BIOLOGICAL ASSAY OF FEED ANTIBIOTIC STABILITY IN THE RUMEN FLUID

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A biological method has been developed to test the stability of virginiamycin and avoparcin in the rumen fluid. Two rumen fistulated wethers were fed virginiamycin (100 mg per animal per day) for 2 months. Rumen fluid was diluted with buffer and incubated anaerobically at 39 °C. Virginiamycin and glucose were added at 10 mg/l and 10 g/l, respectively. The incubation fluid was sampled at 0, 3, 6 and 9 h, centrifuged, and the cell-free supernatant was added to sterile MRS broth at 0, 0.25, 0.50 and 1% (v/v). The broth was inoculated by *Bacillus stearothermophilus* 794B and incubated overnight at 60 °C. This procedure was also used to test the stability of avoparcin in the rumen. Cell-free incubation fluid with virginiamycin had no effect on growth of the indicator organism when added to the MRS broth at 0.25%, but prevented growth at 0.50%. Cell-free incubation fluid containing avoparcin inhibited growth of *B. stearothermophilus* in a dose-dependent manner. No effect on growth was found at 0.20%. Full inhibition of growth was observed at 2% addition. The activity of antibiotics was not influenced by the time of incubation of the rumen fluid with virginiamycin and avoparcin. It can be concluded that both feed antibiotics are stable in the rumen fluid of adapted wethers. The present method, however, cannot detect microbial resistance mechanisms other than the direct inactivation of an antibiotic.

rumen; feed antibiotic; virginiamycin; avoparcin; antibiotic stability

INTRODUCTION

Favourable effects of feeding of antimicrobial feed additives on weight gains and feed efficiency in ruminants have been attributed primarily to changes in ruminal fermentation pattern. Considerable attention has been

given to the use of ionophores in ruminants (reviewed by Nagaraja, 1995). The most consistent fermentation shifts observed when ionophores are fed are increased production of propionate and decreased production of acetate, butyrate and methane. Several studies showed that these effects persisted as long as ionophores were fed (De Jong, Berschauer, 1983; Kobayashi et al., 1988; Šimůnek et al., 1989; Duff et al., 1995; Mbanzamihigo et al., 1995, 1996). Two out of these studies indicated that ionophores inhibited fermentation of fibre in animals non-adapted to ionophores, but a negative effect disappeared within a few weeks (De Jong, Berschauer, 1983; Kobayashi et al., 1988). Whether an adaptation effect exists in antimicrobials other than ionophores is not clear. In our previous study, virginiamycin (a non-ionophore propionate enhancer) significantly altered the fermentation pattern in favour of propionate when rumen fluid was sampled from non-adapted wethers. Most of virginiamycin-induced fermentation shifts disappeared when rumen fluid was taken from wethers adapted to virginiamycin intake for two months (Marounek, Skřivanová, 1993). The adaptation of sheep to virginiamycin was observed also by Nagaraja et al. (1992). The adaptation of rumen microorganisms to feed antibiotics may be caused by inactivation of antibiotics by modifying enzymes or by other mechanisms. In our study, a method for testing of the antibiotic stability in the rumen fluid of adapted wethers is presented. A strain of *Bacillus stearothermophilus* was used as an indicator organism. This bacterium has often been used for the detection of antibiotics in milk. A shortened version of this contribution was presented as a poster at the 5th International Symposium on the Nutrition of Herbivores, held in San Antonio (Texas), in April 1999.

MATERIAL AND METHODS

Two rumen fistulated wethers were fed a commercial concentrate (0.3 kg at 8.00 h), lucerne hay and maize silage *ad libitum*. Once daily, 2 h after the morning feeding, virginiamycin (100 mg) was supplied *per os* in a gelatine capsule. Rumen fluid was sampled two months after the start of virginiamycin feeding, prior to the virginiamycin intake. Rumen fluid of wethers was mixed, diluted with McDougall buffer 1 : 2, and incubated at pH 6.5 in a laboratory LF2 fermentor under $C0_2$ atmosphere, at 39 °C. The fermentor was equipped with mechanical stirring, gas entry and exit, temperature and pH controls. Total volume of the diluted rumen fluid was 1.8 l. Glucose (10 g/l) served as the substrate. Virginiamycin was added at 10 mg/l, final concentration.

The incubation fluid was sampled at 0, 3, 6 and 9 h. The fluid was centrifuged at 4 °C (15 000 g; 20 min). The cell-free supernatant was added to sterile MRS broth (Oxoid) at 0, 0.25, 0.50 and 1.00% (v/v). The broth was inoculated by a culture of *B. stearothermophilus* 794B, and incubated in triplicates overnight at 60 °C. *Bacillus stearothermophilus* was supplied from the Milcom Comp. (Prague, Czech Republic). The bacterium was maintained in 20% (v/v) glycerol between experiments. Optical density of all cultures was measured at 640 nm. Purity of cultures was checked microscopically, after Gram staining. The same procedure was used to test the stability of avoparcin in the rumen fluid. The cell-free supernatant was added to the MRS broth at 0; 0.2; 0.5; 1.0 and 2.0%.

Volatile fatty acids (VFA) were determined by titration after steam distillation in samples of the incubation fluid taken from fermentors. The statistical treatment of data was performed by a one-way analysis of variance.

