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Short Communication

Shedding of *Clostridium difficile* PCR ribotype 078 by zoo animals, and report of an unstable metronidazole-resistant isolate from a zebra foal (*Equus quagga burchellii*)☆

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ABSTRACT

Clostridium difficile is an emerging and potentially zoonotic pathogen, but its prevalence in most animal species, including exhibition animals, is currently unknown. In this study we assessed the prevalence of faecal shedding of *C. difficile* by zoo animals, and determined the ribotype, toxin profile and antimicrobial susceptibility of recovered isolates. A total of 200 samples from 40 animal species (36.5% of which came from plains zebra, *Equus quagga burchellii*) were analysed. *C. difficile* was isolated from 7 samples (3.5% of total), which came from the following animal species: chimpanzee (*Pan troglodytes troglodytes*), dwarf goat (*Capra hircus*), and Iberian ibex (*Capra pyrenaica hispanica*), with one positive sample each; and plains zebra, with 4 positive samples from 3 different individuals. Most recovered isolates (4/7, 57.1%) belonged to the epidemic PCR ribotype 078, produced toxins A and B, and had the genes encoding binary toxin (i.e. $A^+B^+CDT^+$ isolates). The remaining three isolates belonged to PCR ribotypes 039 ($A^-B^-CDT^-$), 042 ($A^+B^+CDT^-$) and 110 ($A^-B^+CDT^-$). Regardless of their ribotype, all isolates displayed high-level resistance to the fluoroquinolones ciprofloxacin, enrofloxacin and levofloxacin. Some isolates were also resistant to meropenem and/or ertapenem. A ribotype 078 isolate recovered from a male zebra foal initially showed in vitro resistance to metronidazole (MIC $\geq$ 256 μg/ml), but lost that trait after subculturing on non-selective media. We conclude that zoo animals belonging to different species can carry ribotype 078 and other toxigenic strains of *C. difficile* showing resistance to antimicrobial compounds commonly used in veterinary and/or human medicine.

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1. Introduction

The emergence of *Clostridium difficile* as a human and animal pathogen has prompted considerable effort in elucidating the epidemiology of this anaerobe. In particular, there is huge interest in clarifying if animals can serve as a reservoir of epidemic strains of *C. difficile*, such as those belonging to PCR ribotypes 027 and 078, and could

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eventually act as a source of community-acquired infection (Rodriguez-Palacios et al., 2013).

Most published studies of *C. difficile* prevalence in animals focus on production and companion species (e.g., Álvarez-Pérez et al., 2009, 2013; Zidaric et al., 2012; Wetterwik et al., 2013). In contrast, surveys of *C. difficile* shedding by exotic animals are still scarce, and mainly limited to a few studies in wildlife (Miller et al., 2010; Jardine et al., 2013; Thakur et al., 2011) and some clinical reports of enteric disease (Bojesen et al., 2006; Silva et al., 2013).

In this article we report the faecal shedding of ribotype 078 and other ribotypes of *C. difficile* by zoo animals, and analyse the antimicrobial susceptibility of recovered isolates. Furthermore, one of the studied ribotype 078 isolates recovered from a male zebra foal was found to display high-level but unstable metronidazole resistance.

2. Materials and methods

2.1. Animals and samples

Sampling of 40 animal species (38 mammals and 2 birds; see Table S1, provided as supplementary data with the online version of this paper) was carried out at the Zoo-Aquarium of Madrid (Madrid, Spain). Animals were handled in strict accordance with good animal practice and national legislation.

Fifty rectal swab samples and 149 fresh faecal samples collected from the ground of animal enclosures were analysed for *C. difficile* presence (Table S1). Additionally, a fragment of intestine from a female chimpanzee which died after having some episodes of haemorrhagic diarrhoea of unknown origin empirically treated with several antimicrobial drugs was also analysed, so as to assess the possible participation of *C. difficile* in the disease. All other sampled animals were in a healthy condition and, in particular, they did not present diarrhoea at the time of sampling and were not under antibiotic treatment.

