Veterinary Parasitology 200 (2014) 284–288

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

Molecular detection and characterization of *Cryptosporidium* species in household dogs, pet shop puppies, and dogs kept in a school of veterinary nursing in Japan

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ARTICLE INFO

Article history:
Received 4 September 2013
Received in revised form
24 December 2013
Accepted 29 December 2013

Keywords:
Cryptosporidium
Dog
Molecular characterization
Puppy

ABSTRACT

Members of *Cryptosporidium* species, which are protozoan parasites, are prevalent worldwide and can cause diarrhoea in both humans and animals, including dogs. In addition, the *Cryptosporidium* species harboured in dogs have the potential for zoonotic transmission. The purpose of the present study was to determine the prevalence of *Cryptosporidium* species infection and perform molecular characterization of isolates in household dogs, pet shop puppies, and dogs kept in a school of veterinary nursing in Japan. Fresh faecal samples were collected once from 529 household dogs (aged from 2 months to 18 years old, from 9 veterinary clinics located in 6 different regions), 471 pet shop puppies (≤3 months old, from 4 pet shops located in 2 different regions), and 98 dogs (aged from 2 to 11 years old) kept in a veterinary nursing school. A nested polymerase chain reaction (PCR) assay targeting the 18S rRNA gene was employed for the detection of *Cryptosporidium* species, and 111 random samples of PCR amplicons (approximately 500-bp) were sequenced for the molecular characterization of the isolates. The prevalences of *Cryptosporidium* species in household dogs, pet shop puppies, and veterinary nursing school dogs were 7.2%, 31.6%, and 18.4%, respectively. In household dogs, no significant correlation was observed between the prevalence of *Cryptosporidium* species and the age (≤6 months vs. >6 months), living conditions (indoor vs. outdoor), faecal conditions (formed vs. unformed), and location of residence. In pet shop puppies, the prevalence of *Cryptosporidium* species was not related to faecal condition; however, the prevalence significantly differed among the pet shops. All of the 111 sequence samples (26 from household dogs, 75 from pet shop puppies, and 10 from veterinary nursing school dogs) were identified as *Cryptosporidium canis*. The present study demonstrates a high prevalence of *Cryptosporidium* species infections in pet shop puppies and dogs of a veterinary nursing school in Japan. However, because *Cryptosporidium hominis* and *Cryptosporidium parvum* are the most common causes of human infections, it is likely that the risk of zoonotic transmission of *Cryptosporidium* species from dogs to humans is low.

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http://dx.doi.org/10.1016/j.vetpar.2013.12.030

1. Introduction

Members of *Cryptosporidium* species, which are major protozoan parasites, are prevalent worldwide and can cause diarrhoea in both humans and animals, including dogs (Scorza and Tangtrongsup, 2010; Bouzid et al., 2013). Currently, it is recognized that the *Cryptosporidium* genus contains at least 20 species and over 40 genotypes, and most of these species and genotypes are host adapted and have a narrow host range (Plutzer and Karanis, 2009; Lucio-Forster et al., 2010; Xiao, 2010). Humans and dogs are most commonly infected with the host-adapted species *Cryptosporidium hominis* and *Cryptosporidium canis*, respectively (Plutzer and Karanis, 2009; Lucio-Forster et al., 2010; Xiao, 2010). Humans can also commonly be infected with the zoonotic *Cryptosporidium parvum* (Plutzer and Karanis, 2009; Xiao, 2010; Bouzid et al., 2013). In addition, other species of *Cryptosporidium* such as *C. canis* are infrequently diagnosed in people with immunodeficiency conditions such as HIV (Plutzer and Karanis, 2009; Xiao, 2010; Bouzid et al., 2013). Similarly, dogs are rarely infected with *C. parvum* (Lucio-Forster et al., 2010; Scorza and Tangtrongsup, 2010). Therefore, *Cryptosporidium* species harboured in dogs generally pose a low risk for zoonotic transmission (Plutzer and Karanis, 2009; Lucio-Forster et al., 2010; Scorza and Tangtrongsup, 2010; Xiao, 2010; Bouzid et al., 2013). However, only a small number of reports are available regarding molecular epidemiological investigations and characterization of *Cryptosporidium* isolates from dogs in Japan (Abe et al., 2002; Satoh et al., 2006; Yoshiuchi et al., 2010). The purpose of the present study was to determine the prevalence of *Cryptosporidium* species infection and perform the molecular characterization of isolates in household dogs, pet shop puppies, and dogs kept in a veterinary nursing school in Japan.

