CLOSTRIDIUM SPIROFORME DRUG SUSCEPTIBILITY

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ABSTRACT

Clostridium spiroforme causes severe rabbit gastroenteritis with subsequently important production losses. Outbreaks are controlled with therapy but field data report frequent failures and suggest *C. spiroforme* being of high drug resistance. In order to establish whether therapy failure is due to intrinsic or acquired drug resistance, 30 field strains of *C. spiroforme* were tested against spiramycin, amoxicillin, doxycycline, sulfadimethoxine, norfloxacin, and tiamulin, respectively representative of the following classes of antimicrobials: macrolides, β-lactams antibiotics, second-generation tetracyclines, sulfonamides, quinolones and diterpenes. The minimum inhibitory concentration was determined with the agar diffusion method (NCCLS M11-A6 manual, 2004). Resulted MICs were: sulfadimethoxine MIC_{50}: 256 μg/ml and MIC_{90}: 256 μg/ml; spiramycin MIC_{50}: 256 μg/ml and MIC_{90}: 256 μg/ml; tiamulin MIC_{50}: 64 μg/ml and MIC_{90}: 128 μg/ml; norfloxacin MIC_{50}: 32 μg/ml and MIC_{90}: 32 μg/ml; amoxicillin MIC_{50}: 0.063 μg/ml and MIC_{90}: 0.063 μg/ml; doxycycline MIC_{50}: 8 μg/ml and MIC_{90}: 16 μg/ml. Among all antimicrobials tested, only doxycycline showed MICs supposed to be of therapeutic efficacy. Results support the field hypothesis of an extensive acquired resistance of *C. spiroforme* to antimicrobials and the presence of intrinsic resistances of importance. Both findings suggest the necessity to give greater priority to prophylaxis of enteritis caused by *C. spiroforme* by attempting to reduce risk factors rather than controlling outbreaks by therapy. The ambitious and complex objective can be pursued by optimising technical management and by a prudent use of antimicrobials.

Key words: *Clostridium spiroforme*, Minimal Inhibitory Concentration (MIC), Drug susceptibility, Rabbit.

INTRODUCTION

Clostridium spiroforme is one of the main pathogens of the rabbit's gastroenteric system and can lead to serious haemorrhagic typhlitis with rapid onset and usually unfortunate ending (Boucher and Nouaille, 2002; Marlier *et al.*, 2003; Songer, 1996). It usually affects rabbits during weaning and can be related to production of a binary toxin similar to the iota toxin of Type E *C. perfringens*. The toxin is composed of independent sub-units "Sa" and "Sb" that are respectively coded by the genes "*sas*" and "*sbs*" (Perelle *et al.*, 1993). In the past, the antigenic resemblance between *C. spiroforme* toxin and *C. perfringens* iota toxin attributed to Type E *C. perfringens* caused the enteric pathology likely to be attributed to *C. spiroforme* (Borriello and Carman, 1983).

The pathogenicity of *C. spiroforme* in rabbit became clear in 1982 (Carman and Boriello, 1982); in the same period it was demonstrated the possibility to experimentally reproduce the disease by oral administration of lincomycin (Yonushonis *et al.*, 1987). The antibiotic-associated origin of mostly of *C. spiroforme* outbreaks can have an indirect evidence in sporadic outbreaks due to accidental rabbit feeding with feed cross contaminated with small amounts of amoxicillin: rabbits developed symptoms of serious intoxication associated with imbalance in caecal microbiota and abnormal proliferation of *C. spiroforme* thanks to destruction of antagonist bacteria due to the β-lactam unintentionally swallowed. A similar mechanism is known in the "antibiotic associated diarrhoea" due to *C. difficile* and affecting several animal species and in humans (Voth and Ballard, 2005). In addition to sporadic and accidentally intoxication, the increased use of antimicrobial agents to control the enzootic rabbit

enteropathy (ERE, a disease causing important losses in rabbit breeding during the last ten years) is for sure a factor increasing *C. spiroforme* clostridiosis that affect nowadays industrial farms. Other trigger factors may include hyper-protein diets that induce an increase trypsin secretion which through the enzymatic scission of the sub-units "Sa" and "Sb" is a potent activator of *C. spiroforme* binary toxin (Ellis *et al.*, 1991). High carbohydrate and low fibre diets can also induce clostridial overload (Percy *et al.*, 1993).

