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Recreational sandboxes for children and dogs can be a source of epidemic ribotypes of Clostridium dificile

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2 3	1	Original Article
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10 11 12	4	epidemic ribotypes of <i>Clostridium dificile</i>
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The sand of public playgrounds can have a role in the transmission of various

In this study we demonstrated that the Gram-positive anaerobe Clostridium difficile is

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Impacts

widely distributed in soils samples from children's and dog's sandboxes located within the metropolitanean area of Madrid. .ne pi .c ribotypes 0. Furthermore, we demonstrated the presence of genetically diverse strains of C. difficile, • including the epidemic PCR ribotypes 014 and 106, in the studied sandboxes.

infections, particularly in children.

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 <i>C. difficile</i> .
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

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

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

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In this study we determined the presence of *C. difficile* in 20 pairs of recreational
sandboxes for children and dogs located in different playgrounds within the Madrid region
(Spain). In addition, we compared the isolates recovered from children's and dog's
sandboxes in terms of genetic characteristics and *in vitro* antimicrobial susceptibility.

78 Materials and methods

79 *Sampling scheme*

Sampling was carried out on two consecutive days (July 1-2, 2015) in 20 pairs of children's and dog's sandboxes located nearby (within 94 m in all cases, mean \pm S.D. = 35.1 \pm 20.5 m; Table 1) in public playgrounds scattered throughout three zones (A, M and V; postal codes: E-28047, E-28222/E-28221/E-28220 and E-28400, respectively) within the Madrid region (central Spain) (Figure S1). Therefore, a total of 40 sandboxes (20 for children and 20 for dogs) were analyzed. The number and distribution of samples per sampling zone and sampling point is indicated in Table 1.

A 200-g sand sample was obtained from each sampling point according to the
procedure described in Córdoba et al. (2002). Briefly, four 50-g sand samples were
collected from different locations within the sampling point using a sterile plastic container
(Nirco, Madrid, Spain). All four sand samples were then thoroughly mixed in a sterile
plastic bag (Nirco), which was transported to the laboratory and kept frozen (-20°C) until
analyzed.

