Molecular characterization of *Giardia* spp. and *Cryptosporidium* spp. from dogs and coyotes in an urban landscape suggests infrequent occurrence of zoonotic genotypes

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ABSTRACT

Giardia spp. and *Cryptosporidium* spp. are common gastrointestinal parasites with the potential for zoonotic transmission. This study aimed to (1) determine the genotypes occurring in dogs and coyotes occupying a similar urban area; (2) determine if these hosts were infected with potentially zoonotic genotypes; (3) provide baseline molecular data. In August and September 2012, 860 dog owners living in neighborhoods bordering six urban parks in Calgary, Alberta, Canada, provided faecal samples from their dogs. From March 2012 through July 2013, 193 coyote faeces were also collected from five of six of the same parks. Direct immunofluorescence microscopy (DFA) indicated that *Giardia* spp. and *Cryptosporidium* spp. infected a total of 64 (7.4%) and 21 (2.4%) dogs, as well as 15 (7.8%) and three (1.6%) coyotes, respectively. Semi-nested, polymerase chain reactions targeting the 16S small-subunit ribosomal ribonucleic acid (SSU rRNA) and 18S SSU rRNA genes of *Giardia* spp. and *Cryptosporidium* spp., respectively, were conducted on samples that screened positive by DFA, and products were sequenced and genotyped. Dogs were infected with *Giardia intestinalis* canid-associated assemblages C (n=14), D (n=13), and *Cryptosporidium canis* (n=3). Similarly, *G. intestinalis* assemblages C (n=1), D (n=1) and *C. canis* (n=1), were detected in coyotes, as well as *G. intestinalis* assemblage A (n=1) and *Cryptosporidium* spp. vole genotype (n=1). Dogs and coyotes were predominantly infected with host-specific genotypes and few potentially zoonotic genotypes, suggesting that they may not represent a significant risk for zoonotic transmission of these parasites in urban areas where these hosts are sympatric.

Keywords: *Giardia* spp., *Cryptosporidium* spp., genotype, dogs, coyotes, humans.

LIST OF ABBREVIATIONS

Abbreviation	Definition
CI	Confidence Interval
Cpg	Cysts per gram
DFA	Direct immunofluorescence /direct fluorescent antibody
DNA	Deoxyribonucleic acid
GI	Gastrointestinal
IAC	Internal amplification control
MLST	Multilocus sequence typing
Opg	Oocysts per gram
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
SSU rRNA	Small-subunit ribosomal ribonucleic acid
UV	Ultraviolet

1. INTRODUCTION

Dogs (*Canis lupus familiaris*) are common companion animals in North America, with an estimated six million owned dogs in Canada and 83 million in the United States (Humane Society of the United States, 1954; Perrin, 2009). Among wild carnivores, coyotes (*Canis latrans*) have experienced the largest range expansion across North America (Laliberte and Ripple, 2004), and are now increasingly common in urban landscapes of US and Canada (Gehrt et al., 2009). Spatial overlap of canids and humans in urban landscapes introduces the possibility of disease transmission among these hosts.

Giardia spp. and *Cryptosporidium* spp. are two of the most common gastrointestinal (GI) parasites to infect humans and other host species (Xiao and Fayer, 2008) and have frequently been reported in dogs (Bryan et al., 2011; Bugg et al., 1999; Fontanarrosa et al., 2006; Gaunt and Carr, 2011; Joffe et al., 2011; Little et al., 2009; Smith et al., 2015; Smith et al., 2014) and coyotes (Liccioli et al., 2012; Thompson et al., 2009) across geographic localities. Infective *Giardia* spp. and *Cryptosporidium* spp. (oo)cysts can persist in the environment (soil and water) for long periods, and can be transmitted directly through faecal-oral routes, or indirectly through the ingestion of contaminated water or food (Feng and Xiao, 2011; Ryan et al., 2014; Xiao and Fayer, 2008).

