



## Recreational sandboxes for children and dogs can be a source of epidemic ribotypes of *Clostridium difficile*

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Complete List of Authors:	Orden, Cristina; FACULTAD DE VETERINARIA. UNIVERSIDAD COMPLUTENSE, SANIDAD ANIMAL Neila, Carlos; FACULTAD DE VETERINARIA. UNIVERSIDAD COMPLUTENSE, SANIDAD ANIMAL BLANCO, JOSE; FACULTAD DE VETERINARIA. UNIVERSIDAD COMPLUTENSE, SANIDAD ANIMAL ALVAREZ-PEREZ, SERGIO; FACULTAD DE VETERINARIA. UNIVERSIDAD COMPLUTENSE, SANIDAD ANIMAL Harmanus, Céline; Leiden University Medical Center Kuijper, Ed; Leiden, Medicine Microbiology GARCIA, MARTA; Universidad Complutense de Madrid, ANIMAL HEALTH
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8 **3 Recreational sandboxes for children and dogs can be a source of**  
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11 **4 epidemic ribotypes of *Clostridium difficile***  
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16 **6 Cristina Orden<sup>1</sup>, Carlos Neila<sup>1</sup>, José L. Blanco<sup>1</sup>, Sergio Álvarez-Pérez<sup>1</sup>, Celine**  
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18 **7 Harmanus<sup>2</sup>, Ed J. Kuijper<sup>2</sup>, and Marta E. García<sup>1</sup>**  
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23 **9 Short title: *C. difficile* in sandboxes**  
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26 **10**

27  
28 **11 Authors and affiliations**  
29

30 <sup>1</sup> *Department of Animal Health, Faculty of Veterinary, Universidad Complutense de*  
31  
32 *Madrid, Madrid, Spain*  
33

34 <sup>2</sup> *Department of Medical Microbiology, Center of Infectious Diseases, Leiden University*  
35  
36 *Medical Center, Leiden, Netherlands*  
37

38  
39 **16**

40  
41 **17 Correspondence:**  
42

43 Prof. José L. Blanco, PhD, DVM. Departamento de Sanidad Animal, Facultad de  
44 Veterinaria, Universidad Complutense de Madrid. Avda. Puerta de Hierro s/n, 28040  
45  
46 Madrid (Spain). Tel.: +34 91 394 3717. E-mail address: [jlblanco@ucm.es](mailto:jlblanco@ucm.es)  
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3 21 **Impacts**  
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- 5 22 • The sand of public playgrounds can have a role in the transmission of various  
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7 23 infections, particularly in children.  
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10 24 • In this study we demonstrated that the Gram-positive anaerobe *Clostridium difficile* is  
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12 25 widely distributed in soils samples from children's and dog's sandboxes located within  
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14 26 the metropolitan area of Madrid.  
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17 27 • Furthermore, we demonstrated the presence of genetically diverse strains of *C. difficile*,  
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19 28 including the epidemic PCR ribotypes 014 and 106, in the studied sandboxes.  
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## 30 **Summary**

31 Different studies have suggested that the sand of public playgrounds could have a role in  
32 the transmission of infections, particularly in children. Furthermore, free access of pets and  
33 other animals to the playgrounds might increase such a risk. We studied the presence of  
34 *Clostridium difficile* in 20 pairs of sandboxes for children and dogs located in different  
35 playgrounds within the Madrid region (Spain). *C. difficile* isolation was performed by  
36 enrichment and selective culture procedures. The genetic (ribotype and amplified fragment  
37 length polymorphism [AFLP]) diversity and antibiotic susceptibility of isolates was also  
38 studied. Overall, 52.5% (21/40) of samples were positive for the presence of *C. difficile*.  
39 Eight of the 20 available isolates belonged to the toxigenic ribotypes 014 ( $n = 5$ ) and 106 ( $n$   
40  $= 2$ ), both regarded as epidemic, and CD047 ( $n = 1$ ). The other 12 isolates were non-  
41 toxigenic, and belonged to ribotypes 009 ( $n = 5$ ), 039 ( $n = 4$ ), and 067, 151 and CD048  
42 (one isolate each). Nevertheless, all isolates (even those of a same ribotype) were classified  
43 into different AFLP genotypes indicating non-relatedness. In conclusion, our results  
44 revealed the presence of epidemic ribotypes of *C. difficile* in children's and dog's  
45 sandboxes located nearby, which constitutes a major health risk.

46  
47 **Keywords:** *Clostridium difficile*; children; dog; epidemic strains; sandboxes.

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## 49 **Introduction**

50 The soil of playgrounds is a reservoir of diverse parasites and infectious agents (Martínez-  
51 Moreno et al., 2007; Dado et al., 2012; Gotkowska-Płachta and Korzeniewska, 2014; Staley  
52 et al., 2016). Furthermore, free access of domestic and wild animals to recreational areas  
53 can increase the burden of microbiological contamination (Haag-Wackernagel and Moch,  
54 2004; Martínez-Moreno et al., 2007; Dado et al., 2012; Gotkowska-Płachta and  
55 Korzeniewska, 2014; Staley et al., 2016). Children are generally regarded as the main  
56 group at risk for environmental exposure to pathogens, not only because they are frequent  
57 users of playgrounds, but also due to the high prevalence of geophagia (i.e. consumption of  
58 sand) within this group, and the immaturity of their immunological, neurological and  
59 digestive systems (Nwachuku and Gerba, 2004; Dado et al., 2012; Gotkowska-Płachta and  
60 Korzeniewska, 2014).

61 *Clostridium difficile* is a Gram-positive, anaerobic bacterium of widespread  
62 distribution in the environment, where it can survive under adverse conditions through the  
63 production of spores (Hensgens et al., 2012; Smits et al., 2016). This bacterial species was  
64 traditionally regarded as a primarily nosocomial pathogen, but this view has been  
65 challenged as the incidence of *C. difficile* infection (CDI) in people outside hospitals started  
66 to increase (Hensgens et al., 2012; Smits et al., 2016). In this context, diverse animal  
67 species, food products and environmental sources have been suggested to play a role in the  
68 transmission of the *C. difficile* and, in particular, of some epidemic genotypes such as  
69 ribotype 078 (Hensgens et al., 2012; Smits et al., 2016). However, to the best of our  
70 knowledge, the presence of *C. difficile* in sandboxes of playgrounds has only been explored  
71 in a limited number of studies (al Saif and Brazier, 1996; Higazi et al. 2011; Båverud et al.,  
72 2003).

