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AUTHORS: Rosanowski, S M; Banica, M; Ellis, E; Farrow, E; Harwood, C; Jordan, B; James, C; McKenna, D; Fox, M; Blake, D P

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S. M. Rosanowski^{a*}, M. Banica^b, E. Ellis^b, E. Farrow^b, C. Harwood^b, B. Jordan^b, C. James^b, D. McKenna^b, M. Fox^a, D. P. Blake^a

The molecular characterisation of *Cryptosporidium* species in relinquished dogs in Great Britain: a novel zoonotic risk?

^a Department of Pathobiology and Population Sciences, The Royal Veterinary College, University of London, North Mymms, Hatfield, Hertfordshire AL9 7TA, United Kingdom. ^b The Royal Veterinary College, University of London, North Mymms, Hatfield, Hertfordshire AL9 7TA, United Kingdom.

Corresponding author

srosanow@cityu.edu.hk

Telephone

+85234425610

*Current address Department of Infectious Diseases and Public Health, College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Hong Kong SAR

ORCID

S.M. Rosanowski 0000-0002-7950-0029

D. Blake: 0000-0003-1077-2306

Abstract

Surveillance was conducted to investigate the occurrence of protozoan parasites of the genus *Cryptosporidium* in dogs newly admitted to a dog rehoming charity in London, Great Britain. Voided faecal samples were collected from all new admissions between 2011 and 2012 during six separate four-week sampling periods. Information on host signalment, including age, breed and reason for submission, and faecal consistency, was collected. Polymerase Chain Reaction (PCR) targeting the 18S ribosomal RNA gene, confirmed by sequencing, was conducted on the faecal samples to detect *Cryptosporidium* genomic DNA and determine *Cryptosporidium* identity. In total, 677 dogs were included in the study. The prevalence of *Cryptosporidium*-positive faecal samples was 4.6% (31/676). There were positive samples in all of the six sampling periods. *Cryptosporidium canis* (n=28), *C. parvum* (n=2) and *C. andersoni* (n=1) were identified. 60 KDa Glycoprotein (gp60) gene amplicon sequencing of the *C. parvum* samples identified genotypes IIaA17G1R1 and IIaA15G2R1 for the first time from a dog. There were no significant associations between signalment data and *Cryptosporidium* status. While this was a study of one rehoming shelter, the presence of the potentially zoonotic *C. parvum* and *C. canis* in dogs highlights a public health concern. Further research is needed to better understand the epidemiology and potential impacts of *Cryptosporidium* infection in dogs.

Keywords: *Cryptosporidium parvum*; *Cryptosporidium andersoni*; dog; 18S rRNA; gp60; epidemiology;

Introduction

Cryptosporidium is a protozoan parasite of the phylum Apicomplexa that is found in wildlife, domestic animals and humans. Zoonotic transmission routes have been identified (Fayer et al. 2000) and due to their close contact with humans, dogs have the potential to act as a vector for zoonotic spread (Bouزيد et al. 2013; Westgarth et al. 2008). The United Kingdom has a high level of companion animal ownership, with between 24% and 31% of households reporting dog ownership (Asher et al. 2011; Murray et al. 2010; Westgarth et al. 2007). Dogs are most often infected with *Cryptosporidium canis* (Osman et al. 2015; Palmer et al. 2008; Rimhanen-Finne et al. 2007), a species primarily associated with dogs but also recognised as an occasional zoonosis (Ryan et al. 2014). Infection of dogs with the potentially zoonotic *Cryptosporidium parvum* has also been reported (Fayer et al. 2001).

The objective of the current study was to investigate the occurrence of *Cryptosporidium* species in dogs recently admitted to a rehoming shelter that provides services to the wider London-area. *Cryptosporidium* status was determined using Polymerase Chain Reaction (PCR), where the sensitivity has been reported to be higher than faecal microscopy for detection of *Cryptosporidium* presence (Abe et al. 2002).

Materials and Methods

Study design and data collection

A cross-sectional survey of all newly arriving dogs at an animal rescue centre in South West London was undertaken during six, 4 to 6 week periods between April 2011 and September 2012. The faecal sampling of newly arriving dogs was done soon after arrival at the rescue centre (most within 5 days), collecting a minimum of 1 gram fresh faecal material from the ground at the time of voiding. The study population consisted of gifted, stray and previously owned dogs, henceforth called unwanted dogs and reflected a population of urban and semi urban dogs that were relinquished during the period of the study. A standardised submission form provided basic information about the individual,

including sex, age, breed, approximate location of origin and reason for submission to the shelter. Faecal consistency was recorded according to the five-point Waltham faecal scoring system, where a score of one represented hard, dry and crumbly, and a score of five watery diarrhoea (Moxham 2001). Samples were stored for up to one week in 2.5% (w/v) potassium dichromate at 4°C prior to processing.

