SUSCEPTIBILITY, RESISTANCE AND ANTIBIOTIC PROFILE OF BACITRACIN AGAINST *CLOSTRIDIUM PERFRINGENS* STRAINS ISOLATED DURING CLINICAL OUTBREAKS OF EPIZOOTIC RABBIT ENTEROPATHY

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ABSTRACT

Bacitracin Minimum Inhibitory Concentration against *Clostridium perfringens* strains isolated during outbreaks of Epizootic Rabbit Enteropathy was determined as an MIC90 of 0.93 µg/ml. No strains were resistant to bacitracin and serial passages in the presence of sub-inhibitory concentrations failed to induce the development of resistant strains. Bacitracin could be described as a concentration-independent bactericidal antibiotic. Bactericidal activity was obtained at a concentration corresponding to twice the MIC, i.e.1,860 µg/l and did not further increase at higher levels. This profile is in favor of continuous administration, e.g. in drinking water.

Key words: Bacitracin, Rabbit, Susceptibility, *Clostridium perfringens*, Resistance.

INTRODUCTION

The veterinary medicinal product BACIVET®-S was recently licensed in France. This water-soluble form contains bacitracin zinc (4,200 IU/g) as active substance and is indicated in growing rabbits to reduce the clinical signs and mortality due to digestive tract infections, e.g. in enteropathies where *Clostridium perfringens* can be isolated. The treatment should be instigated if the facility has previously suffered outbreaks and as soon as the first deaths are confirmed. A regimen corresponding to 420 IU of bacitracin per kg live weight per day is recommended by the oral route in drinking water, for 14 to 21 days. Bacitracin is a polypeptide antibiotic made up of a mixture of several closely related polypeptides. It interferes with bacterial cell wall biosynthesis by inhibiting the pyrophosphatase involved in transporting peptidoglycan precursors through membranes (EMEA, 2001; Chambers, 2001). The zinc salt ensures that the active substance remains stable during storage. Bacitracin is bactericidal against Gram-positive cocci and bacilli, particularly against certain strains of clostridia (Jawatz, 1995). The aim of this study was to determine the *in vitro* pharmacodynamic profile of bacitracin against strains of *Clostridium perfringens* isolated from rabbits during episodes of Epizootic Rabbit Enteropathy (ERE), and including (i) determination of Minimum Inhibitory Concentrations (MIC), (ii) any capacity by bacitracin to induce resistance and (iii) the bactericidal profile of bacitracin.

MATERIALS AND METHODS

Determination of Minimum Inhibitory Concentrations (MIC)

MICs for bacitracin were determined under strictly anaerobic conditions in compliance with the culture standards recommended by the Clinical and Laboratory Standards Institute (CLSI/NCCLS, M11-A6, 2004). In all, 51 strains were collected from rabbit breeding facilities affected by ERE in

Belgium, and 34 other strains were isolated in France. These strains were taken directly from the caecum during the necropsy of animals presenting unambiguous lesions. The strains were independent one from the other and each was isolated during a separate outbreak. The different strains were frozen at -80°C and stored at this temperature pending analysis. After thawing, each strain was subjected to a purity control and a fresh identification (API gallery). The strains were then cultured in duplicate, one in pure Wilkins Chalgren medium (Oxoid, CM643) and the other in Wilkins Chalgren medium supplemented with 1.5% bacto agar (BD24101).

A stock solution of bacitracin zinc was prepared at 5,120 µg/ml. This solution was then diluted in Wilkins Chalgren medium in such a manner to produce a range of final concentrations between 0.25 and 32 µg/ml. Each strain was cultured in Colombia agar containing 5% sheep's blood (BioMérieux 43041) and in Perfringens agar then incubated in an anaerobiosis jar in nitrogen at 37±1°C for 18 to 22 hours before strain purity was checked. One colony from each plate was placed in Wilkins Chalgren broth and incubated in anaerobiosis at 37±1°C for 18 to 22 hours. The next day, the culture obtained was diluted in fresh Wilkins Chalgren medium in such a manner to obtain a bacterial density of 10^7 CFU/ml (optical density of 0.1–0.15). A quantity of autoclaved Wilkins Chalgren agar (18 ml) supplemented with 2 ml of each bacitracin zinc dilution was then placed in Petri dishes. 10 µl of bacterial suspension (i.e. 10^5 cells) were then added to each medium with two antibiotic-free control dishes being included for each strain. Each strain was thus tested in duplicate under strictly anaerobic conditions (jar containing nitrogen) at 8 concentrations (0.25 to 32 µg/ml) as a factor 2 geometric progression, with two negative controls being used to check spontaneous growth and absence of interference.

The minimum inhibitory concentration corresponded to the lowest concentration that inhibited bacterial growth. Concentrations were expressed in µg of bacitracin zinc per ml, considering activity to be 63 IU/mg for the batch used. Cumulated percentage inhibition was calculated for each concentration. The results for each country, followed by the cumulated results, were processed by fitting to a sigmoidal plot. WinNonlin 4.02 (Pharsight, USA) software was used. The relation between the bacitracin zinc concentration and cumulated percentage inhibition was representative of a mass action law which gave the following equation:

$$E= [(Emax \times C^{Gamma}) / (C^{Gamma} + EC50^{Gamma})]$$

where E = effect (percentage inhibition), Emax = maximum theoretical effect (100% in theory), gamma = slope of the response, EC50 = concentration corresponding to the mean response (50%) and C = bacitracin zinc concentration (µg/ml).

