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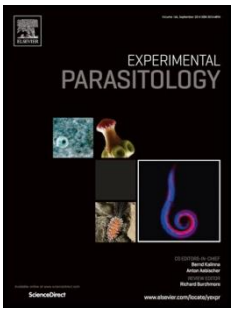

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Comparison of diagnostic techniques for the detection of *Cryptosporidium* oocysts in animal samples

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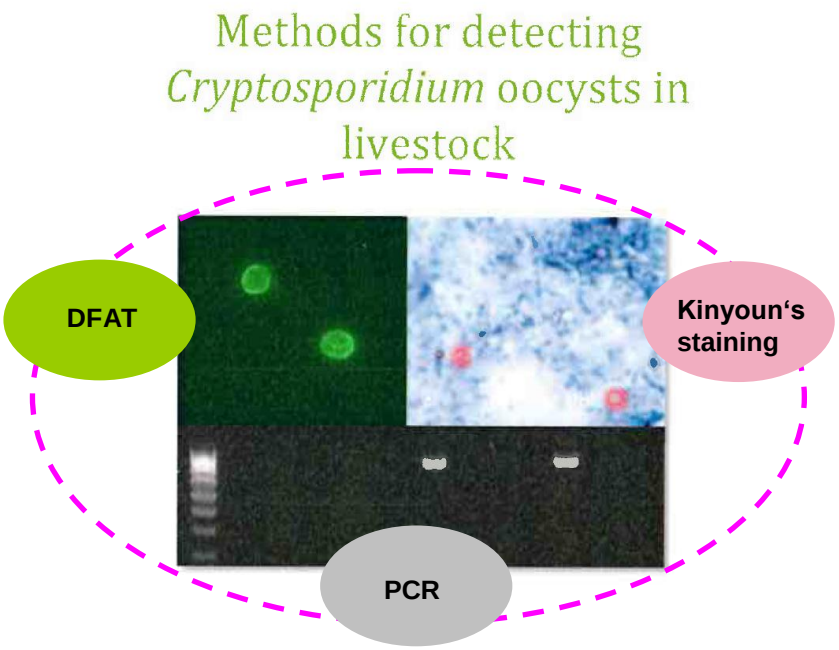
Highlights

- We analysed different diagnostic methods for the detection of *Cryptosporidium* in livestock.
- Latent Class Analysis was used to as a pseudo-gold standard to assess the specificity and sensitivity.
- The three published PCRs were compared to determine their ability to identify the *Cryptosporidium* spp.
- We found the combination of DFAT and the three PCRs are necessary to detect asymptomatic infection.

Graphical Abstract



Abstract

While a large number of laboratory methods for the detection of Cryptosporidium oocysts in faecal samples are now available, their efficacy for identifying asymptomatic cases of cryptosporidiosis is poorly understood. This study was carried out to determine a reliable screening test for epidemiological studies in livestock. In addition, three molecular tests were compared to identify Cryptosporidium species responsible for the infection in cattle, sheep and horses. A variety of diagnostic tests including microscopic (Kinyoun's staining), immunological (Direct Fluorescence Antibody tests or DFAT), enzyme-linked immunosorbent assay (ELISA), and molecular methods (nested PCR) were compared to assess their ability to detect Cryptosporidium in cattle, horse and sheep faecal samples. The results indicate that the sensitivity and specificity of each test is highly dependent on the

input samples; while Kinyoun's and DFAT proved to be reliable screening tools for cattle samples, DFAT and PCR analysis (targeted at the 18S rRNA gene fragment) were more sensitive for screening sheep and horses samples. Finally different PCR primer sets targeted at the same region resulted in the preferential amplification of certain Cryptosporidium species when multiple species were present in the sample. Therefore, for identification of Cryptosporidium spp. in the event of asymptomatic cryptosporidiosis, the combination of different 18S rRNA nested PCR primer sets is recommended for further epidemiological applications and also tracking the sources of infection.

Keywords: Cryptosporidium, cattle, horse, sheep, diagnostic techniques, molecular methods, latent class analysis

1 Introduction

Numerous techniques have been used to detect *Cryptosporidium* infection in humans and animals. These include histology and ultrastructural examination of biopsy material for life-cycle stages, examination of faeces for the presence of oocysts and detection of *Cryptosporidium* antigens or DNA (Smith, 2008). Methods such as direct or indirect immunofluorescence staining techniques (DFAT and IFAT), detection of antigens using enzyme-linked immunosorbent assay (ELISA), as well as various molecular tests such as polymerase chain reaction (PCR), and loop mediated isothermal amplification (LAMP) are widely used to detect the parasite in faecal material (Morgan and Thompson, 1998; Jex et al., 2008; Kaushik et al., 2008; Smith, 2008; Plutzer and Karanis, 2009).