2. Materials and methods

Fresh faecal samples were randomly collected from 529 household dogs (aged from 2 months to 18 years old, from 9 veterinary clinics located in 6 different regions in Japan: one clinic in Hokkaido, three clinics in Tohoku, two clinics in Kanto, one clinic in Kinki, one clinic in Kyushu, and one clinic in Okinawa), 471 pet shop puppies (≤3 months old, from 4 pet shops located in 3 different areas of 2 districts in east Japan: two shops in Aomori prefecture of Tohoku; Shops 1 and 2, one shop in Saitama prefecture of Kanto; Shop 3, and one shop in Ibaraki prefecture of Kanto; Shop 4) and 98 dogs (aged from 2 to 11 years old) kept in a veterinary nursing school (located in Kanto district), with or without a history of illness, on a single occasion between January 2011 and December 2012. All faecal samples were donated by the dog owners and pet shop managers, who granted permission to include their dogs in the survey. The samples were collected immediately after natural defecation and were stored at 4 °C until use (within 3 days). *Cryptosporidium* oocysts were isolated using a sucrose gradient concentration method with a specific gravity of 1.21, and DNA extraction from isolated oocysts was performed using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacture's instructions, as previously described (Abe et al., 2002). The samples of DNA were stored at −20 °C.

A nested polymerase chain reaction (PCR) assay targeting the 18S rRNA gene was employed for the detection of *Cryptosporidium* species. For the primary reaction, the nested PCR protocol utilized the forward primer P1 (5′-ACCTATCAGCTTTAGACGGTAGGGTATTTC-3′) and the reverse primer P2 (5′-TCATAAGGTGCTGAAGGAGTAAGG-3′), which amplified an approximately 750-bp amplicon, and in the secondary reaction, the forward primer P3 (5′-ACAGGGAGGTAGTGACAAGAAATAACA-3′) and P4 (5′-AACTTTCGTTCTTGATTAATGAAAACA-3′) were used, generating an approximately 500-bp amplicon (Xiao et al., 1999; Sevá et al., 2011). For the primary reaction, the PCR mix consisted of 1× buffer containing 3.0 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 1.25 units of GoTaq DNA polymerase (Promega Corporation, Madison, W1), and 3.0 μl of template DNA in a total reaction volume of 25 μl. For the secondary PCR, the PCR mix was the same as for the primary reaction, except the use of 1.5 mM $MgCl_2$ and primary PCR amplicons as template. The primary PCR was performed as follows: after an initial denaturation step of 3 min at 94 °C, a set of 35 cycles was run each consisting of 45 s at 94 °C, 45 s at 60 °C and 1 min at 72 °C, with a final extension of 5 min 72 °C. The secondary PCR conditions were as follows: after an initial denaturation step of 3 min at 94 °C, a set of 35 cycles was run each consisting of 30 s at 94 °C, 90 s at 56 °C and 1 min at 72 °C, with a final extension of 5 min 72 °C.

All PCR products were identified by electrophoresis on 1.5% agarose gels. The specific DNA fragments were confirmed after ethidium bromide staining under UV light using a transilluminator. The random 111 samples (26 from household dogs, 75 from pet shop puppies, and 10 from veterinary nursing school dogs) of secondary PCR amplicons were purified using QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany), and sequenced with the secondary primer set. Sequences were analyzed by a commercial laboratory (FASMAC Co., Ltd. Atsugi, Kanagawa, Japan). Sequence alignment and compilation were performed using the MEGA 5.2.2 (www.megasoftware.net) program. To determine the species, the DNA sequences were compared to GenBank sequences of *Cryptosporidium* species by BLAST search (http://www.ncbi.nlm.nih.gov/), and their similarity was determined based on the degree of sequence identity.

In household dogs, the obtained data were analyzed according to age (≤6 months vs. >6 months), living conditions (indoor vs. outdoor), faecal conditions (formed vs. unformed), and locations of veterinary clinics. In pet shop puppies and veterinary nursing school dogs, the data were compared between the faecal conditions, and the comparison was also performed among pet shops. The results were statistically analyzed using Fisher's exact probability test, with values of $P < 0.05$ considered significant.

3. Results

The prevalence and 95% confidence intervals (CI) are summarized in Table 1. Overall prevalence was significantly different among the dog's origin. The overall