Giardia intestinalis is considered a species complex composed of eight assemblages, A – H (Feng and Xiao, 2011); assemblages C and D most often infect canids, appear largely host-specific, and are rarely detected in humans (Sprong et al., 2009). Conversely, assemblages A and B infect the widest range of host species, are the primary genotypes found to infect humans (Ballweber et al., 2010; Volotao et al., 2007), and also occur in dogs and coyotes (Ballweber et

al., 2010; Thompson et al., 2009; Trout et al., 2006; Volotao et al., 2007). These protozoa are considered potentially zoonotic but predominantly host-specific. However, debate continues over the degree of risk for zoonotic transmission due to varying results from sub-typing analyses and epidemiological studies (Ballweber et al., 2010; Bowman and Lucio-Forster, 2010; Marangi et al., 2009; Ryan et al., 2014; Volotao et al., 2007; Xiao et al., 2007).

The most commonly reported etiological agents of human cryptosporidiosis are *Cryptosporidium hominis* and *Cryptosporidium parvum* (Ryan and Hijjawi, 2015). In canids, there is no evidence of *C. hominis* occurrence, and infrequent reports of *C. parvum* (Lucio-Forster et al., 2010; Smith et al., 2009; Sotiriadou et al., 2013). Dogs and coyotes are typically infected with *Cryptosporidium canis* (Lucio-Forster et al., 2010; Ryan et al., 2014; Thompson et al., 2009; Trout et al., 2006; Xiao et al., 2002), and although this parasite species has rarely been documented in humans (Ryan et al., 2014), immunocompromised persons and children may be at heightened risk for infection (Bowman and Lucio-Forster, 2010; Xiao et al., 2007).

Despite the uncertainty around the risk for zoonotic transmission of some *G. intestinalis* assemblages and *Cryptosporidium* species, the co-existence of dogs and coyotes within urban areas and the ubiquity of these parasites as infectious agents of multiple host species, introduces the question of transmission potential among human and canid hosts. Few known studies from Canada have focused on the molecular characterization of *Giardia* spp. and *Cryptosporidium* spp. infecting dogs (McDowall et al., 2011; Uehlinger et al., 2013), and few from coyotes, overall (Thompson et al., 2009; Trout et al., 2006; Xiao et al., 2002). While previous research allowed us to quantify the prevalence of *Giardia* spp. and *Cryptosporidium* spp. infection in domestic dogs and identify risk factors associated to dog-walking behaviour (Smith et al., 2015;

Smith et al., 2014), parasite assemblage and species identification was not obtained. By genotyping parasite species at the SSU rRNA (small-subunit ribosomal ribonucleic acid), this study aimed to (1) conduct a preliminary assessment of (a) genotypes circulating in urban dogs and coyotes and; (b) assess the occurrence of zoonotic genotypes and; (2) provide baseline molecular data for ongoing research and surveillance.

2. MATERIALS AND METHODS

2.1 Study design and study areas

We used an observational, cross-sectional study design. The study recruited participants living in residential communities surrounding the following six urban parks in Calgary, Alberta, Canada (51°50N, 114°55'W): Bowmont; Fish Creek Provincial; Nosehill; River, Southland; Weaselhead. The faeces from dogs residing in these surrounding communities were collected, and coyote faecal samples were collected from the environment within five of six of these parks, with the exception of River Park. The collection of coyote faeces was largely unsuccessful in River Park, and therefore was excluded as a study site for this species. By targeting these residential communities for the collection of dog faeces, we aimed to maximize the potential for sampling faeces from dogs and coyotes that accessed and/or resided in similar geographic areas, including parks.

2.2 Faecal sampling protocol

2.2.1 Dogs

A total of 860 dog faecal samples were collected from dog-owning residences in August and September 2012, following online questionnaire addressing dog and owner demographics and dog-walking and husbandry practices, as described in detail in Smith et al. (2015).