RESULTS AND DISCUSSION

Ruminal microorganisms cultivated in fermentors in the presence of virginiamycin and avoparcin fermented glucose and produced VFA. Total VFA productions (corrected for amounts present before incubation) were 70.7 and 77.9 mmol/l, respectively. Cell-free incubation fluid containing virginiamycin had no effect on growth of *B. stearothermophilus* 794B when added to the MRS broth at 0.25%, but prevented the growth at 0.50% (Table I). No effect of the time of incubation of virginiamycin with the rumen fluid on the antibiotic activity was observed. Cell-free incubation fluid containing avoparcin inhibited growth of *B. stearothermophilus* 794B in a dose-dependent manner (Table II). No inhibition of growth was observed at 0.20%. Ten times higher incubation fluid addition inhibited growth of *B. stearothermophilus* 794B completely. No contamination of *B. stearothermophilus* 794B cultures was observed.

Biological assays of activities of antibiotics represent inexpensive alternatives to more advanced physicochemical techniques, such as spectrophotofluorimetry and HPLC. Biological assay allows accurate and sensitive assessment of most antimicrobials and remains a commonly used technique. It is an especially precise way of measuring penicillins, where amounts are as low as a few nanograms per millilitre (Bywater, 1982). Usually, an agar-based medium is seeded with a suitable bacterial strains, e.g. *Bacillus subtilis*. Standards and samples are placed in wells in the agar and incubated. The size of the zones in which no bacterial growth occur is proportional to the concentration of antibiotic present. In this study, *B. stearothermophilus* strain was used to test the stability of feed antibiotics in the rumen fluid. *B. stearothermophilus* is sensitive to various antimicrobials and it is an easily available bacterium. Its temperature optimum (60 °C) prevents the growth of contaminating mesophilic flora. Our data show that the antibiotic activity was not destroyed by

I. Effect of time of incubation of rumen fluid[a] with virginiamycin[b] and size of incubation fluid addition to the MRS broth on growth[c] of *Bacillus stearothermophilus* 794B

Incubation fluid addition (%, v/v)	Time of incubation of rumen fluid with virginiamycin (h)			
	0	3	6	9
0	1.10 ± 0.15	1.05 ± 0.18	0.93 ± 0.35	1.17 ± 0.28
0.25	1.13 ± 0.23	1.30 ± 0.26	1.27 ± 0.32	0.95 ± 0.35
0.50	0	0	0	0
1	0	0	0	0

Means ± SD
[a] rumen fluid from adapted wethers
[b] 10 mg/l
[c] measured as optical density at 640 nm

II. Effect of time of incubation of rumen fluid with avoparcin and size of incubation fluid addition to the MRS broth on growth of *Bacillus stearothermophilus* 794B

Incubation fluid addition (%, v/v)	Time of incubation of rumen fluid with avoparcin (h)			
	0	3	6	9
0	0.71 ± 0.07	0.74 ± 0.02	1.06 ± 0.14	0.95 ± 0.13
0.2	0.72 ± 0.04	0.81 ± 0.10	0.97 ± 0.12	0.93 ± 0.17
0.5	0.43 ± 0.10	0.30 ± 0.11	0.57 ± 0.14	0.50 ± 0.12
1	0.18 ± 0.07	0.17 ± 0.08	0.26 ± 0.11	0.18 ± 0.10
2	0	0	0	0

Means ± SD
[a] rumen fluid from adapted wethers
[b] 10 mg/l
[c] measured as optical density at 640 nm

incubation with the rumen fluid of wethers adapted to the virginiamycin and avoparcin intake for two months. It is, however, possible that a resistance mechanism other than the direct inactivation of an antibiotic exists in adapted animals, e.g. alteration of the target site, reduction of the intracellular antibiotic concentration, etc. (Schnappinger, Hillen, 1996).

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Biologické stanovení stability krmných antibiotik v bachorové tekutině.

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Byla vyvinuta biologická metoda ke stanovení stability krmných antibiotik virginiamycinu a avoparcinu v bachorové tekutině. Dvěma skopcům s permanentní bachorovou píštělí byl podáván virginiamycin v množství 100 mg denně po dobu dvou měsíců. Skopcům byla odebrána bachorová tekutina, zředěna pufrem a inkubována za anaerobních podmínek při teplotě 39 °C s přídavkem virginiamycinu (10 mg/l) a glukózy (10 g/l). V čase 0 – 3 – 6 a 9 hodin byly odebrány vzorky inkubační tekutiny, odstředěny a bezbuněčný supernatant byl přidán ke sterilnímu médiu MRS v množství 0 – 0,25 – 0,50 a 1 % (v/v). Toto médium bylo zaočkováno bakterií *Bacillus stearothermophilus* 794B a inkubováno přes noc při 60 °C. Stejný postup

byl použit k testování stability avoparcinu v bachorové tekutině. Bezbuněčná inkubační tekutina obsahující virginiamycin neovlivnila růst bakterie, byla-li přidána k médiu v množství 0,25 %, avšak bránila růstu, byla-li přidána v množství 0,50 %. Bezbuněčná inkubační tekutina s avoparcinem inhibovala růst bakterie *Bacillus stearothermophilus* 794B úměrně přidanému množství. V dávce 0,20 % růst bakterie neovlivnila, v dávce 2 % však růstu zcela zamezila. Aktivita virginiamycinu a avoparcinu nebyla dobou inkubace bachorové tekutiny s antibiotiky nijak ovlivněna. Lze proto učinit závěr, že obě krmná antibiotika jsou v bachorové tekutině adaptovaných skopců stálá. Pokud by vznikla rezistence bachorových mikroorganismů k těmto látkám při jejich delším zkrmování, byla by založena na mechanismech jiných, než je přímá destrukce molekuly antibiotika.

bachor; krmná antibiotika; virginiamycin; avoparcin; stabilita antibiotik

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