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To cite this version:
Sergio Alvarez-Perez, Jose L. Blanco, Emilio Bouza, Patricia Alba, Xavier Gibert, et al.. Prevalence of in diarrhoeic and non-diarrhoeic piglets. Veterinary Microbiology, 2009, 137 (3-4), pp.302. 10.1016/j.vetmic.2009.01.015 . hal-00485531

HAL Id: hal-00485531
https://hal.science/hal-00485531
Submitted on 21 May 2010

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Accepted Manuscript

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PII: S0378-1135(09)00036-4
DOI: doi:10.1016/j.vetmic.2009.01.015
Reference: VETMIC 4330

To appear in: VETMIC

Received date: 29-9-2008
Revised date: 5-1-2009
Accepted date: 6-1-2009

Please cite this article as: Alvarez-Perez, S., Blanco, J.L., Bouza, E., Alba, P., Gibert, X., Maldonado, J., Garcia, M.E., Prevalence of *Clostridium difficile* in diarrhoeic and non-diarrhoeic piglets, Veterinary Microbiology (2008), doi:10.1016/j.vetmic.2009.01.015

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

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ABSTRACT

*Clostridium difficile* is considered to be an important causative agent of porcine neonatal diarrhoea, having taken over from classic bacterial pathogens. However, there are currently no clear data concerning the prevalence of this microorganism in piglets, or about its relative distributions among diarrhoeic and non-diarrhoeic animals. In the present study, we analyzed the presence of *C. difficile* in rectal swabs from 780 piglets from two age groups (newborn and 1–2-month-old pigs) by means of molecular and microbiological procedures. Furthermore DNA was isolated from the bacteria in order to identify toxin A and toxin B genes. *C. difficile* was not found in any of the 239 samples taken from 1- to 2-month-old pigs. Bacteria were, however, recovered from 140 out of 541 newborn piglets (25.9%), including both diarrhoeic and non-diarrhoeic animals, and animals from control farms (free of diarrhoeic animals). Genes for the production of both toxins A and B were identified in 132 of the 140 isolates (A⁺B⁺). Only seven isolates, all from the same farm and from non-diarrhoeic animals, lacked both toxin genes (A⁺B⁻), while one isolate from this same group of animals was A⁻B⁺. This study provides the first report comparing the prevalence of *C. difficile* in large numbers of diarrhoeic and non-diarrhoeic animals. There was no clear link between bacterial isolation and neonatal porcine diarrhoea.

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

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

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

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

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

2.1. Samples

Rectal swabs were obtained from 780 animals: 541 from 1- to 7-day-old piglets (287 non-diarrhoeic and 254 diarrhoeic animals) and 239 from 1- to 2-month-old pigs (187 non-diarrhoeic and 52 diarrhoeic animals). Samples came from 13 pig farms located in three densely swine populated areas in Spain, located in three different Autonomous Communities (zone A [Aragon], zone B [Catalonia] and zone C [Castile-La Mancha]). A total of 13 herds were sampled: Ten had experienced continuous problems with porcine neonatal diarrhoea and, in some cases, they also had 1–2-month-old diarrhoeic animals. The remaining three herds were clinically free of diarrhoea in growing piglets at the time of sampling, and were then considered as controls.

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

2.2. Culture, isolation and identification of C. difficile

In order to select for bacterial spores, the tip of each rectal swab was submerged for 30 s in a 1.5-ml microtube containing 500 µl of 70% ethanol. All the microtubes were incubated at room temperature for 20 min. After incubation, an aliquot of 75 µl was
taken from each tube and inoculated on selective medium (Clostridium difficile agar plate; BioMérieux, Marcy l’Etoile, France). The plates were incubated under anaerobic conditions for 48 h at 37°C.

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

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

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

A modification of the PCR method, described by Kato et al. (1998) was used to detect the non-repeating portion of the *C. difficile* toxin A gene (*tcdA*). Briefly, 3 μl of DNA extract were added to a PCR mixture that contained 2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM of each dNTP, 5.5 pmol of primer NK2 (5’CCCAATAGAAAGATTCAATATTAAGCTT3’) (Isogen Life Science, Maarssen, Netherlands), 5 pmol of primer NK3 (5’GGAAGAAAAGAACTTCTGGCTCACTCAGGT3’) and 0.75 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Madrid, Spain). The final reaction volume was 30 μl. Amplifications were carried out in a GeneAmp PCR System 9700 (Applied
Biosystems, Foster City, USA) thermocycler and consisted of a denaturation step of 5 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. The presence of the *C. difficile* toxin B gene (*tcdB*) was also detected by PCR. The reaction described by Fluit et al. (1991) was adapted to our laboratory conditions. Briefly, 2 µl of DNA extract was added to a PCR mixture that contained 1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 100 µM of each dNTP, 50 pmol of each primer (5’TAATAGAAAAACAGTTAGAAA3’ and 5’TCCAATCCAAAAACAAAATGTA3’) and 2.5 U of AmpliTaq Gold DNA Polymerase in a final volume of 50 µl. Amplifications were carried out under the following conditions: 5 min denaturation at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. Specific amplification products of 252 bp for the *tcdA* fragment and 301 bp for *tcdB* were visualized under UV illumination in a 1.6% agarose gel stained with ethidium bromide (Sigma–Aldrich, Madrid, Spain).