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One hundred and five samples (52.5%) could be assigned to individual animals, while the other 95 samples (47.5%), mostly corresponding to fresh faeces taken from the ground of animal enclosures, remained unassigned (Table S1). Furthermore, as *C. difficile* has been repeatedly isolated from domestic horses (see, e.g., Båverud et al., 2003; Schoster et al., 2012), we were particularly interested in assessing the shedding of the microorganism by other closely related species. Consequently, a significant proportion of the samples analysed in this study ($n = 73$, 36.5%) came from plains zebra. No other equids were available for sampling. Sample size for other animal species ranged from 1 to 14 (mean $\pm$ SD = 3.3 $\pm$ 3.3). Regardless of their origin, all samples were stored at −20 °C and transported in frozen conditions to the laboratory.

2.2. Microbiology procedures

Rectal swab samples were cultured for *C. difficile* isolation by direct plating and using an enrichment procedure, as described in Blanco et al. (2013). In the case of fresh faecal samples, cotton-tipped sterile swabs (Nuova Aptaca, Canelli, Italy) were submerged into the middle of the faeces and then cultured as rectal swabs. The fragment of intestine was introduced into an sterile plastic tube containing 10 ml of the same selective broth used for *C. difficile* enrichment (40 g/l proteose peptone, 5 g/l disodium hydrogen phosphate, 1 g/l potassium dihydrogen phosphate, 0.1 g/l magnesium sulphate, 2 g/l sodium chloride, 6 g/l fructose, 1 g/l sodium taurocholate, 250 mg/l D-cycloserine, 8 mg/l cefoxitin, pH = 7.4; TecLaim, Madrid, Spain) and, after incubation for 7 days at 37 °C under anaerobic conditions, the resulting broth culture was handled as for swab samples (i.e. ethanol shock for spore selection and culturing onto solid medium containing cycloserine and cefoxitin as selective agents).

Bacterial isolates were identified as *C. difficile* by colony morphology (yellowish to white colour, with a ground-glass appearance), the typical odour of this microorganism, and a positive reaction in a rapid specific immunoassay which simultaneously detects the production of glutamate dehydrogenase (GDH), a constitutive antigen of all *C. difficile* isolates (Williams and Spencer, 2009), and toxins A and B (C. Diff Quik Chek Complete; TECHLAB Inc., Blacksburg, VA, USA).

2.3. Molecular characterisation of isolates

The possession of toxin A- and B-encoding genes (*tcdA* and *tcdB*, respectively) was determined by conventional PCR, as described in Álvarez-Pérez et al. (2009). Two additional reactions were used to detect the genes encoding for the enzymatic and binding components of *C. difficile* binary toxin (*cdtA* and *cdtB*, respectively), following the procedures described by Stubbs et al. (2000).

The genetic diversity of *C. difficile* isolates was analysed by PCR ribotyping, using the protocol of Bidet et al. (2000). Ribotypes were designated according to the PHLS Anaerobic Reference Unit (Cardiff, UK) standard nomenclature.

2.4. Antimicrobial susceptibility testing

C. difficile isolates were tested for susceptibility to a panel of 17 antimicrobial agents (Table 1). These assays were performed by the Etest method (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Prereduced Brucella agar supplemented with vitamin K_1 and haemin (bioMérieux) was used as culture medium in all tests, and plates were incubated under anaerobic conditions for 48 h at 37 °C. The breakpoints for antimicrobial resistance were those established by the CLSI for anaerobic bacteria (CLSI, 2007) or used in recent articles on *C. difficile* (Bourgault et al., 2006; Zidaric et al., 2012; Álvarez-Pérez et al., 2013; Peláez et al., 2013), except in the case of enrofloxacin, for which there was no information available and the breakpoint for other fluoroquinolones was assumed.

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