Isolation of *Clostridium difficile* from food animals in Slovenia

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A total of 313 faecal samples from three pig farms and two cattle farms was collected, and *Clostridium difficile* was isolated from 133/257 piglet samples (51.8%) and from 1/56 calf samples (1.8%). Toxins were tested only in calf samples and were positive in 44.6% (25/56). The only bovine isolate belonged to toxinotype XIa (A\(^+\)B\(^-\)CDT\(^+\)). Porcine isolates belonged to toxinotype 0 (A\(^+\)B\(^+\)CDT\(^+\)) and toxinotype V (A\(^+\)B\(^+\)CDT\(^+\)). Of the two ribotypes usually found in toxinotype V, the strains isolated in this study showed a greater similarity to ribotype 066 than to ribotype 078.

**INTRODUCTION**

*Clostridium difficile* is a Gram-positive, spore-forming, anaerobic bacterium that has been well established as a cause of human intestinal disease, mostly following antibiotic treatment (Bartlett, 1992). In recent years, changes have been observed in the epidemiology of *C. difficile*-associated disease in humans, including increased incidence and severity (McDonald *et al*., 2006). *C. difficile* is also gaining importance as an animal pathogen (Songer & Anderson, 2006).

*C. difficile* has been isolated from a variety of domestic and wild animals. Early studies focused mostly on pets (dogs and cats) and horses (Borriello *et al*., 1983; O’Neill *et al*., 1993; Weese *et al*., 2001a, b; Baverud, 2002; Marks & Kather, 2003). More recent reports have also described *C. difficile* in piglets and calves (Songer *et al*., 2000; Kiss & Bilkei, 2005; Rodriguez-Palacios *et al*., 2006; Keel *et al*., 2007).

So far, it is not clear whether these animals are a possible source of human *C. difficile* infections. However, there is a high level of overlap between *C. difficile* types present in humans and animals (Arroyo *et al*., 2005; Rodriguez-Palacios *et al*., 2007; Rupnik, 2007). Two ribotypes associated with outbreaks of severe disease in humans (017 and 027) have been found in animals also (Lefebvre *et al*., 2006a; Rodriguez-Palacios *et al*., 2006). Modes of transmission between animal and human reservoirs could include retail meat (Rodriguez-Palacios *et al*., 2007), dog food (Weese *et al*., 2005) and contact with the hospital environment (Lefebvre *et al*., 2006b).

The objectives of our work were to evaluate the presence of *C. difficile* in food animals in Slovenia and to check whether the same toxinotypes/PCR ribotypes were found in comparison with studies in North America.

**METHODS**

**Farms, animals and sampling.** Rectal swabs from 257 piglets from three different farms (Fig. 1; farms A, B and C) and stool samples from 56 calves from two different farms (Fig. 1; farms D and E) were analysed for the presence of *C. difficile*. The numbers of samples taken at each farm were: 87 (farm A), 95 (farm B), 75 (farm C), 22 (farm D) and 34 (farm E).

Pig farms A and B had the same owner, occasionally exchanged piglets, had a common supply of animals and food, and share veterinary staff. Pig farm C has no connections with farms A and B. Farms D and E, where the calves were sampled, also have a common owner.

The samples were taken from piglets of 1–10 days of age. Only litters with diarrhoeic animals were chosen, and we sampled five individual animals (including symptomatic and asymptomatic animals) per litter. Litters that contained asymptomatic piglets only were not tested. Calves were up to 21 days of age and all of the tested animals had diarrhoea. All samples, either swabs or stool samples, were processed within 4 h.

**Culture and identification.** The samples were inoculated directly onto standard selective medium with cefoxitin and cycloserine (*C. difficile* selective agar; Oxoid) and incubated in anaerobic jars for 48 h. The isolates were identified based on morphological criteria and typical odour. Identification was confirmed by amplification of the *C. difficile*-specific gene *cdd2* using the primer pair Tim6/Struppi6 (Braun *et al*., 1996) and amplification of *C. difficile*-specific toxin genes during toxinoyping (see below).

Calf samples were additionally screened for *C. difficile* toxins A and B using a commercial enzyme immunoassay (Premier toxins A and B; Meridian Diagnostics). The test was performed according to manufacturer’s instructions.

