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A survey of *Clostridium spiroforme* antimicrobial susceptibility in rabbit breeding

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Abstract

Rabbit meat breeding may be heavily affected by enterotoxaemia due to *Clostridium spiroforme*. Data on its antimicrobial susceptibility are insufficient, presumably because of difficulties in cultivating and identifying the pathogen. Our aim is therefore to provide this information to veterinary practitioners by focusing on a panel of therapeutics used in intensive rabbit units. Lincomycin was also checked in order to investigate the origin of resistance to macrolides. Minimal inhibitory concentrations (MICs) were determined with the agar dilution method according to the CLSI M11-A7 protocol (2007). MIC_{50} and MIC_{90} were, respectively, 64 and 64 µg/ml for tiamulin, 32 and 32 µg/ml for norfloxacin, 0.063 and 0.125 µg/ml for amoxicillin, and 8 and 16 µg/ml for doxycycline. MIC_{50} and MIC_{90} were 256 µg/ml for sulphadimethoxine, spiramycin and lincomycin Our results have shown that intrinsic or acquired antimicrobial resistances are diffuse in the *C. spiroforme* population and suggest focusing on prevention rather than on treatment of clostridial overgrowth, by reducing risk factors and using antimicrobials prudently.

Keywords: *Clostridium spiroforme*, Minimal Inhibitory Concentration (MIC), drug susceptibility, rabbit

1. Introduction

Rabbit meat production is important in several European and non-European countries and may also represent a suitable food source in developing countries. However the intensive production system is affected by a high incidence of enteric disorders, mainly in the post-weaning period (Marlier et al., 2003). *Clostridium spiroforme* is an

important agent involved in spontaneous or antibiotic-associated enteritis (Borriello et al., 1983). It has semicircular morphology, a helically coiled multicellular configuration (Borriello et al., 1986) and is classified in Cluster XVIII of the genus *Clostridium* (Collins et al., 1994), together with *Clostridium ramosum* and *Clostridium cocleatum.* Thanks to a binary toxin, similar to the iota toxin of *C. perfringens* type E (Perelle et al., 1993), *C. spiroforme* causes severe enterotoxaemia, usually with rapid onset and frequently a fatal outcome. Treatment is therefore of no help at the individual level but should rather address the prevention of group diffusion.

C. spiroforme is a fastidious micro-organism since it requires enriched, pre-reduced culture media, strictly anaerobic conditions and prolonged incubation time to grow into visible colonies; moreover commercial identification systems are not available. So there are many explanations why this bacterium is not routinely isolated for diagnostic purposes and tested for antimicrobial susceptibility.

In 1991, Carman and Wilkins tested *C. spiroforme* drug susceptibility on a small sample of strains isolated from rabbits reared for research purposes or in extensive units. Knowledge has not been updated since then and no proper survey has been performed on strains emerging from intensive units. Aim of our study was to provide veterinary practitioners with data on drug susceptibility of *C. spiroforme* using a sample of field strains representative of enterotoxaemia outbreaks and antimicrobials currently used in rabbit meat breeding in Europe (Boucher and Nouaille, 2002).

2. Materials and methods

2.1 Sampling

Strains were randomly selected from isolates collected during diagnostic activity. Sixty toxigenic *C. spiroforme* strains isolated from diseased rabbits during outbreaks

occurring in 2007-2008 in 60 Italian farms were used to determine antimicrobial susceptibility.

2.2. Isolation and identification of Clostridium spiroforme

Isolation of *C. spiroforme* was performed from the caecum content of diarrhoeic rabbits that presented semicircular or helicoidal bacteria at microscopic examination. A loop of caecal content was directly streaked on Perfringens Agar Base (Oxoid) supplemented with SFP (Shahidi-Ferguson-Perfringens Selective Supplement, Oxoid), sheep red blood cells (5% v/v) and 12.5 μg/ml rifampicin (Rifampicin powder, Sigma) added as suggested by Carman and Wilkins (1991). To increase selectivity the medium was modified by adding 500 μg/ml tylosin (Tylosin tartrate, Sigma).

In order to check whether tylosin can be used for selective purposes, a pilot survey was performed on *C. spiroforme* reference strains (NCTC 11493 and ATCC 29900) and twenty-five wild strains that had been isolated in medium without adding antimicrobials. Results were MIC = 256 μg/ml for the reference strain of rabbit origin NCTC 11493, MIC = 0.125 μg/ml for the reference strain of human origin ATCC 29900 and MIC_{50} and MIC_{90} = 256 μg/ml for wild strains, thus demonstrating the intrinsic resistance of *C. spiroforme* isolated from rabbit to tylosin (Agnoletti, unpublished data) and validating tylosin's addiction to the selective medium.