This equation was then used to calculate the MIC_{90} (statistical value corresponding to 90% cumulated inhibition) by interpolation.

Induction of resistance

One of the strains isolated in the study described above (MIC = 2 µg/ml) was then cultured in the same medium but containing a subinhibitory concentration of bacitracin zinc (1 µg/ml, i.e. 50% of the MIC). The colonies derived from this culture were then transferred into medium containing 50% of the initial MIC. The strain underwent seven passages in this manner. The MIC was determined after each passage using the method described in the previous section.

Bactericidal profile

Five strains isolated during the MIC determination study were cultured anaerobically at a fixed density of 10^7 CFU/ml in the presence of incremental concentrations of bacitracin zinc ranging from 0.5 to 8 fold the MIC. Bacterial isolates were removed at T0 then after 2, 4, 6 and 24 hours to determine the size of the residual inoculum. The limit of quantification for the counting technique used was 100 (10^2) CFU/ml. In compliance with international conventions, bactericidal activity corresponded to a 1,000 fold decrease in the size of the initial inoculum.

RESULTS AND DISCUSSION

All the strains isolated were inhibited by bacitracin zinc in vitro. Inhibiting concentrations ranged from 0.5 to 2 µg/ml with none of the strains being inhibited at 0.25 µg/ml and all being inhibited at 2 µg/ml. The results of MIC measurements are presented in Table 1 and results given by the % inhibition = f (concentration) relationship in the sigmoidal model are shown in Table 2.

Table 1: Cumulated percentage inhibition plotted against bacitracin zinc concentration in the culture medium

Bacitracin zinc (µg/ml)	France (n=34) (%)	Belgium (n=51) (%)	France + Belgium (n=85) (%)
0.25	0	0	0
0.5	47.1	31.4	37.6
1	94.1	90.2	91.7
2	100	100	100

Table 2: Parameters derived from the equation $E= [(Emax \times C^{Gamma}) / (C^{Gamma} + EC50^{Gamma})]$ for each country and for all the strains (n = 85)

Emax equation parameter	France (n = 34)	Belgium (n = 51)	France + Belgium (n = 85)
Emax (%)	100.3	98.9	99.8
EC50 (µg/ml)	0.60	0.51	0.56
Gamma	4.36	5.0	4.40
MIC_{90} (µg/ml)	0.80	0.99	0.93

As the results were not different in France and Belgium, a common analysis was performed, giving the profile illustrated in Figure 1.

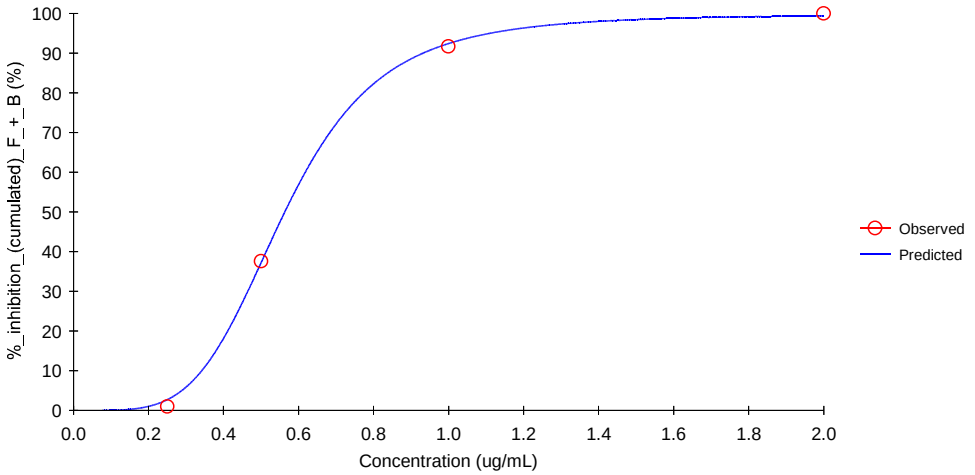

Figure 1: Curve illustrating the sigmoidal model for all strains (France + Belgium, n=85) and demonstrating the close fit between the theoretical and experimental points

These results indicate that the MIC90 of bacitracin zinc is approximately 0.93 µg/ml against field strains of *Clostridium perfringens* isolated under clinical conditions. The tests conducted to detect any induced resistance failed to show any increase in MIC values after 7 passages in sub-inhibitor medium (Table 3).

