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Prevalence of *Clostridium difficile* in diarrhoeic and non-diarrhoeic piglets

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4 **ABSTRACT**

5

6 *Clostridium difficile* is considered to be an important causative agent of porcine
7 neonatal diarrhoea, having taken over from classic bacterial pathogens. However, there
8 are currently no clear data concerning the prevalence of this microorganism in piglets,
9 or about its relative distributions among diarrhoeic and non-diarrhoeic animals. In the
10 present study, we analyzed the presence of *C. difficile* in rectal swabs from 780 piglets
11 from two age groups (newborn and 1–2-month-old pigs) by means of molecular and
12 microbiological procedures. Furthermore DNA was isolated from the bacteria in order
13 to identify toxin A and toxin B genes.

14 *C. difficile* was not found in any of the 239 samples taken from 1- to 2-month-old pigs.
15 Bacteria were, however, recovered from 140 out of 541 newborn piglets (25.9%),
16 including both diarrhoeic and non-diarrhoeic animals, and animals from control farms
17 (free of diarrhoeic animals). Genes for the production of both toxins A and B were
18 identified in 132 of the 140 isolates (A⁺B⁺). Only seven isolates, all from the same farm
19 and from non-diarrhoeic animals, lacked both toxin genes (A⁻B⁻), while one isolate from
20 this same group of animals was A⁻B⁺.

21 This study provides the first report comparing the prevalence of *C. difficile* in large
22 numbers of diarrhoeic and non-diarrhoeic animals. There was no clear link between
23 bacterial isolation and neonatal porcine diarrhoea.

24

25 **Key words:** *Clostridium difficile*, prevalence, pig, diarrhoea, toxin.

26

1

2 **1. Introduction**

3

4 *Clostridium difficile* is an ubiquitous bacterium in the environment and has been
5 recognised as an important emerging pathogen in both humans and animals. *C. difficile*
6 is also the most important cause of antimicrobial-associated and hospital-associated
7 diarrhoea in humans. In recent years, an increase in the incidence of human *C. difficile*
8 infections has been reported, as well as an increase in the virulence and antimicrobial
9 resistance of the isolates (Pepin et al., 2004; McDonald et al., 2005; Kuijper et al., 2006;
10 Jhung et al., 2008).

11 Veterinary medicine has highlighted the role of animals as reservoirs for *C. difficile*, as
12 well as its zoonotic implications and its pathogenicity in different animal species,
13 mainly in equids and swine (Borriello et al., 1983; Arroyo et al., 2005; Rodriguez-
14 Palacios et al., 2006; Rupnik, 2007). The importance of *C. difficile* as an agent involved
15 in porcine neonatal diarrhoea has increased, displacing classic bacterial pathogens
16 (Songer et al., 2000; Yaeger et al., 2002; Songer and Uzal, 2005). However, some
17 questions remain to be answered, such as the role of toxins in the pathogenesis of the
18 microorganism and the susceptibility of swine to *C. difficile* colonization (Waters et al.,
19 1998; Songer, 2004; Songer and Anderson, 2006).

20 In spite of the importance of *C. difficile* as a swine pathogen, there are currently no clear
21 data concerning the prevalence of this microorganism in pig farms in relation to age, or
22 its relative prevalence in animals with and without diarrhoea.

23 The aims of this study were to analyse the prevalence of *C. difficile* in two age groups of
24 diarrhoeic and non-diarrhoeic pigs (newborn pigs (1–7 days old) and 1–2-month-old
25 pigs), and to determine the presence of *C. difficile* toxin A and B genes in the isolates.

1

2

3 **2. Materials and methods**

4

5 *2.1. Samples*

6

7 Rectal swabs were obtained from 780 animals: 541 from 1- to 7-day-old piglets (287
8 non-diarrhoeic and 254 diarrhoeic animals) and 239 from 1- to 2-month-old pigs (187
9 non-diarrhoeic and 52 diarrhoeic animals). Samples came from 13 pig farms located in
10 three densely swine populated areas in Spain, located in three different Autonomous
11 Communities (zone A [Aragon], zone B [Catalonia] and zone C [Castile-La Mancha]).

12 A total of 13 herds were sampled: Ten had experienced continuous problems with
13 porcine neonatal diarrhoea and, in some cases, they also had 1–2-month-old diarrhoeic
14 animals. The remaining three herds were clinically free of diarrhoea in growing piglets
15 at the time of sampling, and were then considered as controls.