2.2.2 Coyotes

Coyote faeces were initially collected from the ground in parks from March 2012 to July 2013 as described by Liccioli *et al*. (2012). A total of 193 coyote faeces collected within this timeframe were selected in order to coincide as much as possible with the collection of samples from dogs, while maximizing sample size. All faeces collected over this time period and estimated to be between 2-5 days old (Liccioli et al., 2012) were selected for the current study.

The faecal samples were kept at -80° C for 72 hours to inactivate *Echinococcus* spp. eggs (Veit et al., 1995) and then transferred to -20° C until laboratory analysis.

2.3 Laboratory analysis

2.3.1 Microscopy

Two grams of faeces were prepared for direct immunofluorescence (DFA) microscopy using a modified version of the manufacturer's instructions (Waterborne Inc., New Orleans, LA, USA) for detection of *Giardia* spp. and *Cryptosporidium* spp. Faeces were homogenized in phosphate-buffered saline (PBS), strained through double-layered cheesecloth, and centrifuged for five minutes at 528 x *g* to isolate (oo)cysts. The majority of supernatant was discarded, apart from 1.5 ml used to homogenize sediment. For each sample, 20 µl from 1.5 ml of homogenized sediment was transferred to a microscope slide, dried, and mounted with 20 µl of Aqua-Glo™ (20X) containing *Giardia* spp. and *Cryptosporidium* spp. fluorescein-labeled monoclonal antibodies. Meanwhile, 200 µl of homogenized sediment from each sample was transferred separately to a microcentrifuge tube in preparation for deoxyribonucleic acid (DNA) extraction. Positive samples were identified as those containing (oo)cysts that were within correct size, shape, and colour parameters (United States Environmental Protection Agency, 2005). Presence or absence

of *Giardia* spp. or *Cryptosporidium* spp. (oo)cysts, as well as the number of (oo)cysts per microscope slide were recorded.

Parasite prevalence was calculated based on results of DFA microscopy and confidence intervals (CI) were calculated using the 95% CI Sterne estimator (Santner and Duffy, 1989). The number of cysts per gram (cpg) or oocysts per gram (opg) were estimated (and rounded up to the nearest whole number) by extrapolating the number of (oo)cysts counted per microscope slide to the volume of homogenized sediment sourced from two grams of faeces and median and upper and lower limits reported. A chi-square exact test (Good, 2005) was used to look for significant differences of *Giardia* spp. and *Cryptosporidium* spp. prevalence in dogs versus coyotes. Differences of median intensities of *Giardia* spp, and *Cryptosporidium* spp. between dogs and coyotes were compared using the Mann–Whitney Test for two independent samples (Good, 2005). Additional prevalence values were calculated to account for the potential of re-sampling coyotes ('Data quality control measures' in Supplementary Material). All statistical analyses were conducted using Statistical Package for the Social Sciences version 20.0 (SPSS Inc., Armonk, NY, USA).

2.3.2 Molecular analysis

Samples positive for (oo)cysts were prepared for DNA extraction using the 200 µl of homogenized sediment set aside during preparation for microscopy. Faecal sediment was first exposed to five rounds of freeze/thaw (dry ice with 95% ethanol and thawing at 70ºC) to fracture (oo)cysts. The remaining DNA extraction procedure followed the QIAamp DNA Stool protocol (Qiagen, Toronto, ON, Canada) yielding 200 µl elution volumes. In addition to positive faecal specimens, the following negative controls were extracted to account for possible contamination

and to assess synchrony of negatives using DFA and polymerase chain reaction (PCR): DNA lysis buffer and a sub-sample of faecal specimens that had screened DFA negative for *Giardia* spp. and *Cryptosporidium* spp. Positive controls were extracted to determine extraction efficiency using aliquots of 50 *Cryptosporidium muris* oocysts (Waterborne Inc.).