2.4. Analysis of results

A χ²-test was used to compare the prevalence of *C. difficile* among the different production areas and to study the possible association between *C. difficile* isolation and the presence or absence of diarrhoea. We used statistical software (Statgraphics Plus 5.0 for Windows, Statistical Graphics Corp.) and applied Yates’ correction when necessary. P-values <0.05 were considered significant.

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

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

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

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

Both tcdA and tcdB genes were amplified by PCR (A^+^B^+^ isolates) in 132 out of the 140 (94.3%) *C. difficile* isolates. Only seven isolates, all from non-diarrhoeic piglets from farm B3, showed no amplification of either of these genes (A^−^B^−^ isolates). In one isolate,
also recovered from non-diarrhoeic piglets from farm B3, tcdB but not tcdA was amplified by PCR (A'B+ isolate).

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

4. Discussion

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

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

The carrier rate of *C. difficile* seems to vary among asymptomatic individuals by species and, within the same species, depending on age and other population characteristics (Keel and Songer, 2006). Our results confirm these observations.
Although most *C. difficile* strains produce both toxin A and toxin B, some strains produce only toxin B, or no toxins at all (Songer and Uzal, 2005). Both *tdaA* and *tddB* genes that code for the essential virulence factors of *C. difficile* (Songer et al., 2000; Keel and Songer, 2006), were identified in most isolates recovered in the present study. In contrast, *A*B and *A*B isolates were relatively rare in the sampled farms. The exception to this was farm B3, where 50% of the asymptomatic carriers of *C. difficile* were piglets colonized by *A*B strains, and the other 50% by *A*B or *A*B strains. Previous studies in pigs have shown results similar to those demonstrated here. For example, the overall prevalence of *C. difficile* in piglets from 10 herds in North Carolina (USA) was 47.6%, and ranged from 0 to 97% across the herds (Songer, 2004). Also in the United States, *C. difficile* toxins were detected in the colonic content of 29% of 1–7-day-old piglets with neonatal diarrhoea (Yaeger et al., 2002). Also, *C. difficile* toxins were detected in the colonic content of 23/29 (79%) apparently healthy piglets (Yaeger et al., 2007). Similar results have also been found in other animal species: for example, no association has been found between *C. difficile* isolation and canine diarrhoea, and no significant differences in the percentage of *A*B* C. difficile* isolates recovered from diarrhoeic and non-diarrhoeic dogs were detected (Perrin et al., 1993; Marks et al., 2002). On the other hand, studies in calves detected a higher prevalence of *C. difficile* in faeces from healthy control animals than in faeces from calves with diarrhoea (Rodriguez-Palacios et al., 2006). Finally, *C. difficile* was isolated from approximately 30% of the faecal samples from healthy 0–13-day-old foals, but not from foals 1–6 months of age (Båverud, 2003). However, in this last case, only 35.7% of the isolates recovered from the 0–13-day-old foals were positive for toxin A and toxin B by PCR (Båverud, 2003).
In humans, *C. difficile* infection is associated almost exclusively with antibiotic usage (Bartlett and Perl, 2005). In domestic animals this association is less clear, as infection by this bacterium has been observed in animals not treated with antibiotics (Waters et al., 1998; Yaeger et al., 2002). Moreover, it has been suggested that other factors may contribute to the establishment of *C. difficile* infection, including diet, environmental conditions in some production farms, animal stress, and the disruption of the intestinal microenvironment by other pathogens (Yaeger et al., 2002; Nagy and Bilkei, 2003; Kiss and Bilkei, 2005).

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

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

Acknowledgements
This work was partially supported by grant FAU2006-00005-C02 INIA from the Spanish Ministry of Education and Science. This work was partially supported by LABORATORIOS HIPRA, S.A. Sergio Álvarez-Pérez acknowledges a grant from the FPU programme (ref. AP 2005-1034), Spanish Ministry of Education and Science.

Conflict of interest statement: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


1

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

3

4
Table 1: Isolation of *C. difficile* from 1- to 7-day-old piglet faecal samples and PCR detection of *tcdA* and *tcdB* genes

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<td>A'B⁺</td>
<td>A'B⁺</td>
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