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Research paper

Comparison of five diagnostic tests for *Giardia duodenalis* in fecal samples from young dogs

Fabienne D. Uehlinger[a,*], S. Ali Naqvi[e], Spencer J. Greenwood[b], J. Trenton McClure[a], Gary Conboy[c], Ryan O'Handley[d], Herman W. Barkema[e]

[a] Department of Health Management and Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island C1A 4P3, Canada
[b] Department of Biomedical Sciences and Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island C1A 4P3, Canada
[c] Department of Pathology & Microbiology, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, Prince Edward Island C1A 4P3, Canada
[d] School of Animal and Veterinary Sciences, Roseworthy Campus, The University of Adelaide, Roseworthy, SA 5371, Australia
[e] Department of Production Animal Health, Faculties of Veterinary Medicine and Medicine, University of Calgary, 3300 University Drive N.W., Calgary, Alberta T2N 4N1, Canada

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ABSTRACT

Five diagnostic tests were compared for the diagnosis of *Giardia duodenalis* in fecal samples of young dogs. Fecal samples were collected from 136 healthy dogs < 1 year old and examined using immunofluorescence antibody microscopy (IFA) after sucrose gradient centrifugation, zinc sulfate centrifugal flotation technique (ZSCT), SNAP® *Giardia* test, and ProSpecT® *Giardia* EZ Microplate assay. In addition, polymerase chain reaction (PCR) of the 16S rRNA gene was performed. Kappa (κ) statistic was calculated to assess diagnostic agreement between the IFA and each test. Using the IFA as the gold standard, the relative sensitivity and specificity of each test were determined. Subsequently, a Bayesian approach was used to estimate the sensitivity and specificity of each test in comparison to the IFA results. *Giardia duodenalis* was detected in 41% of the samples examined by IFA. The ZSCT resulted in 37% of positive samples, with a relative sensitivity and specificity of 86 and 98%, respectively. The SNAP® *Giardia* test was positive in 40% of the samples, with a relative sensitivity and specificity of 91 and 96%, respectively. The ProSpecT® test was positive in 51% of the samples, with a relative sensitivity and specificity of 100 and 83%, respectively. The relative sensitivity and specificity for PCR were 58 and 56%, respectively, with 55% of samples being PCR-positive. While the sensitivity and specificity estimates of each test in comparison to the IFA changed when using a Bayesian approach, the conclusions remained the same. While the ProSpecT® test was the most sensitive test in this study, it is not designed for dogs and more costly than the other tests. The SNAP® *Giardia* test performed similar to the ZSCT but may be more favorable because it is fast and easy to perform. Performance of the PCR was poor and the benefit of PCR may be in determining genotypes for evaluating zoonotic transfer between dogs and humans.

1. Introduction

Giardia duodenalis is a common protozoan parasite in dogs and is worldwide in distribution. The need for accurate diagnosis of *G. duodenalis* infection is heightened due to zoonotic potential of dogs to serve as a source for human infections (Thompson, 2004; Uehlinger et al., 2013). Until recently, diagnosis of *G. duodenalis* mainly involved detection of cysts by microscopy following $ZnSO_4$ centrifugal fecal flotation technique (ZSCT)[1] and less frequently detection of trophozoites by direct saline fecal smear. Centrifugal flotation and simple flotation techniques, using various salts or sugar solutions, followed by light microscopy, are widely used to detect a wide range of helminth eggs and protozoan cysts (Barr et al., 1992; Dryden et al., 2005). Recognition of cysts recovered by flotation under the microscope can be difficult due to the presence of yeasts, plant remnants and debris in the fecal sample with similar appearance to *G. duodenalis* (Dryden et al., 2006). The small size (8–12 μm x 7–10 μm) and the nature of intermittent shedding further add to the difficulties in the correct diagnosis of this parasite.