Faecal processing, DNA purification and polymerase chain reaction (PCR) detection

The technique used to enrich and recover *Cryptosporidium* oocysts from faecal samples has been described previously (Sangster et al. 2016), following a process adapted from Abe et al. (2002) for use with canine faecal samples which included a flotation step to improve sensitivity. Subsequently, each sample was boiled for five minutes, centrifuged at 10,000 × g for one minute and the supernatant recovered for DNA extraction using a Qiagen DNeasy Blood and Tissue Kit (Qiagen®, Germany) following the manufacturer's protocol. Samples were stored at -20°C prior to ethanol precipitation (0.1 volume sodium acetate, 3 M pH 5.2; 2.5 volumes ice cold 100% ethanol, 1 µl glycogen as a carrier; centrifugation at 10,000 × g for 15 min; washed in one volume 70% ethanol and re-suspended in 20 µl molecular grade water). The purified DNA was tested for the presence of *Cryptosporidium* spp. genomic DNA using conventional PCR targeting the 18S rRNA gene (Morgan et al. 1997) in a total volume of 25 µl. A *Cryptosporidium parvum* 18S rDNA amplicon derived previously from an infection in a domestic dog and cloned into pGEM-T easy (Promega, Southampton, UK) was used as a positive control, with molecular grade water used as a no template negative control. PCR reactions were resolved by agarose gel electrophoresis (2% w/v Ultrapure™ agarose powder in 0.5x Tris-Borate-EDTA buffer, stained with 0.01% v/v SafeView nucleic acid stain (NBS Biologicals, U.K.) and visualised under ultraviolet light using an U:Genius Image Capture gel documentation system (Syngene, U.K)).

Diagnostic confirmation and genotyping of positive samples

All positive 18S rRNA gene PCR amplicons were purified using a Qiagen MinElute Purification Kit (as recommended by the manufacturer) and sequenced in each direction (GATC Biotech, Cologne, Germany) using the same primers employed for the original PCR. Sequence identity was confirmed by BLASTn against the National Centre for Biotechnology Information (NCBI) non-redundant nucleotide collection. Samples positive for *C. parvum* were subtyped by PCR amplification and sequencing of a fragment from the *Cryptosporidium gp60* gene as described previously (Sangster et al. 2016), in the absence of a *gp60* locus *C. canis* reference sequence (Stensvold et al. 2015).

Statistical analysis

Survey data were recorded in pro-forma recording sheets and stored in an Excel spreadsheet (Microsoft). Data were checked for errors and encoded into categories where appropriate (i.e. dog breed). The demographic variables were stratified by the presence or absence of *Cryptosporidium* by 18S rRNA gene PCR for comparison. Denominator data were not the same for all demographic variables, as some data were missing. Normally distributed continuous data were described as means and standard deviations, non-normally distributed data as medians and interquartile ranges (IQR). All other data were described as counts, percentages and 95% confidence intervals.

Statistical analyses were performed using Stata IC version 13.1 (StataCorp, Station College, TX, US) and significance was assigned when $P < 0.05$. The outcome was whether the sample was positive for *Cryptosporidium* by 18S PCR (0 or 1). Association between sampling occasion was tested using Fisher's exact test. Associations between categorical signalment variables (age (adult ≥ 1 year and young < 1 year), sex, neutered status, reason for submission and faecal consistency score) were tested using logistic regression.

Ethical Approval

This study was reviewed and approved by the Royal Veterinary College Clinical Research Ethical Review Board, approval number M2014 0047.

Results

In total, 677 dogs were sampled during the six survey periods (Table 1). The signalment and demographics of the dogs is presented in Online Resource 1. The time between submission and sample collection was a median of 4 (IQR 2 to 8 days; n=648) and at this time 49.3% (330/670) of dogs had a faecal consistency of 3 and 25.8% (173/670) had a faecal consistency of 2.