16 A fresh stool sample was taken from each animal by introducing a sterile swab into the
17 rectum. Swabs without transport media were stored at -20°C in the herds until all
18 samples of the herd were collected. Then they were transported in frozen conditions to
19 the laboratory to be analysed.

20

21 *2.2. Culture, isolation and identification of C. difficile*

22

23 In order to select for bacterial spores, the tip of each rectal swab was submerged for 30 s
24 in a 1.5-ml microtube containing 500 µl of 70% ethanol. All the microtubes were
25 incubated at room temperature for 20 min. After incubation, an aliquot of 75 µl was

1 taken from each tube and inoculated on selective medium (*Clostridium difficile* agar
2 plate; BioMérieux, Marcy l'Etoile, France). The plates were incubated under anaerobic
3 conditions for 48 h at 37°C.

4 *C. difficile* isolates were identified by colony morphology, Gram stain and the typical
5 horse-manure odour of this microorganism. Their identification was confirmed using
6 the API rapid ID 32 A system (BioMérieux), following the manufacturer's instructions.

7

8 *2.3. DNA isolation and polymerase chain reaction (PCR) detection of C. difficile toxin*
9 *A and toxin B genes*

10

11 In order to isolate *C. difficile* DNA, a single colony was taken from a pure culture of
12 each isolate and was suspended in 1 ml of sterile milli-Q water. Microtubes were
13 incubated at 100°C for 20 min to lyse the bacterial cells and then centrifuged for 2 min
14 at 8000 g. Two hundred microliters of each supernatant were transferred to a new
15 microtube and stored at -20°C until used in PCR assays.

16 A modification of the PCR method, described by Kato et al. (1998) was used to detect
17 the non-repeating portion of the *C. difficile* toxin A gene (*tcdA*). Briefly, 3 µl of DNA
18 extract were added to a PCR mixture that contained 2.5 mM MgCl₂, 50 mM KCl, 10
19 mM Tris-HCl (pH 8.3), 200 µM of each dNTP, 5.5 pmol of primer NK2
20 (5'CCCAATAGAAGATTCAATATTAAGCTT3') (Isogen Life Science, Maarssen,
21 Netherlands), 5 pmol of primer NK3
22 (5'GGAAGAAAAGAACTTCTGGCTCACTCAGGT3') and 0.75 U of AmpliTaq Gold
23 DNA Polymerase (Applied Biosystems, Madrid, Spain). The final reaction volume was
24 30 µl. Amplifications were carried out in a GeneAmp PCR System 9700 (Applied

1 Biosystems, Foster City, USA) thermocycler and consisted of a denaturation step of 5
2 min at 95°C, followed by 35 cycles of 15 s at 95°C, 20 s at 50°C and 40 s at 72°C.
3 The presence of the *C. difficile* toxin B gene (*tcdB*) was also detected by PCR. The
4 reaction described by Fluit et al. (1991) was adapted to our laboratory conditions.
5 Briefly, 2 µl of DNA extract was added to a PCR mixture that contained 1.5 mM
6 MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 100 µM of each dNTP, 50 pmol of each
7 primer (5'TAATAGAAAACAGTTAGAAA3' and
8 5'TCCAATCCAAACAAAATGTA3') and 2.5 U of AmpliTaq Gold DNA Polymerase
9 in a final volume of 50 µl. Amplifications were carried out under the following
10 conditions: 5 min denaturation at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at
11 50°C and 1 min at 72°C.
12 Specific amplification products of 252 bp for the *tcdA* fragment and 301 bp for *tcdB*
13 were visualized under UV illumination in a 1.6% agarose gel stained with ethidium
14 bromide (Sigma–Aldrich, Madrid, Spain).

15

16 2.4. Analysis of results

17

18 A χ^2 -test was used to compare the prevalence of *C. difficile* among the different
19 production areas and to study the possible association between *C. difficile* isolation and
20 the presence or absence of diarrhoea. We used statistical software (Statgraphics Plus 5.0
21 for Windows, Statistical Graphics Corp.) and applied Yates' correction when necessary.
22 P-values <0.05 were considered significant.

23

24

25 3. Results

1
2 The results of *C. difficile* isolation from 1- to 7-day-old piglet faecal samples are shown
3 in Table 1. Table 1 also shows the results of the PCR detection of *C. difficile tcdA* and
4 *tcdB* genes. *C. difficile* was recovered from 140 out of 541 (25.9%) rectal swabs taken
5 from 1- to 7-day-old piglets. Moreover, *C. difficile* was isolated from at least one non-
6 diarrhoeic piglet from this age group from all the sampled farms, including the control
7 farms. Conversely, at one farm (C2), *C. difficile* was not isolated from any piglet with
8 diarrhoea.