As faecal samples from clinical cases generally contain large numbers of oocysts and parasite antigenic material, even methods that have a low sensitivity can provide a positive diagnosis. In contrast, when testing samples containing few oocysts, as may be required for an epidemiological investigation, the use of an initial screening method (e.g. staining and microscopic analysis of slides), followed by a confirmatory method such as immunofluorescence or molecular approaches can augment confidence in the diagnosis (Smith, 2008). For this purpose the immunofluorescent staining of oocysts with fluorescein isothiocyanate-conjugated anti-*Cryptosporidium*

monoclonal antibody (FITC-C-mAb) has been reported to be particularly specific (96-100%) and sensitive (98.5-100%) (Sterling and Arrowood, 1986; Jex et al., 2008). On the other hand, *Cryptosporidium* coproantigen can be detected in faecal samples even before excretion of oocysts has commenced. There are numerous studies on different ELISA's and immunochoromographic (IC) tests specific for coproantigen with a reported specificity and sensitivity of between 97 and 100% (Robert et al., 1990; Ungar, 1990; Newman et al., 1993; Garcia and Shimizu, 1997; Chan et al., 2000; Johnston et al., 2003; Chalmers et al., 2011). A further advantage of these coproantigen detection assays is that they can be used to test large numbers of samples in a rapid and cost-effective manner. However, for more detailed epidemiological studies, the assays are not suitable because they do not provide any information on the species or genotype of *Cryptosporidium* present (Garcia et al., 2003; Johnston et al., 2003; Jex et al., 2008). To date 29 *Cryptosporidium* genotypes have been described among which *C. parvum*, *C. xiaoi*, *C. bovis*, *C. ryanae*, *C. andersoni* and *C. ubiquitum* are known to be infective to livestock and horses.

More than two decades have passed since the first report of describing the detection of *Cryptosporidium parvum* by PCR (Laxer et al., 1991). These techniques have been developed to detect and differentiate *Cryptosporidium* species at species/genotype and subtype level (Widmer, 1998; Widmer et al., 1998; Sulaiman et al., 1999; Morgan et al., 1995). While it is well established that PCR assays aimed at different regions of the *Cryptosporidium* genome have different sensitivities and specificities, little is known about the behaviour and efficiency of different primer pairs aimed at the same target region (Smith, 2008).

A review by Plutzer and Karanis (2009) emphasizes the importance of molecular tools to assess the zoonotic potential of various *Cryptosporidium* species and the sources of human infection. Application of various molecular approaches, their specificity and sensitivity in the detection of human cryptosporidiosis have already been studied (Jiang and Xiao, 2003; Smith, 2008). In addition a number of genomic loci have been identified as targets for the detection of species as well as for genotype identification of different *Cryptosporidium* isolates (Leetz et al., 2007; Plutzer et al., 2010).

There has been no direct evaluation of all assays used routinely in Ireland for testing asymptomatic cryptosporidiosis in animals. In addition to that the specificity and sensitivity of different molecular tests has not been applied for testing animal species such as cattle, sheep, and horses specifically in veterinary research. Therefore, our study aimed to evaluate commonly used assays such as microscopic examination, ELISA and PCR for detecting asymptomatic cryptosporidiosis in cattle, horse and sheep. Since there is no gold standard technique for detection of *Cryptosporidium* oocysts (Smith, 2008), we used latent class analysis (LCA) to construct a pseudo-gold standard to estimate the sensitivity and specificity of each test for the detection of *Cryptosporidium* oocysts.

Three published PCR protocols targeting the 18S rRNA gene fragment were compared to gain a better understanding of *Cryptosporidium* genotypes present in sub-clinical cases of horse, sheep, and cattle.

2 Materials and Methods

2.1 Experimental design

2.1.1 Comparison of conventional, immunological and molecular screening methods

First the sensitivity and specificity of one standard microscopic, two antibody- based and three nested PCR assays (targeted at the 18S rRNA gene locus) for the detection of *Cryptosporidium* in faecal samples (n=182) from asymptomatic sheep (n=66), cattle (n=80), and horses (n=36) were compared. Due to the absence of a gold standard for the detection of *Cryptosporidium* oocysts in faecal samples, the LCA statistical model was used to calculate the specificity and sensitivity of each test.