* Corresponding author. Present address: Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, SK S7N 5B4, Canada.
E-mail address: f.uehlinger@usask.ca (F.D. Uehlinger).
[1] Zinc sulfate centrifugation technique (ZSCT).

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Microscopic detection and accurate identification of cysts requires a high level of expertise, limiting the use to experienced laboratories (Dixon et al., 1997). A one-time negative microscopy result may not be an accurate assessment of *G. duodenalis* presence or absence in dogs and several repeated fecal examinations may be required for definitive diagnosis (Zimmer and Burrington, 1986; Decock et al., 2003).

Immunofluorescence antibody microscopy (IFA) has made identification of *G. duodenalis* easier through monoclonal antibody staining of cysts and is widely used in detecting *G. duodenalis* primarily in research samples (O'Handley et al., 1999; Geurden et al., 2004; Budu-Amoako et al., 2012; Uehlinger et al., 2013). It has also been demonstrated that IFA is a sensitive and specific test for the detection of *G. duodenalis* in dog feces (Geurden et al., 2008). Although the preparation time may be longer than for conventional microscopy, the actual analysis is easier due to the fluorescein-isothiocyanate-labeled monoclonal antibodies that emit fluorescence when excited at a particular wavelength. Furthermore, the test is more specific through the use of monoclonal antibodies (Dixon et al., 1997). However, the need for expensive specialized equipment (fluorescence microscope) make it impractical for routine laboratory diagnosis. Diagnostic fecal antigen detection kits may be more convenient as they are quick and cyst identification is no longer required. Furthermore, these tests may detect cyst shedding when *G. duodenalis* cyst concentration in feces is very low or when only antigen is present, thereby overcoming a disadvantage in microscopic methods (Vidal and Catapani, 2005). Such commercially available tests are the SNAP® *Giardia* test (IDEXX Laboratories Inc., Westbrook, ME, USA) and the Rapid® Giardia Ag test (BioNote, Gyeonggi-do, Korea) designed for use in dogs and the ProSpecT® *Giardia* EZ Microplate Assay (Oxoid Inc., Nepean, ON, Canada), an enzyme-linked immunosorbent assay (ELISA) designed for use in humans. Polymerase chain reaction (PCR) has been used for epidemiological prevalence estimations of *G. duodenalis* in cattle, sheep and dogs (Scaramozzino et al., 2009; Yang et al., 2009; Ng et al., 2011; Santín et al., 2012), and only one study evaluated its performance in dogs using a Bayesian approach (Traub et al., 2009). Bayesian analysis is indicated when evaluating diagnostic test characteristics in the absence of a validated gold standard test. In addition to its use by Traub et al. (2009), only two other studies have been identified which applied Bayesian methodology in the evaluation of diagnostic tests for *Giardia* spp. in dogs (Geurden et al., 2008; Papini et al., 2013). However, no study has compared all 5 diagnostic tests used here. Therefore, the objectives of this study were to: a) determine the relative sensitivity and specificity of the ZSCT, SNAP® Giardia test, the ProSpecT® test and PCR in reference to the IFA; and b) to estimate the sensitivity and specificity of these four tests in comparison to the IFA results using a Bayesian approach for the diagnosis of *G. duodenalis* in dogs.

2. Materials and methods

2.1. Sample collection

Fecal samples were collected from healthy dogs < 1 yr of age from different sources on Prince Edward Island (PEI), Canada. Age was based on the owner's information or determined by characteristics such as teeth eruption, wear and condition as well as muscle mass. Samples were put in individual, sealed containers and stored at 4 °C until shipment to the AVC laboratory. For dogs presenting to veterinary clinics and the pet store dogs, samples were stored at 4 °C and submitted within 72 h of collection and samples from dogs housed at the AVC animal resources department were submitted immediately after collection. Each fecal sample was analyzed by IFA after sucrose gradient centrifugation, light microscopy following ZSCT, SNAP® *Giardia*, and the ProSpecT® test. A subset of samples was available for analysis by PCR.