**Toxinotyping, binary toxin gene detection and PCR ribotyping.**

Toxinotypes were determined in all strains using a standard method based on amplification and subsequent restriction of the toxin A and B genes, *tcdA* (A3 fragment) and *tcdB* (B1 fragment) (Rupnik *et al*., 1998). The presence of a functional gene for the binding component
of the binary toxin (CDTb) was tested using a standard PCR (Stubbs et al., 2000).

Six randomly selected strains from farms A and B and seven from farm C were ribotyped. Ribotyping was carried out using two well-established methods (Bidet et al., 1999; Stubbs et al., 1999). Electrophoresis was performed using certified low range ultra agarose (Bio-Rad) at 80 V for 3 h. The control strains for ribotyping were 51377 (ribotype 066) and R7605 (ribotype 078) (Rupnik et al., 2001).

RESULTS AND DISCUSSION

A total of 313 faecal samples comprising 257 samples from piglets and 56 samples from calves was analysed and Clostridium difficile was isolated from 133 (51.8%) of the piglet samples and from 1 (1.8%) of the calf samples. The low isolation rate in calves was not in agreement with results of toxin tests performed on the same samples, which were positive in 44.6% (25/56) of the samples. All toxin-positive samples were culture negative. Discrepancy between culture results and toxin testing was also noted in a Canadian study, although their reported isolation rates (11.2%) were higher than ours (Rodriguez-Palacios et al., 2007). It is likely that a higher number of carriers is present in the Slovenian calf population, suggesting the need for changes in isolation procedure and the use of enrichment media. On the other hand, these results could also indicate the lack of specificity of the commercial toxin A/B test for calf samples, as it has been validated only for humans. Therefore, alternative toxin testing, such as cytotoxicity, could be used in future studies.

The only culture-positive calf sample was negative in the toxin test, which is not surprising as it was typed as toxinotype Xla. This variant toxinotype is positive for binary toxin, but negative for TcdA and TcdB (A−B−CDT+) and correlates with ribotype 033 (Rupnik et al., 2001). The same ribotype (033) has been reported in calf/bovine strains in Canada (Rodriguez-Palacios et al., 2006) and the USA (Keel et al., 2007).

In comparison with calves, the number of isolated strains from piglets was much larger. However, the variability among strains was low. Piglet isolates belonged to two toxinotypes. All piglet isolates from farms A and B (102 strains) were of toxinotype V (A+B+CDT+), whilst isolates from farm C (31 strains) belonged to toxinotype 0 (A−B−CDT−).

Toxinotype 0 can include strains from more than 150 different PCR ribotypes (Rupnik et al., 2001). To test whether our isolates of toxinotype 0 from farm C belong to a single or to different ribotypes, PCR ribotyping was performed on representative strains. All tested strains showed an identical ribotype, but its identity according to Cardiff numbering could not be determined (data not shown).

Toxinotype V, found in farms A and B, could belong to either ribotype 066 or 078 (Rupnik et al., 2001). We therefore compared representatives from both farms with known ribotype 066 and 078 strains using two established ribotyping methods (Bidet et al., 1999; Stubbs et al., 1999). Both methods grouped our piglet strains together with 066 representatives and not with ribotype 078 (Fig. 2). In contrast to our data, type 078 was recently reported in the majority of C. difficile strains from food animals. PCR ribotype 078 accounted for 94% of bovine and 83% of swine isolates in the study of Keel et al. (2007), and represented a large proportion of strains isolated from calves in Canada (Rodriguez-Palacios et al., 2006).

The overall heterogeneity of bovine and pig strains was low in the USA study of Keel et al. (2007) with three to four ribotypes per animal species, despite the dispersed geographical origin of the animals. The Canadian study
included 102 dairy farms and had a somewhat larger variety, with 8 different ribotypes (Rodriguez-Palacios et al., 2006). We also found only one ribotype per farm and overall only two different types of C. difficile strains in pigs. Farms A and B, with an identical C. difficile type (066), also had the same owner, same veterinarian staff and the occasional exchange of animals between the two farms.

In summary, C. difficile is present in large Slovenian farms of food animals. The toxino-types found in piglets and calves are the same as reported in North American animal studies, although the ribotyping data do not agree entirely.

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REFERENCES