An internal amplification control (IAC) was initially used to determine and control for the level of PCR inhibition in dog and coyote faecal specimens screening positive for *Giardia* spp. and/or *Cryptosporidium* spp. using DFA ('Data quality control measures' in Supplementary Material). A 292 bp fragment of the 16S SSU rRNA (Hopkins et al., 1997) and 840bp fragment of the 18S SSU rRNA gene (Xiao et al., 2001) were subsequently targeted for detection of *Giardia* spp. and *Cryptosporidium* spp., respectively. Coyote and dog faecal specimens DFA positive for *Giardia* spp. and/or *Cryptosporidium* spp., as well as negative and positive controls were analysed using semi-nested PCR. Faecal DNA extracts were analysed in replicates of five and negative and positive controls in replicates of three. Water was used as an additional negative control on all PCR plates.

The *Giardia* spp. assay was a semi-nested reaction and used a 25 µl reaction volume for the primary reaction and contained the following: five µl of template, 1.1 µl of water, 1X Maxima Hot Start PCR Mastermix (Thermo Fisher, Waltham, MA, USA), 1M Betaine (Sigma-Aldrich, St. Louis, MO, USA), 400 ng/µL bovine serum albumin (BSA, Sigma-Aldrich), and primer concentrations of 0.8 µM each (high-performance liquid chromatography–HPLC purified, Thermo Fisher). The secondary reaction used five µl of template from the primary reaction and similar reagents and volumes. A previously published forward primer sequence was used for both the primary and secondary reactions (Hopkins et al., 1997) and the following reverse primer

for the primary reaction: 5'- TTG GAT GTG CGG GCC GTC TCG CA- 3'. The reverse primer for the secondary reaction used was: 5' - GTC GAA CCC TGA TTC TCC GCC AG – 3'. PCR cycling conditions were as follows: 95ºC for 4 minutes, and 35 cycles of 95ºC for 20 seconds, 59ºC for 30 seconds, 72ºC for 40 seconds, and a final elongation step of 72ºC for 7 minutes (Applied Biosystems, 2720 thermalcycler). Products were separated on 2.5% agarose gels comprised of 1X TAE buffer and visualized using SYBR® Safe (Thermo Fisher) under ultraviolet (UV) light.

Previously published primer sequences and a PCR assay validated for several *Cryptosporidium* genera described by Ruecker et al. (2011) were used to detect *Cryptosporidium* spp.

Giardia spp. PCR amplicons were cut from gels and purified using a QIAquick Gel Extraction Kit (Qiagen). Amplicons were bi-directionally sequenced with Sanger sequencing at Macrogen (Seoul, South Korea), using the same nested primers used for PCR. Multiple alignments using ClustalW were conducted with reference sequences for *G. intestinalis* assemblages A [GenBank: M54878], B [GenBank: AF199447], and C-G [GenBank: AF199449, AF199443, AF199448, AF199444, AF199450] published by Sprong *et al*. (2009). Alternate sources were used for the following reference sequences: *Giardia ardeae* [GenBank: Z17210], *Giardia muris* [GenBank: X65063] (van Keulen et al., 1993), *Giardia microti* [GenBank: AF006677] (van Keulen et al., 1998), and *Giardia psittaci* [GenBank: AF473853] (van Keulen et al., 2002). *Giardia* spp. was identified using polymorphic regions at the positions 22/23/24/38/44/45/63/75/95/170/175 (Table S1 in Supplementary Material), where the first base of the forward primer was considered bp one.

The sequences were cropped to the edge of the primer region and then aligned using ClustalW. For sequences without interpretable variants at indicative polymorphic positions, GenBank using BLAST (GenBank, 1982) was searched for the closest sequences. Query sequences confirmed by BLAST to be most highly identical to *Giardia* spp. were aligned to reference sequences using ClustalW. Percent identity scores >98% were considered *Giardia* spp. All *Giardia* spp. bioinformatics work was conducted using Geneious 7 (Biomatters Ltd., Auckland, New Zealand).

Cryptosporidium spp. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced bi-directionally at the University of Calgary DNA Sequencing and Genomics Analysis Laboratory with the same nested primers used for PCR (Ruecker et al., 2011). Consensus sequences were generated from forward and reverse DNA trace sequences. Multiple alignments were conducted using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) with reference sequences for *Cryptosporidium* species and genotypes sourced from Ruecker *et al*.(2013; 2012), as well as sequences more recently added to the sequence library using methods described in Ruecker *et al*.(2012). For alignments with less than 100% identity, bases were scanned for alignment anomalies, and identified.