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94 Microbiological analyses

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

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2 3 4	118	Molecular characterization of isolates
5 6	119	Possession of <i>tcdA</i> and <i>tcdB</i> genes (which encode for toxins A and B, respectively), and
7 8	120	cdtA and cdtB (which encode for the two components of binary toxin (CDT), respectively),
9 10 11	121	was analyzed by conventional PCR protocols (Álvarez-Pérez et al. 2009, 2014, 2015).
12 13	122	Genotyping of isolates was performed by high-resolution capillary gel-based
14 15	123	electrophoresis PCR-ribotyping, following the procedures described in Fawley et al.
16 17 18	124	(2015). Ribotypes were designated according to the nomenclature of the Leiden (Prof. Ed
19 20	125	Kuijper; The Netherlands)-Leeds (Dr. Warren Fawley and Prof. Mark Wilcox; UK)
21 22 22	126	database. Novel ribotypes were named using internal reference codes (prefix 'CD' followed
23 24 25	127	by a number).
26 27	128	Isolates were further genetically characterized by amplified fragment length
28 29 30	129	polynorphism (AFLP) fingerprinting, using the protocol detailed in Álvarez-Pérez et al.
31 32	130	(2017). A binary 0/1 matrix was created based on the absence/presence of AFLP markers
33 34	131	and a dendrogram of AFLP patterns was created with PAST v.3.11 software (Hammer et
35 36 27	132	al., 2001) using Pearson's correlation coefficients and the unweighted-pair group method
37 38 39	133	with arithmetic averages (UPGMA) clustering algorithm. Isolates clustering with <86%
40 41	134	similarity were considered to represent different AFLP genotypes (Killgore et al., 2008;
42 43	135	Álvarez-Pérez et al., 2017).
44 45 46	136	
47 48	137	Antimicrobial susceptibility testing
49 50	138	In vitro susceptibility of isolates was determined by the Etest (bioMérieux) on prereduced
51 52 53	139	Brucella agar supplemented with vitamin K1 and haemin (bioMérieux), according to the
54 55	140	manufacturer's instructions. Plates were incubated anaerobically at 37°C and examined at
56 57	141	48 h. Tested antimicrobial compounds and breakpoints for antimicrobial resistance were as
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2 3 4	142	follows: penicillin G, $\geq 2 \ \mu g/ml$; teicoplanin, $\geq 2 \ \mu g/ml$; rifampicin, $\geq 4 \ \mu g/ml$; linezolid and
5 6	143	tigecycline, >4 μ g/ml; clindamycin, erythromycin and levofloxacin, ≥8 μ g/ml; imipenem,
7 8	144	minocycline and tetracycline, $\geq 16 \ \mu g/ml$; amoxicillin/clavulanic acid, $\geq 16/8 \ \mu g/ml$; and
9 10 11	145	metronidazole and vancomycin, ≥32 µg/ml. (CLSI, 2012; Álvarez-Pérez et al., 2013, 2014,
12 13	146	2015, 2017; Peláez et al. 2013).
14 15	147	In order to detect possible metronidazole heteroresistance, which is manifested as a
16 17 18	148	slow growth of resistant subpopulations within the inhibition halo in the Etest at
19 20	149	concentrations above the resistance breakpoint, metronidazole test plates were further
21 22	150	incubated anaerobically at 37°C for five additional days (Peláez et al., 2008).
23 24 25	151	
26 27	152	Data analysis
28 29	153	Fisher's exact test and Pearson's chi-square test were used for statistical analysis of
30 31 22	154	categorical data where appropriate. P -values of <0.05 were considered to be statistically
32 33 34	155	significant in all cases.
35 36	156	
37 38	157	Results
39 40 41	158	Clostridium difficile was recovered from 21 (52.5%) of the sand samples analyzed,
42 43	159	collected from 12 and 9 sandboxes located in recreational areas for dogs and children,
44 45	160	respectively (Table 1). The distribution of isolates by sampling (sub)zone and type of
46 47 48	161	sample (children's or dog's sandboxes) is shown in Table 1. There was no difference in C.
49 50	162	<i>difficile</i> prevalence between children's and dog's sandboxes ($P = 0.527$) or among
51 52	163	sampling zones ($P = 0.203$). A positive culture result for both samples of each pair was
53 54	164	obtained in five cases, whereas C. difficile was recovered only from one sandbox of the pair
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3 4	165	in 11 cases (four from children's sandboxes and seven from dog's sandboxes) and a
5 6	166	negative culture result for both samples was obtained in four cases (Table 1).
7 8	167	One C. difficile isolate (obtained from a children's sandbox in zone A [sample A-N-
9 10	168	2], Table 1) was lost during subculturing in the laboratory. Eight of the 20 remaining
11 12 13	169	isolates (six from dog's and two from children's sandboxes) were toxigenic and belonged
14 15	170	to ribotypes 014 ($A^+B^+CDT^-$, $n = 5$), 106 ($A^+B^+CDT^-$, $n = 2$) and CD047 (isolate M-P-4,
16 17	171	A ⁺ B ⁺ CDT ⁻) (Tables 1 and S1, Figure 1). The other 12 isolates were non-toxigenic (i.e. A ⁻ B ⁻
18 19	172	CDT) and belonged to ribotypes 009 ($n = 5$), 039 ($n = 4$), and 067, 151 and CD048 (one
20 21 22	173	isolate each) (Tables 1 and S1, Figure 1). Further genetic characterization of isolates by
22 23 24	174	AFLP fingerprinting classified each one of these into a different genotype (Figure 1 and
25 26	175	Table S1) Notably clustering of isolates in the UPGMA dendrogram obtained from AFLP
27 28	176	data was independent from the origin (both at the '(sub)zone' and 'children vs. dog areas'
29 30	170	levele) and ribetime of iceletes (Figure 1)
31 22	1//	levels) and fibotype of isolates (Figure 1).
32 33 34	178	Regardless of their origin and genotype, all studied isolates showed resistance to
35 36	179	imipenem and levofloxacin (Figure 1 and Table S1). Additionally, the isolates of ribotypes
37 38	180	CD048 and 151 (A-N-8 and V-N-1, respectively) displayed resistance to clindamycin and
39 40	181	erythromycin, and a ribotype 014 isolate (A-P-3) was resistant to penicillin (Figure 1 and
41 42 42	182	Table S1). MICs to the other antimicrobial compound tested were generally low, and fell
43 44 45	183	below the resistance breakpoint in all cases (Table S1).
46 47	184	Notably, the samples obtained from a pair of children's and dog's sandboxes in zone
48 49	185	V (V-N-2/V-P-2; Figure 2) yielded <i>C. difficile</i> isolates of a same toxigenic ribotype (014)
50 51	186	and which showed a similar antimicrobial susceptibility profile, but the AFLP profiles of
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54 55	187	such isolates displayed limited similarity (Pearson's correlation = 0.126) (Figure 1). In
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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-N189 1/V-P-1) yielded *C. difficile* isolates of different ribotypes.