Agar plates were incubated in anaerobic conditions (10% CO_2, 10% H_2, 80% N_2) at 37°C for 24-48 hrs. Colonies with the characteristic morphology were subcultured on Columbia Agar (Oxoid) with the addition of sheep red blood cells (5% v/v). Ultimate identification of bacterial isolates and the detection of toxin genes were performed by PCR according to methods described by Drigo et al. (2008).

Finally, bacterial strains, collected in Reinforced Clostridial Medium (Oxoid) diluted 1:2 in sterile glycerol, were stored in cryogenic vials (Nalgene) at –80°C until determination of the MICs .

2.3. Antimicrobial susceptibility test

MICs were evaluated for spiramycin (Spiramycin from Streptomyces sp., Sigma), amoxicillin (Amoxicillin, Sigma), doxycycline (Doxycycline hyclate, Sigma), sulphadimethoxine (Sulphadimethoxine, Sigma) and tiamulin (Tiamulin fumarate, Sigma) as these antimicrobials are members of the macrolide, β-lactam, second-generation tetracycline, sulphonamide and diterpene classes, which are commonly used in the treatment of either enteric or respiratory disorders in rabbits (Boucher and Nouaille, 2002); all but amoxicillin are usually administered orally. Tiamulin is a semi-synthetic pleuromutilin derivative intended for anti-clostridial purposes in rabbit therapy. Norfloxacin (Norfloxacin, Sigma), which displays efficacy against clostridia of human origin (Behra-Miellet et al., 2002; Yao et al., 2003) was also tested, Second generation quinolones are second choice drugs for rabbit colibacillosis treatment. Lincomycin (Lincomycin hydrochloride, Sigma) was also checked in order to investigate the origin of resistance to macrolides.

Standard powders were solubilized according to the manufacturers' instructions, whereas the agar dilution method described in the CLSI M11-A7 (2007) manual was adopted for MIC determination; drug concentrations ranged from 0.06 to 256 µg/ml. The medium used was the Brucella Agar (Oxoid) supplemented with hemin (5 µg/ml), vitamin K_1 (1 µg/ml) and laked sheep blood (5% v/v).

Two batches of MIC tests were performed and, in order to estimate repeatability, three reference strains (*Clostridium spiroforme* ATCC 29900, of human origin, *Clostridium*

perfringens ATCC 13124 and *Bacteroides fragilis* ATCC 25285) and three *C. spiroforme* wild strains randomly selected from examined strains, were run in each batch. MICs for *C. spiroforme* reference strain NCTC 11493 of rabbit origin were also determined to compare wild-type results.

2.4 Data analysis

MIC values were expressed as µg/ml; when necessary, the commonly adopted international units were converted (Sweetman, 2007). To describe the results, the 50th and 90th percentile (referred to as MIC_{50} and MIC_{90},) and the geometric means of the MICs were calculated.

3. Results

In vitro susceptibility of sixty toxigenic *C. spiroforme* field strains was tested against spiramycin, amoxicillin, doxycycline, sulphadimethoxine, norfloxacin, tiamulin and lincomycin. MIC distribution and MIC_{50} and MIC_{90} against wild strains are reported in Table 1. MICs for *C. spiroforme* reference strains of human (ATCC 29900) and rabbit origin (NCTC 11493) are listed in Table 2 and compared with the MIC geometric mean of field strains. Results were MIC_{50} and MIC_{90} = 256 µg/ml for sulphadimethoxine, spiramycin and lincomycin; MIC_{50} and MIC_{90} = 64 µg/ml for tiamulin; MIC_{50} and MIC_{90} = 32 µg/ml for norfloxacin; MIC_{50} = 0.063 and MIC_{90} = 0.125 µg/ml for amoxicillin; MIC_{50} = 8 and MIC_{90} = 16 µg/ml for doxycycline.

Variance between the two performed batches was very low considering that 74% (31/42) of MIC values were in full agreement and 26% differed by just one dilution.

