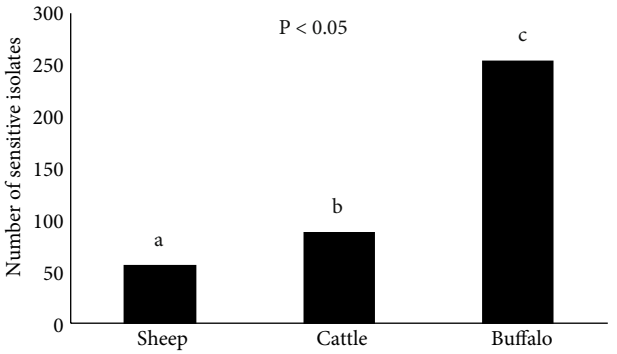

Figure 1. Number of sensitive isolates of the different ruminant species in response to all antibiotics (P < 0.05). a, b, c: Superscripts show differences among ruminant species in their mutant values at P < 0.05.

permeability of the cell wall or by efflux of the antibiotic from the cell (21).

The ability of microbes to tolerate antibiotics at the same doses that inhibit sensitive bacteria is highly species-specific. Resistance of bacterial isolates (i.e. mutants) to some antibiotics, such as amikacin and ciprofloxacin, appears to be mediated by extracellular polysaccharides (i.e. glycocalyx) that repel antibiotics from the cell membrane (4). Some reports indicate that extracellular polysaccharide plays a key role in the ionophore antibiotic resistance of some ruminal bacterial species. When *Prevotella bryantii* B14 (22) and *Clostridium aminophilum* F (5) 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 lysozymes (positively charged proteins). Because the resistant cells did not persist after the ionophore was withdrawn, there was little indication that resistance was mediated by a traditional mechanism

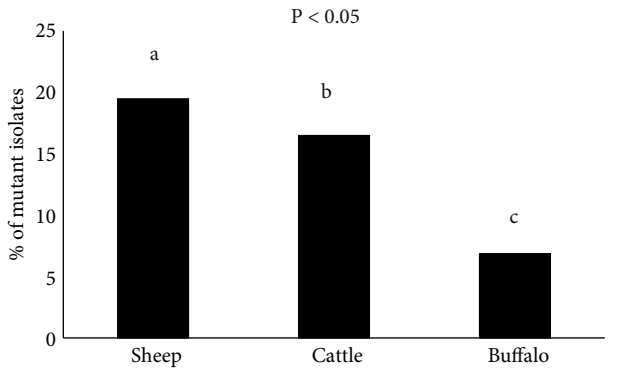

Figure 2. Average of the detected mutant colonies (as percentage of sensitive isolates) in response to all antibiotics in the different ruminant species (P < 0.05). a, b, c: Superscripts show differences among ruminant species in their mutant values at P < 0.05.

(e.g., a degradative enzyme or an ion pump that expelled antibiotics). Little is known about the genetics of extracellular polysaccharide production in ruminal bacterial species, but studies with nonruminal bacterial species indicate that it is encoded by a large number of inducible genes (23).

The susceptibility and resistance patterns of ruminal bacteria can be determined on the basis of the major fermentation products produced. In general, ruminal bacteria that produce lactic acid, butyric acid, formic acid, or hydrogen as major end products were susceptible, while bacteria producing succinic acid or ferment lactic acid were resistant (24).

In conclusion, the simulated effects of the studied antibiotics on the ability of isolated bacterial populations to form mutant colonies were higher in buffalo and cattle than in sheep. Mutant resistant colony formation ranked as follows: amikacin > ciprofloxacin (least toxic; more mutant colonies) > ciprofloxacin > cefoperazone > streptomycin > cefotaxime = piperacillin > vancomycin > erythromycin > gentamicin = chloramphenicol = polymyxin = roxithromycin > metronidazole = duricef (most toxic; no mutant colonies). In vivo experiments are required to confirm our results and investigate how to reduce rumen mutant colonies caused by antibiotic use.

