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Research paper

Detection and molecular diversity of *Giardia duodenalis* and *Cryptosporidium* spp. in sheltered dogs and cats in Northern Spain

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ABSTRACT

Domestic dogs and cats may act as natural reservoirs of a large number of zoonotic pathogens, including the enteric parasites *Giardia duodenalis* and *Cryptosporidium* spp., the most relevant protozoan species causing gastrointestinal disease worldwide. A cross-sectional epidemiological study aiming to assess the prevalence and molecular diversity of *G. duodenalis* and *Cryptosporidium* spp. was conducted in an animal rescue centre in the province of Álava (Northern Spain). A total of 194 and 65 faecal dropping samples from individual dogs and cats, respectively, were collected between November 2013 and June 2016. *G. duodenalis* cysts and *Cryptosporidium* spp. oocysts were detected by direct fluorescence microscopy and PCR-based methods targeting the small subunit ribosomal RNA gene of these parasites.

Overall, *G. duodenalis* and *Cryptosporidium* spp. were detected in 33% (63/194) and 4.1% (8/194) of dogs, and 9.2% (6/65) and 4.6% (3/65) of cats, respectively. *G. duodenalis* and *Cryptosporidium* co-infections were observed in 1.5% (3/194) of dogs, but not in cats. No significant differences in infection rates could be demonstrated among dogs or cats according to their sex, age group, status, or geographical origin. Multi-locus sequence-based genotyping of the glutamate dehydrogenase and β-giardin genes of *G. duodenalis* allowed the characterization of 19 canine isolates that were unambiguously assigned to sub-assemblages AII ($n = 7$), BIII ($n = 1$), and BIV ($n = 7$), and assemblages C ($n = 3$) and D ($n = 1$). Two feline isolates were genotyped as assemblages A and F, respectively. No mixed assemblage or sub-assemblage infections were identified. *C. canis* ($n = 5$) and *C. hominis* ($n = 1$) were the *Cryptosporidium* species found in dogs, whereas *C. felis* ($n = 1$) was identified in cats. The finding of *G. duodenalis* sub-assemblages AII, BIII, and BIV circulating in dogs (but not cats) may have zoonotic potential, although most of the AII and BIV isolates sub-genotyped corresponded to genetic variants not previously found in Spanish human populations. Dogs may also act as novel suitable hosts for *C. hominis*. We recommend to considerer companion animals as sentinel surveillance system for zoonotic giardiasis and cryptosporidiosis in order to minimize the risk of spreading of these parasitic diseases among the human population.

1. Introduction

Cats, dogs, and humans have shared a close relationship due to companionship, recreation, protection, and occupational reasons, for over 10,000 years. Pet ownership has been recognizably proven to exert beneficial effects on human health by improving physical condition and mental and emotional well-being (Hodgson et al., 2015). Consequently, pets are being increasingly used as an effective therapeutic option in healthcare facilities (Wells, 2007). However, cats and dogs may also act as natural reservoirs of human infections by pathogenic bacteria, viruses, and parasites (Chomel, 2014), including the enteric protozoan *Giardia duodenalis* and *Cryptosporidium* spp. (Esch and Petersen, 2013).

G. duodenalis is the only *Giardia* species infective to dogs, cats, and humans. Of the eight genetic variants (assemblages A–H) forming part of *G. duodenalis*, dogs and cats are predominantly infected by canine-specific (C–D) or feline-specific (F) assemblages. Zoonotic assemblages A and B (particularly the former) are responsible for a lower proportion of cases in pets (Ryan and Cacciò, 2013). The genus *Cryptosporidium*

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encompasses at least 26 valid species, of which *C. canis* and *C. felis* cause the vast majority of infections in dogs and cats, respectively (Ryan et al., 2014). Both species are considered of low zoonotic risk to humans. Additionally, novel *Cryptosporidium* species and genotypes have been proposed in recent years (Jezkova et al., 2016; Kváč et al., 2016).

Although the zoonotic potential of *G. duodenalis* and *Cryptosporidium* spp. is not questioned, the extent and frequency of such transmission events remain a topic of intense debate (Xiao and Feng, 2008; Ballweber et al., 2010; Lucio-Forster et al., 2010). Large household- or community based molecular epidemiological surveys coincide in concluding that domestic cats and dogs play a minor role as source of human giardiasis or cryptosporidiosis (Cooper et al., 2010; Inpankaew et al., 2014). A similar conclusion has been reached by seasonal models analysing large time series of human and canine *Giardia* cases in USA (Mohamed et al., 2014). However, other studies suggest that zoonotic transmission may occur sporadically (Xiao et al., 2007; Beser et al., 2015) or under certain epidemiological conditions (Inpankaew et al., 2007; Volotão et al., 2007).

Both *G. duodenalis* and *Cryptosporidium* spp. have been reported in European cats and dogs with prevalence rates ranging from <1–15% in asymptomatic animals (Overgaauw et al., 2009; Osman et al., 2015; Paoletti et al., 2015) to up to 25% in symptomatic populations (Batchelor et al., 2008; Epe et al., 2010). In Spain, *G. duodenalis* has been detected in 15% and 1–38% of the studied feline and canine populations, respectively (reviewed in Carmena et al., 2012 and Bouzid et al., 2015). Infection by *Cryptosporidium* spp. has only been documented in 7–15% of the dogs investigated (reviewed in Navarro-i-Martinez et al., 2011). Genotyping data are only available from few molecular studies carried out in the Autonomous Region of Madrid (Dado et al., 2012b) and Catalonia (Ortuño et al., 2014).