2.1.2 Comparison of three nested PCR protocols targeting the 18S rRNA gene locus

A separate set of samples (22 positive samples from cattle, 17 from horse, and 10 from sheep, respectively) were analysed with the same three PCR assays used above in order to determine their ability to amplify subclinical infections in the various animal hosts and identify the *Cryptosporidium* species present. The 49 samples had been found positive for the presence of *Cryptosporidium* oocysts by direct fluorescent antibody test (DFAT).

2.2 Sample collection, oocysts staining/labelling and microscopic examination

Overall 16 farms were sampled on a monthly basis from March-June 2009 and 2010. Nine farms including six mixed cattle and sheep and three mixed cattle and horse farms were located in the east of Ireland and seven farms (2 cattle, 3 sheep and 2 mixed cattle/sheep farms) in the west of the country. Faecal samples were collected on

the ground and *Cryptosporidium* oocysts were concentrated using Sheather's flotation method (Smith, 2008). As it was mentioned in the study design section, 182/231 (sheep (n=66), cattle (n=80) and horses (n=36)) were submitted for analysis of different diagnostic methods and 49/231 were selected for analysis of three different 18S rRNA PCR protocols.

2.3 Kinyoun's Carbol-Fuchsin acid fast staining

Five µl of concentrated sample were added to a ten-well glass microscope slide (C.A.Hendley (Essex) Ltd., United Kingdom) and allowed to dry. Following fixing (100% methanol, two min), the slides were flooded with Kinyoun's carbol-fuchsin for 5 min. After a brief rinse with tap water, the slide was decolorized with acid alcohol (45-60 sec) and rinsed again. Alcoholic methylene blue (1%, 1 min) was used as counterstain (Smith, 2008). All slides were screened at a magnification of 100 X. In each slide a positive control containing *Cryptosporidium* oocysts isolated from a positive horse sample was included in one of the wells.

2.4 Direct Fluorescent Antibody Test (DFAT)

Five µl concentrated sample were transferred to each well of a ten-well glass microscope slide and fixed by methanol. 25 µl FITC-labelled anti-*Cryptosporidium* monoclonal antibody (CellLabs, Australia) were added to each well, the slides incubated at 37 °C in a humid chamber for 30 min and then rinsed in a bath of PBS. Subsequently the slide was drained and mounted with glycerol-based mounting fluid while still wet. All slides were screened using a fluorescence microscope (Nikon E400), initially at 20 X magnification, then at 40 X for confirmation. In each slide, a positive control with *Cryptosporidium* oocysts isolated from a positive-tested horse was included. For the negative control, the FITC antibody was added to a blank well

157 on the slide (Smith, 2008). Spherical and sub-spherical structures, 4-8 µm in size, that
158 stained a bright green fluorescent colour were identified as *Cryptosporidium* oocysts.
159 All samples that contained one or more putative *Cryptosporidium* oocysts were
160 considered positive. Together with samples that contained structures similar to
161 *Cryptosporidium* oocysts, regardless of size, they were processed further for
162 molecular analysis. The viablity of observed oocysts was not assessed in this
163 experminet.

164 **2.5 Enzyme-linked immunosorbent assay (ELISA)**

165 A commercial ELISA kit (BIO-X Diagnostics, Belgium) was used to detect
166 *Cryptosporidium* antigens following the manufacturer's instructions. The test was
167 performed on faecal samples within a month after collection (until then they were
168 maintained at 4 °C without preservative). The plate is coated with monoclonal
169 antibody. Faecal samples were diluted and added to each coated well. After one hour
170 incubation at 37 °C the conjugated monoclonal antibody was added. Following the
171 incubation, the reaction was visualized by Tetramethylbenzidine (TMB) and the
172 results were read at 450 nm using Microplate ELISA reader (Thermo Scientific,
173 USA).

174 **2.6 DNA extraction**

175 For the molecular tests DNA was extracted according to the method published by
176 Boom et al., (1990) and McLauchlin et al., (1999). Briefly, approx. 200 µl of
177 concentrated oocyst suspension mixed with 900 µl 10 M guanidinium thiocyanate in
178 0.1 M Tris-HCl (pH 6.4)-0.2 M EDTA (pH 8.0)-2% (w/v) Triton X-100, 0.3 g 0.5 mm
179 diameter glass beads (Stratech Scientific, UK) and 60 µl isoamyl alcohol, were
180 homogenized in a Mini-Beadbeater (Stratech Scientific) for 2 min. The mixture was

