PREVALENCE AND MOLECULAR CHARACTERIZATION OF *CLOSTRIDIUM DIFFICILE* ISOLATED FROM RABBITS AND DETECTION OF ITS MAIN TOXINS

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ABSTRACT

This study investigated the prevalence of *Clostridium difficile* and its toxins in 132 Italian rabbit farms. Intestinal contents were collected from the small intestine and the caecum of 317 rabbits affected by enteric diseases and 80 control rabbits. Samples were stratified by age (<35 days, 35-55, >55, breeders) and enteric lesions (caecal constipation or fluid-filled caecum). The intestinal contents were cultured on selective media and screened for *C. difficile* toxins A and B production using a commercial ELISA. *C. difficile* isolates were tested by multiplex PCR to confirm the biochemical identification and to assess the presence of *tcdA, tcdB, cdtA, cdtB* genes encoding for toxin A, toxin B, and the two components of the binary-toxin respectively. The isolates were analyzed by PCR-ribotyping to investigate their genetic relatedness. *C. difficile* was recovered from the intestinal content of 10 rabbits older than 35 days with enteric disorders in 7 farms (prevalence=5.1%). *C. difficile* was not isolated from control rabbits. Eight strains resulted positive for both *tcdA* and *tcdB* genes. One strain was $tcdA^-/tcdB^-$, whereas another resulted $tcdA^-/tcdB^+$ and was also positive for both genes encoding for the binary-toxin. Toxin A and B were detected in four of the 10 samples positive for *C. difficile*. Typing analysis demonstrated that 5 strains isolated in two different farms belonged to the same clonal group that is also responsible for enteric disease in humans. The results show that *C. difficile* is occasionally involved in outbreaks of enteric disease in rabbits.

Key words: Rabbit, *Clostridium difficile*, Toxins, PCR-ribotype.

INTRODUCTION

Clostridium difficile (CD) is indicated in literature as an agent of serious pseudo-membranous colitis in humans, particularly in hospitalized patients, and in numerous species of animal (Voth and Ballard, 2005). In both human and animal, CD is considered responsible for enteritis following prolonged treatment with antibiotics (AAD: antibiotic associated diarrhea) (Voth and Ballard, 2005), even if in rabbits cases of clostridiosis caused by CD have been reported also in untreated subjects from 35 to 55 days of age, in which enterocolitis with liquid caecal content was observed (Perkins *et al.*, 1995). Although CD appears to be frequently isolated in rabbit farms in France (Bouvier *et al*., 2005), its presence has not been reported in Italy. The greatest factors of CD virulence are represented by 2 exotoxins with cytotoxic activity: Toxin A (or enterotoxin) and Toxin B (or cytotoxin) (Voth and Ballard, 2005). The presence of Toxins A and B can be readily detected in intestinal content through commercially available immunoenzymatic tests. There is also a third toxin, the binary toxin, which is similar to the iota-toxin of *C. perfringens* and *C. spiroforme* without cytotoxic activity but able to modify actin filaments by its ADP-ribosiltransferase activity (Voth and Ballard, 2005). The biochemical identification of CD using commercially available kits is particularly laborious and often scarcely reliable, whereas the introduction of molecular biology in diagnostic laboratories permits the simultaneous determination of the presence of species-specific DNA fragments and the encoding

genes for Toxins A (*tcd*A) and B (*tcd*B) (Lemee *et al*., 2004). This study was conducted to acquire information on the role of *C. difficile* and its toxins in enteric syndromes in rabbits by observing its spread in healthy and diseased populations.

MATERIALS AND METHODS

The study was conducted on 397 rabbits taken from 132 industrial farms distributed throughout Italy, from October 2006 to July 2007. A total of 317 animals were affected by lesions of the gastroenteric tract, while 80 rabbits without enteric lesions composed the control group. The samples were stratified in classes according to the age of the animals and the postmortem examination performed (Table 1). From the same farm no more than three groups of different age, in different times were included in the study.