3. RESULTS

3.1 Laboratory analysis

3.1.1 Microscopy

There was no significant difference in *Giardia* spp. prevalence in dogs and coyotes (7.4% vs. 7.8%, respectively; χ^2= 0.025, D.F. = 1, P_{exact}= 0.880, n = 1053 – Table 1) or *Cryptosporidium* spp.

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A total of 860 dog faecal samples were collected from dog-owning residences in Calgary, and ¶
193 coyote faecal samples were collected from Calgary parks.¶

(2.4% vs. 1.6%, respectively; $\chi^2 = 0.557$, D.F. = 1, $P_{exact} = 0.599$, n = 1053 – Table 1). Median *Giardia* spp. infection intensity was significantly higher in dogs than coyotes (Mann–Whitney $U = 231$, $n_{dogs} = 64 = n_{coyotes} = 15$, $P < 0.002$ – Table 1) and median *Cryptosporidium* spp. infection intensity was significantly higher in coyotes than dogs (Mann–Whitney $U = 61$, $n_{dogs} = 21 = n_{coyotes} = 3$, $P_{exact} < 0.004$ – Table 1).

3.1.2 Molecular analysis

DNA was extracted from 64 dog and 15 coyote faecal specimens that screened DFA positive for *Giardia* spp., and 21 dog and three coyote specimens that screened DFA positive for *Cryptosporidium* spp. (total of 94 extracts that accounted for mixed *Giardia* spp. - *Cryptosporidium* spp. infections). Controls and additional samples extracted included 18 negative samples (DNA lysis buffer), 18 faecal specimens that screened DFA negative for *Giardia* spp. and *Cryptosporidium* spp., and six positive controls.

Of the dog and coyote faecal specimens screening DFA positive for *Giardia* spp. and that were analysed using PCR, 34/63 (54.0%) and 7/14 (50.0%) were also PCR positive, respectively (Figure 1a). Note that two samples analysed by DFA (from one dog and one coyote) were not analysed by PCR due to the insignificant quantities of biological sample remaining after DFA (and, consequently, inability to extract DNA). Of the dog and coyote faecal specimens screening DFA positive for *Cryptosporidium* spp. that were analysed using PCR, 3/21 (14.3%) and 1/3 (33.3%) were also PCR positive, respectively (Figure 1b). The large majority of negative controls screened PCR negative for both parasites. A single dog and coyote faecal specimen that were negative using DFA, were positive for *Giardia* spp. and *Cryptosporidium* spp. using PCR,

respectively. The majority of *Cryptosporidium* spp. positive extraction controls were affirmative for all three replicates.

Of the dog and coyote faecal samples PCR positive and sequenced for *Giardia* spp., 27/33 (82%) and 3/7 (43%) returned sequences interpretable using discriminatory polymorphic nucleotides for dog and coyotes, respectively (Table 2). Of the 27 dog faeces successfully sequenced for *Giardia* spp., the majority was sourced from park-attending dogs (n=25/27). Of the interpretable *G. intestinalis* sequences from dogs, 20 were a 100% identity match to the reference sequences for assemblages C and D for at least one of two duplicate samples from the same individual. The remaining 7 interpretable sequences had percent identity values of over 99% apart from one dog sample with a 98.9% identity match value to *G. intestinalis* assemblage D (Table 2). The majority of base pair anomalies in all samples with identity scores less than 100% appeared to be due to polymerase errors, showing obscured peaks on at least one trace sequence, as well as potential single nucleotide polymorphisms (SNP) in four samples. For the six samples that were not identifiable using polymorphic regions of the 16S SSU rRNA gene, GenBank searches using BLAST returned five as *Giardia* spp. Multiple alignments to reference sequences using ClustalW produced two higher identity scores of 90.1% and 85.5% to *G. intestinalis* assemblage C, the former was the DFA negative dog faecal specimen that screened PCR positive for *G. intestinalis*. The remaining three *Giardia* spp. samples produced identity scores between 38% and 65%. The one remaining dog sample searched in GenBank using BLAST returned results consistent with a microorganism other than *Giardia* spp., suggesting primer cross-reactivity. Three *Giardia* spp. sequences from coyotes were 100% identical to reference sequences. Three uninterpretable sequences returned GenBank matches to microorganisms other than *Giardia* spp., and one did not return results.