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3 73 In this study we determined the presence of *C. difficile* in 20 pairs of recreational  
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5 74 sandboxes for children and dogs located in different playgrounds within the Madrid region  
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8 75 (Spain). In addition, we compared the isolates recovered from children's and dog's  
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10 76 sandboxes in terms of genetic characteristics and *in vitro* antimicrobial susceptibility.  
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## 78 **Materials and methods**

### 79 *Sampling scheme*

19 80 Sampling was carried out on two consecutive days (July 1-2, 2015) in 20 pairs of children's  
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21 81 and dog's sandboxes located nearby (within 94 m in all cases, mean  $\pm$  S.D. = 35.1  $\pm$  20.5  
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24 82 m; Table 1) in public playgrounds scattered throughout three zones (A, M and V; postal  
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26 83 codes: E-28047, E-28222/E-28221/E-28220 and E-28400, respectively) within the Madrid  
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28 84 region (central Spain) (Figure S1). Therefore, a total of 40 sandboxes (20 for children and  
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31 85 20 for dogs) were analyzed. The number and distribution of samples per sampling zone and  
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33 86 sampling point is indicated in Table 1.  
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35 87 A 200-g sand sample was obtained from each sampling point according to the  
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37 88 procedure described in Córdoba et al. (2002). Briefly, four 50-g sand samples were  
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40 89 collected from different locations within the sampling point using a sterile plastic container  
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42 90 (Nirco, Madrid, Spain). All four sand samples were then thoroughly mixed in a sterile  
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45 91 plastic bag (Nirco), which was transported to the laboratory and kept frozen (-20°C) until  
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47 92 analyzed.  
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3 94 *Microbiological analyses*  
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5 95 Sand samples (50 g each, taken and aseptically weighted from the 200-g mixtures kept in  
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7 96 the freezer) were transferred into sterile one-liter glass bottles, diluted 1:10 in peptone  
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10 97 water (Laboratorios Conda, Madrid, Spain) and incubated under agitation (200 rpm) for 15  
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12 98 min at room temperature. These suspensions were then allowed to settle for 5 min and the  
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14 99 supernatants were filtered through filter membranes (0.45 µm of pore size; Filter Lab,  
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17 100 Barcelona, Spain) following the procedure detailed in Álvarez-Pérez et al. (2016). Filter  
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19 101 membranes were then introduced into 10-ml glass tubes containing 5 ml of selective broth  
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21 102 for enrichment of *C. difficile* (TecLaim, Madrid, Spain; see recipe in Blanco et al., 2013).  
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23 103 After seven days of incubation at 37°C under anaerobiosis, 2 ml of the enrichment culture  
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25 104 were mixed 1:1 with absolute ethanol (Panreac, Barcelona, Spain) in 5 ml sterile plastic  
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27 105 tubes (Nirco) and left for 1 hour under agitation (200 rpm) at room temperature. Finally,  
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29 106 tubes were centrifuged at 1520 g for 10 min, the supernatants were discarded and  
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31 107 precipitates were spread with a sterile cotton-tipped swab (Nirco) onto a plate of CLO agar  
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33 108 (bioMérieux, Marcy l'Etoile, France), which contains cycloserine and cefoxitin as selective  
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35 109 agents. Inoculated plates were incubated under anaerobic conditions for 72 h at 37°C and  
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37 110 suspected colonies were identified as *C. difficile* by colony morphology, the typical odor of  
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39 111 this microorganism, and a positive result in a rapid specific immunoassay for detection of  
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41 112 the constitutive antigen glutamate dehydrogenase (GDH) (C. Diff Quik Chek Complete;  
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43 113 TECHLAB Inc., Blacksburg, VA, USA). The same immunoassay was used to determine  
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45 114 the toxigenic/non toxigenic status of isolates, as it detects production of *C. difficile* toxins A  
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47 115 and B. A single *C. difficile* isolate was selected from each primary culture and sub-cultured  
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49 116 on CLO agar to obtain axenic cultures that could be used in subsequent tests.  
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3 118 *Molecular characterization of isolates*

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5 119 Possession of *tcdA* and *tcdB* genes (which encode for toxins A and B, respectively), and  
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7 120 *cdtA* and *cdtB* (which encode for the two components of binary toxin (CDT), respectively),  
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9 121 was analyzed by conventional PCR protocols (Álvarez-Pérez et al. 2009, 2014, 2015).  
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11 122 Genotyping of isolates was performed by high-resolution capillary gel-based  
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13 123 electrophoresis PCR-ribotyping, following the procedures described in Fawley et al.  
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15 124 (2015). Ribotypes were designated according to the nomenclature of the Leiden (Prof. Ed  
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17 125 Kuijper; The Netherlands)-Leeds (Dr. Warren Fawley and Prof. Mark Wilcox; UK)  
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19 126 database. Novel ribotypes were named using internal reference codes (prefix 'CD' followed  
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21 127 by a number).  
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26 128 Isolates were further genetically characterized by amplified fragment length  
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28 129 polymorphism (AFLP) fingerprinting, using the protocol detailed in Álvarez-Pérez et al.  
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30 130 (2017). A binary 0/1 matrix was created based on the absence/presence of AFLP markers  
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32 131 and a dendrogram of AFLP patterns was created with PAST v.3.11 software (Hammer et  
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34 132 al., 2001) using Pearson's correlation coefficients and the unweighted-pair group method  
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36 133 with arithmetic averages (UPGMA) clustering algorithm. Isolates clustering with <86%  
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38 134 similarity were considered to represent different AFLP genotypes (Killgore et al., 2008;  
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40 135 Álvarez-Pérez et al., 2017).  
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47 137 *Antimicrobial susceptibility testing*

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49 138 In vitro susceptibility of isolates was determined by the Etest (bioMérieux) on prereduced  
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51 139 Brucella agar supplemented with vitamin K1 and haemin (bioMérieux), according to the  
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53 140 manufacturer's instructions. Plates were incubated anaerobically at 37°C and examined at  
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55 141 48 h. Tested antimicrobial compounds and breakpoints for antimicrobial resistance were as  
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3 142 follows: penicillin G,  $\geq 2$   $\mu\text{g/ml}$ ; teicoplanin,  $> 2$   $\mu\text{g/ml}$ ; rifampicin,  $\geq 4$   $\mu\text{g/ml}$ ; linezolid and  
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5 143 tigecycline,  $> 4$   $\mu\text{g/ml}$ ; clindamycin, erythromycin and levofloxacin,  $\geq 8$   $\mu\text{g/ml}$ ; imipenem,  
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7 144 minocycline and tetracycline,  $\geq 16$   $\mu\text{g/ml}$ ; amoxicillin/clavulanic acid,  $\geq 16/8$   $\mu\text{g/ml}$ ; and  
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10 145 metronidazole and vancomycin,  $\geq 32$   $\mu\text{g/ml}$ . (CLSI, 2012; Álvarez-Pérez et al., 2013, 2014,  
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12 146 2015, 2017; Peláez et al. 2013).

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15 147 In order to detect possible metronidazole heteroresistance, which is manifested as a  
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17 148 slow growth of resistant subpopulations within the inhibition halo in the Etest at  
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19 149 concentrations above the resistance breakpoint, metronidazole test plates were further  
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21 150 incubated anaerobically at 37°C for five additional days (Peláez et al., 2008).

#### 22 23 24 151 25 26 152 *Data analysis*

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28 153 Fisher's exact test and Pearson's chi-square test were used for statistical analysis of  
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30 154 categorical data where appropriate. *P*-values of  $< 0.05$  were considered to be statistically  
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32 155 significant in all cases.

