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Original Article

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

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Short title: *C. difficile* in sandboxes

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Impacts

- The sand of public playgrounds can have a role in the transmission of various infections, particularly in children.
- In this study we demonstrated that the Gram-positive anaerobe *Clostridium difficile* is widely distributed in soils samples from children's and dog's sandboxes located within the metropolitanean area of Madrid.
- Furthermore, we demonstrated the presence of genetically diverse strains of *C. difficile*, including the epidemic PCR ribotypes 014 and 106, in the studied sandboxes.

Summary

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

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

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).

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 and dog's sandboxes located nearby (within 94 m in all cases, mean ± S.D. = 35.1 ± 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 (-20ºC) until analyzed.

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.

Molecular characterization of isolates

Possession of *tcdA* and *tcdB* genes (which encode for toxins A and B, respectively), and *cdtA* and *cdtB* (which encode for the two components of binary toxin (CDT), respectively), was analyzed by conventional PCR protocols (Álvarez-Pérez et al. 2009, 2014, 2015). Genotyping of isolates was performed by high-resolution capillary gel-based electrophoresis PCR-ribotyping, following the procedures described in Fawley et al. (2015). Ribotypes were designated according to the nomenclature of the Leiden (Prof. Ed Kuijper; The Netherlands)-Leeds (Dr. Warren Fawley and Prof. Mark Wilcox; UK) database. Novel ribotypes were named using internal reference codes (prefix 'CD' followed by a number).

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

Antimicrobial susceptibility testing

In vitro susceptibility of isolates was determined by the Etest (bioMérieux) on prereduced Brucella agar supplemented with vitamin K1 and haemin (bioMérieux), according to the manufacturer's instructions. Plates were incubated anaerobically at 37°C and examined at 48 h. Tested antimicrobial compounds and breakpoints for antimicrobial resistance were as

follows: penicillin G, ≥2 µg/ml; teicoplanin, >2 µg/ml; rifampicin, ≥4 µg/ml; linezolid and tigecycline, >4 µg/ml; clindamycin, erythromycin and levofloxacin, ≥8 µg/ml; imipenem, minocycline and tetracycline, ≥16 µg/ml; amoxicillin/clavulanic acid, ≥16/8 µg/ml; and metronidazole and vancomycin, ≥32 µg/ml. (CLSI, 2012; Álvarez-Pérez et al., 2013, 2014, 2015, 2017; Peláez et al. 2013).

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

Data analysis

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

Results

Clostridium difficile was recovered from 21 (52.5%) of the sand samples analyzed, collected from 12 and 9 sandboxes located in recreational areas for dogs and children, respectively (Table 1). The distribution of isolates by sampling (sub)zone and type of sample (children's or dog's sandboxes) is shown in Table 1. There was no difference in *C. difficile* prevalence between children's and dog's sandboxes ($P = 0.527$) or among sampling zones ($P = 0.203$). A positive culture result for both samples of each pair was obtained in five cases, whereas *C. difficile* was recovered only from one sandbox of the pair

in 11 cases (four from children's sandboxes and seven from dog's sandboxes) and a negative culture result for both samples was obtained in four cases (Table 1).

One *C. difficile* isolate (obtained from a children's sandbox in zone A [sample A-N-2], Table 1) was lost during subculturing in the laboratory. Eight of the 20 remaining isolates (six from dog's and two from children's sandboxes) were toxigenic and belonged to ribotypes 014 ($A^+B^+CDT^-$, $n = 5$), 106 ($A^+B^+CDT^-$, $n = 2$) and CD047 (isolate M-P-4, $A^+B^+CDT^-$) (Tables 1 and S1, Figure 1). The other 12 isolates were non-toxigenic (i.e. $A^-B^-CDT^-$) and belonged to ribotypes 009 ($n = 5$), 039 ($n = 4$), and 067, 151 and CD048 (one isolate each) (Tables 1 and S1, Figure 1). Further genetic characterization of isolates by AFLP fingerprinting classified each one of these into a different genotype (Figure 1 and Table S1). Notably, clustering of isolates in the UPGMA dendrogram obtained from AFLP data was independent from the origin (both at the '(sub)zone' and 'children vs. dog areas' levels) and ribotype of isolates (Figure 1).

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

Notably, the samples obtained from a pair of children's and dog's sandboxes in zone V (V-N-2/V-P-2; Figure 2) yielded *C. difficile* isolates of a same toxigenic ribotype (014) and which showed a similar antimicrobial susceptibility profile, but the AFLP profiles of such isolates displayed limited similarity (Pearson's correlation = 0.126) (Figure 1). In

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-1/V-P-1) yielded *C. difficile* isolates of different ribotypes.

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

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; 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

et al., 2017). Curiously, despite the frequent shedding of *C. difficile* ribotype 078 by animals previously observed in Spain (Peláez et al., 2013; Álvarez-Pérez et al., 2013, 2014, 2015) and many other countries (Janezic et al., 2014) we did not found any isolate of this epidemic ribotype in the present study. Nevertheless, as a single *C. difficile* isolate from each primary culture was selected for detailed phenotypic and genetic characterization, we cannot discard the possibility that this and other ribotypes might have been overlooked.