The seriousness of *C. spiroforme* pathology is heightened by difficulties in therapeutic control. Field evidences suggest *C. spiroforme* being of high drug resistance, differently to *C. perfringens* which diplays good susceptibility to several antimicrobials (Dubreuil and Neut, 2004; Bouvier *et al.*, 2005; Agnoletti *et al.*, 2007) and is involved in rabbit "enteritis complex" (Peeters *et al.*, 1986).

C. spiroforme is a fastidious micro-organism and requires enriched selective media and strict anaerobiosis growing. Furthermore the biochemical identification and the interpretative procedure proposed by Kaneuchi *et al*. (1979) is difficult to apply. Lastly, the Clinical and Laboratory Standard Institute (CLSI, previously named NCCLS) indicates the determination of the Minimum Inhibitory Concentration (MIC) through diffusion in agar as the reference method for the determination of the drug resistance of anaerobic micro-organisms (NCCLS, 2004); the method is complex and time consuming.

So many difficulties may be responsible for the lack of data on drug resistance of field strains of *C. spiroforme*. At the present one study conducted on a limited number of bacterial strains and with antimicrobials that are mostly of no longer nowadays used in farms is available (Carman and Wilkins, 1991).

Our study aims to update the knowledge on drug susceptibility of *C. spiroforme*, both reference and field rabbits isolates, by determining MICs of antimicrobials used in rabbit nowadays therapy.

MATERIALS AND METHODS

We examined 30 field strains of *Clostridium spiroforme* taken from rabbits affected by enteric pathologies in 30 different Italian farms in 2007.
Using the selective medium described by Agnoletti *et al.* (2004) the strains isolated were stored in cryogenic vials (Nalgene) at –80°C until the moment of test execution; the bacterial film was collected in a Reinforced Clostridial Medium (Oxoid) diluted 1:2 in sterile glycerol.

Each strain, appropriately cloned and preliminarily identified on the basis of morphology of the colonies and the characteristic microscopic aspect (Borriello *et al.*, 1986), was identified by PCR (Drigo *et al.*, 2007). The strains selected were then tested positive for the *Sbs* and *Sba* genes that code for the two sub-units of the binary toxin by PCR multiplex (Drigo *et al.*, 2007) for a further confirmation of their pathogenicity.

The following antimicrobial agents were used: spiramycin (Spiramycin from *Streptomyces* sp., Sigma), amoxicillin (Amoxicillin, Sigma), doxycycline (Doxycycline hyclate, Sigma), sulfadimethoxine (Sulfadimethoxine, Sigma), norfloxacin (Norfloxacin, Sigma), tiamulin (Tiamulin fumarate, Sigma), respectively members of the following classes of antimicrobial: macrolides, β-lactams antibiotics, second-generation tetracyclines, sulfonamides, quinolones and diterpenes. The instructions provided in the respective analysis certificates were followed to solubilize the standard powders, whereas the agar diffusion method provided in the NCCLS/CLSI M11-A6 (2004) manual was adopted for MIC determination. This was followed by the calculation of the MIC_{50}, MIC_{90}, the geometrical mean of the MICs and the MIC range. All MIC values were expressed in μg/ml, when necessary the international units commonly adopted were converted (Sweetman, 2007).

Each test batch featured the inclusion of 3 reference strains (*C. spiroforme* ATCC 2290, *C. perfringens* ATCC 13124 and *Bacteroides fragilis* ATCC 25285), even if the Clinical and Laboratory Standard Institute does not indicate acceptable MIC ranges for the active principals and reference strains used in the study.

RESULTS AND DISCUSSION

The genus *Clostridium* is fairly heterogeneous in terms of drug resistance and for this reason permits the identification of three different groups; *C. perfringens*, which is usually susceptible to the antimicrobials, *C. difficile*, considered to be highly resistant, and the other species of the genus that present intermediate degrees of susceptibility. The susceptibility of *C. spiroforme* has only been scarcely documented and for such reason it has not yet been positioned in any of the three groups above.

The results of the MIC determination for the 6 antimicrobial products tested against both the field strains of *C. spiroforme* and the reference strains are respectively provided in Tables 1 and 2, whereas Table 3 provides summarized MIC value data, indicating MIC_{50}, MIC_{90}, and the geometrical mean MIC values and the MIC range.