#### 33 34 35 156 36 37 157 **Results**

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39 158 *Clostridium difficile* was recovered from 21 (52.5%) of the sand samples analyzed,  
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41 159 collected from 12 and 9 sandboxes located in recreational areas for dogs and children,  
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43 160 respectively (Table 1). The distribution of isolates by sampling (sub)zone and type of  
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45 161 sample (children's or dog's sandboxes) is shown in Table 1. There was no difference in *C.*  
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47 162 *difficile* prevalence between children's and dog's sandboxes ( $P = 0.527$ ) or among  
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49 163 sampling zones ( $P = 0.203$ ). A positive culture result for both samples of each pair was  
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51 164 obtained in five cases, whereas *C. difficile* was recovered only from one sandbox of the pair  
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3 165 in 11 cases (four from children's sandboxes and seven from dog's sandboxes) and a  
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5 166 negative culture result for both samples was obtained in four cases (Table 1).  
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8 167 One *C. difficile* isolate (obtained from a children's sandbox in zone A [sample A-N-  
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10 168 2], Table 1) was lost during subculturing in the laboratory. Eight of the 20 remaining  
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12 169 isolates (six from dog's and two from children's sandboxes) were toxigenic and belonged  
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14 170 to ribotypes 014 ( $A^+B^+CDT^-$ ,  $n = 5$ ), 106 ( $A^+B^+CDT^-$ ,  $n = 2$ ) and CD047 (isolate M-P-4,  
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17 171  $A^+B^+CDT^-$ ) (Tables 1 and S1, Figure 1). The other 12 isolates were non-toxigenic (i.e.  $A^-B^-$   
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19 172  $CDT^-$ ) and belonged to ribotypes 009 ( $n = 5$ ), 039 ( $n = 4$ ), and 067, 151 and CD048 (one  
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21 173 isolate each) (Tables 1 and S1, Figure 1). Further genetic characterization of isolates by  
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23 174 AFLP fingerprinting classified each one of these into a different genotype (Figure 1 and  
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25 175 Table S1). Notably, clustering of isolates in the UPGMA dendrogram obtained from AFLP  
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27 176 data was independent from the origin (both at the '(sub)zone' and 'children vs. dog areas'  
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29 177 levels) and ribotype of isolates (Figure 1).  
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33 178 Regardless of their origin and genotype, all studied isolates showed resistance to  
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35 179 imipenem and levofloxacin (Figure 1 and Table S1). Additionally, the isolates of ribotypes  
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37 180 CD048 and 151 (A-N-8 and V-N-1, respectively) displayed resistance to clindamycin and  
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39 181 erythromycin, and a ribotype 014 isolate (A-P-3) was resistant to penicillin (Figure 1 and  
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41 182 Table S1). MICs to the other antimicrobial compound tested were generally low, and fell  
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43 183 below the resistance breakpoint in all cases (Table S1).  
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47 184 Notably, the samples obtained from a pair of children's and dog's sandboxes in zone  
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49 185 V (V-N-2/V-P-2; Figure 2) yielded *C. difficile* isolates of a same toxigenic ribotype (014)  
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51 186 and which showed a similar antimicrobial susceptibility profile, but the AFLP profiles of  
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53 187 such isolates displayed limited similarity (Pearson's correlation = 0.126) (Figure 1). In  
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3 188 contrast, four pairs of sand samples (A-N-3/A-P-3, A-N-4/A-P-4, A-N-5/A-P-5 and V-N-  
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5 189 1/V-P-1) yielded *C. difficile* isolates of different ribotypes.  
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## 9 10 191 **Discussion**

11  
12 192 The growing number of pets and other animals leaving excrements in the sandboxes of  
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14 193 playgrounds and other recreational areas constitute a serious epidemiological threat  
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16 194 (Martínez-Moreno et al., 2007; Gotkowska-Płachta and Korzeniewska, 2014; Staley et al.,  
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18 195 2016). Current tests for assessing the sanitary conditions of sandboxes focus on detecting  
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20 196 some select pathogenic parasites and bacterial indicators of fecal contamination (Martínez-  
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22 197 Moreno et al., 2007; Gotkowska-Płachta and Korzeniewska, 2014; Staley et al., 2016), but  
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24 198 mostly neglect the possible presence of other emerging pathogens such as *C. difficile*.  
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28 199 Reports of *C. difficile* presence in recreational sandboxes are still limited in number  
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30 200 and of variable scope. For example, Al-Saif and Brazier (1996) reported the isolation of *C.*  
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32 201 *difficile* from a 21% of soil samples taken from public parks, gardens, playgrounds and  
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34 202 other locations in the suburbs of Cardiff, UK. Subsequent characterization of some of those  
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36 203 soil isolates by PCR ribotyping and pyrolysis mass spectrometry (PyMS) fingerprinting  
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38 204 revealed the presence of toxin-producers and different ribotypes (Al Saif et al., 1998).  
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40 205 Similarly, Higazi et al. (2011) investigated by a PCR-based approach the presence of *C.*  
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42 206 *difficile* in soil samples from public parks and elementary school playgrounds in a  
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44 207 Midwestern town of the USA and reported an overall prevalence of 6.5%, but bacterial  
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46 208 isolates were only obtained in some cases and these were not genotyped nor tested for  
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48 209 antimicrobial resistance. Finally, Båverud et al. (2013) observed an overall *C. difficile*  
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50 210 prevalence of 4% in soil samples obtained from public parks, playgrounds, gardens and  
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3 211 cultivated fields, but the origin and characteristics of recovered isolates were not detailed in  
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5 212 their paper.

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7 213 In this study, we demonstrated that *C. difficile* is widely distributed in soils samples  
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9 214 from both children's and dog's sandboxes located within the metropolitan area of  
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11 215 Madrid. Furthermore, our results revealed that recovered isolates were genetically diverse  
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13 216 and displayed resistance to several antibiotics ( $\geq 2$  drugs, including in all cases imipenem  
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15 217 and levofloxacin). Notably, analysis of AFLP fingerprinting results showed high genetic  
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17 218 variation even among isolates obtained from a same sampling (sub)zone.

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19 219 Most *C. difficile* isolates recovered in this study from sandboxes belonged to  
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21 220 ribotypes 014 and 009. The toxigenic ribotype 014 is one of the most prevalent genotypes  
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23 221 isolated from human patients and animals in Europe (including Spain) and other countries  
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25 222 such as Australia, Brazil and the USA (Bauer et al., 2011; Koene et al. 2012; Alcalá et al.  
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27 223 2012, 2015; Janezic et al., 2012, 2014; Tickler et al., 2014; Freeman et al., 2015; Knight et  
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29 224 al., 2015a,b; Silva et al. 2015). Non-toxigenic ribotype 009 is also prevalent in both human  
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31 225 and animal hosts in some countries including Brazil (Silva et al. 2015), but it is rarely  
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33 226 reported in Spain and the rest of Europe (e.g. Koene et al. 2012; Wetterwik et al., 2013;  
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35 227 Álvarez-Pérez et al., 2015).

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37 228 Other ribotypes found in this study such as 039 and 106 are also frequently isolated  
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39 229 from human and/or animal fecal samples (Bauer et al., 2011; Alcalá et al., 2012, 2015;  
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41 230 Koene et al., 2012; Tickler et al., 2014; Freeman, 2015). In particular, ribotype 106 has  
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43 231 been implicated in outbreaks of human disease in the UK (Ratnayake et al., 2011) and is  
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45 232 also relatively common in continental Europe and North America (Bauer et al., 2011;  
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47 233 Alcalá et al., 2012, 2015; Tickler et al., 2014; Freeman et al., 2015). We recently obtained  
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49 234 several ribotype 106 isolates from the feces of dogs with diverse digestive disorders (Orden  
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3 235 et al., 2017). Curiously, despite the frequent shedding of *C. difficile* ribotype 078 by  
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5 236 animals previously observed in Spain (Peláez et al., 2013; Álvarez-Pérez et al., 2013, 2014,  
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7 237 2015) and many other countries (Janezic et al., 2014) we did not found any isolate of this  
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9 238 epidemic ribotype in the present study. Nevertheless, as a single *C. difficile* isolate from  
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11 239 each primary culture was selected for detailed phenotypic and genetic characterization, we  
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13 240 cannot discard the possibility that this and other ribotypes might have been overlooked.  
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17 241 Finally, all isolates characterized in this study displayed high-level in vitro  
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19 242 resistance to imipenem and levofloxacin, a phenotype which is fairly common among  
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21 243 diverse ribotypes of *C. difficile* from different geographic locations (Alcalá et al., 2012;  
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23 244 Keessen et al., 2013; Pirš et al., 2013; Freeman et al., 2015). As carbapenems and  
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25 245 fluoroquinolones are widely used in human and veterinary medicine to treat a diversity of  
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27 246 infections (Papich, 2011; Papp-Wallace et al., 2011; Redgrave et al., 2014), monitoring the  
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29 247 resistance to these compounds in *C. difficile* and other emerging pathogens should be a  
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31 248 priority. Furthermore, some isolates were found to be resistant to erythromycin,  
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33 249 clindamycin and penicillin G, all of which are of common use in clinical practice (Papich,  
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35 250 2011). Although we did not detect any isolate with decreased susceptibility or  
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37 251 heterogeneous resistance to metronidazole, we recommend to determine MIC values to this  
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39 252 antibiotic even for environmental isolates, as metronidazole is still considered a first-line  
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41 253 drug for the treatment of anaerobe infections in human and animal medicine (Dhand and  
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43 254 Snyderman, 2009; Löfmark et al., 2010; Papich, 2016) and (hetero)resistant strains of *C.*  
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45 255 *difficile* and other clostridia have been reported by different authors (Peláez et al., 2008,  
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47 256 2013; Álvarez-Pérez et al., 2013, 2014, 2015, 2017; Wetterwik et al., 2013).  
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## 56 258 **Conclusions**