left at room temperature for 5 min, and then centrifuged (18, 000 × g, 2 min). The supernatant was incubated with 100 µl of coarse activated silica at room temperature for 10 min with gentle agitation. Subsequently, the silica pellet was washed twice with 200 µl 10 M guanidinium thiocyanate in 0.1 M Tris-HCl (pH 6.4), twice with 200 µl of ice-cold 80 % ethanol, and once with 200 µl ice-cold acetone at 13,000 × g for 20 s. After the final wash the pellet was dried under a vacuum at 45 °C for 10 min. The DNA was eluted into 150 µl nuclease free water after vortex mixing and incubation at 56 °C for 5 min. Following centrifugation at 18,000 × g for 2 min the supernatant was collected and stored in -20 °C. Prior to PCR amplification, all DNA extracts were purified by PVP (polyvinylpyrrolidone, Sigma) precipitation as follows: 50 µl extracted DNA was incubated with 150 µl PVP-TE (10% [w/v] PVP in TE buffer) for 10 min at room temperature. Subsequently 100 µl 2 M ammonium acetate and 600 µl isopropanol were added to the mixture and the DNA precipitated by incubating at -20 °C for 30 min. The DNA was pelleted by centrifugation (11,000 × g, 10 min), dried and reconstituted in 50 µl of water.

2.7 PCR amplification

Samples were screened using three nested PCR protocols, all of which target the same region of the 18S rRNA. Primer sequences and PCR conditions are provided in Table 1, taq polymerases were selected based on the published protocols by authors (Xiao et al. 1999; 2001, Ryan et al., 2003 and Nichols et al., 2003; 2010).

All nested PCR products of the correct size were sent to GATC Biotech (Germany) for sequencing using the internal forward primers. If the sequence results were of poor quality, sequencing was repeated in the reverse. All sequences were compared against published data using a BLASTn search on PubMed (Altschul et al., 1990) to identify

Cryptosporidium species. For further identification sequences were aligned with selected reference sequences using ClustalW program (Larkin et al., 2007)

2.8 Measurement of sensitivity of molecular tests

The sensitivity of each PCR protocol was determined based on the number of positive results observed from each test versus the overall number of positive results observed by combining all PCR protocols. PCR was considered positive if at least one of the three PCR protocols reported positive.

2.9 Statistical models

PASW Statistics (formerly SPSS, Version 18) was used to measure the level of agreement between the methods. Pearson's chi-square and Kappa tests were performed to measure the agreement between each diagnostic technique. Chi-squared test is significant when p-value is less than 0.05; Kappa test is significant if kappa values are close to 1. Contingency tables and percentage agreements were used to determine total-, positive-, and negative-agreement between the tests.

As there is no gold standard for the detection of *Cryptosporidium* to measure the sensitivity and specificity of each test, LCA was performed to obtain the sensitivity and specificity of each test. LCA was constructed based on the status of *Cryptosporidium* infection (infected versus non-infected animals), which can be regarded as a pseudo-gold standard or as presumed true status of infection. LCA was conducted using the poLCA package in the statistics software R version 2.15 (R Core Team., 2012). LCA is based on the concept that observed results of different imperfect tests for the same infection are influenced by a latent common variable, the true infection status, which cannot be directly measured. In basic LCA models, the

observed variables are assumed to be conditionally independent. In a group of hosts with unknown infection status, for whom results from several diagnostic tests are available, LCA models the probability of each combination of test results on the latent class and thereby provides an estimate of sensitivity and specificity for each of the diagnostic tests evaluated (Hui and Walter, 1980; Rindskopf and Rindskopf, 1986).

In order to understand the level of agreement between pairs of tests, raw measures of agreement between each individual test were measured using the P-values of chi-squared test, the Kappa value of Kappa's test (reported from SPSS output), the proportion of overall (P_O), positive (P_A) and agreement (P_N) (Table 4). The measures of raw agreement (i.e. negative agreement (P_N), positive agreement (P_A) and overall agreement (P_O) indicated the number of instances in which two tests were in agreement regarding the status of a sample (i.e. positive or negative). The Kappa test is used to assess the level of agreement between two tests. Pearson's chi-squared test was applied to evaluate how likely it is that any observed difference between the sets arose by chance. Both tests showed the statistical significant agreement between the results.