 191 Discussion

The growing number of pets and other animals leaving excrements in the sandboxes of playgrounds and other recreational areas constitute a serious epidemiological threat (Martínez-Moreno et al., 2007; Gotkowska-Płachta and Korzeniewska, 2014; Staley et al., 2016). Current tests for assessing the sanitary conditions of sandboxes focus on detecting some select pathogenic parasites and bacterial indicators of fecal contamination (Martínez-Moreno et al., 2007; Gotkowska-Płachta and Korzeniewska, 2014; Staley et al., 2016), but mostly neglect the possible presence of other emerging pathogens such as C. difficile. Reports of *C. difficile* presence in recreational sandboxes are still limited in number and of variable scope. For example, Al-Saif and Brazier (1996) reported the isolation of C. *difficile* from a 21% of soil samples taken from public parks, gardens, playgrounds and other locations in the suburbs of Cardiff, UK. Subsequent characterization of some of those soil isolates by PCR ribotyping and pyrolysis mass spectrometry (PyMS) fingerprinting revealed the presence of toxin-producers and different ribotypes (Al Saif et al., 1998). Similarly, Higazi et al. (2011) investigated by a PCR-based approach the presence of C. *difficile* in soil samples from public parks and elementary school playgrounds in a Midwestern town of the USA and reported an overall prevalence of 6.5%, but bacterial isolates were only obtained in some cases and these were not genotyped nor tested for antimicrobial resistance. Finally, Båverud et al. (2013) observed an overall C. difficile prevalence of 4% in soil samples obtained from public parks, playgrounds, gardens and

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cultivated fields, but the origin and characteristics of recovered isolates were not detailed intheir paper.

In this study, we demonstrated that *C. difficile* is widely distributed in soils samples from both children's and dog's sandboxes located within the metropolitanean area of Madrid. Furthermore, our results revealed that recovered isolates were genetically diverse and displayed resistance to several antibiotics (≥ 2 drugs, including in all cases imipenem and levofloxacin). Notably, analysis of AFLP fingerprinting results showed high genetic variation even among isolates obtained from a same sampling (sub)zone. Most *C. difficile* isolates recovered in this study from sandboxes belonged to

ribotypes 014 and 009. The toxigenic ribotype 014 is one of the most prevalent genotypes 220 221 isolated from human patients and animals in Europe (including Spain) and other countries such as Australia, Brazil and the USA (Bauer et al., 2011; Koene et al. 2012; Alcalá et al. 222 223 2012, 2015; Janezic et al., 2012, 2014; Tickler et al., 2014; Freeman et al., 2015; Knight et al., 2015a,b; Silva et al. 2015). Non-toxigenic ribotype 009 is also prevalent in both human 224 225 and animal hosts in some countries including Brazil (Silva et al. 2015), but it is rarely 226 reported in Spain and the rest of Europe (e.g. Koene et al. 2012; Wetterwik et al., 2013; Álvarez-Pérez et al., 2015). 227

Other ribotypes found in this study such as 039 and 106 are also frequently isolated from human and/or animal fecal samples (Bauer et al., 2011; Alcalá et al., 2012, 2015; Koene et al., 2012; Tickler et al., 2014; Freeman, 2015). In particular, ribotype 106 has been implicated in outbreaks of human disease in the UK (Ratnayake et al., 2011) and is also relatively common in continental Europe and North America (Bauer et al., 2011; Alcalá et al., 2012, 2015; Tickler et al., 2014; Freeman et al., 2015). We recently obtained several ribotype 106 isolates from the feces of dogs with diverse digestive disorders (Orden