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3 259 In summary, our results revealed the presence of epidemic ribotypes of *C. difficile* in  
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5 260 children's and dog's sandboxes, which constitutes a major health risk. Due to the zoonotic  
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7 261 potential attributed to some ribotypes of *C. difficile*, the possible presence of this emerging  
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10 262 pathogen should be considered in any environmental risk assessment.  
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27  
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#### 32 33 272 **Declaration of interest**

34  
35 273 None of the authors of this paper has a financial or personal relationship with other people  
36  
37 274 or organizations that could inappropriately influence or bias the content of the paper.  
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437 **List of Tables**

438 **Table 1.** Overview of the samples analyzed in this study and the *Clostridium difficile*  
439 isolates obtained from them.

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For Review Only

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3 441 **Figure Legends**  
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5 442 **Figure 1.** Dendrogram of AFLP profiles obtained for the 20 *Clostridium difficile* isolates  
6  
7 443 characterized in this study. The dendrogram was created by unweighted pair group method  
8  
9 444 with arithmetic averages (UPGMA) clustering using Pearson's correlation coefficients.  
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11 445 Individual AFLP genotypes are distinguished at  $\geq 86\%$  similarity (red dotted vertical line).  
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13 446 Isolates obtained from children's and dog's sandboxes are indicated by blue and yellow  
14  
15 447 backgrounds, respectively. Colored squares at the tip of branches indicate the ribotype (see  
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17 448 color legend on the lower left corner). In vitro resistance to clindamycin (C), erythromycin  
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19 449 (E), imipenem (I), levofloxacin (L) and/or penicillin G (P) is denoted by the red letters next  
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21 450 to strain names.  
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26 451 **Figure 2.** Image showing the children's and dog's sandboxes from zone V which yielded  
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28 452 ribotype 014 *Clostridium difficile* isolates (see details in Results).  
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3 455 **Supporting Information**  
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5 456 Additional Supporting Information may be found in the online version of this article:  
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8 457 **Table S1.** Characteristics of the *Clostridium difficile* isolates analyzed in this study.  
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10 458 **Figure S1.** Schematic representation of the Madrid region (central Spain), indicating the  
11  
12 459 approximate location of the zones from which sand samples were obtained in this study.  
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3 **1 Original Article**  
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8 **3 Recreational sandboxes for children and dogs can be a source of**  
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11 **4 epidemic ribotypes of *Clostridium difficile***  
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16 **6 Cristina Orden<sup>1</sup>, Carlos Neila<sup>1</sup>, José L. Blanco<sup>1</sup>, Sergio Álvarez-Pérez<sup>1</sup>, Celine**  
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18 **7 Harmanus<sup>2</sup>, Ed J. Kuijper<sup>2</sup>, and Marta E. García<sup>1</sup>**  
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23 **9 Short title:** *C. difficile* in sandboxes  
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28 **11 Authors and affiliations**  
29

30 <sup>1</sup> *Department of Animal Health, Faculty of Veterinary, Universidad Complutense de*  
31  
32 *Madrid, Madrid, Spain*  
33

34 <sup>2</sup> *Department of Medical Microbiology, Center of Infectious Diseases, Leiden University*  
35  
36 *Medical Center, Leiden, Netherlands*  
37

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39 16

40  
41 **17 Correspondence:**  
42

43  
44 Prof. José L. Blanco, PhD, DVM. Departamento de Sanidad Animal, Facultad de  
45  
46 Veterinaria, Universidad Complutense de Madrid. Avda. Puerta de Hierro s/n, 28040  
47  
48 Madrid (Spain). Tel.: +34 91 394 3717. E-mail address: [jlblanco@ucm.es](mailto:jlblanco@ucm.es)  
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## 21 **Impacts**

- 22 • The sand of public playgrounds can have a role in the transmission of various  
23 infections, particularly in children. ~~However, most studies published so far have~~  
24 ~~focused on select pathogenic parasites and fecal bacteria.~~
- 25 • In this study we demonstrated that the Gram-positive anaerobe *Clostridium difficile* is  
26 widely distributed in soils samples from children's and dog's sandboxes located within  
27 the metropolitan area of Madrid.
- 28 • Furthermore, we demonstrated the presence of genetically diverse strains of *C. difficile*,  
29 including the epidemic PCR ribotypes 014 and 106, in the studied sandboxes.

## 31 Summary

32 Different studies have suggested that the sand of public playgrounds could have a role in  
33 the transmission of infections, particularly in children. Furthermore, free access of pets and  
34 other animals to the playgrounds might increase such a risk. We studied the presence of  
35 *Clostridium difficile* in 20 pairs of sandboxes for children and dogs located in different  
36 playgrounds within the Madrid region (Spain). *C. difficile* isolation was performed  
37 ~~according to standard~~by enrichment and selective culture procedures. The genetic (ribotype  
38 and amplified fragment length polymorphism [AFLP]) diversity and antibiotic  
39 susceptibility of isolates was also studied. Overall, 52.5% (21/40) of samples were positive  
40 for the presence of *C. difficile*. Eight of the 20 available isolates belonged to the toxigenic  
41 ribotypes 014 ( $n = 5$ ) and 106 ( $n = 2$ ), both regarded as epidemic, and CD047 ( $n = 1$ ). The  
42 other 12 isolates were non-toxigenic, and belonged to ribotypes 009 ( $n = 5$ ), 039 ( $n = 4$ ),  
43 and 067, 151 and CD048 (one isolate each). Nevertheless, all isolates (even those of a same  
44 ribotype) were classified into different AFLP genotypes indicating non-relatedness. In  
45 conclusion, our results revealed the presence of epidemic ribotypes of *C. difficile* in  
46 children's and dog's sandboxes located nearby, which constitutes a major health risk.

47

48 **Keywords:** *Clostridium difficile*; children; dog; epidemic strains; sandboxes.

49

## 50 **Introduction**

51 The soil of playgrounds is a reservoir of diverse parasites and infectious agents (Martínez-  
52 Moreno et al., 2007; Dado et al., 2012; Gotkowska-Płachta and Korzeniewska, 2014; Staley  
53 et al., 2016). Furthermore, free access of domestic and wild animals to recreational areas  
54 can increase the burden of microbiological contamination (Haag-Wackernagel and Moch,  
55 2004; Martínez-Moreno et al., 2007; Dado et al., 2012; Gotkowska-Płachta and  
56 Korzeniewska, 2014; Staley et al., 2016). Children are generally regarded as the main  
57 group at risk for environmental exposure to pathogens, not only because they are frequent  
58 users of playgrounds, but also due to the high prevalence of geophagia (i.e. consumption of  
59 sand) within this group, and the immaturity of their immunological, neurological and  
60 digestive systems (Nwachuku and Gerba, 2004; Dado et al., 2012; Gotkowska-Płachta and  
61 Korzeniewska, 2014).

