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Brazilian Journal of Microbiology 44, 2, 485-491 (2013)
ISSN 1678-4405
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www.sbmicrobiologia.org.br

Research Paper

Identification of pathogens and virulence profile of *Rhodococcus equi* and *Escherichia coli* strains obtained from sand of parks

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Submitted: March 19, 2011; Approved: September 10, 2012.

Abstract

The identification of pathogens of viral (Rotavirus, Coronavirus), parasitic (*Toxocara* spp.) and bacterial (*Escherichia coli*, Salmonella spp., *Rhodococcus equi*) origin shed in feces, and the virulence profile of *R. equi* and *E. coli* isolates were investigated in 200 samples of sand obtained from 40 parks, located in central region of state of Sao Paulo, Brazil, using different diagnostic methods. From 200 samples analyzed, 23 (11.5%) strains of *R. equi* were isolated. None of the *R. equi* isolates showed a virulent (*vapA* gene) or intermediately virulent (*vapB* gene) profiles. Sixty-three (31.5%) strains of *E. coli* were identified. The following genes encoding virulence factors were identified in *E. coli*: *eae*, *bfp*, *saa*, *iucD*, *papGI*, *sfa* and *hly*. Phylogenetic classification showed that 63 *E. coli* isolates belonged to groups B1 (52.4%), A (25.4%) and B2 (22.2%). No *E. coli* serotype O157:H7 was identified. Eggs of *Toxocara* sp. were found in three parks and genetic material of bovine Coronavirus was identified in one sample of one park. No *Salmonella* spp. and Rotavirus isolates were identified in the samples of sand. The presence of *R. equi*, *Toxocara* sp, bovine Coronavirus and virulent E. coli isolates in the environment of parks indicates that the sanitary conditions of the sand should be improved in order to reduce the risks of fecal transmission of pathogens of zoonotic potential to humans in these places.

Key words: enteric pathogens, virulence, sand, feces, parks, *E. coli*, *R. equi*.

Introduction

Enteric pathogens are a major group of organisms related to infections in humans and animals. These pathogens are resistant to adverse environmental conditions and are frequently transmitted by oral route due to fecal contamination of foods, water, vegetables and fruits (Acha and Szyfres, 2003).

Sandboxes are commonly found in parks all over the world, and are mainly used by children and adolescents. Access of companion animals, birds and occasionally livestock, inadequate hygiene of the sandboxes, precarious hygiene habits of children, and lack of knowledge about the

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risks posed by microorganisms eliminated in feces of animals favor the transmission of pathogens of animal origin to humans in these places (Matsuo and Nakashio, 2005).

The present study investigated the presence of pathogens of viral (Coronavirus, Rotavirus), parasitic (*Toxocara* spp.) and bacterial (*E. coli*, *Salmonella* spp., *R. equi*) origin eliminated in feces of animals, and the virulence profile of *R. equi* and *E. coli* isolates, obtained from the sand of parks located in the central region of state of Sao Paulo, Brazil.

Materials and Methods

Collection of samples

Two hundred samples of sand from 40 parks were analyzed. The strains were collected between 2008 and 2009. All parks were located in the central region of state of Sao Paulo, Brazil. After superficial dirt was removed, about 250 g of soil were collected 10-15 cm deep. Samples were placed in individual plastic bags and taken to laboratory under refrigeration (4-8 °C).

Culture, identification and storage of bacterial isolates

All samples were processed in the Laboratory of Microbiology and Infectious Diseases of Animals, Department of Veterinary Hygiene and Public Health, School of Veterinary Medicine, UNESP - Universidade Estadual Paulista, Botucatu, Sao Paulo, Brazil. Samples were kept under refrigeration (4-8 °C) or frozen (-20 °C) until they were analyzed.

Samples (25 g) of feces from all parks were inoculated aseptically in 225 mL sterilized distilled water. After homogenization, 0.03 μL of material was inoculated in defibrinated bovine blood agar (5%) and MacConkey agar for *E. coli* isolation. Plates were incubated at 37 °C for three days and were assessed every day for bacterial growth. Simultaneously, 0.03 μL of these samples were cultured in NANAT selective media for *R. equi* (Takai *et al.*, 1996). Microorganisms were identified by colony morphology, staining methods, and biochemical tests (Quinn *et al.*, 1994). Isolates of bacterial origin were stored in Lignièris agar at 25 °C.

Identification of *Salmonella* spp.