3 Results

3.1 Comparison of conventional, immunological and molecular screening methods by host

The total numbers of positive samples, according to the various screening methods, are shown in Table 2. As it is illustrated, there is an association between the results obtained from both DFAT and PCR in cattle and sheep at 0.05 level of significance. Kinyoun's and DFAT showed to be associated in assessing cattle samples for presence of *Cryptosporidium* species. However, none of the diagnostic tests results illustrated

significant agreement with each other to detect *Cryptosporidium* oocysts in horse samples. The number of instances in which the two tests agreed to report a positive or negative sample for detection of oocysts is presented in Table 4.

3.1.1 Cattle

According to the LCA model (Table 3), DFAT showed the best specificity (93%) for the detection of oocysts followed by PCR (84%), ELISA (82%) and microscopy of Kinyoun's stained slides (78%); PCR was the most sensitive test (78%), followed by examination of slides stained with Kinyoun's (76%) which in turn appeared to be more sensitive than DFAT (58%), or ELISA (22%) in cattle.

3.1.2 Horse

Veronesi et al. (2010) demonstrated the effectiveness of DFAT for detecting *Cryptosporidium* oocysts in horse faecal samples. In the present study we found DFAT to be the most sensitive (100%) test but the specificity was somewhat lower (31%) for the detection of *Cryptosporidium* oocysts in horses. The highest specificity was observed by PCR (56%). However, measuring the level of agreement between DFAT and PCR for analyzing horse faecal samples, we found a low level of agreement in reporting positive samples.

3.1.3 Sheep

PCR found to be the the most sensitive and specific test to detect *Cryptosporidium* oocysts in all three hosts. As illustrated in Table 3 Kinyoun's staining showed the highest specificity in sheep compared to horse and cattle. However, the sensitivity of

this test for assessing sheep samples is lower than the sensitivity observed in testing cattle and horses. DFAT provides the better reliability in screening sheep samples in terms of sensitivity (80%) and specificity (90%).

3.2 Comparison of different nested PCR protocols targeting the 18S rRNA gene locus

The sensitivity of the molecular tests was compared using DNA extracted from a different set of samples (horse, n=17, sheep, n=10, and cattle, n=22). These samples were reported positive by DFAT screening. The combination of all three PCR assays identified 32 positive samples in total. PCR protocol 2 produced positive results in 31 samples whereas PCR protocol 1 and 3 resulted in amplification of cryptosporidial DNA in 11 and 10 samples, respectively.

Sequencing of the amplicons obtained with the three nested PCR protocols revealed the presence of a wide range of *Cryptosporidium* species in the samples. A number of samples (4/49) investigated in the present study appeared to carry multiple infections (Table 5). We found PCR protocol 2 provides a better sensitivity compared to protocols 1 and 3 in genotyping *Cryptosporidium* spp. in asymptomatic animal samples.

4 Discussion

4.1 Identification of a reliable test for the screening of animal faecal samples

Based on our findings, it is recommended to combine PCR and DFAT to screen livestock to maximize the chance of *Cryptosporidium* detection in asymptomatic cases. Our study also found reduced capacity of ELISA to identify presence of

301 *Cryptosporidium* antigens in samples with low numbers of oocysts as well as false
302 positive test results. This coincides with findings of Doing et al. (1999) and Johnston
303 et al. (2003) both of which examined human stools samples.

304 Although traditional staining techniques have been reported to be less specific and
305 sensitive (Quilez et al., 1996; Morgan et al., 1998; Clark, 1999), the present study
306 shows examination of concentrated samples following Kinyoun's to be more specific
307 in sheep (84%) than horses and cattle.

308 Clearly, the usefulness of antibody-based *Cryptosporidium* assays for detecting
309 infections in animal samples requires more research, especially in light of the wide
310 range of *Cryptosporidium* species that can infect animals. Most commercial assays
311 were developed specifically for the detection of *C. parvum* and it is to be expected that
312 their ability to detect other species or genotypes is highly variable. This study aimed
313 to detect the whole range of *Cryptosporidium* species in three host animals and was
314 not only focused on detection of *C. parvum.*

315 In agreement with the previous reports (Smith, 2008), our findings underline the
316 better reliability of DFAT as a primary screening tool in veterinary diagnostics for the
317 detection of *Cryptosporidium* oocysts in animal faecal samples compared to other
318 methods assessed in this study. As was to be expected, the combination of three PCR
319 protocols was the most sensitive and specific test for detecting *Cryptosporidium*
320 oocysts in sheep and horse samples. For cattle samples, DFAT was found to be the
321 most specific test while PCR was the most sensitive one.