62 *Clostridium difficile* is a Gram-positive, anaerobic bacterium of widespread  
63 distribution in the environment, where it can survive under adverse conditions through the  
64 production of spores (Hensgens et al., 2012; Smits et al., 2016). This bacterial species was  
65 traditionally regarded as a primarily nosocomial pathogen, but this view has been  
66 challenged as the incidence of *C. difficile* infection (CDI) in people outside hospitals started  
67 to increase (Hensgens et al., 2012; Smits et al., 2016). In this context, diverse animal  
68 species, food products and environmental sources have been suggested to play a role in the  
69 transmission of the *C. difficile* and, in particular, of some epidemic genotypes such as  
70 ribotype 078 (Hensgens et al., 2012; Smits et al., 2016). However, to the best of our  
71 knowledge, the presence of *C. difficile* in sandboxes of playgrounds has only been explored  
72 in a limited number of studies (al Saif and Brazier, 1996; Higazi et al. 2011; Båverud et al.,  
73 2003).

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3 74 In this study we determined the presence of *C. difficile* in 20 pairs of recreational  
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5 75 sandboxes for children and dogs located in different playgrounds within the Madrid region  
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8 76 (Spain). In addition, we compared the isolates recovered from children's and dog's  
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10 77 sandboxes in terms of genetic characteristics and *in vitro* antimicrobial susceptibility.  
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## 14 79 **Materials and methods**

### 16 80 *Sampling scheme*

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18  
19 81 Sampling was carried out on two consecutive days (July 1-2, 2015) in 20 pairs of children's  
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21 82 and dog's sandboxes located nearby (within 94 m in all cases, mean  $\pm$  S.D. = 35.1  $\pm$  20.5  
22  
23 83 m; Table 1) in public playgrounds scattered throughout three zones (A, M and V; postal  
24  
25 84 codes: E-28047, E-28222/E-28221/E-28220 and E-28400, respectively) within the Madrid  
26  
27 85 region (central Spain) (Figure S1). Therefore, a total of 40 sandboxes (20 for children and  
28  
29 86 20 for dogs) were analyzed. The number and distribution of samples per sampling zone and  
30  
31 87 sampling point is indicated in Table 1.  
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35 88 A 200-g sand sample was obtained from each sampling point according to the  
36  
37 89 procedure described in Córdoba et al. (2002). Briefly, four 50-g sand samples were  
38  
39 90 collected from different locations within the sampling point using a sterile plastic container  
40  
41 91 (Nirco, Madrid, Spain). All four sand samples were then thoroughly mixed in a sterile  
42  
43 92 plastic bag (Nirco), which was transported to the laboratory and kept frozen (-20°C) until  
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45 93 analyzed, ~~which took place within 24 h.~~  
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95 *Microbiological analyses*

96 Sand samples (50 g each, taken and aseptically weighted from the 200-g mixtures kept in  
97 the freezer) were transferred into sterile one-liter glass bottles, diluted 1:10 in peptone  
98 water (Laboratorios Conda, Madrid, Spain) and incubated under agitation (200 rpm) for 15  
99 min at room temperature. These suspensions were then allowed to settle for 5 min and the  
100 supernatants were filtered through filter ~~papers-membranes~~ (0.45 µm of pore size; Filter Lab,  
101 Barcelona, Spain) following the procedure detailed in Álvarez-Pérez et al. (2016). Filter  
102 ~~papers-membranes~~ were then introduced into 10-ml glass tubes containing 5 ml of selective  
103 broth for enrichment of *C. difficile* (TecLaim, Madrid, Spain; see recipe in Blanco et al.,  
104 2013). After seven days of incubation at 37°C under anaerobiosis, 2 ml of the enrichment  
105 culture were mixed 1:1 with absolute ethanol (Panreac, Barcelona, Spain) in 5 ml sterile  
106 plastic tubes (Nirco, ~~Madrid, Spain~~) and left for 1 hour under agitation (200 rpm) at room  
107 temperature. Finally, tubes were centrifuged at 1520 g for 10 min, the supernatants were  
108 discarded and precipitates were spread with a sterile cotton-tipped swab (Nirco) onto a  
109 plate of CLO agar (bioMérieux, Marcy l'Etoile, France), which contains cycloserine and  
110 cefoxitin as selective agents. Inoculated plates were incubated under anaerobic conditions  
111 for 72 h at 37°C and suspected colonies were identified as *C. difficile* by colony  
112 morphology, the typical odor of this microorganism, and a positive result in a rapid specific  
113 immunoassay for detection of the constitutive antigen glutamate dehydrogenase (GDH) (C.  
114 Diff Quik Chek Complete; TECHLAB Inc., Blacksburg, VA, USA). The same  
115 immunoassay was used to determine the toxigenic/non toxigenic status of isolates, as it  
116 detects production of *C. difficile* toxins A and B. A single *C. difficile* isolate was selected  
117 from each primary culture and sub-cultured on CLO agar to obtain axenic cultures that  
118 could be used in subsequent tests.

119

120 *Molecular characterization of isolates*

121 Possession of *tcdA* and *tcdB* genes (which encode for toxins A and B, respectively), and  
122 *cdtA* and *cdtB* (which encode for the two components of binary toxin (CDT), respectively),  
123 was analyzed by conventional PCR protocols (Álvarez-Pérez et al. 2009, 2014, 2015).

124 Genotyping of isolates was performed by high-resolution capillary gel-based  
125 electrophoresis PCR-ribotyping, following the procedures described in Fawley et al.

126 (2015). Ribotypes were designated according to the nomenclature of the Leiden ([Prof. Ed](#)

127 [Kuijper; The Netherlands](#))-Leeds ([Dr. Warren Fawley and Prof. Mark Wilcox; UK](#))

128 database (~~The Netherlands~~). ~~If a matching PCR ribotype was not found, the electrophoresis~~  
129 ~~profile was sent to Leeds for a search in the Leeds database of more than 600 PCR~~

130 ~~ribotypes (Dr. Warren Fawley and Prof. Mark Wilcox, Leeds).~~ Novel ribotypes were named  
131 using internal reference codes (prefix 'CD' followed by a number).

132 Isolates were further genetically characterized by amplified fragment length  
133 polymorphism (AFLP) fingerprinting, using the protocol detailed in Álvarez-Pérez et al.  
134 (2017). A binary 0/1 matrix was created based on the absence/presence of AFLP markers  
135 and a dendrogram of AFLP patterns was created with PAST v.3.11 software (Hammer et  
136 al., 2001) using Pearson's correlation coefficients and the unweighted-pair group method  
137 with arithmetic averages (UPGMA) clustering algorithm. Isolates clustering with <86%  
138 similarity were considered to represent different AFLP genotypes (Killgore et al., 2008;  
139 Álvarez-Pérez et al., 2017).