Briefly, samples (25 g each) were inoculated into 250 mL of peptone water 1.0% (Oxoid) and incubated at 35 °C for 24 h. Aliquots of 0.1 mL and 1 mL were inoculated each into 10 mL of Rappaport-Vassiliadis (RV) (Oxoid) and Tetrathionate (TT) (Oxoid) broth, respectively, and incubated at 42 °C (RV) and 35 °C (TT) for 24 h. A loopful of each broth culture was inoculated simultaneously in xylose-lysine-desoxycholate agar (XLD) (Oxoid) and bismuth sulfite agar (BS) (Oxoid), followed by incubation at 35 °C for 24 h. Colonies suggestive of *Salmonella* were inoculated in triple sugar iron (TSI) and lysine iron (LIA) agar slants. Tubes were incubated for 35 °C for 24 h. Colonies suggestive of *Salmonella* spp. in at least one of the culture media (TSI or LIA) were submitted to biochemical tests, agglutination test using polyvalent anti-*Salmonella* serum (Probac) (Quinn *et al.*, 1994; Andrews *et al.*, 1998), and serotype identification(Popoff and Le Minor, 1992).

Virulence of *R. equi*

Isolation of plasmid DNA was obtained by using an alkalin lysis method (Takai *et al.*, 2003). Target DNA for PCR amplification was based on the genes encoding a 15-17 kDa antigen (*vapA* gene), and a 20 kDa antigen (*vapB* gene) sequence. Plasmid DNA was digested with restriction endonucleases (EcoRI, EcoT22I and HinddIII). Primer 1 (5'-GACTCTTCACAAGACGGT-3') and primer 2 (5'-TAGGCGTTGTGCCAGCTA-3') were used to detect virulent (*vapA* gene) strains and the 569-552 bp expected product. Primer 3 (5'-AACGTAGTCGCGGTGAG AA-3') and primer 4 (5'-ACCGAGACTTGAGCGACTA--3') were used for intermediately virulent (*vapB* gene) isolates to detect the 1066-1048 bp expected product. Samples were submitted to 30 cycles of amplification as follows: denaturation for 90 s at 94 °C, annealing for 1 min at 55 °C, and extension for 2 min at 72 °C (Takai *et al.*, 1996; Takai *et al.*, 2003). Characterization of plasmid virulence was performed in Kitasato University, Japan.

E. coli serotypes and virulence factors

Sorbitol-negative O157:H7 serotypes were submitted to agglutination test with O157 and H7 sera (Probac). Reference strains were *E. coli* FVL2 (sfa, pap, iucD, hly, cnf-1), FV35 (afa, iucD, cnf-1), J96 (*papGII, papGIII*), O157:H7 (vt1, vt2, eae), 2348/69 (eae, bfp, eaf), IANO (stb, lt1), EAEC O42 (eaec), B90 (cnf-2), FVL16 (cnf-1, hly, pap, sfa, iucD), ETEC13 (sta), EIEC (ipaH), supplied by the Laboratory of Bacterial Antigens, Campinas State University, Brazil. *E. coli* DH5α strain was used as a negative control. First, primers for virulence factor genes were determined individually using a template DNA from appropriate positive and negative control strains. The presence of the following groups of genes were analyzed by PCR: *papC* and *papG* alleles (P fimbria), *sfaC/D* (S fimbria), *afaB/C* (afimbrial adhesin), *saa* (self-agglutinating adhesin), *iucD* (aerobactin), *cnf-1* and *cnf-2* (cytotoxic necrotizing factor type 1 and 2), *hly* (α-hemolysin), *vt1* and *vt2* (verotoxins), *sta* and *stb* (heat stable toxins), *lt1* (heat labile toxins), *eaec* (E. coli EAEC), *ipaH* (*E. coli* EIEC), and *eae*, *eaf* and *bfp* (*E. coli* EPEC). Appropriate primer sequences, annealing temperature, and size of amplified fragment (base pairs - bp) for these genes were determined in previous studies (Schmidt *et al.*, 1995; Yamamoto *et al.*, 1995; Blanco *et al.*, 1996; Blanco *et al.*, 1997; Karkkainen *et al.*, 1998; Aranda *et al.*, 2004; Villareal *et al.*, 2006). Phylogenetic classifica-

tion (chuA, yjaA, TspE4.C2) in groups A, B1, B2 and D was performed by PCR (Emödy *et al.*, 2003).

Identification of *Toxocara* spp.

Flotation-centrifugation with sodium nitrate (Na_2NO_3) 1.20 g/cm^3 was used for the recovery of *Toxocara* spp. eggs. Centrifugation was performed at 2.500 rpm (679 g) for 5 min. After that, the supernatant of each tube was placed in microscope slides, covered with coverslips, and examined under a light microscope (10x). This process was repeated three times for each sample (Santarém *et al.*, 1998).