Finally, all isolates characterized in this study displayed high-level in vitro resistance to imipenem and levofloxacin, a phenotype which is fairly common among diverse ribotypes of *C. difficile* from different geographic locations (Alcalá et al., 2012; Keessen et al., 2013; Pirš et al., 2013; Freeman et al., 2015). As carbapenems and fluoroquinolones are widely used in human and veterinary medicine to treat a diversity of infections (Papich, 2011; Papp-Wallace et al., 2011; Redgrave et al., 2014), monitoring the resistance to these compounds in *C. difficile* and other emerging pathogens should be a priority. Furthermore, some isolates were found to be resistant to erythromycin, clindamycin and penicillin G, all of which are of common use in clinical practice (Papich, 2011). Although we did not detect any isolate with decreased susceptibility or heterogeneous resistance to metronidazole, we recommend to determine MIC values to this antibiotic even for environmental isolates, as metronidazole is still considered a first-line drug for the treatment of anaerobe infections in human and animal medicine (Dhand and Snydman, 2009; Löfmark et al., 2010; Papich, 2016) and (hetero)resistant strains of *C. difficile* and other clostridia have been reported by different authors (Peláez et al., 2008, 2013; Álvarez-Pérez et al., 2013, 2014, 2015, 2017; Wetterwik et al., 2013).

Conclusions

In summary, our results revealed the presence of epidemic ribotypes of *C. difficile* in 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 pathogen should be considered in any environmental risk assessment.

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Declaration of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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

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 ≥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 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.

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).

Supporting Information

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.

Figure S1. Schematic representation of the Madrid region (central Spain), indicating the approximate location of the zones from which sand samples were obtained in this study.

Original Article

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

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Short title: *C. difficile* in sandboxes

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Impacts

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

Summary

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

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

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).

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 and dog's sandboxes located nearby (within 94 m in all cases, mean ± S.D. = 35.1 ± 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 (-20ºC) until analyzed~~, which took place within 24 h~~.

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 ~~papers~~ membranes (0.45 µm of pore size; Filter Lab, Barcelona, Spain) following the procedure detailed in Álvarez-Pérez et al. (2016). Filter ~~papers~~ 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~~, Madrid, Spain~~) 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.

Molecular characterization of isolates

Possession of *tcdA* and *tcdB* genes (which encode for toxins A and B, respectively), and *cdtA* and *cdtB* (which encode for the two components of binary toxin (CDT), respectively), was analyzed by conventional PCR protocols (Álvarez-Pérez et al. 2009, 2014, 2015). Genotyping of isolates was performed by high-resolution capillary gel-based electrophoresis PCR-ribotyping, following the procedures described in Fawley et al. (2015). Ribotypes were designated according to the nomenclature of the Leiden (Prof. Ed Kuijper; The Netherlands)-Leeds (Dr. Warren Fawley and Prof. Mark Wilcox; UK) database ~~(The Netherlands)~~. ~~If a matching PCR ribotype was not found, the electrophoresis profile was sent to Leeds for a search in the Leeds database of more than 600 PCR ribotypes (Dr. Warren Fawley and Prof. Mark Wilcox, Leeds).~~Novel ribotypes were named using internal reference codes (prefix 'CD' followed by a number).

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

Antimicrobial susceptibility testing

In vitro susceptibility of isolates was determined by the Etest (bioMérieux) on prereduced Brucella agar supplemented with vitamin K1 and haemin (bioMérieux), according to the manufacturer's instructions. Plates were incubated anaerobically at 37°C and examined at 48 h. Tested antimicrobial compounds and breakpoints for antimicrobial resistance were as follows: penicillin G, ≥2 µg/ml; teicoplanin, >2 µg/ml; rifampicin, ≥4 µg/ml; linezolid and tigecycline, >4 µg/ml; clindamycin, erythromycin and levofloxacin, ≥8 µg/ml; imipenem, minocycline and tetracycline, ≥16 µg/ml; amoxicillin/clavulanic acid, ≥16/8 µg/ml; and metronidazole and vancomycin, ≥32 µg/ml. (CLSI, 2012; Álvarez-Pérez et al., 2013, 2014, 2015, 2017; Peláez et al. 2013).

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

Data analysis

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

Results

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

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

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

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

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

and which showed a similar antimicrobial susceptibility profile, but the AFLP profiles of such isolates displayed limited similarity (Pearson's correlation = 0.126) (Figure 1). In 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-1/V-P-1) yielded *C. difficile* isolates of different ribotypes.

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 ~~mostly~~ 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 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;

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 et al., 2017). Curiously, despite the frequent shedding of *C. difficile* ribotype 078 by animals previously observed in Spain (Peláez et al., 2013; Álvarez-Pérez et al., 2013, 2014, 2015) and many other countries (Janezic et al., 2014) we did not found any isolate of this epidemic ribotype in the present study. Nevertheless, as a single *C. difficile* isolate from each primary culture was selected ~~from each primary culture~~ for detailed phenotypic and genetic characterization, we cannot discard the possibility that this and other ribotypes might have been overlooked.