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141 *Antimicrobial susceptibility testing*

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3 142 In vitro susceptibility of isolates was determined by the Etest (bioMérieux) on prereduced  
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5 143 Brucella agar supplemented with vitamin K1 and haemin (bioMérieux), according to the  
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7 144 manufacturer's instructions. Plates were incubated anaerobically at 37°C and examined at  
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10 145 48 h. Tested antimicrobial compounds and breakpoints for antimicrobial resistance were as  
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12 146 follows: penicillin G,  $\geq 2$   $\mu\text{g/ml}$ ; teicoplanin,  $> 2$   $\mu\text{g/ml}$ ; rifampicin,  $\geq 4$   $\mu\text{g/ml}$ ; linezolid and  
13  
14 147 tigecycline,  $> 4$   $\mu\text{g/ml}$ ; clindamycin, erythromycin and levofloxacin,  $\geq 8$   $\mu\text{g/ml}$ ; imipenem,  
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16 148 minocycline and tetracycline,  $\geq 16$   $\mu\text{g/ml}$ ; amoxicillin/clavulanic acid,  $\geq 16/8$   $\mu\text{g/ml}$ ; and  
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18 149 metronidazole and vancomycin,  $\geq 32$   $\mu\text{g/ml}$ . (CLSI, 2012; Álvarez-Pérez et al., 2013, 2014,  
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20 150 2015, 2017; Peláez et al. 2013).

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23  
24 151 In order to detect possible metronidazole heteroresistance, which is manifested as a  
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26 152 slow growth of resistant subpopulations within the inhibition halo in the Etest at  
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28 153 concentrations above the resistance breakpoint, metronidazole test plates were further  
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30 154 incubated anaerobically at 37°C for five additional days (Peláez et al., 2008).

### 31 155 32 33 34 35 156 *Data analysis*

36  
37 157 Fisher's exact test and Pearson's chi-square test were used for statistical analysis of  
38  
39 158 categorical data where appropriate. *P*-values of  $< 0.05$  were considered to be statistically  
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41 159 significant in all cases.  
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45 160

## 46 161 **Results**

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48 162 *Clostridium difficile* was recovered from 21 (52.5%) of the sand samples analyzed,  
49  
50 163 collected from 12 and 9 sandboxes located in recreational areas for dogs and children,  
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52 164 respectively (Table 1). The distribution of isolates by sampling (sub)zone and type of  
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54 165 sample (children's or dog's sandboxes) is shown in Table 1. There was no difference in *C.*



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3 166 *difficile* prevalence between children's and dog's sandboxes ( $P = 0.527$ ) or among  
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5 167 sampling zones ( $P = 0.203$ ). A positive culture result for both samples of each pair was  
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7 168 obtained in five cases, whereas *C. difficile* was recovered only from one sandbox of the pair  
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9 169 in 11 cases (four from children's sandboxes and seven from dog's sandboxes) and a  
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11 170 negative culture result for both samples was obtained in four cases (Table 1).

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14 171 One *C. difficile* isolate (obtained from a children's sandbox in zone A [sample A-N-  
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16 172 2], Table 1) was lost during subculturing in the laboratory. Eight of the 20 remaining  
17  
18 173 isolates (~~seven~~six from dog's and ~~four~~two from children's sandboxes) were toxigenic and  
19  
20 174 belonged to ribotypes 014 ( $A^+B^+CDT^-$ ,  $n = 5$ ), 106 ( $A^+B^+CDT^-$ ,  $n = 2$ ) and CD047 (isolate  
21  
22 175 M-P-4,  $A^+B^+CDT^-$ ) (Tables 1 and S1, Figure 1). The other ~~eight~~12 isolates were non-  
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24 176 toxigenic (i.e.  $A^-B^-CDT^-$ ) and belonged to ribotypes 009 ( $n = 5$ ), 039 ( $n = 4$ ), and 067, 151  
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26 177 and CD048 (one isolate each) (Tables 1 and S1, Figure 1). Further genetic characterization  
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28 178 of isolates by AFLP fingerprinting classified each one of these into a different genotype  
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30 179 (Figure 1 and Table S1). Notably, clustering of isolates in the UPGMA dendrogram  
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32 180 obtained from AFLP data was independent from the origin (both at the '(sub)zone' and  
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34 181 'children vs. dog areas' levels) and ribotype of isolates (Figure 1).

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36 182 Regardless of their origin and genotype, all studied isolates showed resistance to  
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38 183 imipenem and levofloxacin (Figure 1 and Table S1). Additionally, the isolates of ribotypes  
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40 184 CD048 and 151 (A-N-8 and V-N-1, respectively) displayed- resistance to clindamycin and  
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42 185 erythromycin, and a ribotype 014 isolate (A-P-3) was resistant to penicillin (Figure 1 and  
43  
44 186 Table S1). MICs to the other antimicrobial compound tested were generally low, and fell  
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46 187 below the resistance breakpoint in all cases (Table S1).

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48  
49 188 Notably, the samples obtained from a pair of children's and dog's sandboxes in zone  
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51 189 V (V-N-2/V-P-2; Figure 2) yielded *C. difficile* isolates of a same toxigenic ribotype (014)

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3 190 and which showed a similar antimicrobial susceptibility profile, but the AFLP profiles of  
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5 191 such isolates displayed limited similarity (Pearson's correlation = 0.126) (Figure 1). In  
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7 192 contrast, four pairs of sand samples (A-N-3/A-P-3, A-N-4/A-P-4, A-N-5/A-P-5 and V-N-  
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9 193 1/V-P-1) yielded *C. difficile* isolates of different ribotypes.

## 14 195 **Discussion**

16  
17 196 The growing number of pets and other animals leaving excrements in the sandboxes of  
18  
19 197 playgrounds and other recreational areas constitute a serious epidemiological threat  
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21 198 (Martínez-Moreno et al., 2007; Gotkowska-Płachta and Korzeniewska, 2014; Staley et al.,  
22  
23 199 2016). Current tests for assessing the sanitary conditions of sandboxes ~~mostly~~ focus on  
24  
25 200 detecting some select pathogenic parasites and bacterial indicators of fecal contamination  
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27 201 (Martínez-Moreno et al., 2007; Gotkowska-Płachta and Korzeniewska, 2014; Staley et al.,  
28  
29 202 2016), but mostly neglect the possible presence of other emerging pathogens such as *C.*  
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31 203 *difficile*.

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35 204 Reports of *C. difficile* presence in recreational sandboxes are still limited in number  
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37 205 and of variable scope. For example, Al-Saif and Brazier (1996) reported the isolation of *C.*  
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39 206 *difficile* from a 21% of soil samples taken from public parks, gardens, playgrounds and  
40  
41 207 other locations in the suburbs of Cardiff, UK. Subsequent characterization of some of those  
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43 208 soil isolates by PCR ribotyping and pyrolysis mass spectrometry (PyMS) fingerprinting  
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45 209 revealed the presence of toxin-producers and different ribotypes (Al Saif et al., 1998).  
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47 210 Similarly, Higazi et al. (2011) investigated by a PCR-based approach the presence of *C.*  
48  
49 211 *difficile* in soil samples from public parks and elementary school playgrounds in a  
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51 212 Midwestern town of the USA and reported an overall prevalence of 6.5%, but bacterial  
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53 213 isolates were only obtained in some cases and these were not genotyped nor tested for

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3 214 antimicrobial resistance. Finally, Båverud et al. (2013) observed an overall *C. difficile*  
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5 215 prevalence of 4% in soil samples obtained from public parks, playgrounds, gardens and  
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7 216 cultivated fields, but the origin and characteristics of recovered isolates were not detailed in  
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9  
10 217 their paper.

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12 218 In this study, we demonstrated that *C. difficile* is widely distributed in soils samples  
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14 219 from both children's and dog's sandboxes located within the metropolitan area of  
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17 220 Madrid. Furthermore, our results revealed that recovered isolates were genetically diverse  
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19 221 and displayed resistance to several antibiotics ( $\geq 2$  drugs, including in all cases imipenem  
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21 222 and levofloxacin). Notably, analysis of AFLP fingerprinting results showed high genetic  
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23 223 variation even among isolates obtained from a same sampling (sub)zone.