Diagnosis of bovine coronavirus (BCoV)

Samples were tested for the presence of BCoV with a group II coronavirus specific RT-PCR assay targeted to the RNA-dependent RNA-polymerase gene (RdRp) with a 136-bp predicted product (Brandão *et al.*, 2005). BCoV Kakegawa strain (Akashi *et al.*, 1980) and PBS were used as positive and negative controls, respectively.

Diagnosis of rotavirus

Samples were analyzed for the presence of rotavirus 11-segment RNA using polyacrylamide gel electrophoresis-PAGE (Herring *et al.*, 1982). NCDV group A rotavirus strain was used as the positive control.

Statistical analysis

Chi-square test (Epi-Info, 6.4) was used to evaluate the differences in the presence of different pathogens in the parks, considering $p < 0.05$ (Triola, 2005).

Results

The frequency of pathogens identified in samples of sand obtained from parks is shown in Table 1. There was no statistical difference ($p > 0.05$) between the presence of the different pathogens in the parks sampled.

E. coli and *R. equi* strains were the most common pathogens isolated throughout the study. *R. equi* strains were isolated in 23 (11.5%) sand samples. None of the *R. equi* isolates showed virulent (*vapA* gene) or intermediately virulent (*vapB* gene) plasmid profiles. Sixty-three (31.5%) strains of *E. coli* were identified. The following virulence factor genes were identified in the *E. coli* strains: *eae*, *bfp*, *saa*, *iucD*, *papGI*, *sfa* and *hly*. Phylogenetic classification showed that the 63 *E. coli* isolates belonged to groups B1 (52.4%), A (25.4%) and B2 (22.2%). No *E. coli* serotype O157:H7 was identified (Table 2).

Eggs of *Toxocara* spp. were recovered only in three of the parks.

Genetic material of bovine Coronavirus was identified in one public park (Table 1), as suggested by the sequencing analysis of the 136 bp amplicon obtained in one sample (data not show). No *Salmonella* spp. or Rotavirus isolates were identified in the samples of sand.

Discussion

Rhodococcus equi is a well-recognized Gram positive intracellular bacterium associated with different clinical manifestations in humans and animals. The organism is widespread in soil, particularly in feces of foals, other herbivores and their environment (Prescott, 1991). The virulence mechanism of this pathogen is related with the presence of virulence-associated plasmids-Vap (Takai *et al.*, 1991), and three levels of virulence are currently recognized: virulent, intermediately virulent and avirulent (Takai, 1997). Virulent *R. equi* strains contain a large plasmid of 85-90 kb, responsible for encoding the 15-17-kDa antigens (VapA) that are considered the major causes of suppurative pneumonia in foals (Ribeiro *et al.*, 2005). VapB or intermediately virulent isolates present 20-kDa antigens and a 79-100 kb plasmid (Takai, 1997). They are frequently observed in swine lymphadenitis (Takai *et al.*, 1996) and patients infected by acquired immunodeficiency syndrome-AIDS (Takai *et al.*, 2003). In contrast, avirulent strains show no evidence of either *vapA* or *vapB* genes. These strains are found in the soil of areas where foals are raised, in soil and/or sand of human dwelling, mainly in yards and parks, and in humans with rhodococcosis co-infected by AIDS virus (Takai *et al.*, 1996; Takai, 1997).

Table 1 - Identification of pathogens from fecal origin in sand of parks. Brazil, 2010.

Pathogens	Frequency	p value
	Isolates identified / total of specimens (%)	
Escherichia coli	23/200 (11.5)	0.33
Rhodococcus equi	63/200 (31.5)	0.27
Toxocara spp.	3/200 (1.5)	0.5
bovine Coronavirus	1/200 (0.5)	0.5

Nº = number.
$p < 0.05$ indicates statistical differences between microorganisms.

Table 2 - Phylogenetic classification and virulence factors of *Escherichia coli* strains isolated from sand samples of parks. Brazil, 2010.

