

ANTIMICROBIAL SUSCEPTIBILITY TO ZINC BACITRACIN OF *Clostridium perfringens* OF RABBIT ORIGIN

Agnoletti F.*, Bacchin C.*, Bano L.*, Passera A.†, Favretti M.†, Mazzolini E.†

*Lab. di Treviso, Ist. Zooprofilattico Sperimentale delle Venezie. Treviso, Italy.

†Lab. di Udine, Ist. Zooprofilattico Sperimentale delle Venezie. Campoformido, Udine, Italy.

ABSTRACT: Zinc bacitracin is widely used in Italian rabbit farms to control both Epizootic Rabbit Enteropathy (ERE) and clostridiosis, and field results demonstrate useful activity. Nevertheless, data regarding the *in vitro* efficacy of zinc bacitracin against clostridia of rabbit origin are not available. In this study, the minimal inhibitory concentrations (MICs) of zinc bacitracin were evaluated in 123 *C. perfringens* strains isolated from rabbits in Italian fattening units. The agar dilution method was performed in Brucella Agar supplemented with laked sheep blood, haemin and vitamin K₁, as recommended in NCCLS document M11-A6. Most strains (94.3%) had low MIC values ($\leq 0.5 \mu\text{g/ml}$), and a few strains (4%) were inhibited by a concentration of $1 \mu\text{g/ml}$. Two isolates (1.6%) had a MIC value of $16 \mu\text{g/ml}$. The MIC values of ATCC reference strains showed a good fit between each batch. MIC required to inhibit the 90% of organisms was $0.5 \mu\text{g/ml}$ and the presence of only two strains with MIC= $16 \mu\text{g/ml}$ revealed the susceptibility to zinc bacitracin of Italian isolates of *C. perfringens* from rabbit and the absence of acquired resistance.

Key words: Zinc bacitracin; *Clostridium perfringens*; minimum inhibitory concentration (MIC); rabbit

INTRODUCTION

Zinc bacitracin is a polypeptide antibiotic with a range of action against Gram-positive bacteria. It is widely used in Italian commercial rabbit farms because of its proven field efficacy against clostridiosis and Epizootic Rabbit Enteropathy (ERE) (Mercier and Richard, 2001; Duperray *et al.*, 2003; Coudert and Licois, 2004). Its administration in the feed or water of fattening rabbits significantly reduces mortality and improves both growth rate and slaughtering weight in ERE infected units (King, 1980; Boisot *et al.*, 2004).

Gram-positive bacteria belonging to the genus *Clostridium* are commonly involved in the “enteritis complex”, and rabbit enterotoxaemia, due to clostridial overload induced by antibiotic treatment and high-carbohydrate/low-fibre diets, is well-known (Percy *et al.*, 1993). *C. spiroforme* and *C. perfringens* are commonly isolated in rabbit, but whereas the former is the proven primary agent of rabbit enteropathy (Carman and Evans, 1984), the etiological role of *C. perfringens* is not yet well understood and deserves further in-depth study. In spite of this, *C. perfringens* is considered by practitioners an important cause of losses in fattening rabbits and a target for therapeutic and prophylactic treatment in feed or water.

Some authors (e.g., Dewree *et al.*, 2003; Le Normand *et al.*, 2003) have compared bacteriological findings and the presence of clostridial toxins in animals with ERE, and have hypothesised an etiopathological link between *C. perfringens* and ERE. Enterotoxigenic strains of *C. perfringens* do

Correspondence: F. Agnoletti, fagnoletti@izsvenezie.it

Received April 2006 - Accepted November 2006.

turn out to be the most common bacteria isolated from ERE-affected animals, and greater frequency of α -toxins has also been found in the caecal contents of rabbits dying of ERE than in those dying of other enteric pathologies (Dewree *et al.*, 2003; Le Normand *et al.*, 2003). Even the most recent studies, although confirming the role of Gram-positive micro-organisms in the etiology of ERE, seem to minimise the involvement of *C. perfringens*, the presence of which in caecal contents may be a consequence, rather than a cause, of the intestinal disorder caused by ERE (Marlier *et al.*, 2005; Szalo *et al.*, 2006).