Table 1: Frequency of MICs of *Clostridium spiroforme*

	MIC (μg/ml)														
	0.016	0.032	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Spiramycin											1				29
Amoxicillin		11	18	1											
Doxycycline									1	20	9				
Sulfadimethoxine															30
Norfloxacin											2	28			
Tiamulin						1					4	7	14	4	

Table 2: Distribution of MICs for ATCC reference strains (μg/ml)

	C. spiroforme ATCC 2290	*C. perfringens* ATCC 13124	*Bacteroides fragilis* ATCC 25285
Spiramycin	0.125	16	64
Amoxicillin	0.125	0.125	32
Doxycycline	0.25	1	4
Sulfadimethoxine	256	256	256
Norfloxacin	8	1	16
Tiamulin	0.063	0.063	0.125

Table 3: Summarized MIC values (μg/ml)

	MIC_{50}	MIC_{90}	Geometric mean	MIC range
Spiramycin	256	256	233.4	16-256
Amoxicillin	0.063	0.063	0.051	0.032-0.125
Doxycycline	8	16	9.6	4-16
Sulfadimethoxine	256	256	256	256
Norfloxacin	32	32	30.5	16-32
Tiamulin	64	128	42.2	0.5-128

With a MIC value geometric mean of 233 and 256 μg/ml respectively, spiramycin and sulfadimethoxine, appear to have no therapeutic value whatsoever. *C. spiroforme* seems intrinsically resistant to spiramycin and sulfadimethoxine, whereas the gap between the strain ATCC and field strain MIC values measured for spiramycin suggests that the resistance to this macrolide is acquired, not intrinsic.

Intermediate susceptibility values were observed for norfloxacin and tiamulin, which showed geometric mean MIC values of respectively 30.5 and 42.2 μg/ml. Fluoroquinolones were shown to exert pharmacological action even in regard to Gram-positive bacteria, such as genus C*lostridium*.

Samples of *C. perfringens* isolated from humans provide values of MIC_{90} <1 when subjected to testing with numerous fluoroquinolones, whereas up to 26% of the strains of *C. difficile* of human origin proved resistant (Dubreuil and Neut, 2004).

In any case however, fluoroquinolones are not usually used to treat enteric pathologies in the rabbit, and are considered drugs of second choice in particularly for serious cases of colibacillosis. Of greater concern, however, is the extent of resistance to tiamulin, semi-synthetic pleuromutilin derivative, which is still one of today's leading veterinary drugs against clostridia. The distribution of the MICs and the susceptibility of strain ATCC confirm this to be an acquired and not intrinsic form of resistance; tiamulin, in fact, has been used for many years, but progressive increases in dosage in the field have become necessary in order to achieve the therapeutic results desired. The β-lactams represented by amoxicillin are considered the antimicrobials of preference in the treatment of human clostridiosis and show extremely high efficacy *in vitro* even against *C. spiroforme.* These molecules cannot be administered orally to rabbits, however, because they are particularly toxic in this form and therefore cannot be used in the control of enteric pathologies. Lastly, despite the facts that tetracycline is commonly administered orally in rabbit farms and that this class of antibiotic is characterized by phenomena of crossed resistance, doxycycline, a second generation semi-synthetic tetracycline, provides the lowest MIC_{50} and MIC_{90} values of all the antimicrobial products tested and may be one of the few pharmacological tools available for the control of *C. spiroforme*. The MIC values observed demonstrate the elevated resistance to drugs vaunted by *C. spiroforme*, confirming the evidence from the field regarding the difficulty of controlling cases of enteric pathology caused by *C. spiroforme* through the use of antimicrobials. From this point of view, *C. spiroforme* differs from other forms of clostridia, particularly *C. perfringens*, which is considered highly susceptible to pharmaceutical treatment. This difference may be related to the phylogenetic distance of *C. spiroforme* from *C. perfringens*. The latter, in fact, is taxonomically positioned in Cluster I that contains the types of clostridia considered to be the "core species" of the genus *Clostridium;* instead of *C. spiroforme*, together with *Cl. ramosum* and *Cl. cocleatum*, which are classed in Cluster XVIII (Collins *et al.*, 1994) which is phylogenetically so remote from the former as to justify the hypothesis of taxonomic reclassification in a different genus (Euzéby, 2007).

CONCLUSIONS

The results obtained demonstrate the range of resistance to antimicrobials of field strains of *C. spiroforme*. The gap between the MIC values measured for strain ATCC 2290 and field strains suggests – with the exception of sulfadimethoxine – that the resistances observed are acquired, and not intrinsic in nature. Doxycycline is the only drug that presents MIC values compatible with therapeutic use, due to the fact that amoxicillin, extremely efficacious *in vitro*, is highly toxic when administered to rabbits orally. Although the *in vitro* susceptibility of *C. spiroforme* to other active principals must be tested in the future, the results obtained suggest the need to grant greater priority to *C. spiroforme* prophylaxis than therapeutic control by attempting to reduce the trigger factors primarily through the careful and prudent use of antimicrobials.

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