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3 4	235	et al., 2017). Curiously, despite the frequent shedding of C. difficile ribotype 078 by
5 6	236	animals previously observed in Spain (Peláez et al., 2013; Álvarez-Pérez et al., 2013, 2014,
7 8	237	2015) and many other countries (Janezic et al., 2014) we did not found any isolate of this
9 10	238	epidemic ribotype in the present study. Nevertheless, as a single C. difficile isolate from
11 12 13	239	each primary culture was selected for detailed phenotypic and genetic characterization, we
14 15	240	cannot discard the possibility that this and other ribotypes might have been overlooked.
16 17	241	Finally, all isolates characterized in this study displayed high-level in vitro
18 19 20	242	resistance to imipenem and levofloxacin, a phenotype which is fairly common among
20 21 22	243	diverse ribotypes of C. difficile from different geographic locations (Alcalá et al., 2012;
23 24	244	Keessen et al., 2013; Pirš et al., 2013; Freeman et al., 2015). As carbapenems and
25 26 27	245	fluoroquinolones are widely used in human and veterinary medicine to treat a diversity of
27 28 29	246	infections (Papich, 2011; Papp-Wallace et al., 2011; Redgrave et al., 2014), monitoring the
30 31	247	resistance to these compounds in C. difficile and other emerging pathogens should be a
32 33	248	priority. Furthermore, some isolates were found to be resistant to erythromycin,
34 35 36	249	clindamycin and penicillin G, all of which are of common use in clinical practice (Papich,
37 38	250	2011). Although we did not detect any isolate with decreased susceptibility or
39 40	251	heterogeneous resistance to metronidazole, we recommend to determine MIC values to this
41 42 42	252	antibiotic even for environmental isolates, as metronidazole is still considered a first-line
44 45	253	drug for the treatment of anaerobe infections in human and animal medicine (Dhand and
46 47	254	Snydman, 2009; Löfmark et al., 2010; Papich, 2016) and (hetero)resistant strains of C.
48 49	255	difficile and other clostridia have been reported by different authors (Peláez et al., 2008,
50 51 52	256	2013; Álvarez-Pérez et al., 2013, 2014, 2015, 2017; Wetterwik et al., 2013).
53 54	257	
55 56	258	Conclusions
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Declaration of interest

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In summary, our results revealed the presence of epidemic ribotypes of C. difficile in

pathogen should be considered in any environmental risk assessment.

children's and dog's sandboxes, which constitutes a major health risk. Due to the zoonotic

potential attributed to some ribotypes of *C. difficile*, the possible presence of this emerging

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or organizations that could inappropriately influence or bias the content of the paper.

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2 3 4	437	List of Tables
5 6	438	Table 1. Overview of the samples analyzed in this study and the Clostridium difficile
7 8	439	isolates obtained from them.
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 950 51 52	439	isolates obtained from them.
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441 Figure Legends

442 Figure 1. Dendrogram of AFLP profiles obtained for the 20 *Clostridium difficile* isolates characterized in this study. The dendrogram was created by unweighted pair group method 443 with arithmetic averages (UPGMA) clustering using Pearson's correlation coefficients. 444 445 Individual AFLP genotypes are distinguished at \geq 86% similarity (red dotted vertical line). Isolates obtained from children's and dog's sandboxes are indicated by blue and yellow 446 backgrounds, respectively. Colored squares at the tip of branches indicate the ribotype (see 447 color legend on the lower left corner). In vitro resistance to clindamycin (C), erythromycin 448 (E), imipenem (I), levofloxacin (L) and/or penicillin G (P) is denoted by the red letters next 449 450 to strain names. Figure 2. Image showing the children's and dog's sandboxes from zone V which yielded 451 ribotype 014 Clostridium difficile isolates (see details in Results). 452 453 454 22 Zoonoses and Public Health

455 Supporting Information

456 Additional Supporting Information may be found in the online version of this article:

- **Table S1.** Characteristics of the *Clostridium difficile* isolates analyzed in this study.
- 458 Figure S1. Schematic representation of the Madrid region (central Spain), indicating the
- 459 approximate location of the zones from which sand samples were obtained in this study.