2.2. Laboratory analyses

Giardia duodenalis cysts were isolated from fecal samples with a sucrose gradient centrifugation technique (O'Handley et al., 1999), with the modification that only 1–2 g of feces was used, depending on the initial amount submitted. *Giardia duodenalis* cysts were identified in 20 µl of re-suspended pellet by direct immunofluorescence microscopy (Uehlinger et al., 2006; Uehlinger et al., 2013). One slide was examined per sample. The remaining concentrated pellets were frozen at −20 °C for PCR analysis.

To assess the recovery efficiency of the cyst isolation procedure used in this study, seeding experiments were performed. To ascertain that no *G. duodenalis* cyst were present in the fecal sample used for seeding with known numbers of cysts, five fresh (< 24 h old; unpreserved) 1–2 g fecal samples were processed from one dog using sucrose gradient centrifugation and analyzed by IFA as described above. For seeding, two fresh (< 24 h; unpreserved) fecal samples from two dogs which were shedding high numbers of cysts were used. Of these samples, five 1–2 g samples were processed and analyzed as described above. Intact cysts were enumerated in each slide and the average number of cysts per gram of feces found in each of the five samples was used to calculate the amount needed for seeding the negative samples with a known number of cysts per gram of feces. *Giardia duodenalis* cysts were recovered in 5/5 samples seeded with approximately 30 cysts per g of fresh feces, while cysts were recovered from 3/5 samples seeded with approximately 10 cysts per g of fresh feces. No samples were seeded with less than 10 cysts per g of feces. This demonstrates that the cyst isolation procedure applied to canine fecal samples in this study has a detection limit of approximately 10 *G. duodenalis* cysts per g of feces.

The ZSCT followed by microscopy was performed according to Zajac and Conboy (2012, pp. 4–8) except that 1–2 g of feces was used and centrifugation occurred at 800 × *g*. One slide was examined per sample.

The SNAP® *Giardia* test was performed according to manufacturer's instructions.

The IFA, the ZSCT and the SNAP® *Giardia* tests were performed within 24 h of each other and within 24 h of receipt at the laboratory; refrigerated feces were used for these tests. The fecal samples were then frozen at −20 °C until analysis with the ProSpecT® *Giardia* EZ Microplate Assay. According to the manufacturer's instructions, this test is compatible with samples frozen at −20 to −70 °C within 48 h of collection. For logistical reasons, this was not possible in the study here but all samples were frozen within 96 h of collection. Compatible duration of freezing is not stated by the manufacturer, but freezing did not exceed two weeks for any sample in this study. The samples were thawed at room temperature prior to analysis with the ProSpecT® test on the same day. The manufacturer's instructions were followed and plates were read spectrophotometrically at 450 nm. The plate reader was set to blank on the negative control so that the negative control well optical density (OD) value was automatically subtracted from all of the other readings. Therefore, a sample was considered positive if its OD value was ≥0.050 and negative if it was < 0.050.

For PCR, DNA was extracted from the concentrated pellet obtained previously by sucrose gradient centrifugation using the QIAmp Stool Kit (Qiagen Inc., Mississauga, ON, Canada). The manufacturer's protocol was adhered to with the following modifications: briefly, 200 µl of sample was added to the lysis buffer and subjected to five freeze/thaw cycles with liquid nitrogen and boiling water for one minute each. Supernatant was treated with 'InibitEx' tablets (Qiagen Inc., Mississauga, ON, Canada), followed by proteinase K.

A previously published nested PCR protocol was employed for amplification of a fragment of the *Giardia* 16S rDNA based on Read et al. (2002). Samples were considered PCR-positive for *G. duodenalis* when a band signal of appropriate molecular weight in the gel electrophoresis was obtained in comparison to a positive control. For quality control, a known *G. duodenalis*-positive dog fecal sample and PCR grade water (as