Phylogenetic classification	Gene	Number of strains
A	*saa*	6
A	*papGI, sfa, hly*	1
B1	*saa*	3
B1	*eae*	1
B1	*iucD*	1
B1	*eae, bfp*	1
B1	*saa, eae, bfp*	1
B2	*saa*	2

All our *R. equi* strains were classified as avirulent. These results are in agreement with similar study in Japan, which also reported the presence of avirulent *R. equi* in the soil of parks and yards (Takai *et al.*, 1996). Avirulent strains have been frequently identified in the environment of domestic animals, particularly foals (Takai, 1997). Currently, *R. equi* has emerged as a pulmonary pathogen among immunosuppressed patients, mainly those infected by AIDS virus (Acha and Szyfres, 2003). A recent survey of *R. equi* virulence profile in 20 humans in Brazil showed 11 patients infected with avirulent strains (Ribeiro *et al.*, 2011). Plasmid virulence of *R. equi* strains isolated in Brazil was characterized in foals (Ribeiro *et al.*, 2005) and a dog (Farias *et al.*, 2007). The present study was the first investigation in this country about virulence profile of *R. equi* strains isolated from park sand. Beside the absence of virulent or intermediately virulent *R. equi* strains, the presence of this microorganism in the sand of parks constitutes a public health problem. This risk is particularly important to children and immunocompromised people, especially HIV-positive patients, because avirulent *R. equi* may cause the disease in immunosuppressed and non-immunosuppressed patients (Takai *et al.*, 2003), including in Brazil (Ribeiro *et al.*, 2011).

E. coli is a very diverse species of bacteria found both in the intestinal tract of humans and animals, and in the environment. The microorganism is classified in six different pathotypes based on enteric manifestations, as follows: enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC), enteroaggregative (EAEC), and diffusely adherent (DAEC). Pathogenic manifestations of *E. coli* are closely related with different virulence factors, including enterotoxins, cytotoxins, fimbriae, adhesins, and iron chelation mechanisms (Kaper *et al.*, 2004).

Gene *eae* encodes intimin, which mediates the intimate attachment of EPEC and EHEC to epithelial cells, and stimulates mucosal immune response and intestinal crypt hyperplasia. Gene *eae* and *bfp* were found in three strains isolated from the sand of parks in the present study, and are generally related with atypic enteropathogenic *E. coli*. This class of *E. coli* EPEC causes diarrhea in children younger one year of age, mainly in emerging countries (Kaper *et al.*, 2004). In Brazil, there was a case of concurrent infection of a child and a dog by enteropathogenic *E. coli* that showed *eae* gene and was isolated from feces (Rodrigues *et al.*, 2004). Atypic EPEC isolated from parks constitutes a public health risk, especially for children and immunosuppressed humans.

P fimbriae are known to contribute for *E. coli* pathogenesis by promoting colonization of host tissues and stimulating injurious inflammatory response in the host (Kuehn *et al.*, 1994). PapG adhesin is located on P fimbria. Three classes of PapG (PapGI, PapGII and PapGIII) are recognized: papG Class I are predominantly found in fecal strains; allele GII in strains involved in pyelonephritis and bacteremia cases; and allele GIII in isolates responsible for cystitis cases in humans and animals (Bergsten *et al.*, 2005). S pillus is constituted of subunits: sfaS subunit mediates *E. coli* interaction with intestinal and other epithelial cells. *sfaS* gene is associated with human pyelonephritis, meningitis and sepsis (Féria *et al.*, 2001). Haemolysin is a pore-forming cytotoxin that lyses erythrocytes, leukocytes, and endothelial and epithelial cells of mammals. The *hly* genes are frequently found in extraintestinal *E. coli* infections in humans and animals (Johnson *et al.*, 1991). One of our isolates harbored the genes that encode *papGI*, *sfa* and *hly*. The identification of these virulence factors in a same isolate may be explained by the presence of a pathogenicity island (PAI), which enhances the infectivity of the microorganism. PAIs have been frequently found in *E. coli* responsible for human extraintestinal infections (Kurazono *et al.*, 2000). In Brazil, genes that encode *papG* adhesins, as well as *hly* and *sfa* genes, were found in *E. coli* strains isolated from pyometra, urinary tract infections, and feces of dogs (Siqueira *et al.*, 2009). Free access of dogs to parks increases the risk of human infection with virulent *E. coli*. These animals may act as reservoirs, harboring pathogenic strains with virulence factors such as *papG*, *hly*, and *sfa* genes.

Iron is essential for bacterial metabolism. *E. coli* uses this ion for the transport and storage of both electrons and oxygen, and for DNA synthesis (Emödy *et al.*, 2003). Growth of bacteria under restricted iron concentrations make them use successfully competitive mechanisms to obtain this ion from the host. Aerobactin is the most effective iron chelation system employed by *E. coli* for iron acquisition, mediated by *iu*c genes types A, B, C and D (Griffiths, 1997). In humans, this virulence factor is intimately associated with urinary infections and septicemia (Torres *et al.*, 2001). The *iucD* genes were found in only one isolate of our study. Currently, *iuc* genes have been found in dogs with pyometra (Coogan *et al.*, 2004), urinary tract infections, and in dog feces in Brazil (Siqueira *et al.*, 2009). Like other virulence factors, the presence of *iucD* gene in *E. coli* strains isolated from sand represents a risk to the population visiting these parks.