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1 Original Article

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3	Recreational sandboxes for children and dogs can be a source of
4	epidemic ribotypes of <i>Clostridium dificile</i>
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6	Cristina Orden ¹ , Carlos Neila ¹ , José L. Blanco ¹ , Sergio Álvarez-Pérez ¹ , Celine
7	Harmanus ² , Ed J. Kuijper ² , and Marta E. García ¹
8	
9	Short title: C. difficile in sandboxes
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2 3 4	21	Impacts
5 6	22	• The sand of public playgrounds can have a role in the transmission of various
7 8	23	infections, particularly in children. However, most studies published so far have
9 10 11	24	focused on select pathogenic parasites and fecal bacteria.
12 13	25	• In this study we demonstrated that the Gram-positive anaerobe <i>Clostridium difficile</i> is
14 15	26	widely distributed in soils samples from children's and dog's sandboxes located within
16 17 18	27	the metropolitanean area of Madrid.
19 20	28	• Furthermore, we demonstrated the presence of genetically diverse strains of <i>C. difficile</i> ,
21 22	29	including the epidemic PCR ribotypes 014 and 106, in the studied sandboxes.
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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 standardby 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.
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48	Keywords: Clostridium difficile; children; dog; epidemic strains; sandboxes.

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50 Introduction

51 The soil of playgrounds is a reservoir of diverse parasites and infectious agents (Martínez-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 54 can increase the burden of microbiological contamination (Haag-Wackernagel and Moch, 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 73 2003).

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In this study we determined the presence of *C. difficile* in 20 pairs of recreational sandboxes for children and dogs located in different playgrounds within the Madrid region (Spain). In addition, we compared the isolates recovered from children's and dog's sandboxes in terms of genetic characteristics and *in vitro* antimicrobial susceptibility. Materials and methods Sampling scheme

Sampling was carried out on two consecutive days (July 1-2, 2015) in 20 pairs of children's 81 and dog's sandboxes located nearby (within 94 m in all cases, mean \pm S.D. = 35.1 \pm 20.5 82 m; Table 1) in public playgrounds scattered throughout three zones (A, M and V; postal 83 codes: E-28047, E-28222/E-28221/E-28220 and E-28400, respectively) within the Madrid 84 region (central Spain) (Figure S1). Therefore, a total of 40 sandboxes (20 for children and 85 20 for dogs) were analyzed. The number and distribution of samples per sampling zone and 86 sampling point is indicated in Table 1. 87 A 200-g sand sample was obtained from each sampling point according to the 88 procedure described in Córdoba et al. (2002). Briefly, four 50-g sand samples were 89

collected from different locations within the sampling point using a sterile plastic container
(Nirco, Madrid, Spain). All four sand samples were then thoroughly mixed in a sterile
plastic bag (Nirco), which was transported to the laboratory and kept frozen (-20°C) until
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 the freezer) were transferred into sterile one-liter glass bottles, diluted 1:10 in peptone 97 water (Laboratorios Conda, Madrid, Spain) and incubated under agitation (200 rpm) for 15 98 99 min at room temperature. These suspensions were then allowed to settle for 5 min and the supernatants were filtered though filter papers-membranes (0.45 µm of pore size; Filter Lab, 100 Barcelona, Spain) following the procedure detailed in Álvarez-Pérez et al. (2016). Filter 101 papers membranes were then introduced into 10-ml glass tubes containing 5 ml of selective 102 broth for enrichment of C. difficile (TecLaim, Madrid, Spain; see recipe in Blanco et al., 103 2013). After seven days of incubation at 37°C under anaerobiosis, 2 ml of the enrichment 104 culture were mixed 1:1 with absolute ethanol (Panreac, Barcelona, Spain) in 5 ml sterile 105 plastic tubes (Nirco, Madrid, Spain) and left for 1 hour under agitation (200 rpm) at room 106 107 temperature. Finally, tubes were centrifuged at 1520 g for 10 min, the supernatants were discarded and precipitates were spread with a sterile cotton-tipped swab (Nirco) onto a 108 plate of CLO agar (bioMérieux, Marcy l'Etoile, France), which contains cycloserine and 109 110 cefoxitin as selective agents. Inoculated plates were incubated under anaerobic conditions for 72 h at 37°C and suspected colonies were identified as *C. difficile* by colony 111 morphology, the typical odor of this microorganism, and a positive result in a rapid specific 112 immunoassay for detection of the constitutive antigen glutamate dehydrogenase (GDH) (C. 113 Diff Quik Chek Complete; TECHLAB Inc., Blacksburg, VA, USA). The same 114 115 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 from each primary culture and sub-cultured on CLO agar to obtain axenic cultures that 117 could be used in subsequent tests. 118