Table 3: Results of MIC measurements after 7 passages in sub-inhibitor medium, with no notable change in MIC

Passage No.	MIC (µg/ml)
0	2
1	3
2	3
3	3
4	4
5	3
6	2
7	2

The bactericidal profile was similar for the 5 strains tested. A typical plot is given for one of the strains (BW 19, MIC = 1 µg/ml) in Figure 2. The profile obtained shows a bactericidal effect characterized by a 1000-fold decrease in the size of the initial inoculum (decrease therefore from $10^{7.5}$ CFU/ml to $10^{4.5}$ CFU/ml). This effect was marked from a bacitracin concentration corresponding to 2 fold the MIC (2 µg/ml in this example). Higher concentrations did not cause any further, substantial decrease in bacterial density, except at the highest concentration at the T+24 hours time point. This profile is characteristic of concentration-independent (time-dependent) bactericidal antibiotics.

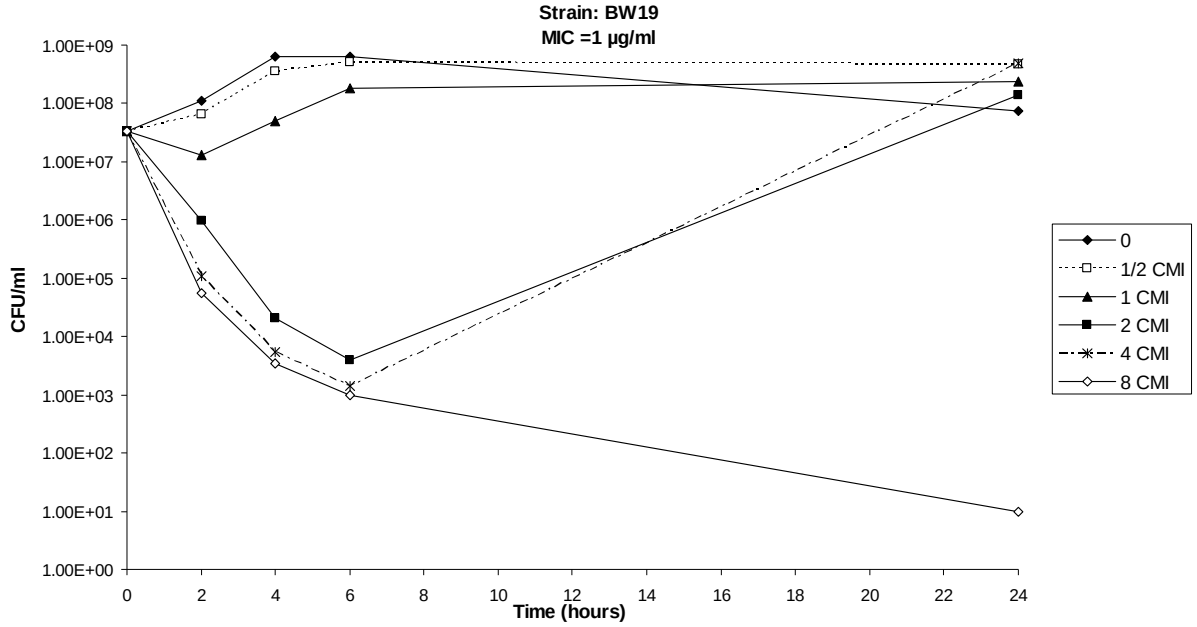

Figure 2: Typical plot showing bacitracin bactericidal kinetics against *C. perfringens.* Each point corresponds to residual bacterial density after exposure to concentrations ranging from 0 to 8 fold the MIC, i.e. from 0 to 8 µg/ml for the strain tested

CONCLUSIONS

This study showed that bacitracin zinc exerted remarkably homogeneous antibiotic potency against strains of *Clostridium perfringens* isolated from sick rabbits. MIC values ranged from 0.5 to 2.0 µg/ml, corresponding to only three factor 2 dilutions in the *in vitro* approach employed. No significant difference was observed between France and Belgium, confirming that the field strains encountered were very homogeneous in terms of susceptibility. The absence of any resistance in these two countries is hardly surprising. Attempts to induce resistance by repeated passages in the presence of subinhibitory concentrations of bacitracin zinc (50% of the MIC), i.e. conditions that generally predispose to emergence, suggest that the risk is very low when Bacivet®-S is used under clinical conditions. This could nevertheless be revised in the light of more prolonged use. The results obtained *in vitro* also showed that the product exerts a concentration-independent (time-dependent) bactericidal

effect from a concentration corresponding to twice the MIC. On the basis of an MIC_{90} value of 0.9 µg/ml in France and Belgium (value similar to that measured for instance for tiamulin by Bouvier *et al.*, 2005), it is therefore possible to predict a bactericidal concentration (MBC90) of about 1.8 µg/ml for the target strains. Bacitracin zinc concentrations present at the site of action (cecum in the rabbit) should therefore be maintained at this value throughout the treatment period, consistent with the pharmacodynamic profile evidenced in this study. Such a constraint will also even further reduce the very low risk of inducing resistance. The calculation of an optimal dosing regimen on the basis of pharmacokinetic/pharmacodynamic data (PK/PD, Richez *et al.*, 2008) should therefore take account of this pharmacodynamic requirement (McKellar *et al.*, 2004) in the light of the results obtained in this study.

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