4. Discussion

Several technical improvements optimized the quality of our collection of *C. spiroforme* strains and enabled us to perform a proper antimicrobial survey for a pathogen that is

147 currently affecting intensive rabbit meat production. The availability of selective culture
148 media for routine diagnostic purposes increased our ability to collect *C. spiroforme* wild
149 strains. Moreover, since a PCR for *C. spiroforme* identification had become available
150 (Drigo et al., 2008), we were able to replace Kaneuchi's biochemical identification
151 method (1979), which is time-consuming and needs technical experience in the
152 manipulation of anaerobic bacteria. It also enabled us to differentiate *C. spiroforme*
153 from *C. cocleatum;* the latter displays high genetic homology with similar morphology
154 and differs biochemically solely in galactose fermentation (Kaneuchi et al., 1979;
155 Euzéby, 1998). Finally, once tested, Supplemented Brucella Agar proved to efficiently
156 support *C. spiroforme* growth and we therefore applied the agar dilution method to
157 evaluate MICs of anaerobes in accordance with the CLSI (2007) protocol.
158 Our results showed that *C. spiroforme* is resistant *in vitro* or has low susceptibility to
159 sulphadimethoxine, spiramycin, norfloxacin, doxycycline, tiamulin and lincomycin
160 (Table 1). On comparing *C. spiroforme* field strains with the NCTC 11493 reference
161 strain of rabbit origin, our findings seemed to corroborate that *C. spiroforme* might be
162 intrinsically resistant to sulphadimethoxine, spiramycin, lincomycin, norfloxacin and
163 tiamulin since reference and field strains displayed the same high MIC values (Table 2).
164 The *C. spiroforme* ATCC 29900 strain of human origin is susceptible to spiramycin and
165 tiamulin (MICs≤0.25 μg/ml); one possible explanation for the difference in behavior is
166 that the strain differs genetically from those of rabbit origin (Gilchrist et al., 1995) thus
167 emphasizing the appropriateness of using proper reference strains to draw conclusions.
168 Norfloxacin MIC_{90} showed *in vitro* inefficacy against *C. spiroforme,* similarly to what
169 is observed in human therapy with *C. difficile* and in spite of the fact that *C. perfringens*
170 is susceptible to second generation quinolones (Behra-Miellet et al., 2002; Yao et al.,

171 2003). One plausible explanation is that *C. spiroforme* and *C. difficile,* which are
172 clustered in XVIII and XI of the *Clostridium* genus, share a similar antimicrobial
173 pattern and other features (e.g. both are fastidious micro-organisms), but differ from *C.*
174 *perfringens,* which is a member of cluster I (Collins et al., 1994).

175 Tetracyclines are commonly administered orally to treat enteritis or respiratory
176 diseases. The entire class of antibiotic is characterized by phenomena of crossed
177 resistance which could explain why doxycycline, a second generation semi-synthetic
178 tetracycline, provides MIC_{50} and MIC_{90} values (8 and 16 μg/ml, respectively) of low
179 therapeutic efficacy. Amoxicillin had the lowest MICs (geometric mean = 0.081) but is
180 of no use, as β-lactam antibiotics are extremely toxic when orally administered to
181 rabbits (Harcourt-Brown, 2002).

182 In conclusion, while we are aware of the need to test antimicrobials other than the ones
183 assessed by us, our *in vitro* evaluation of drug susceptibility revealed that *C. spiroforme*
184 has a wide resistance to antimicrobial classes normally used in rabbit therapy. In field
185 conditions, acquired or intrinsic resistances were present in almost all the herds we
186 examined, thus explaining why the disease is considered a therapeutic challenge for
187 veterinary practitioners and suggesting that it would be appropriate to develop
188 alternative preventive strategies.

189 **References**

190 Behra-Miellet, J., Dubreuil, L., Jumas-Bilak, E., 2002. Antianaerobic activity of
191 moxifloxacin compared with that of ofloxacin, ciprofloxacin, clindamycin,
192 metronidazole and β-lactams. Int. J. Antimicrob. Ag. 5, 366-374.