Cryptosporidium status

The prevalence of *Cryptosporidium* positive faecal samples was 4.6% (31/677); including *Cryptosporidium canis* (n=28), *C. parvum* (n=2) and *C. andersoni* (n=1) (Table 1; 18S rRNA gene amplicon sequences deposited with GenBank under the accession numbers LT839057-LT839087). Genotyping using the *gp60* the locus revealed subtypes IIaA17G1R1 and IIaA15G2R1 (accession numbers LT839088-LT839089). There were positive samples in all of the six survey periods, Survey 1 5.8%, Survey 2 5.0%, Survey 3 3.8%, Survey 4 3.6%, Survey 5 3.1% and Survey 6 6.8% (Table 1). There was no significant difference in the number of positive samples and sampling occasion (P=0.85).

The odds of a positive sample, described by dog signalment is presented in Online Resource 2. There were no significant associations between signalment and *Cryptosporidium* status.

Of the 26 *Cryptosporidium* positive dogs with complete information, the median time between arrival at the rehoming charity and testing was 4.5 days (interquartile range (IQR) 2-6.8; max 39). In three dogs with positive samples that arrived at the charity between the 11th and 21st of March 2012, time between submission and faecal sampling was delayed (median 26 days; IQR 20.5-32.5).

Discussion

We report here the first occurrence of two *C. parvum* gp60 zoonotic subtypes in dogs: IlaA17G1R1 and IlaA15G2R1, indicating a new risk for zoonotic transmission. Both subtypes have been detected previously in the United Kingdom and elsewhere, where they were dominant among human cases of cryptosporidiosis (Leoni et al. 2006; Xiao 2010), as well as commonly identified among livestock (Chalmers et al. 2011; Waldron and Power 2011; Wielinga et al. 2008). Further this study identified the first occurrence of *C. andersoni* (Lindsay et al. 2000) in a dog faecal sample. Reports of human infection with *C. andersoni* is very rare (Leoni et al. 2006), although one report has suggested a role in clinical disease in China (Jiang et al. 2014). It should be noted, however, that dogs may not be able to host patent infections of *C. andersoni*, as this species has previously been reported to be highly host-adapted (Lindsay et al. 2000). Evidence of parasite replication would be required before the dog can be considered to be a new host for *C. andersoni*, rather than transport host. Further research in this area is required.

The molecular methods used in this study revealed a low level of *Cryptosporidium* infection in dogs submitted to the rehoming shelter, with less than 5% presenting positive results. This is similar to the prevalence of infection (between 1.0% and 14.7%) identified in previous studies of dogs from various sources (e.g. stray, kennelled or pet dogs) in the United Kingdom and Europe (Dubná et al. 2007; Grimason et al. 1993; Osman et al. 2015; Overgaauw et al. 2009), North America (el-Ahraf et al. 1991; Smith et al. 2014), Japan (Abe et al. 2002), China (Jian et al. 2014), and Australia (Milstein and Goldsmid 1995). The majority of positive cases in this study were identified as the host adapted *C. canis*. *C. canis* has been reported as the only species infecting dogs in the majority of studies where PCR typing has been undertaken (de Lucio et al. 2017; Lucio-Forster et al. 2010; Morgan et al. 1997; Osman et al. 2015).

Sampling dogs from rescue shelters may exaggerate the true prevalence of *Cryptosporidium*. Palmer et al. (2008) identified a higher risk of *Giardia* infection in dogs from rescue shelters, which was theorised to be due to direct contact with other dogs, faeces and environmental contamination or stress, factors likely to exert a comparable influence on *Cryptosporidium*. The shedding of

Cryptosporidium oocysts in faeces due to chronic or subclinical infection may be increased due to the immunosuppressive effect of stress (Thompson et al. 2005). While the study by Palmer et al. (2008) included dogs already resident at shelters, the relinquishment or submission of stray dogs to a shelter would be a stressful event, predicating shedding if infection was present. Additionally, some of the dogs arriving at the shelter may have been roaming as strays prior to submission, with the potential that the environment was more contaminated than for owned dogs. Here, dogs were sampled on average 4 days after arrival at the shelter (arguably the most stressful time, and not enough time to develop patent shelter-gained infections). However, sampling was delayed for three dogs that subsequently had positive samples. These three dogs may provide evidence of within kennel transmission of *C. canis*. Genotyping the *C. canis* isolates detected here might have provided some clarification.

While the prevalence of *Cryptosporidium* in dogs submitted to a rehoming shelter was low, indicating infection is uncommon, the presence of the potentially zoonotic *C. parvum* and *C. andersoni* in dogs highlights a public health concern. Further research is needed to understand better the epidemiology, source and potential impacts of *Cryptosporidium* infection in dogs.

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