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120	Molecular characterization of isolates
121	Possession of <i>tcdA</i> and <i>tcdB</i> 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	polynorphism (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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2 3 4	142	In vitro susceptibility of isolates was determined by the Etest (bioMérieux) on prereduced
5 6	143	Brucella agar supplemented with vitamin K1 and haemin (bioMérieux), according to the
7 8 9	144	manufacturer's instructions. Plates were incubated anaerobically at 37°C and examined at
) 10 11	145	48 h. Tested antimicrobial compounds and breakpoints for antimicrobial resistance were as
12 13	146	follows: penicillin G, $\geq 2 \ \mu g/ml$; teicoplanin, $\geq 2 \ \mu g/ml$; rifampicin, $\geq 4 \ \mu g/ml$; linezolid and
14 15	147	tigecycline, >4 μ g/ml; clindamycin, erythromycin and levofloxacin, ≥8 μ g/ml; imipenem,
16 17 18	148	minocycline and tetracycline, $\geq 16 \ \mu g/ml$; amoxicillin/clavulanic acid, $\geq 16/8 \ \mu g/ml$; and
19 20	149	metronidazole and vancomycin, ≥32 µg/ml. (CLSI, 2012; Álvarez-Pérez et al., 2013, 2014,
21 22	150	2015, 2017; Peláez et al. 2013).
23 24 25	151	In order to detect possible metronidazole heteroresistance, which is manifested as a
25 26 27	152	slow growth of resistant subpopulations within the inhibition halo in the Etest at
28 29	153	concentrations above the resistance breakpoint, metronidazole test plates were further
30 31	154	incubated anaerobically at 37°C for five additional days (Peláez et al., 2008).
32 33 34	155	
35 36	156	Data analysis
37 38	157	Fisher's exact test and Pearson's chi-square test were used for statistical analysis of
39 40 41	158	categorical data where appropriate. <i>P</i> -values of <0.05 were considered to be statistically
41 42 43	159	significant in all cases.
44 45	160	
46 47	161	Results
48 49 50	162	Clostridium difficile was recovered from 21 (52.5%) of the sand samples analyzed,
50 51 52	163	collected from 12 and 9 sandboxes located in recreational areas for dogs and children,
53 54	164	respectively (Table 1). The distribution of isolates by sampling (sub)zone and type of
55 56 57	165	sample (children's or dog's sandboxes) is shown in Table 1. There was no difference in C.
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166	<i>difficile</i> prevalence between children's and dog's sandboxes ($P = 0.527$) or among
167	sampling zones ($P = 0.203$). A positive culture result for both samples of each pair was
168	obtained in five cases, whereas C. difficile was recovered only from one sandbox of the pair
169	in 11 cases (four from children's sandboxes and seven from dog's sandboxes) and a
170	negative culture result for both samples was obtained in four cases (Table 1).
171	One C. difficile isolate (obtained from a children's sandbox in zone A [sample A-N-
172	2], Table 1) was lost during subculturing in the laboratory. Eight of the 20 remaining
173	isolates (seven-six from dog's and four-two from children's sandboxes) were toxigenic and
174	belonged to ribotypes 014 ($A^+B^+CDT^-$, $n = 5$), 106 ($A^+B^+CDT^-$, $n = 2$) and CD047 (isolate
175	M-P-4, A ⁺ B ⁺ CDT ⁻) (Tables 1 and S1, Figure 1). The other eight <u>12</u> isolates were non-
176	toxigenic (i.e. A ⁻ B ⁻ CDT ⁻) and belonged to ribotypes 009 ($n = 5$), 039 ($n = 4$), and 067, 151
177	and CD048 (one isolate each) (Tables 1 and S1, Figure 1). Further genetic characterization
178	of isolates by AFLP fingerprinting classified each one of these into a different genotype
179	(Figure 1 and Table S1). Notably, clustering of isolates in the UPGMA dendrogram
180	obtained from AFLP data was independent from the origin (both at the '(sub)zone' and
181	'children vs. dog areas' levels) and ribotype of isolates (Figure 1).
182	Regardless of their origin and genotype, all studied isolates showed resistance to
183	imipenem and levofloxacin (Figure 1 and Table S1). Additionally, the isolates of ribotypes
184	CD048 and 151 (A-N-8 and V-N-1, respectively) displayed- resistance to clindamycin and
185	erythromycin, and a ribotype 014 isolate (A-P-3) was resistant to penicillin (Figure 1 and
186	Table S1). MICs to the other antimicrobial compound tested were generally low, and fell
187	below the resistance breakpoint in all cases (Table S1).
188	Notably, the samples obtained from a pair of children's and dog's sandboxes in zone
189	V (V-N-2/V-P-2; Figure 2) yielded C. difficile isolates of a same toxigenic ribotype (014)