9 In zone B was detected a higher prevalence of *C. difficile* in non-diarrhoeic animals, that
10 was a statistically significant relationship ($p < 0.05$). However, when the 13 farms were
11 considered individually, a significant association between the variables was only found
12 at farm B1 ($p < 0.05$). In the other two zones, A and C, no association was found between
13 *C. difficile* isolation and the presence or absence of diarrhoea ($p > 0.05$, in both cases).

14 In the 1–7-day-old non-diarrhoeic piglets, a higher prevalence of *C. difficile* was seen in
15 zone B than in zones A or C, or in the control farms ($p < 0.01$). There were no
16 statistically significant differences between zones A and C, or between these zones and
17 the control farms. When the data for *C. difficile* prevalence in the different farms within
18 each sampled zone were analysed, significant differences were only observed among the
19 farms located in zone C ($p < 0.05$). There were no significant differences in prevalence
20 among the three control farms.

21 When a similar analysis was performed for the 1–7-day-old diarrhoeic piglets,
22 statistically significant differences were only found between zones B and C ($p < 0.01$).

23 Both *tcdA* and *tcdB* genes were amplified by PCR (A^+B^+ isolates) in 132 out of the 140
24 (94.3%) *C. difficile* isolates. Only seven isolates, all from non-diarrhoeic piglets from
25 farm B3, showed no amplification of either of these genes (A^-B^- isolates). In one isolate,

1 also recovered from non-diarrhoeic piglets from farm B3, *tcdB* but not *tcdA* was
2 amplified by PCR (A⁻B⁺ isolate).

3 *C. difficile* was not isolated from any of the 239 samples taken from 1- to 2-month-old
4 pigs, regardless of the presence or absence of diarrhoea. Of these 239 samples, 187 were
5 from non-diarrhoeic pigs and 52 were from diarrhoeic animals.

6

7

8 **4. Discussion**

9

10 In the present work, *C. difficile* was not isolated from any 1–2-month-old pig. However,
11 this bacterium was recovered from a considerable proportion of 1–7-day-old piglets,
12 regardless of the presence or absence of diarrhoea. Moreover, differences in *C. difficile*
13 prevalence in piglets were observed among the three production areas sampled, though
14 significant differences were only found between zone B and other zones, or the control
15 farms.

16 It has been suggested that *C. difficile* may be currently the most important uncontrolled
17 cause of neonatal diarrhoea in pigs (Songer, 2004; Songer and Anderson, 2006), having
18 displaced other bacterial and viral agents as the main cause of this disease (Yaeger et
19 al., 2002). Although *C. difficile*-associated infection affects mainly 1–7-day-old piglets
20 (Songer et al., 2000; Songer and Uzal, 2005). Furthermore, outbreaks in adult pigs have
21 also been described (Kiss and Bilkei, 2005).

22 The carrier rate of *C. difficile* seems to vary among asymptomatic individuals by species
23 and, within the same species, depending on age and other population characteristics
24 (Keel and Songer, 2006). Our results confirm these observations.

1 Although most *C. difficile* strains produce both toxin A and toxin B, some strains
2 produce only toxin B, or no toxins at all (Songer and Uzal, 2005). Both *tcdA* and *tcdB*
3 genes that code for the essential virulence factors of *C. difficile* (Songer et al., 2000;
4 Keel and Songer, 2006), were identified in most isolates recovered in the present study.
5 In contrast, A⁻B⁻ and A⁻B⁺ isolates were relatively rare in the sampled farms. The
6 exception to this was farm B3, where 50% of the asymptomatic carriers of *C. difficile*
7 were piglets colonized by A⁺B⁺ strains, and the other 50% by A⁻B⁺ or A⁻B⁻ strains.
8 Previous studies in pigs have shown results similar to those demonstrated here. For
9 example, the overall prevalence of *C. difficile* in piglets from 10 herds in North Carolina
10 (USA) was 47.6%, and ranged from 0 to 97% across the herds (Songer, 2004). Also in
11 the United States, *C. difficile* toxins were detected in the colonic content of 29% of 1–7-
12 day-old piglets with neonatal diarrhoea (Yaeger et al., 2002). Also, *C. difficile* toxins
13 were detected in the colonic content of 23/29 (79%) apparently healthy piglets (Yaeger
14 et al., 2007).