4.2 Comparison of 3 PCR protocols

In a study by Leetz et al. (2007) it was concluded that PCR detection protocols for *Cryptosporidium* are not capable of detecting all isolates particularly in samples with low numbers of oocysts. The findings of this study and Leetz et al. (2007) highlight the difficulties in dealing with low oocyst numbers and/or low cryptosporidial DNA when working with environmental and/or faecal samples.

Since we are analysing three animal hosts, it is important to select a locus, which is conservative and can be easily amplified in a wide range of *Cryptosporidium* genotypes. On the other hand, too much similarity in the target sequence can make identification challenging.

Cryptosporidium genotypes have been characterised based on sequence differences in the small-subunit rRNAgene locus (18S rRNA), actin, COWP, and the 70-kDa heat shock protein (Plutzer and Karanis, 2009). Xiao et al. (1999) showed intraspecific variations in the nucleotide sequences of *Cryptosporidium* species and found differences within *C. parvum* isolates of human and bovine origin in four regions of 18S rRNA gene. Smith (2008) also stated the usefulness of 18S rRNA based molecular tests for identification of a wide range of *Cryptosporidium* genotypes. Although the locations of the primer sets within the 18S rRNA gene used in this study are very close to each other and in some cases even overlap (Figure 1), different sensitivity of each protocol was obtained in this study. Analysing animal samples using protocol 2 (developed by Ryan et al., 2003) was the most sensitive test (96%) followed by nested PCR protocols 1 (34%) and 3 (31%). PCR products of the three protocols were sequenced to confirm the results and determine the specificity of all molecular tests for identification of *Cryptosporidium* species. Interestingly, different

346 *Cryptosporidium* genotypes were amplified using each protocol (Table 5). PCR
347 protocol 1 was repeated twice in order to increase the number of positive results, the
348 results are combined together and presented in Table 5. The repeat resulted in an
349 overall increase in the number of samples detected positive for *Cryptosporidiu*m
350 species which was still lower than the number of positives by protocol 2.

351 We recommend application of DFAT and PCR for screening of sub-clinical horse
352 samples. In addition to DFAT, Kinyoun's was found to be a suitable method for
353 examination of cattle and sheep. This study also suggests that a combination of three
354 nested PCR assays (protocols 1, 2, and 3) targeting the 18S rRNA gene locus provides
355 a better understanding of the diversity of species in subclinical infections in livestock
356 as well as the presence of mixed infections. However, the primers developed by Ryan
357 et al. (2003) (protocol 2) was found to be the most reliable compared to the other
358 primer sets (used in protocols 1 and 3) for screening animal faecal samples.
359 Sequencing of the PCR products also confirmed the reliability of this protocol (Ryan
360 et al., 2003). Our study highlights the difficulties in dealing with low oocyst numbers
361 and/or low cryptosporidial DNA when working with environmental and/or faecal
362 samples. Thus, the validation of protocols remains an important issue for further
363 epidemiologic studies.

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6 Bibliography

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403-410

Boom, R., Sol, C.J., Salimans, M.M., Jansen, C.L., Wertheim-van Dillen, P.M., Van Der Noordda, J. (1990) Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28, 495- 503.

Chalmers, R.M., Campbell, B.M., Crouch, N., Charlett, A., Davies, A.P., 2011. Comparison of diagnostic sensitivity and specificity of seven *Cryptosporidium* assays used in the UK. J. Med. Microbiol. 60, 1598-1604.

Chan, R., Chen, J., York, M.K., Setijono, N., Kaplan, R.L., Graham, F., Tanowitz, H.B., 2000. Evaluation of a combination rapid immunoassay for detection of *Giardia* and *Cryptosporidium* antigens. J. Clin. Microbiol. 38, 393-394.

Clark, D.P., 1999. New insights into human cryptosporidiosis. Clin. Microbiol. Rev. 12, 554-563.

Doing, K.M., Hamm, J.L., Jellison, J.A., Marquis, J.A., Kingsbury, C., 1999. False-positive results obtained with the Alexon ProSpecT *Cryptosporidium* enzyme immunoassay. J. Clin. Microbiol 37, 1582-1583.

Garcia, L.S., Shimizu, R.Y., 1997. Evaluation of nine immunoassay kits (enzyme immunoassay and direct fluorescence) for detection of *Giardia lamblia* and *Cryptosporidium parvum* in human fecal specimens. J. Clin. Microbiol. 35, 1526-1529.

Garcia, L.S., Shimizu, R.Y., Novak, S., Carroll, M., Chan, F., 2003. Commercial assay for detection of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens by rapid solid-phase qualitative immunochromatography. J. Clin. Microbiol. 41, 209-212.