193 Borriello, S.P., Carman, R.J., 1983. Association of iota-like toxin and *Clostridium*
194 *spiroforme* with spontaneous and antibiotic-associated diarrhea and colitis in
195 rabbits. J. Clin. Microbiol. 3, 414-418.
196 Borriello, S.P., Davies, H.A., Carman R.J., 1986. Cellular morphology of *Clostridium*
197 *spiroforme*. Vet. Microbiol. 11, 191-195.
198 Boucher, S., Nouaille, L., 2002. Maladies des lapins. 2nd ed., Groupe France Agricole,
199 Paris Cedex, France, pp. 56-61, 246-255.
200 Carman, R.J., Wilkins, T.D., 1991. *In vitro* susceptibility of rabbit strains of *Clostridium*
201 *spiroforme* to antimicrobial agents. Vet. Microbiol. 28, 391-397.
202 Clinical and Laboratory Standards Institute, 2007. Methods for antimicrobial
203 susceptibility testing of anaerobic bacteria; approved standard - Seventh edition.
204 CLSI Document M11-A7, Vol. 27 N° 2. CLSI, Wayne, Pennsylvania 19087-
205 1898, USA.
206 Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Cai,
207 J., Hippe, H., Farrow, J.A.E., 1994. The phylogeny of the genus *Clostridium*:
208 proposal of five new genera and eleven new species combinations. Int. J. Syst.
209 Bacteriol. 44, 812-826.
210 Drigo, I., Bacchin, C., Cocchi, M., Bano, L., Agnoletti, F., 2008. Development of PCR
211 protocols for specific identification of *Clostridium spiroforme* and detection of
212 *sas* and *sbs* genes. Vet. Microbiol. 131, 414-418.
213 Euzéby, J.P., 1998. Dictionnaire de Bactériologie Vétérinaire.
214 http://www.bacterio.cict.fr/bacdico/ cc/spiroforme.html.

215 Gilchrist, M.J.R., Carman, R.J., Kralovic, S.M., Linnemann C.C. Jr, 1995. Bacteremia
216 due to a multicenter, spirally aggregated *Clostridium* strain. Clin. Infect. Dis. 2
217 Suppl., S233-S236.
218 Harcourt-Brown, F., 2002. Textbook of rabbit medicine. Butterworth Heinemann, pp.
219 94-120.
220 Kaneuchi, C., Toshimitsu, M., Toshiharu, S., Tomotari, M., 1979. Taxonomic study of
221 helically coiled, spore forming anaerobes isolated from the intestine of humans
222 and other animals: *Clostridium cocleatum* sp. nov. and *Clostridium spiroforme*
223 sp. nov. Int. J. Syst. Bacteriol. 29, 1-12.
224 Leclercq, R., 2002. Mechanisms of resistance to macrolides and lincosamides: nature of
225 the resistance elements and their clinical implications. Clin. Infect. Dis. 34, 482-
226 492.
227 Marlier, D., Dewrée, R., Delleur, V., Licois, D., Lassence, C., Poulipoulis, A.,
228 Vindevogel, H., 2003. Description des principales étiologies de maladies
229 digestives chez le lapin européen (*Oryctolagus cuniculus*). Ann. Méd. Vét. 147,
230 385-392.
231 Perelle, S., Gibert, M., Boquete, P., Popoff, M.R., 1993. Characterization of *Clostridium*
232 *spiroforme* iota-toxin genes and expression in *Escherichia coli*. Infect. Immun.
233 63, 5147-5156.
234 Sweetman, S.C., 2007. Martindale: the complete drug reference. 35th ed., RPS
235 Publishing, London.
236 Yao, J.D.C., Moellering R.C. Jr. , 2003. Antibacterial agents. In: Murray, P.R.,
237 Baron, E.J., Jorgensen, J.H., Pfaller, M.A., Yolken, R.H. (Eds), Manual of
238 clinical microbiology, ASM Press,Washington D.C. USA, pp.1039-1073.

239 **Table 1.** Distribution of minimal inhibitory concentrations, MIC_{50} and $MIC_{90,}$ of 7
240 antimicrobial agents for 60 *Clostridium spiroforme* field strains.

	MIC (µg/ml)																
	0.016	0.032	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	MIC_{50}	MIC_{90}
spiramycin											1		1		58	256	256
amoxicillin		19	34	5		2										0.063	0.125
doxycycline									1	39	20					8	16
sulphadimeth.															60	256	256
norfloxacin											5	54	1			32	32
tiamulin											12	13	29	6		64	64
lincomycin											2				58	256	256

241
242

243 **Table 2.** Comparison of MICs of antimicrobials for *C. spiroforme* reference and field

244 strains.

C. spiroforme	spiramycin	amoxicillin	doxycycline	sulphadimeth.	norfloxacin	tiamulin	lincomycin
ATCC 29900*	0.12-0.25	0.12	0.06-0.12	256	8	0.12-0.25	0.5-1
NCTC 11493**	256	0.063	0.5	256	32	32	256
field strains***	238.8	0.081	9.9	256	32.7	44.7	233.4

245 * Human origin; ** Rabbit origin; *** MIC geometric mean