15 Similar results have also been found in other animal species: for example, no
16 association has been found between *C. difficile* isolation and canine diarrhoea, and no
17 significant differences in the percentage of A⁺B⁺ *C. difficile* isolates recovered from
18 diarrhoeic and non-diarrhoeic dogs were detected (Perrin et al., 1993; Marks et al.,
19 2002). On the other hand, studies in calves detected a higher prevalence of *C. difficile* in
20 faeces from healthy control animals than in faeces from calves with diarrhoea
21 (Rodriguez-Palacios et al., 2006). Finally, *C. difficile* was isolated from approximately
22 30% of the faecal samples from healthy 0–13-day-old foals, but not from foals 1–6
23 months of age (Båverud, 2003). However, in this last case, only 35.7% of the isolates
24 recovered from the 0–13-day-old foals were positive for toxin A and toxin B by PCR
25 (Båverud, 2003).

1 In humans, *C. difficile* infection is associated almost exclusively with antibiotic usage
2 (Bartlett and Perl, 2005). In domestic animals this association is less clear, as infection
3 by this bacterium has been observed in animals not treated with antibiotics (Waters et
4 al., 1998; Yaeger et al., 2002). Moreover, it has been suggested that other factors may
5 contribute to the establishment of *C. difficile* infection, including diet, environmental
6 conditions in some production farms, animal stress, and the disruption of the intestinal
7 microenvironment by other pathogens (Yaeger et al., 2002; Nagy and Bilkei, 2003; Kiss
8 and Bilkei, 2005).

9 Although our study did not cover the entire country, the three sampled areas account for
10 approximately 50% of the Spanish piglet production (data from the Ministry of
11 Agriculture, Fisheries and Foods,
12 <http://www.mapa.es/es/estadistica/pags/anuario/introduccion.htm>), being Spain the
13 second largest pig producer country in the European Union. The fact that zone B
14 (Catalonia) (which is the main pig producing area in Spain) had the highest percentage
15 of asymptomatic carrier piglets deserves further investigation. It is also necessary to
16 clarify the relationship between the colonization and possible infection of the pig
17 intestinal tract by *C. difficile* and the different factors affecting the microbial flora.

18

19 In conclusion, this is the first report comparing the prevalence of *C. difficile* in large
20 numbers of diarrhoeic and non-diarrhoeic pigs. No clear link between the isolation of
21 this bacteria and neonatal porcine diarrhoea was identified, in agreement with results
22 from other species, including humans.

23

24

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1

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8

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2 **Table 1:** Isolation of *C. difficile* from 1- to 7-day-old piglet faecal samples and PCR3 detection of *tcdA* and *tcdB* genes

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Table 1: Isolation of *C. difficile* from 1- to 7-day-old piglet faecal samples and PCR detection of *tcdA* and *tcdB* genes

Zone	Farm	Non-diarrhoeic animals					Diarrhoeic animals				
		n	Isolation of <i>C. difficile</i>	A ⁻ B ⁻	A ⁻ B ⁺	A ⁺ B ⁺	n	Isolation of <i>C. difficile</i>	A ⁻ B ⁻	A ⁻ B ⁺	A ⁺ B ⁺
A	A1	27	5 (18.5 %)	0	0	5	27	7 (25.9 %)	0	0	7
	A2	26	8 (30.8 %)	0	0	8	27	7 (25.9 %)	0	0	7
	A3	27	7 (25.9 %)	0	0	7	27	8 (29.6 %)	0	0	8
	Total	80	20 (25 %)	0	0	20	81	22 (27.2 %)	0	0	22
B	B1	25	11 (44 %)	0	0	11	25	3 (12 %)	0	0	3
	B2	27	15 (55.5 %)	0	0	15	27	10 (37 %)	0	0	10
	B3	25	16 (64 %)	7	1	8	21	10 (47.6 %)	0	0	10
	Total	77	42 (54.5 %)	7	1	34	73	23 (31.5 %)	0	0	23
C	C1	25	2 (8 %)	0	0	2	25	3 (12 %)	0	0	3
	C2	25	4 (16 %)	0	0	4	25	0 (0 %)	0	0	0
	C3	25	7 (28 %)	0	0	7	25	7 (28 %)	0	0	7
	C4	25	1 (4 %)	0	0	1	25	3 (12 %)	0	0	3
	Total	100	14 (14 %)	0	0	14	100	13 (13 %)	0	0	13
Control farms	I	10	3 (30 %)	0	0	3					
	II	10	2 (20 %)	0	0	2					
	III	10	1 (10 %)	0	0	1					
	Total	30	6 (20 %)	0	0	6					