Hui, S.L., Walter, S.D., 1980. Estimating the error rates of diagnostic tests. Biometrics 36, 167-171.

Jex, A., Smith, H., Monis, P., Campbell, B., Gasser, R., 2008. *Cryptosporidium*--biotechnological advances in the detection, diagnosis and analysis of genetic variation. Biotech. Adv. 26, 304-317.

Jiang, J., Xiao, L., 2003. An evaluation of molecular diagnostic tools for the detection and differentiation of human pathogenic *Cryptosporidium* spp. J. Euk. Microbiol. 50, 542-547.

Johnston, S.P., Ballard, M.M., Beach, M.J., Causer, L., Wilkins, P.P., 2003. Evaluation of three commercial assays for detection of *Giardia* and *Cryptosporidium* organisms in fecal specimens. J. Clin. Microbiol. 41, 623-626.

Kaushik, K., Khurana, S., Wanchu, A., Malla, N., 2008. Evaluation of staining techniques, antigen detection and nested PCR for the diagnosis of cryptosporidiosis in HIV seropositive and seronegative patients. Acta. Trop. 107, 1-7.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ and Higgins DG., 2007. ClustalW and ClustalX version 2. Bioinformatics 23(21), 2947-2948.

Laxer, M.A., Timblin, B.K., Patel, R.J., 1991. DNA sequences for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction. The Am. J. Trop. Med. Hyg 45, 688-694.

Leetz, A.S., Sotiriadou, I., Ongerth, J., Karanis, P., 2007. An evaluation of primers amplifying DNA targets for the detection of *Cryptosporidium* spp. using *C. parvum* HNJ-1 Japanese isolate in water samples. Parasitol. Res. 101, 951-962.

McLauchlin, J., Pedraza-Diaz, S., Amar-Hoetzeneder, C., Nichols, G., 1999. Genetic Characterization of *Cryptosporidium* Strains from 218 Patients with diarrhea diagnosed as having sporadic cryptosporidiosis. J. Clin. Microbiol. 37, 3153-3158.

Morgan, U.M., Constantine, C.C., O'Donoghue, P., Meloni, B.P., O'Brien, P.A., Thompson, R., 1995. Molecular characterization of *Cryptosporidium* isolates from humans and other animals using random amplified polymorphic DNA analysis. Am. J. Trop. Med. Hyg. 52, 559-564.

433 Morgan, U., Thompson, R., 1998. PCR detection of *Cryptosporidium*: The way
434 forward? Parasitol. Today. 14, 241-245.
435 Morgan, U.M., Pallant, L., Dwyer, B., Forbes, D., Rich, G., Thompson, R., 1998.
436 Comparison of PCR and microscopy for detection of *Cryptosporidium parvum*
437 in human fecal specimens: clinical trial. J. Clin. Microbiol. 36, 995-998.
438 Newman, R., Jaeger, K., Wuhib, T., Lima, A., Guerrant, R., Sears, C., 1993.
439 Evaluation of an antigen capture enzyme-linked immunosorbent assay for
440 detection of *Cryptosporidium* oocysts. J. Clin. Microbiol. 31, 2080-2084.
441 Nichols, R., Campbell, B., Smith, H., 2003. Identification of *Cryptosporidium* spp.
442 oocysts in United Kingdom noncarbonated natural mineral waters and drinking
443 waters by using a modified nested PCR-restriction fragment length
444 polymorphism assay. Appl. Enviro. Microbiol. 69, 4183-4189.
445 Nichols, R., Connelly, L., Sullivan, C., Smith, H., 2010. Identification of
446 *Cryptosporidium* species and genotypes in Scottish raw and drinking waters
447 during a one-year monitoring period. Appl. Environ. Microbiol. 76, 5977-
448 5988.
449 Plutzer, J., Karanis, P., 2009. Genetic polymorphism in *Cryptosporidium* species: An
450 update. Vet. Parasitol. 165, 187-199.
451 Plutzer, J., Törökné, A., Karanis, P., 2010. Combination of ARAD microfibre
452 filtration and LAMP methodology for simple, rapid and cost effective
453 detection of human pathogenic *Giardia duodenalis* and *Cryptosporidium* spp.
454 in drinking water. Lett. App. Microbiol. 50, 82-88.
455 Quilez, J., Sánchez-Acebo, C., Clavel, A., Del Cacho, E., Lopez-Bernad, F., 1996.
456 Comparison of an acid-fast stain and a monoclonal antibody-based
457 immunofluorescence reagent for the detection of *Cryptosporidium* oocysts in
458 faecal specimens from cattle and pigs. Vet. Parasitol. 67, 75-81.
459 R Core Team., 2012. R: A Language and Environment for Statistical Computing
460 (Vienna, Austria, R Foundation for Statistical Computing).
461 Rindskopf, D., Rindskopf, W., 1986. The value of latent class analysis in medical
462 diagnosis. Stat. Med 5, 21-27.
463 Robert, B., Ginter, A., Antoine, H., Collard, A., Coppe, P., 1990. Diagnosis of bovine
464 cryptosporidiosis by an enzyme-linked immunosorbent assay. Vet. Parasitol.
465 37, 1-8.