In recent years, our research group has conducted a series of community-based and field studies aiming to characterize the transmission dynamics of *G. duodenalis* and *Cryptosporidium* spp. in the province of Álava, Northern Spain. Molecular data was, therefore, gathered from human (Cardona et al., 2011), livestock (Cardona et al., 2011, 2015), and wild animal (Cano et al., 2016) populations, and from environmental water samples (Carmena et al., 2007). Additionally, these studies also evidenced that ownership of domestic dogs or cats tended to increase the prevalence odds of human giardiasis and cryptosporidiosis (Cardona et al., 2011). In an attempt to complete our understanding of the epidemiology of these parasitic diseases in Álava, we report here the prevalence, molecular diversity and frequency of *G. duodenalis* and *Cryptosporidium* species in sheltered cats and dogs from this region. Additionally, generated genotyping information were used to evaluate the potential role of pet animals as suitable reservoirs of human disease.

2. Material and methods

2.1. Study area and faecal sample collection

The province of Álava (Northern Spain) numbers 51 municipalities distributed in seven administrative regions. Although no official pet census is currently available, it is estimated that >7000 dogs and 5000 cats are kept as companion animals only in the capital city Vitoria-Gasteiz. Stray, abandoned, or surrendered animals in the province are sent and held at the Armentia Municipal Animal Shelter (AMAS). In average, the AMAS provides care for 1250 dogs and 350 cats every year, and its adoption program allows finding a new home for 76%–94% of these animals.

A total of 194 and 65 faecal dropping samples from individual dogs and cats, respectively, were regularly collected as soon as practicably possible after defecation between November 2013 and June 2016 by the AMAS personnel. Faecal material was obtained within 24 h after the animals entered the AMAS in order to prevent acquired infections in the centre through continuous exposure to already infected animals or potential high environmental contamination. Faecal specimens were placed in screw-topped specimen containers and uniquely labelled indicating identification number and date of collection. Information regarding sex, age, breed, and geographical origin of the animal was also consigned. Faecal samples were stored at −20 °C and shipped to the Spanish National Centre for Microbiology for further diagnostic and molecular analyses.

2.2. Direct fluorescent antibody test

A direct fluorescent antibody test (DFAT) was used to detect *Giardia* cysts and *Cryptosporidium* oocysts by fluorescence microscopy. Briefly, ~1 g of faecal material was processed using the concentration system PARASEP Midi® (Grifols Movaco, Barcelona, Spain) according to the manufacturer's instructions. Five microliter of concentrated faecal material were placed on welled slides. Smears were air-dried, methanol fixed, stained with fluorescein-labelled mouse monoclonal antibodies (Crypto/Giardia Cel, Cellabs, Sydney, Australia), and examined at 400× magnification. The burden of the infection was estimated by counting the number of (oo)cysts per well.

2.3. DNA extraction and purification

Total DNA was extracted from a new aliquot (~200 mg) of each faecal sample using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified DNA samples (200 μL) were stored at −20 °C for further downstream molecular analysis. A water extraction control was routinely included in each sample batch processed.

2.4. Molecular detection of Giardia duodenalis

Detection of *G. duodenalis* DNA was achieved using a real-time PCR method targeting a 62-bp region of the small subunit ribosomal RNA (*ssu* rRNA) gene of the parasite (Verweij et al., 2003). Amplification reactions were conducted in a volume of 25 μL containing 3 μL of template DNA, 12.5 pmol of primers Gd-80F and Gd-127R, 10 pmol of probe (Supplemental content 1), and 12.5 μL TaqMan® Gene Expression Master Mix (Applied Biosystems, CA, USA). Detection of parasitic DNA was performed on a Corbett Rotor-Gene 6000 real-time PCR cycler (Qiagen Corbett, Hilden, Germany) using an amplification protocol consisting on an initial hold step of 2 min at 55 °C and 15 min at 95 °C followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The ramping of the machine was 10 °C/s in every step. Ten-fold dilutions of a DNA isolate obtained from a human stool sample with a known number of *G. duodenalis* cysts were included in each experiment for sensitivity and quantification purposes (see below). No-template water (negative) and DNA (positive) controls of genomic DNA were included in each PCR run.

2.5. Molecular characterization of Giardia duodenalis isolates

G. duodenalis isolates that tested positive by real-time PCR were subsequently assessed at the glutamate dehydrogenase (*gdh*) and β-giardin (*bg*) loci. The semi-nested-PCR protocol proposed by Read et al. (2004) with minor modifications was used to amplify a ~432-bp fragment of the *gdh* gene. PCR reaction mixtures (25 μL) consisted of 5 μL of template DNA, 0.5 μM of each primer (GDHeF/GDHiR in the primary reaction and GDHiF/GDHiR in the secondary reaction, respectively, Supplemental content 1), 2.5 units of MyTAQ™ DNA polymerase (Bioline GmbH, Luckenwalde, Germany), and 5 μL of MyTAQ™ Reaction Buffer containing 5 mM dNTPs and 15 mM $MgCl_2$. Both amplification protocols consisted of an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension of 72 °C.

Similarly, a ~511-bp fragment of the *bg* gene of *G. duodenalis* was amplified using the nested-PCR protocol described by Lalle et al.