Despite the wide field use in Europe of this antimicrobial agent, there is little information about the *in vitro* susceptibility to zinc bacitracin of clostridia isolated from rabbit. This may be due to the need for anaerobes of an minimal inhibitory concentration (MIC) evaluation with the recommended methods of agar or broth dilution: both methods are time-consuming, and commercial micro-methods are not available for veterinary use. Data regarding the efficacy of bacitracin against *C. perfringens* in poultry and swine (Dutta *et al.*, 1983; Devriese *et al.*, 1993; Watkins *et al.*, 1997; Johansson *et al.*, 2004) are available but, as far as we know, no data focusing on the *in vitro* susceptibility of strains of rabbit origin have been published. The present study was undertaken to explore this aspect.

MATERIAL AND METHODS

Bacterial isolates

To evaluate the *in vitro* activity of zinc bacitracin, a total sample of 123 *C. perfringens* field strains isolated over a two-year period (2003-2004) and originating from 72 Italian rabbit herds was examined. Most of the herds (96%) were located in Northern Italian regions. One isolate from one rabbit affected with enteric syndrome was considered a field strain. A single outbreak was sampled in 66 herds; in 6 units, 2-4 enteric outbreaks were followed up. Outbreaks were considered to be different when at least one month distanced the isolates.

C. perfringens was isolated from the caecal content by cultivating on Perfringens Agar Base (Oxoid), supplemented with 5% (v/v) sheep red blood cells, polymixin B sulphate and kanamycin (S.F.P. supplement, Oxoid); plates were incubated in anaerobic conditions (10% CO₂, 10% H₂, 80% N₂) at 37°C for 24 h. Colonies with the characteristic morphology, black colour and a double haemolytic halo, were subcultured on Columbia Agar containing 5% of sheep red blood cells. Identification of isolates was performed with a standard biochemical test (API 20 A - bioMérieux). Field strains were stored at -80°C in Schaedler Anaerobe Broth (Oxoid) until use.

Antimicrobial susceptibility test

MICs were determined using the agar dilution test in Brucella Agar, supplemented with laked sheep

Table 1: Distribution of MICs¹ for ATCC reference strains (* *C. perfringens*; ** *Bacteroides fragilis*; *** *Bacteroides thetaiotaomicron*) obtained in the four test batches.

No. ATCC	MIC (μ g/ml)												
	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
No.													
3628*			3	1									
10543*		3	1										
13124*			1	3									
25285**								1	3				
29741***									3	1			

¹MICs: minimal inhibitory concentrations.

Table 2: Frequency of MICs¹ of zinc-bacitracin for 123 *C. perfringens* isolates from rabbit.

MIC ($\mu\text{g/ml}$)	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
No. strains		5	36	75	5				2				

¹MICs: minimal inhibitory concentrations.

blood, haemin and vitamin K₁, as recommended in NCCLS document M11-A6 (2004). Zinc bacitracin standard powder was solubilised, as indicated by the supplier (Alpharma AS, Oslo, Norway) and diluted in sterile distilled water, and the stock solution was used to supplement Brucella Agar plates with zinc bacitracin concentrations ranging from 0.06 to 256 $\mu\text{g/ml}$.

Stored strains of *C. perfringens* were revitalised in supplemented Brucella Agar. The MIC inocula was 2 μl of *C. perfringens* broth cultures containing approximately 1×10^5 CFU. MICs were recorded after 48 h of incubation (37°C) in anaerobic conditions, by means of the interpretative criteria of NCCLS (2004).

Internal reproducibility of the test

In order to check the internal reproducibility of the method, five reference strains (*C. perfringens* ATCC 3628, ATCC 10543, ATCC 13124, *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741) were used and included in each test batch.