Table 1: Number of sampled rabbits subdivided according to age and enteric lesions

Lesions / Age (days)	Fluid filled caecum	Constipation	Control rabbits	Total
< 35	27	7	20	54
35-55	94	35	18	147
> 55	82	35	17	134
breeders	28	9	25	62
Total	231	86	80	397

Samples of intestinal content (2 g if solid, 2 ml if liquid) taken from both the small and large intestine were introduced in two different sterile containers. The first sample was cultured on selective media for CD (*Clostridium Difficile* Agar Base, Oxoid) following shock in alcohol as described in the literature (Jousimies-Somer *et al*., 2002). When a bacterial growth was observed, the strictly anaerobic colonies were isolated and biochemically identified using a commercially available kit (API ANA, BioMerieux). The biochemical identification of CD and its toxigenic potential were confirmed by two multiplex-PCRs: the first amplified the species-specific *tpi* (triose phosphate isomerase) gene and the two toxin genes *tcdA* and *tcdB* (Lemee *et al*., 2004), the second amplified both *cdtA* and *cdtB* genes coding for the two sub-units of the CD binary-toxin (Stubbs *et al*., 2000). The CD strains isolated were subjected to PCR-ribotyping (Bidet *et al*., 1999) and the resulting patterns of PCR-fragments were compared with those of three ribotypes already observed in some Italian clinical isolates from human (Spigaglia *et al*., 2001) and with the pattern of the ribotype 027 that characterized the epidemic clone which recently caused severe outbreaks in USA, Canada and Europe (Kuijper *et al*., 2006). To search toxin A and B in the intestinal contents, the second sample was analyzed by means of commercially available immuno-enzymatic test kits (ELISA - *C. difficile* tox A/B II™ , Techlab) in accordance with the producer's instructions.

RESULTS AND DISCUSSION

Clostridium difficile or its toxins were observed in a total of 14 subjects with enteric syndromes (14/317, 4.4%) and two healthy subjects in 12 different farms (12/132, 9%). The results are summarized in Table 2.

The results obtained demonstrate a lesser involvement of CD in the enteric symptoms observed in rabbit farms in Italy than in France (Bouvier *et al.*, 2005). The presence of CD was observed only in subjects older than 35 days in agreement with the case studies described above. The presence of CD toxins was observed in all the classes of age taken into consideration regardless of whether the subjects had disease or not, whereas CD was isolated only from subjects with gastroenteric lesions regardless of type of lesion observed. The presence of potentially toxigenic CD strains isolated from intestinal contents resulted negative to the toxin immuno-enzymatic test might be explained by the low sensitivity of commercial available ELISA kits (85-92%) compared to that of the cytotoxicity assay, considered as the gold standard in CD toxins detection (Reyes *et al*., 2007).

Table 2: Age, enteric lesions and presence of attendant pathogens in subjects tested positive for *C. difficile* and/or its toxins

Farm	sample	Age (d)	Lesions	Bact. exam.	*tpi*	*tcdA*	*tcdB*	*cdtA*	*cdtB*	Tox A/B	*CP*	*CS*	REPEC	Cocc.
A	1	> 55	FFC	+	+	+	+	-	-	+	+	-	-	-
B	2	35-55	C	+	+	-	-	-	-	-	+	-	-	-
C	3	breeder	C	+	+	+	+	-	-	-	+	-	-	-
D	4	> 55	C	+	+	V	+	+	+	-	-	+	-	-
E	5	> 55	C	+	+	+	+	-	-	-	-	+	+	-
F	6	35 -55	FFC	+	+	+	+	-	-	+	-	-	+	-
F	7	35- 55	FFC	+	+	+	+	-	-	-	-	-	+	-
G	8	> 55	FFC	+	+	+	+	-	-	+	-	+	+	-
G	9	35 -55	FFC	+	+	+	+	-	-	+	-	-	+	+
G	10	35 -55	FFC	+	+	+	+	-	-	-	-	-	+	+
H	11	<35	FFC	-	nd	nd	nd	nd	nd	+	-	-	-	-
I	12	<35	C	-	nd	nd	nd	nd	nd	+	-	-	-	-
J	13	35-55	FFC	-	nd	nd	nd	nd	nd	+	-	+	-	-
D	14	35-55	NEL	-	nd	nd	nd	nd	nd	+	-	-	-	+
K	15	>55	NEL	-	nd	nd	nd	nd	nd	+	+	-	-	-
L	16	breeder	FFC	-	nd	nd	nd	nd	nd	+	-	+	-	-