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190	and which showed a similar antimicrobial susceptibility profile, but the AFLP profiles of
191	such isolates displayed limited similarity (Pearson's correlation = 0.126) (Figure 1). In
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-
193	1/V-P-1) yielded C. difficile isolates of different ribotypes.
194	
195	Discussion
196	The growing number of pets and other animals leaving excrements in the sandboxes of
197	playgrounds and other recreational areas constitute a serious epidemiological threat
198	(Martínez-Moreno et al., 2007; Gotkowska-Płachta and Korzeniewska, 2014; Staley et al.,
199	2016). Current tests for assessing the sanitary conditions of sandboxes mostly focus on
200	detecting some select pathogenic parasites and bacterial indicators of fecal contamination
201	(Martínez-Moreno et al., 2007; Gotkowska-Płachta and Korzeniewska, 2014; Staley et al.,
202	2016), but mostly neglect the possible presence of other emerging pathogens such as C .
203	difficile.
204	Reports of C. difficile presence in recreational sandboxes are still limited in number
205	and of variable scope. For example, Al-Saif and Brazier (1996) reported the isolation of <i>C</i> .
206	difficile from a 21% of soil samples taken from public parks, gardens, playgrounds and
207	other locations in the suburbs of Cardiff, UK. Subsequent characterization of some of those
208	soil isolates by PCR ribotyping and pyrolysis mass spectrometry (PyMS) fingerprinting
209	revealed the presence of toxin-producers and different ribotypes (Al Saif et al., 1998).
210	Similarly, Higazi et al. (2011) investigated by a PCR-based approach the presence of C.
211	difficile in soil samples from public parks and elementary school playgrounds in a
212	Midwestern town of the USA and reported an overall prevalence of 6.5%, but bacterial
213	isolates were only obtained in some cases and these were not genotyped nor tested for

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

In this study, we demonstrated that *C. difficile* is widely distributed in soils samples
from both children's and dog's sandboxes located within the metropolitanean area of
Madrid. Furthermore, our results revealed that recovered isolates were genetically diverse
and displayed resistance to several antibiotics (≥2 drugs, including in all cases imipenem
and levofloxacin). Notably, analysis of AFLP fingerprinting results showed high genetic
variation even among isolates obtained from a same sampling (sub)zone.

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

Other ribotypes found in this study such as 039 and 106 are also frequently isolated from human and/or animal fecal samples (Bauer et al., 2011; Alcalá et al., 2012, 2015; Koene et al., 2012; Tickler et al., 2014; Freeman, 2015). In particular, ribotype 106 has been implicated in outbreaks of human disease in the UK (Ratnayake et al., 2011) and is also relatively common in continental Europe and North America (Bauer et al., 2011;