RESULTS

The MIC values of the *C. perfringens* ATCC reference strains obtained in the four test batches are listed in Table 1. The fit between each batch was good, since a variation of the endpoint of ± 1 dilution is considered by NCCLS normal variability of the MIC test (NCCLS, 2004).

The MIC values of field strains are listed in Table 2; most (94.3%) had MIC ≤ 0.5 $\mu\text{g/ml}$, a few (4%) had MIC = 1 $\mu\text{g/ml}$, and only two (1.6%) had MIC = 16 $\mu\text{g/ml}$.

DISCUSSION

Bacitracin is a bactericidal peptide antibiotic which inhibits proper cell wall synthesis and has additional effects on bacterial membranes (Benning and Mathers, 1999; Butaye *et al.*, 2003).

Data on the *in vitro* susceptibility of anaerobic bacteria to antimicrobial agents for veterinary use are quite difficult to produce, due to technical requirements and the lack of standardisation among laboratories. The agar disk diffusion method is not currently approved by NCCLS (2004) for antimicrobial susceptibility testing of anaerobic bacteria, and proper commercial ready-to-use micro-methods are not available for veterinary antimicrobials. At present, updating the susceptibility or resistance of anaerobes is a research effort of specialised laboratories, which monitor the activity of antibiotics commonly used in veterinary therapy.

In the present study, *C. perfringens* displayed high *in vitro* susceptibility to zinc bacitracin, as 90% of field strains were inhibited by 0.5 $\mu\text{g/ml}$ of the drug.

Nearly all the study units (96%) were located in Northern Italian regions, but results may be considered representative of the national situation, as most rabbit production originates from these regions. No overclustering of samples of field strains occurred either, as isolates were mostly collected from different units and different enteric outbreaks. Despite the wide use of zinc bacitracin in Italian fattening rabbit units, the spread of acquired resistance between rabbit isolates does not seem to be relevant. A MIC required to inhibit the growth of 90% of organisms (MIC₉₀) of 0.5 $\mu\text{g/ml}$ was obtained,

fitting the MIC values described by Devriese *et al.* (1993) with strains of poultry, porcine and bovine origin, and by Benning and Mathers (1999) with *C. perfringens* of swine origin.

In 1997, Watkins *et al.* reported different results, but they used a micro-broth dilution test and found a MIC₉₀ of > 256 µg/ml for poultry isolates and 16 µg/ml for turkey strains. On one hand, these differences may reflect the varying use of bacitracin in different countries and species; on the other, they may be attributed to the different methods and media used: as reported by Benning and Mathers (1999), the micro-broth dilution method overrates MICs. In addition, the interaction of red blood cells and zinc bacitracin should be investigated (Benning and Mathers, 1999).

CONCLUSIONS

The agar dilution method used to evaluate MICs to zinc bacitracin of *C. perfringens* strains of rabbit origin gave results indicating good reproducibility. The value of MIC₉₀ = 0.5 µg/ml obtained in this study revealed the high *in vitro* susceptibility of *C. perfringens* field strains isolated from rabbits and the absence of acquired resistance phenomena in Italian rabbit populations.

Acknowledgements: We would like to thank Alpharma AS, Oslo, Norway, which kindly supplied the zinc bacitracin standard.