306 Of the dog and coyote faecal samples PCR positive and sequenced for *Cryptosporidium* spp., all
307 of them returned interpretable sequences (Table 2). Two of three dog faeces successfully
308 sequenced for *Cryptosporidium* spp. were sourced from park-attending dogs. All three dogs
309 PCR positive for *Cryptosporidium* spp. were infected with *C. canis* (Table 2). Two of these were
310 100% identical to GenBank: AB210854 and the third had one potential SNP. One coyote was
311 infected with *C. canis* [99.7% match to GenBank: AB210854, one indel accounting for the 0.3%
312 difference] and the coyote sample DFA negative but PCR positive for *Cryptosporidium* spp. was
313 carrying the vole genotype [100% identity match to GenBank: JQ178298].
314
315 **4. DISCUSSION**
316 Our characterization of *G.intestinalis* assemblages and *Cryptosporidium* species using an SSU
317 rRNA target locus suggested that dogs and coyotes were primarily infected with host-specific
318 genotypes. While there is the potential for transmission of these parasites among sympatric dogs
319 and coyotes, the potential for zoonotic transmission appeared relatively low.
320
321 The predominance of host-specific assemblages of *G. intestinalis* and *Cryptosporidium* species
322 infecting dogs and coyotes found in the current study is not surprising, and has been reported for
323 dogs globally, spanning research locales in Italy (Scaramozzino et al., 2009; Zanzani et al.,
324 2014), Poland (Solarczyk and Majewska, 2010), the United States (Wang et al., 2012), Japan
325 (Abe et al., 2002), Finland (Rimhanen-Finne et al., 2007), and Australia (Palmer et al., 2008),
326 and Canada (McDowall et al., 2011; Uehlinger et al., 2013). Despite the predominance of host-
327 specific genotypes often reported for dogs, there are opposing accounts of assemblages A/B as
328 the dominant genotypes present (Leonhard et al., 2007; Marangi et al., 2009; Sotiriadou et al.,
329 2013; Thompson et al., 2008; Volotao et al., 2007). Not all of the studies targeted the same

genetic loci or investigated sub-assemblages of A/B, making direct comparison difficult, but it does suggest a high frequency of potentially zoonotic genotypes in *G. intestinalis*-infected dogs in some locales. The absence of *C. parvum* detection in dogs in our study is supported by literature (Lucio-Forster et al., 2010; Smith et al., 2009; Sotiriadou et al., 2013). Prevalence of *C. parvum* likely also varies among dogs geographically and may be higher in regions where dogs have access to human infected faeces or livestock (e.g. rural, farming communities), although no known studies have been conducted to compare *C. parvum* occurrence among these two host populations.

Assemblages of *G. intestinalis* and *Cryptosporidium* species infecting coyotes have been investigated less often than for dogs, but published studies indicate the occurrence of both host-associated and zoonotic genotypes (Thompson et al., 2009; Trout et al., 2006; Xiao et al., 2002). In our study, we detected only two non-host-specific genotypes of *G. intestinalis* and *Cryptosporidium* spp. infecting coyotes: one *G. intestinalis* assemblage A, and the other, the *Cryptosporidium* spp. vole genotype. *Giardia intestinalis* assemblage A was previously reported in coyotes by Thompson et al. (2009), but this is the first known report of the *Cryptosporidium* spp. vole genotype, of which is likely a result of coyote predation of co-habiting rodent species - a principal component of coyotes' diet in our study area (Liccioli et al., 2013; Morey et al., 2007).