Finally, all isolates characterized in this study displayed high-level ~~I~~in vitro resistance to imipenem and levofloxacin, a phenotype which ~~-~~is fairly common among ~~clinical *C. difficile* isolates of~~ diverse ribotypes of *C. difficile* from different geographic locations (Alcalá et al., 2012; Keessen et al., 2013; Pirš et al., 2013; Freeman et al., 2015). As ~~-~~carbapenems and fluoroquinolones are widely used in human and veterinary medicine to treat a diversity of infections (Papich, 2011; Papp-Wallace et al., 2011; Redgrave et al., 2014), monitoring the resistance to these compounds in *C. difficile* and other emerging pathogens should be a priority. Furthermore, some isolates were found to be resistant to erythromycin, clindamycin and penicillin G, all of which are of common use in clinical practice (Papich, 2011). Although we did not detect any isolate with decreased susceptibility or heterogeneous resistance to metronidazole, we recommend to determine MIC values to this antibiotic even for environmental isolates, as metronidazole is still considered a first-line drug for the treatment of anaerobe infections in human and animal medicine (Dhand and Snydman, 2009; Löfmark et al., 2010; Papich, 2016) and (hetero)resistant strains of *C. difficile* and other clostridia have been reported by different

authors (Peláez et al., 2008, 2013; Álvarez-Pérez et al., 2013, 2014, 2015, 2017; Wetterwik et al., 2013).

Conclusions

In summary, our results revealed the presence of epidemic ribotypes of *C. difficile* in 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 pathogen should be considered in any environmental risk assessment.

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Declaration of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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

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 ≥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 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.

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).

Supporting Information

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.

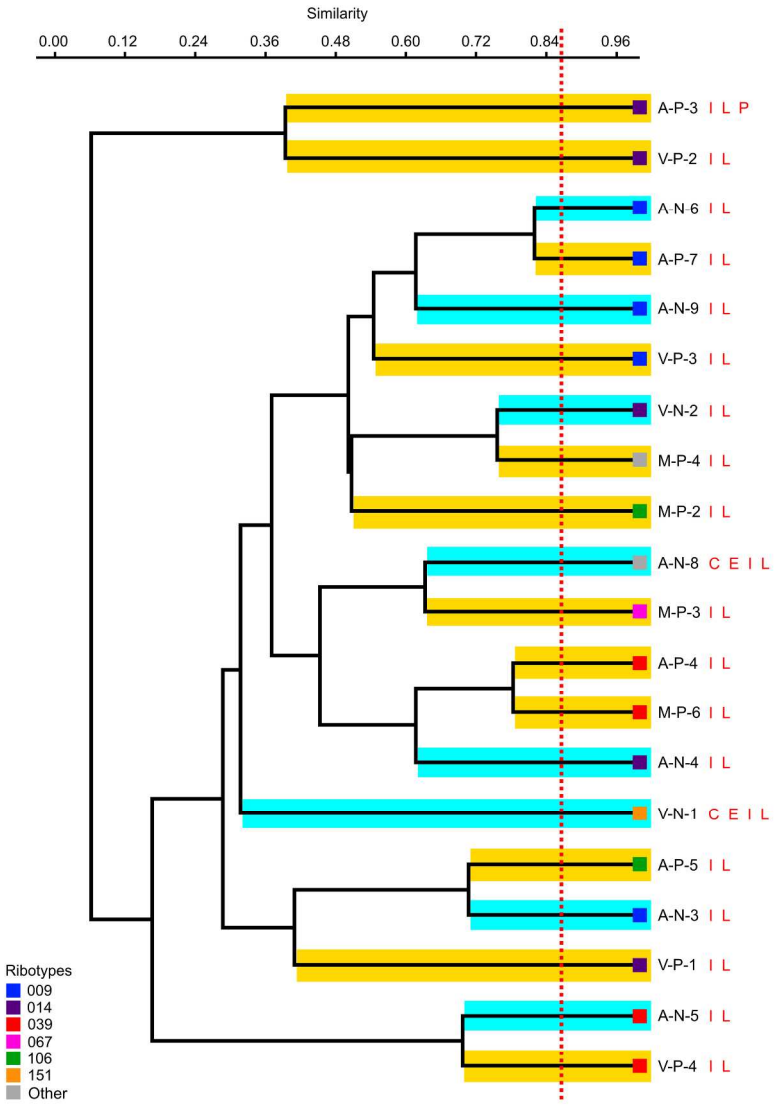
Figure S1. Schematic representation of the Madrid region (central Spain), indicating the approximate location of the zones from which sand samples were obtained in this study.

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 *C. difficile*?	Ribotype (toxin profile)	Sample's code	Positive for *C. difficile*?	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 ($A^-B^-CDT^-$)	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
M								
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

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

† 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)

210x297mm (128 x 128 DPI)