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26 224 Most *C. difficile* isolates recovered in this study from sandboxes belonged to  
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28 225 ribotypes 014 and 009. The toxigenic ribotype 014 is one of the most prevalent genotypes  
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30 226 isolated from human patients and animals in Europe (including Spain) and other countries  
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32 227 such as Australia, Brazil and the USA (Bauer et al., 2011; Koene et al. 2012; Alcalá et al.  
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35 228 2012, 2015; Janezic et al., 2012, 2014; Tickler et al., 2014; Freeman et al., 2015; Knight et  
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37 229 al., 2015a,b; Silva et al. 2015). Non-toxigenic ribotype 009 is also prevalent in both human  
38  
39 230 and animal hosts in some countries including Brazil (Silva et al. 2015), but it is rarely  
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41 231 reported in Spain and the rest of Europe (e.g. Koene et al. 2012; Wetterwik et al., 2013;  
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43 232 Álvarez-Pérez et al., 2015).

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46 233 Other ribotypes found in this study such as 039 and 106 are also frequently isolated  
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48 234 from human and/or animal fecal samples (Bauer et al., 2011; Alcalá et al., 2012, 2015;  
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50 235 Koene et al., 2012; Tickler et al., 2014; Freeman, 2015). In particular, ribotype 106 has  
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52 236 been implicated in outbreaks of human disease in the UK (Ratnayake et al., 2011) and is  
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54 237 also relatively common in continental Europe and North America (Bauer et al., 2011;  
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3 238 Alcalá et al., 2012, 2015; Tickler et al., 2014; Freeman et al., 2015). We recently obtained  
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5 239 several ribotype 106 isolates from the feces of dogs with diverse digestive disorders (Orden  
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7 240 et al., 2017). Curiously, despite the frequent shedding of *C. difficile* ribotype 078 by  
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9 241 animals previously observed in Spain (Peláez et al., 2013; Álvarez-Pérez et al., 2013, 2014,  
10  
11 242 2015) and many other countries (Janezic et al., 2014) we did not find any isolate of this  
12  
13 243 epidemic ribotype in the present study. Nevertheless, as a single *C. difficile* isolate from  
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15 244 each primary culture was selected ~~from each primary culture~~ for detailed phenotypic and  
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17 245 genetic characterization, we cannot discard the possibility that this and other ribotypes  
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19 246 might have been overlooked.

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24 247 Finally, all isolates characterized in this study displayed high-level ~~in~~ in vitro  
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26 248 resistance to imipenem and levofloxacin, a phenotype which -is fairly common among  
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28 249 ~~clinical *C. difficile* isolates of~~ diverse ribotypes of *C. difficile* from different geographic  
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30 250 locations (Alcalá et al., 2012; Keessen et al., 2013; Pirš et al., 2013; Freeman et al., 2015).  
31  
32 251 As -carbapenems and fluoroquinolones are widely used in human and veterinary medicine  
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34 252 to treat a diversity of infections (Papich, 2011; Papp-Wallace et al., 2011; Redgrave et al.,  
35  
36 253 2014), monitoring the resistance to these compounds in *C. difficile* and other emerging  
37  
38 254 pathogens should be a priority. Furthermore, some isolates were found to be resistant to  
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40 255 erythromycin, clindamycin and penicillin G, all of which are of common use in clinical  
41  
42 256 practice (Papich, 2011). Although we did not detect any isolate with decreased  
43  
44 257 susceptibility or heterogeneous resistance to metronidazole, we recommend to determine  
45  
46 258 MIC values to this antibiotic even for environmental isolates, as metronidazole is still  
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48 259 considered a first-line drug for the treatment of anaerobe infections in human and animal  
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50 260 medicine (Dhand and Snyderman, 2009; Löfmark et al., 2010; Papich, 2016) and  
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52 261 (hetero)resistant strains of *C. difficile* and other clostridia have been reported by different  
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3 262 authors (Peláez et al., 2008, 2013; Álvarez-Pérez et al., 2013, 2014, 2015, 2017; Wetterwik  
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5 263 et al., 2013).  
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## 9 10 265 **Conclusions**

11  
12 266 In summary, our results revealed the presence of epidemic ribotypes of *C. difficile* in  
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14 267 children's and dog's sandboxes, which constitutes a major health risk. Due to the zoonotic  
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17 268 potential attributed to some ribotypes of *C. difficile*, the possible presence of this emerging  
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19 269 pathogen should be considered in any environmental risk assessment.  
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22 270

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31  
32  
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34  
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36  
37 277 excellent technical assistance.  
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40 278

## 41 42 279 **Declaration of interest**

43  
44 280 None of the authors of this paper has a financial or personal relationship with other people  
45  
46 281 or organizations that could inappropriately influence or bias the content of the paper.  
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48  
49 282

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3 445 **List of Tables**  
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5 446 **Table 1.** Overview of the samples analyzed in this study and the *Clostridium difficile*  
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8 447 isolates obtained from them.  
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3 449 **Figure Legends**  
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5 450 **Figure 1.** Dendrogram of AFLP profiles obtained for the 20 *Clostridium difficile* isolates  
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7 451 characterized in this study. The dendrogram was created by unweighted pair group method  
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9 452 with arithmetic averages (UPGMA) clustering using Pearson's correlation coefficients.  
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11 453 Individual AFLP genotypes are distinguished at  $\geq 86\%$  similarity (red dotted vertical line).

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14 454 Isolates obtained from children's and dog's sandboxes are indicated by blue and yellow  
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16 backgrounds, respectively. Colored squares at the tip of branches indicate the ribotype (see  
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18 455 color legend on the lower left corner). In vitro resistance to clindamycin (C), erythromycin  
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20 456 (E), imipenem (I), levofloxacin (L) and/or penicillin G (P) is denoted by the red letters next  
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22 457 to strain names.  
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26 459 **Figure 2.** Image showing the children's and dog's sandboxes from zone V which yielded  
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28 460 ribotype 014 *Clostridium difficile* isolates (see details in Results).  
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3 463 **Supporting Information**  
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5 464 Additional Supporting Information may be found in the online version of this article:  
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7  
8 465 **Table S1.** Characteristics of the *Clostridium difficile* isolates analyzed in this study.  
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10 466 **Figure S1.** Schematic representation of the Madrid region (central Spain), indicating the  
11  
12 467 approximate location of the zones from which sand samples were obtained in this study.  
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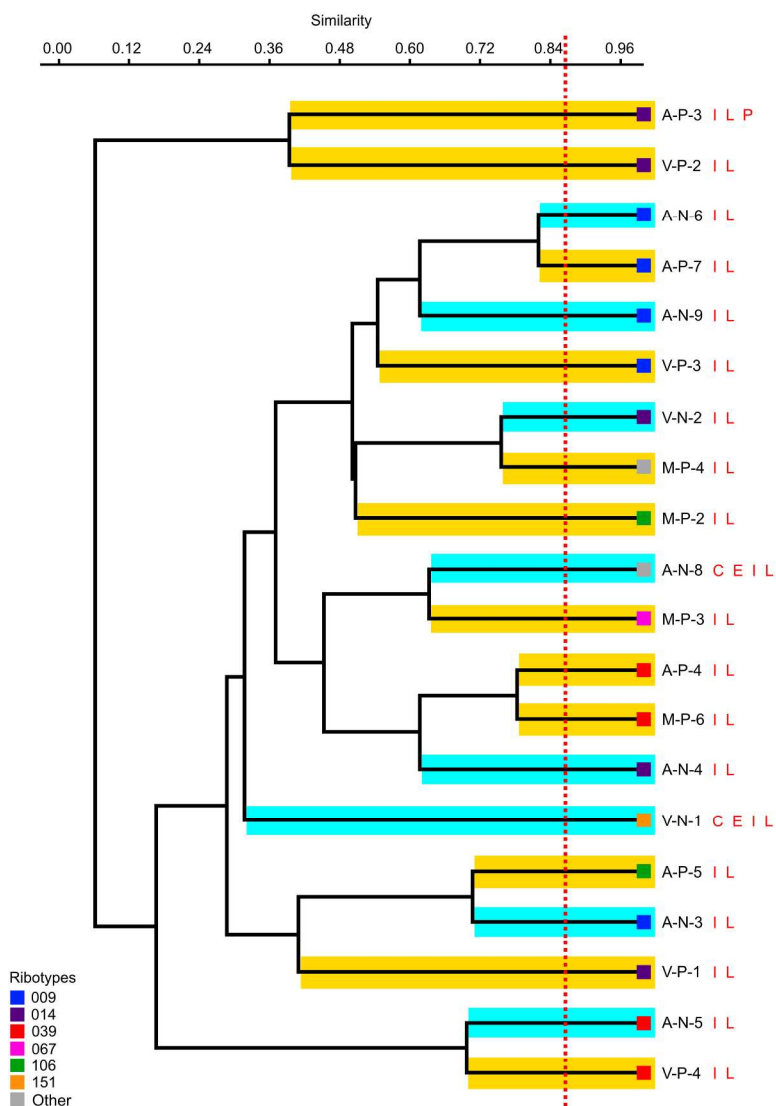
1 **Table 1.** Overview of the samples analyzed in this study and the *Clostridium difficile* isolates obtained from them.