It is possible that we underestimated the occurrence of some *G. intestinalis* assemblages (e.g. assemblages C/D). Notable SNPs in one or both 16S reverse primer regions for several *G. intestinalis* assemblages and *Giardia* species (Figure S1 in Supplementary Material), excluding

G. intestinalis assemblage A, suggests variable sensitivity of the assay. Additional SNPs may also occur within the 16S forward primer region, however, for many *G. intestinalis* assemblages and *Giardia* species, lack of sequence data upstream of the this forward primer region (5') renders this inconclusive. Unfortunately, GenBank 16S DNA sequence data related to *G. intestinalis* assemblages, other than those represented by assemblages A (Healey et al., 1990; Prabhu et al., 2007)/B (van Keulen et al., 1995), originates within the forward primers themselves – a problem since even mis-matched forward primer sequences are artificially amplified through the sequencing reactions (and subsequently entered into GenBank as confirmed sequence in the primer regions). The two other *Giardia* species for which 16S DNA sequences exist upstream of the forward primer region include *G.muris* (van Keulen et al., 1992; van Keulen et al., 1993) and *G. ardeae* (van Keulen et al., 1993), and both contain a SNP in this region, and SNPs within both reverse primer regions. Reduced sensitivity of the assay from SNPs within primer regions is demonstrated by our own data that shows a nearly 50-fold decrease in PCR method sensitivity against *G. muris* (Table S2 in Supplementary Material). The discrepant detection of *G. intestinalis* seen by PCR versus DFA in this study, may be partially attributed to the application of a PCR assay with potentially heightened sensitivity to assemblages A/B relative to assemblages C/D. The antibodies used in the DFA assay are able to cross-react to various *G. intestinalis* assemblages and *Cryptosporidium* species (Waterborne Inc., New Orleans, LA, USA), and therefore may have detected some positive cases that the PCR assay did not.

This *Giardia* PCR assay highly sensitive for *G. intestinalis* assemblages A/B (i.e. LOD_{95}=10.0 template copies per reaction, CI=25.2 – 4.0, Table S2 in Supplementary Material), suggests a lower relative abundance of zoonotic than host-specific genotypes within *G. intestinalis*

infections of dogs and coyotes in our study. Although *C. canis* is potentially zoonotic, the risk to human health is considered minimal, as few cases of *C. canis* have been documented in humans, overall (Lucio-Forster et al., 2010; Xiao, 2010). In extremely rare cases, children and the immunocompromised individuals represent the susceptible group (Bowman and Lucio-Forster, 2010; Marangi et al., 2009; Volotao et al., 2007; Xiao et al., 2007). It is possible that zoonotic *G. intestinalis* assemblages and *Cryptosporidium* spp. were present within mixed infections and went undetected because they did not predominate the mixed infection; for *Cryptosporidium* spp., loss of detection has been demonstrated to occur most often when the concentration ratio among genotypes is high (Ruecker et al., 2011).

There are known limitations of using a single-locus approach for *G. intestinalis* assemblage differentiation; multilocus sequence typing (MLST) is recommended to confirm genotypes ascertained using SSU rRNA (Feng and Xiao, 2011). While some of the more advanced molecular methods (including MLST) also help to distinguish sub-genotypes, resolve mixed infections, and provide higher discriminatory power to assist with interpreting zoonotic transmission potential for both *G. intestinalis* and *Cryptosporidium* spp. (Feng and Xiao, 2011; Ruecker et al., 2011; Thompson and Ash, 2016), our study targeted the SSU rRNA to preliminarily assess the parasitic genotypes circulating in dogs and coyotes. Allelic sequence heterozygosity within *G. intestinalis* assemblages at identifying positions could also influence assemblage-based typing results, although this phenomenon is uncommon for this species (Sprong et al., 2009). Our study contributes to the international body of research pertaining to genotypes of *Giardia* spp. and *Cryptosporidium* spp. from dogs and coyotes, and provides preliminary, baseline molecular data for ongoing research and surveillance. Follow up studies using a combined epidemiological and MLST approach would provide a relevant confirmatory

measure and help to further clarify public and animal health significance of *Giardia* spp. and *Cryptosporidium* spp. circulating in wild and domestic canids.