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References

1. Hartung J, Kietzmann M. Use of antibiotics in animal production. In: Proceedings of the Max Rubner Conference 2012, Antibiotics in the Food Chain. Karlsruhe, Germany: MRI; 2012. pp. 5–6.
2. Landers TF, Cohen B, Wittum TE, Larson EL. A review of antibiotic use in food animals: perspective, policy, and potential. Public Health Rep 2012; 127: 4–22.
3. Fisher MJP, Scott R. Evaluating and controlling pharmaceutical emissions from dairy farms: a critical first step in developing a preventative management approach. J Cleaner Prod 2008; 16: 1437–1446.
4. Russell JB, Houlihan AJ. Ionophore resistance of ruminal bacteria and its potential impact on human health. FEMS Microbiol Rev 2003; 27: 65–74.
5. Rychlik JL, Russell JB. The adaptation and resistance of *Clostridium aminophilum* F to the butyrivibriocin-like substance of *Butyrivibrio fibrisolvens* JL5 and monensin. FEMS Microbiol Lett 2002; 209: 93–98.
6. Newbold CJ, Rode LM. Dietary additives to control methanogenesis in the rumen. International Congress Series 2006; 1293: 138–147.
7. Salem AZM, Gohar YM, Lopez S, Ronquillo MG, Cerrillo MA. Sensitivity of ruminal bacterial isolates of sheep, cattle and buffalo to 13 therapeutic antibiotics. Afr J Microbiol Res 2012; 6: 4727–4733.
8. Official Journal of the European Union. Regulation (EC) No. 1831/2003 of the European Parliament and of the Council of 22 September 2003 on Additives for Use in Animal Nutrition, 2013. pp. L268/29–L268/43.
9. Levinson W, Jawetz E. Review of Medical Microbiology and Immunology. New York, NY, USA: Lange Medical Book/McGraw-Hill; 2000.
10. Peach KC, Bray WM, Winslow D, Linington PF, Linington RG. Mechanism of action-based classification of antibiotics using high-content bacterial image analysis. Mol Biosyst 2013; 9: 1837–1848.
11. Merck & Co. Thioglycolate broth. In: Merck Microbiology Culture Media Manual. Whitehouse Station, NJ, USA: Merck; 1982. p. 161.
12. National Institutes of Health Circular: Culture Media for the Sterility Test, 2nd rev. Bethesda, MD, USA: National Institutes of Health; 1946.
13. Moolman GJJ, Wyk MV. Kirby-Bauer disc diffusion susceptibility testing as a screening procedure for cephalosporin resistance in *Streptococcus pneumoniae* - correlation with the E-test. South Afr J Epidemiol Infect 2004; 19: 55–59.
14. Steel RGD, Torrie JH. Principles and Procedures of Statistics: A Biometrical Approach. 2nd ed. New York, NY, USA: McGraw-Hill International; 1980.
15. SAS. SAS/STAT User's Guide (Version 8). Cary, NC, USA: SAS Institute Inc.; 1999.
16. Van Soest PJ. Nutritional Ecology of the Ruminant. 2nd ed. New York, NY, USA: Cornell University Press; 1994.
17. McCuddin ZP, Carlson SA, Rasmussen MA, Franklin SK. *Klebsiella* to *Salmonella* gene transfer within rumen protozoa: implications for antibiotic resistance and rumen defaunation. Vet Microbiol 2006; 114: 275–284.
18. Sengupta N, Alam SI, Kumar RB, Singh L. Diversity and antibiotic susceptibility pattern of cultivable anaerobic bacteria from soil and sewage samples of India. Infect Genet Evol 2011; 11: 64–77.
19. Russell JB, Strobel HJ. Mini review: Effect of ionophores on ruminal fermentation. Appl Environ Microbiol 1989; 55: 1–6.
20. Fulghum RS, Baldwin BB, Williams PP. Antibiotic susceptibility of anaerobic ruminal bacteria. Appl Microbiol 1968; 16: 301–307.
21. Costa SS, Viveiros M, Amaral L, Couto I. Multidrug efflux pumps in *Staphylococcus aureus*: an update. Open Microbiol 2013; 7: 59–71.

22. Callaway TR, Russell JB. Selection of a highly monensin-resistant *Prevotella bryantii* sub-population with altered outer membrane characteristics. Appl Environ Microbiol 1999; 65: 4753–4759.

23. Roberts IS. The biochemistry and genetics of capsular polysaccharide production in bacteria. Annu Rev Microbiol 1996; 50: 285–315.

24. Nagaraja TG, Taylor MB. Susceptibility and resistance of ruminal bacteria to antimicrobial feed additives. Appl Environ Microbiol 1987; 53: 1620–1625.