Sampling zone (subzones)	Sampling point	Children’s sandboxes			Dog’s sandboxes			Distance between sandboxes†	
		Sample’s code	Positive for <i>C. difficile</i> ?	Ribotype (toxin profile)	Sample’s code	Positive for <i>C. difficile</i> ?	Ribotype (toxin profile)		
A	1	A-N-1	No		A-P-1	No		36 m	
	2	A-N-2*	Yes	ND (+)*	A-P-2	No		26 m	
	3	A-N-3	Yes	009 (A <sup>+</sup> B <sup>-</sup> CDT <sup>-</sup> )	A-P-3	Yes	014 (A <sup>+</sup> B <sup>+</sup> CDT <sup>-</sup> )	60 m	
	4	A-N-4	Yes	014 (A <sup>+</sup> B <sup>+</sup> CDT <sup>-</sup> )	A-P-4	Yes	039 (A <sup>-</sup> B <sup>-</sup> CDT <sup>-</sup> )	0 m	
	5	A-N-5	Yes	039 (A <sup>-</sup> B <sup>-</sup> CDT <sup>-</sup> )	A-P-5	Yes	106 (A <sup>+</sup> B <sup>+</sup> CDT <sup>-</sup> )	0 m	
	6	A-N-6	Yes	009 (A <sup>-</sup> B <sup>-</sup> CDT <sup>-</sup> )	A-P-6	No		20 m	
	7	A-N-7	No		A-P-7	Yes	009 (A <sup>-</sup> B <sup>-</sup> CDT <sup>-</sup> )	50 m	
	8	A-N-8	Yes	CD048 (A <sup>-</sup> B <sup>-</sup> CDT <sup>-</sup> )	A-P-8	No		50 m	
	9	A-N-9	Yes	009 (A <sup>-</sup> B <sup>-</sup> CDT <sup>-</sup> )	A-P-9	No		40 m	
	10	A-N-10	No		A-P-10	No		30 m	
M	M.1	M-N-1	No		M-P-1	No		25 m	
		M-N-2	No		M-P-2	Yes	106 (A <sup>+</sup> B <sup>+</sup> CDT <sup>-</sup> )	20 m	
		M-N-3	No		M-P-3	Yes	067 (A <sup>-</sup> B <sup>-</sup> CDT <sup>-</sup> )	94 m	
	M.2	5	M-N-5	No	M-P-5	No		40 m	
	M.3	4	M-N-4	No		M-P-4	Yes	CD047 (A <sup>+</sup> B <sup>+</sup> CDT <sup>-</sup> )	46 m
		6	M-N-6	No		M-P-6	Yes	039 (A <sup>-</sup> B <sup>-</sup> CDT <sup>-</sup> )	17 m
V	1	V-N-1	Yes	151 (A <sup>-</sup> B <sup>-</sup> CDT <sup>-</sup> )	V-P-1	Yes	014 (A <sup>+</sup> B <sup>+</sup> CDT <sup>-</sup> )	30 m	
	2	V-N-2	Yes	014 (A <sup>+</sup> B <sup>+</sup> CDT <sup>-</sup> )	V-P-2	Yes	014 (A <sup>+</sup> B <sup>+</sup> CDT <sup>-</sup> )	46 m	
	3	V-N-3	No		V-P-3	Yes	009 (A <sup>-</sup> B <sup>-</sup> CDT <sup>-</sup> )	42 m	
	4	V-N-4	No		V-P-4	Yes	039 (A <sup>-</sup> B <sup>-</sup> CDT <sup>-</sup> )	30 m	

2 \* ND: not determined (this isolates was lost during subculturing in the laboratory).

3 † Distance between the children’s and dog’s sandboxes of each sampling point.





Dendrogram of AFLP profiles obtained for the 20 *Clostridium difficile* isolates characterized in this study. The dendrogram was created by unweighted pair group method with arithmetic averages (UPGMA) clustering using Pearson’s correlation coefficients. Individual AFLP genotypes are distinguished at  $\geq 86\%$  similarity (red dotted vertical line). Colored squares at the tip of branches indicate the ribotype (see color legend on the lower left corner). In vitro resistance to clindamycin (C), erythromycin (E), imipenem (I), levofloxacin (L) and/or penicillin G (P) is denoted by the red letters next to strain names.

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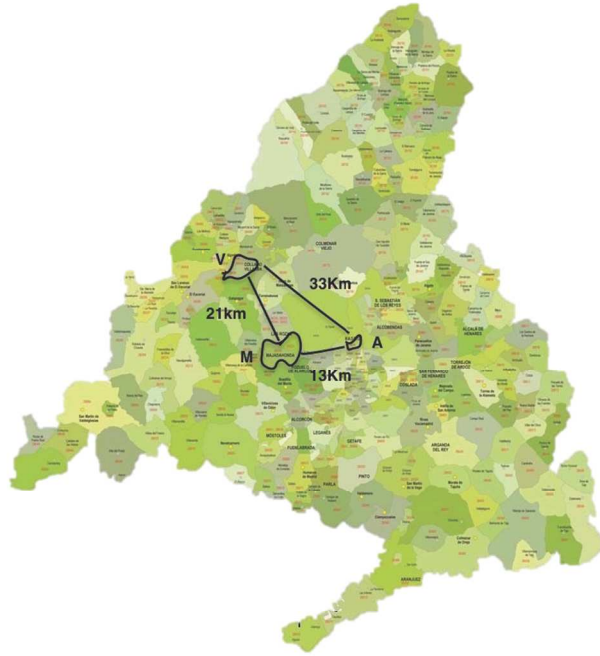


Image showing the children's and dog's sandboxes from zone V which yielded ribotype 014 Clostridium difficile isolates (see details in Results).

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