FFC: fluid filled caecum; C: caecal constipation; NEL: no enteric lesions; V: variant gene; nd: not done; Bact. exam.: bacteriological examination; *tpi:* triose phosphate isomerase gene; *Tox* A/B: elisa test for CD toxin A and B; CP: *Clostridium perfringens*; CS: *Clostridium spiroforme*; REPEC: rabbit enteropathogenic *Escherichia coli;* Cocc.: coccidia

Vice versa, the detection of CD toxins in 6 intestinal contents in which CD was not isolated, might be related to the insufficient quantity of CD alive spores that can be detected by the bacteriological examination or to the low specificity of the ELISA test (86%) (Post *et al*., 2002). This last assumption is supported by the fact that 4 of the 6 ELISA positive samples, showed an optical density very close to the inferior cut-off value of the ELISA kit. Particularly worthy of note is the potential pathogenicity of nearly all the strains isolated due to the presence of genes encoding for toxins A and B. Only one strain isolated from a subject with caecal constipation carried the genes encoding the binary-toxin's two sub-units. The presence of both bacteriological positivity and ELISA test positivity in 4 cases out of 10 is also particularly interesting, and leads to the supposition that CD plays an active role in triggering the gastroenteric symptoms observed. It is also worth noting that the presence of CD in these four subjects was observed together with other bacteria entero-pathogenic for rabbits (Table 2), and this supports the hypothesis that also enterocolitis caused by CD must be included among the enteric syndromes of multi-factorial etiology in rabbits. Even if performed in a limited number of isolates, the results obtained from the PCR-ribotyping of CD strains lead to a series of interesting considerations (Figure 1). First of all, most of these isolates belong to ribotypes that have also been detected in humans (strains 6, 7, 8, 9, 10 to PCR-ribotype D and strain 4 to PCR-ribotype A), which suggests the possibility of transfer from rabbits to humans and vice versa. Furthermore, in the same farm was isolated the same ribotype in rabbits of different age and at different times. This observation suggests the circulation of a specific ribotype over time. The recent isolation of two other CD strains belonging to the same ribotype, even if not related to those observed in human, from the same farm seems to support further this hypothesis.