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3 4	238	Alcalá et al., 2012, 2015; Tickler et al., 2014; Freeman et al., 2015). We recently obtained
5 6	239	several ribotype 106 isolates from the feces of dogs with diverse digestive disorders (Orden
7 8	240	et al., 2017). Curiously, despite the frequent shedding of C. difficile ribotype 078 by
9 10 11	241	animals previously observed in Spain (Peláez et al., 2013; Álvarez-Pérez et al., 2013, 2014,
12 13	242	2015) and many other countries (Janezic et al., 2014) we did not found any isolate of this
14 15	243	epidemic ribotype in the present study. Nevertheless, as a single <i>C. difficile</i> isolate from
16 17 18	244	each primary culture was selected from each primary culture for detailed phenotypic and
19 20	245	genetic characterization, we cannot discard the possibility that this and other ribotypes
21 22	246	might have been overlooked.
23 24 25	247	Finally, all isolates characterized in this study displayed high-level Iin vitro
26 27	248	resistance to imipenem and levofloxacin, a phenotype which -is fairly common among
28 29	249	elinical C. difficile isolates of diverse ribotypes of C. difficile from different geographic
30 31	250	locations (Alcalá et al., 2012; Keessen et al., 2013; Pirš et al., 2013; Freeman et al., 2015).
32 33 34	251	As -carbapenems and fluoroquinolones are widely used in human and veterinary medicine
35 36	252	to treat a diversity of infections (Papich, 2011; Papp-Wallace et al., 2011; Redgrave et al.,
37 38	253	2014), monitoring the resistance to these compounds in C. difficile and other emerging
39 40 41	254	pathogens should be a priority. Furthermore, some isolates were found to be resistant to
42 43	255	erythromycin, clindamycin and penicillin G, all of which are of common use in clinical
44 45	256	practice (Papich, 2011). Although we did not detect any isolate with decreased
46 47 48	257	susceptibility or heterogeneous resistance to metronidazole, we recommend to determine
49 50	258	MIC values to this antibiotic even for environmental isolates, as metronidazole is still
51 52	259	considered a first-line drug for the treatment of anaerobe infections in human and animal
53 54	260	medicine (Dhand and Snydman, 2009; Löfmark et al., 2010; Papich, 2016) and
55 56 57	261	(hetero)resistant strains of C. difficile and other clostridia have been reported by different
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3 4	262	authors (Peláez et al., 2008, 2013; Álvarez-Pérez et al., 2013, 2014, 2015, 2017; Wetterwik
5 6	263	et al., 2013).
7 8	264	
9 10 11	265	Conclusions
12 13	266	In summary, our results revealed the presence of epidemic ribotypes of C. difficile in
14 15	267	children's and dog's sandboxes, which constitutes a major health risk. Due to the zoonotic
16 17 18	268	potential attributed to some ribotypes of <i>C. difficile</i> , the possible presence of this emerging
19 20	269	pathogen should be considered in any environmental risk assessment.
21 22	270	
23 24 25	271	Acknowledgements
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33 34	275	collection and interpretation, or the decision to submit the work for publication. We thank
35 36	276	the staff of the Genomics Service at Universidad Complutense de Madrid for providing
37 38	277	excellent technical assistance.
39 40 41	278	
42 43	279	Declaration of interest
44 45	280	None of the authors of this paper has a financial or personal relationship with other people
46 47 48	281	or organizations that could inappropriately influence or bias the content of the paper.
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445	List of Tables
446	Table 1. Overview of the samples analyzed in this study and the Clostridium difficile
447	isolates obtained from them.
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	Figure Legends
	Figure 1. 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).
	Isolates obtained from children's and dog's sandboxes are indicated by blue and yellow
	backgrounds, respectively. Colored squares at the tip of branches indicate the ribotype (see
I	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.
I	Figure 2. Image showing the children's and dog's sandboxes from zone V which yielded
	ribotype 014 Clostridium difficile isolates (see details in Results).

463 Supporting Information

464 Additional Supporting Information may be found in the online version of this article:

- **Table S1.** Characteristics of the *Clostridium difficile* isolates analyzed in this study.
- 466 Figure S1. Schematic representation of the Madrid region (central Spain), indicating the
- 467 approximate location of the zones from which sand samples were obtained in this study.

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

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

* 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 ≥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.

207x277mm (300 x 300 DPI)



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

176x132mm (150 x 150 DPI)

33Km

21km



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210x297mm (128 x 128 DPI)