The presence of a self-agglutinating adhesin (saa) in *E. coli* has been previously described (Paton *et al.*, 2001). Virulence of this adhesin to humans and domestic animals remains unclear. However, *saa* gene was found in 19.0% of *E. coli* strains obtained from the sand of parks in the current study. This result suggests that further studies should be carried out in order to investigate the role of this adhesin as an *E. coli* virulence factor.

E. coli have been phylogenetically classified in four groups named A, B1, B2 and D. *E. coli* strains belonging to groups B2 and D are commonly pathogenic for humans and animals, whereas A and B1 are less pathogenic (Clermont *et al.*, 2000). Based on phylogenetic systematics, *E. coli*

isolates obtained from the sand of parks were classified in A and B1 groups. Although these groups are predominantly related with non-pathogenic *E. coli* strains, these results indicate fecal contamination of the environment.

Toxocariasis is a cosmopolitan parasitic zoonosis. *Toxocara* spp. is one of the most common parasites of young dogs and cats. Eggs of the parasite are frequently shed in large amounts in the feces of companion animals. Toxocariasis in human occur by spread of the larvae, leading to different clinical forms of disease. Clinical manifestations involve serious neurological, ophthalmologic, pulmonary, and/or cutaneous signs (Acha and Szyfres, 2003). The presence of eggs of *Toxocara* spp. in the sand of parks have been reported in several countries (Dubná *et al.*, 2007; Matsuo and Nakashio, 2005), including in Brazil(Santarém *et al.*, 1998). Three parks had positive samples for eggs of this parasite. These results suggest environmental contamination by feces of companion animals and indicate risk of toxocariasis to humans that use these parks, particularly children.

Coronavirus infections in animals were reviewed elsewhere (Brandão *et al.*, 2001). In Brazil, previous studies have identified Coronavirus in feces of cattle and dogs with and without diarrhea (Brandão *et al.*, 2005, 2007). Identification of bovine Coronavirus in the sand from parks in Brazil is uncommon, although it also represents fecal contamination of the environment.

Rotavirus was detected in the feces of domestic animals (Rodriguez *et al.*, 2004; Ruiz *et al.*, 2009) and chickens (Villarreal *et al.*, 2006) with and without diarrhea in Brazil. Likewise, different *Salmonella* spp. serotypes were detected in feces of livestock (Ribeiro *et al.*, 2010), birds and chickens (Hofer *et al.*, 1997) in this country. None of sand samples collected in our parks showed Rotavirus and *Salmonella* spp. In contrast, an epidemiological study involving human patients with salmonellosis in several European countries revealed that one the major risk factors for the disease was the access of children up to four years of age to the sand of parks (Doorduyn *et al.*, 2006). These findings indicate that similar studies must be performed in other regions in Brazil in order to investigate the occurrence of *Salmonella* spp. and Rotavirus in the sand of parks. Despite the absence of *Salmonella* spp. and Rotavirus in the samples analyzed, these pathogens should be included in microbiological testing required to determine the sanitary conditions of the sand used in parks, as they may be shed in the feces of birds and domestic animals.

The identification of *R. equi*, *E. coli* EPEC, bovine Coronavirus, and *Toxocara* spp. are indicators of fecal contamination of the sand of the parks sampled. Contamination may have been caused by feces from domestic animals (Takai *et al.*, 1996), birds (Prescott, 1991), or contaminated shoes of people who visit these places. Our results suggest the need to introduce control measures to prevent contamination of the sand by pathogens eliminated in animal feces. In fact, the risks of the transmission of pathogens shed in animal feces in parks may reduce if access of domestic animals to these places is prevented, fecal material is daily removed from the sand, sand is periodically tested for sanitary quality and replaced with material of known origin, and people are continuously educated on hygiene habits before using parks.

The presence of *R. equi*, *E. coli* EPEC, *Toxocara* spp. and bovine Coronavirus identified in parks studied indicates environmental contamination by microorganisms found in feces of domestic animals, birds, and/or contaminated shoes of people. These results represent a risk for the transmission of pathogens with zoonotic potential to humans in these places, particularly to children.

Acknowledgments

This work was supported by FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil (2007/57781-3)

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