5. CONCLUSIONS

Although risk for zoonotic transmission cannot be excluded, our infrequent detection of zoonotic genotypes using this *Giardia* spp. PCR assay sensitive to assemblages A/B, and validated for multiple genera of *Cryptosporidium* spp. (Ruecker et al., 2011), supports the conclusion that dogs and coyotes in our study area present a relatively low concern for zoonotic transmission of these parasites.

Our previous work demonstrated the potential for *Giardia* spp. transmission among dogs is highest when they are off-leash, likely influenced by increased interaction and accessibility to environmental sources of infection, e.g. soil and water (Smith et al., 2015). Dog owners should note the relevance of off-leash activity for transmission of gastrointestinal parasites (Smith et al., 2015; Smith et al., 2014) among dogs and coyotes, including the host-specific *Giardia* and *Cryptosporidium* genotypes found to occur in these canid populations.

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Deleted: particularly young dogs that may be most susceptible to infection (Bugg et al., 1999; Fontanarrosa et al., 2006; Smith et al., 2014)

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8. DECLARATION OF INTEREST

None.

453 **Table 1.** Prevalence (%) and median intensities (#(oo)cysts/g) of *G. intestinalis* and *Cryptosporidium* genotypes occurring
454 in dog and coyote host species.

	***Giardia* spp.**				***Cryptosporidium* spp.**				**Overall**	
Host Species	**No. of cysts/g[a]**	**Infection intensity range[b]**	**No. (%[c]) of cases**	**CI[d]**	**No. of oocysts/g**	**Infection intensity range**	**No. (%) of cases**	**CI**	**No. (%) of cases**	**CI**
Dog (*n*=860)	1.0 x 10^{4}	3.8 x 10^{1}-1.6 x 10^{5}	64(7.4)	5.9-9.4	3.8 x 10^{1}	3.8 x 10^{1}-1.6 x 10^{3}	21(2.4)	1.5-3.7	79(9.2)	7.4-1.1 x 10^{3}
Coyote (*n*=193)	4.9 x 10^{2}	3.8 x 10^{1}-6.0 x 10^{3}	15(7.8)	4.6-1.2 x 10^{3}	4.2 x 10^{3}	4.5 x 10^{2}-5.6 x 10^{3}	3(1.6)	4.0 x 10^{1}-4.6	15(7.8)	4.6-1.2 x 10^{3}

455 [a]Median intensity values
456 [b]Upperand lower limits of (oo)cysts per gram
457 [c]Prevalence based on DFA microscopy from faecal samples
458 [d]95% confidence intervals

459 **Table 2.** *Giardia* spp. and *Cryptosporidium* spp. genotypes infecting dog and coyotes.
460

	G. intestinalis		***Cryptosporidium* spp.**	
Host Species	**Assemblage**	**No. (%)**	**Species/Genotype**	**No. (%)**
Dog	Assemblage C	14(51.9)	*C. canis*	3(100.0)
	Assemblage D	13(48.1)	(*n*=3)	
	(*n*=27)			
Coyote	Assemblage A	1(33.3)	*C. canis*	1(50.0)
	Assemblage C	1(33.3)	Vole genotype	1(50.0)
	Assemblage D	1(33.3)	(*n*=2)	
	(*n*=3)			

461

462 **Figure Legends**

463 **Figure 1. Two images depicting results from repetitive nested PCR.**

464 (a) Four replicates of amplified *G. intestinalis* 16S SSU rRNA gene product (292bp) from a dog

465 (sample number B38) (b) Two replicates of amplified *C. canis* 18S SSU rRNA gene product

466 (800bp) from a dog (sample number W122). MW indicates a 100bp molecular weight ladder.

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