REFERENCES

- Benning V.R., Mathers J.J. 1999. Comparison of agar dilution and broth microdilution methods of anaerobic antimicrobial susceptibility testing using several veterinary antibiotics against *Clostridium perfringens* strains originating from porcine and avian sources. *Anaerobe*, 5, 561-569.
- Boisot P., Duperray J., Guyonvarch A., Richard A., Licois D., Coudert P. 2004. Evaluation of the effectiveness of soluble bacitracin (Bacivet S®) in drinking water compared to bacitracin in the feed (Albac®), during an experimental reproduction of epizootic rabbit enteropathy syndrome. In Proc.: *8th World Rabbit Cong., Puebla Sept.*, 457-462.
- Butaye P., Devriese L.A., Haesebrouck F. 2003. Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. *Clin. Microbiol. Rev.*, 16, 175-188.
- Carman R.J., Evans R.H. 1984. Experimental and spontaneous clostridial enteropathies of laboratory and free living lagomorphs. *Lab. Anim. Sci.*, 34, 443-452.
- Coudert P., Licois D. 2004. Study of early phenomena during experimental epizootic rabbit enteropathy: preliminary results. In Proc.: *8th World Rabbit Congr., Puebla Sept.*, 520-525.
- Devriese L.A., Daube G., Homme J., Haesebrouck F. 1993. *In vitro* susceptibility of *Clostridium perfringens* isolated from farm animals to growth-enhancing antibiotics. *J. Appl. Bacteriol.*, 75, 55-57.
- Dewree R., Licois D., Coudert P., Lassence C., Vindevogel H., Marlier D., 2003. L'entéropathie épizootique du lapin (EEL): étude du rôle des infections par *Clostridium perfringens* dans l'étiopathogénie de ce syndrome. In Proc.: *10^{èmes} Journées de la Recherche Cunicole, Paris Nov.*, 251-254.
- Duperray J., Boisot P., Guyonvarch A., Richard A. 2003. Persistence de l'efficacité de la bacitracine pour lutter contre l'entéropathie épizootique du lapin (EEL) après quatre années d'utilisation sur le terrain. In Proc.: *10^{èmes} Journées de la Recherche Cunicole, Paris Nov.*, 271-274.
- Dutta G.N., Devriese L.A., Van Assche P.F. 1983. Susceptibility of clostridia from farm animals to 21 antimicrobial agents including some used for growth promotion. *J. Antimicrob. Chemother.*, 12, 347-356.
- Johansson A., Greko C., Engström B.E., Karlsson M., 2004. Antimicrobial susceptibility of Swedish, Norwegian and Danish isolates of *Clostridium perfringens* from poultry, and distribution of tetracycline resistance genes. *Vet. Microbiol.*, 99, 251-257.
- King J.O.L. 1980. Effects of feeding zinc bacitracin on the fertility of rabbit does and the development of young rabbits. *Br. Vet. J.*, 136, 240-244.
- Le Normand B., Le Guenec J., Moalic P.Y. 2003. Contribution à l'étude toxinotypique des souches de *Clostridium perfringens* isolées dans l'entéropathie épizootique du lapin (EEL). Relation avec la clinique observée. In Proc.: *10^{èmes} Journées de la Recherche Cunicole, Paris Nov.*, 243-246.
- Marlier D., Dewrée R., Lassence C., Licois D., Mainil J., Coudert P., Meulemans L., Ducatelle R., Vindevogel H. 2005. Infectious agents associated with epizootic rabbit enteropathy: isolation and attempts to reproduce the syndrome. *Vet. J.* In press. doi:10.1016/j.tvjl.2005.07.011.
- Mercier P., Richard A. 2001. Contrôle d'efficacité d'une supplémentation en bacitracine-zinc chez le lapin en engraissement. In Proc.: *10^{èmes} Journées de la Recherche Cunicole, Paris Nov.*, 127-130.
- National Committee for Clinical Laboratory Standards. 2004. Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard - 6th edition. *NCCLS Document M11-A6, Vol. 24 N° 2. NCCLS, Wayne, PA.*
- Percy D.H., Muckle C.A., Hampson R.J., Brash M.L. 1993. The enteritis complex in domestic rabbits: a field study. *Can. Vet. J.*, 34, 95-102.
- Szalo I.M., Lassence C., Licois D., Coudert P., Poulipoulis A., Vindevogel H., Marlier D. 2006. Fractionation of the reference inoculum of epizootic rabbit enteropathy in discontinuous gradient identifies aetiological agents in high density fractions. *Vet. J.* In press. doi:10.1016/j.tvjl.2005.12.013.
- Watkins K.L., Shryock T.R., Dearth R.N., Saif Y.M. 1997. *In-vitro* antimicrobial susceptibility of *Clostridium perfringens* from commercial turkey and broiler chicken origin. *Vet. Microbiol.*, 54, 195-200.