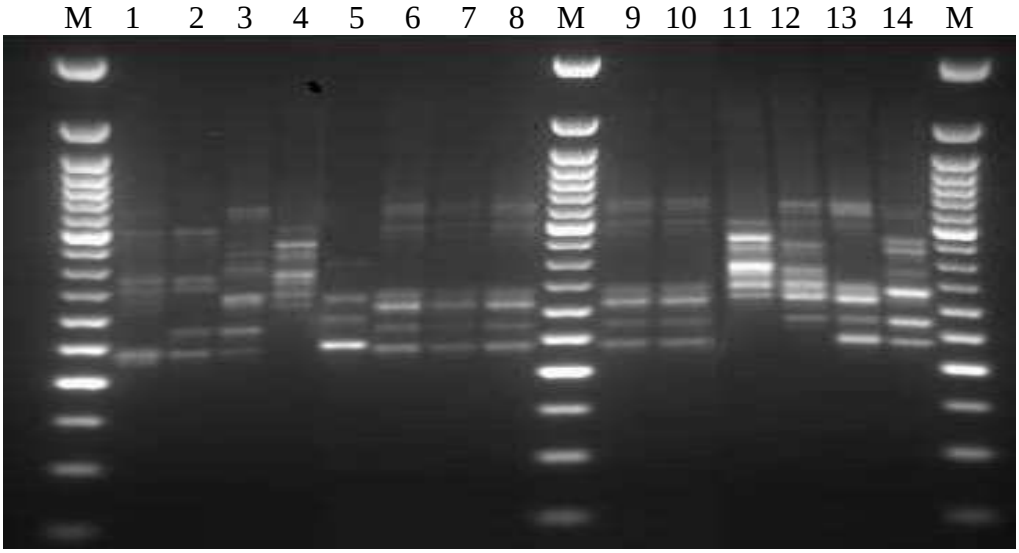

Figure 1: PCR-ribotyping. M: marker 50bp ladder; lines 1-10: strains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10; lines 11-14: control strains for PCR ribotype A, R, D, 027, respectively

REFERENCES

Bidet P., Barbut F., Lalande V., Burghoffer B., Petit J.C. 1999. Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. *FEMS Microbiol Lett., 175, 261-6.*

Bouvier A.C., Jacquinet C., Manco B. 2005. Etude récente sur la sensibilité de différentes souches de *Clostridium* prélevées sur des lapins avec signes cliniques d'EEL, vis-à-vis de la tiamuline. *In: Proc. 11èmes Journées de la Recherche Cunicole, 2005 November, Paris, France, 253-256.*

Jousimies-Somer H., Summanen P., Citron D.M., Jo Baron E., Wexler H.M., Finegold S.M. 2002. Laboratory tests for diagnosis of *Clostridium difficile* enteric disease. In: *Wadsworth – KTL, Anaerobic bacteriology manual. Star publishing company, Belmont, California, USA, pp. 133-141.*

Kuijper E.J., Coignard B., Tull P. 2006. Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin. Microbiol. Infect., 12 Suppl, 6, 2-18.*

Lemee L., Dhalluin A., Testelin S., Mattrat M.A., Maillard K., Lemeland J.F., Pons J.L. 2004. Multiplex PCR targeting *tpi* (triose phosphate isomerase), *tcdA* (toxin A), and *tcdB* (toxin B) genes for toxigenic culture of *Clostridium difficile. J. Clin. Microbiol., 42, 5710-5714.*

Perkins S.E., Fox J.G., Taylor N.S., Green D.L., Lipman N.S. 1995. Detection of *Clostridium difficile* toxins from the small intestine and cecum of rabbits with naturally acquired enterotoxemia*. Lab. Anim. Sci., 45, 379-384.*

Post K.W., Jost B.H., Songer J.G. 2002 Evaluation of a test for *Clostridium difficile* toxins A and B for the diagnosis of neonatal swine enteritis. *J. Vet. Diagn. Invest., 14, 258-259.*

Reyes R.C., John M.A., Ayotte D.L., Covacich A., Milburn S., Hussain Z. 2007. Performance of TechLab C. DIFF QUIK CHEK and TechLab C. DIFFICILE TOX A/B II for the detection of *Clostridium difficile* in stool samples. *Diagn. Microbiol. Infect., 59, 33-7.*

Spigaglia P., Cardines R., Rossi S., Menozzi M.G., Mastrantonio P. 2001. Molecular typing and long-term comparison of *Clostridium difficile* strains by pulsed-field gel electrophoresis and PCR-ribotyping. *J. Med. Microbiol., 50, 407-414.*

Stubbs S., Rupnik M., Gilbert M., Brazier J., Duerden B., Popoff M.R. 2000. Production of an actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile. FEMS Microbiol. Lett., 186, 307–312.*

Voth D.E., Ballard J.D. 2005. *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin. Microbiol. Rev., 18, 247-263.*