Ryan, U., Xiao, L., Read, C., Zhou, L., Lal, A.A., Pavlasek, I., 2003. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. App. Env. Microbiol. 69, 4302-4307.

Smith, H., 2008. Diagnostics, In: Fayer, R., Xiao, L. (Eds.) *Cryptosporidium* and Cryptosporidiosis CRC Press, Boca Raton, FL pp. 173-207.

Sterling, C.R., Arrowood, M.J., 1986. Detection of *Cryptosporidium* sp. infections using a direct immunofluorescent assay. Pediatr. Infect. Dis. 5, 139-142.

Sulaiman, I.M., Xiao, L., Lal, A.A., 1999. Evaluation of *Cryptosporidium parvum* Genotyping Techniques. App. Env. Microbiol. 65, 4431-4435.

Ungar, B., 1990. Enzyme-linked immunoassay for detection of *Cryptosporidium* antigens in fecal specimens. J. Clin. Microbiol. 28, 2491-2495.

Veronesi, F., Passamonti, F., Cacciò, S., Diaferia, M., Piergili Fioretti, D., 2010. Epidemiological survey on equine *Cryptosporidium* and *Giardia* infections in Italy and molecular characterization of isolates. Zoonoses. Public. Health. 57, 510-517.

Widmer, G., 1998. Genetic heterogeneity and PCR detection of *Cryptosporidium parvum*. Adv. Parasitol. 40, 223-239.

Widmer, G., Tzipori, S., Fichtenbaum, C., Griffiths, J., 1998. Genotypic and phenotypic characterization of *Cryptosporidium parvum* isolates from people with AIDS. J. Infect. Dis. 178, 834-840.

Xiao, L., Morgan, U.M., Limor, J., Escalante, A., Arrowood, M., Shulaw, W., Thompson, R., Fayer, R., Lal, A.A., 1999. Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium* species. App. Environ. Microbiol. 65, 3386-3391.

Xiao, L., Bern, C., Limor, J., Sulaiman, I., Roberts, J., Checkley, W., Cabrera, L., Gilman, R.H., Lal, A.A., 2001. Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J. Infect. Dis. 183, 492-497.

Figure 1 Location of primers on 18 S rRNA gene fragment of *C. parvum* Acc. AY204230.1

Tables

Table 1- Primers selected for amplification of 18S rRNA

Primer pair (product size)	Primer	Primer sequence (5'-3')	Ref
Protocol 1 (840 bp)	XF1(Outer) fw	TTC-TAG-AGC-TAA-TAC-ATG-CG	**Xiao et al., 1999, 2001**
	XR1(Outer) rev	CCC-ATT-TCC-TTC-GAA-ACA-GGA	
	XF2(Inner) fw	GGA-AGG-GTT-GTA-TTT-ATT-AGA-TAA-AG	
	XR2(Inner) rev	AAG-GAG-TAA-GGA-ACA-ACC-TCC-A	
Protocol 2 (587 bp)	18SiCF2(outer) fw	GAC-ATA-TCA-TTC-AAG-TTT-CTG-ACC	**Ryan et al., 2003**
	18SiCR2(outer) rev	CTG-AAG-GAG-TAA-GGA-ACA-ACC	
	18SiCF1(Inner) fw	CCT-ATC- AGC-TTT-AGA-CGG-TAG-G	
	18SiCR1(Inner) rev	TCT-AAG-AAT-TTC-ACC-TCT-GAC-TG	
Protocol 3 (435 bp)	WR494 F(Outer) fw	TGA-GTK-AAG-TAT-AAA-CCC-CTT- TAC	**Nichols et al., 2003, 2010**
	XR1(Outer) rev	CCC-ATT-TCC-TTC-GAA-ACA-GGA	
	CPB-DIAGF(Inner) fw	AAG-CTC-GTA-GTT-GGA-TTT-CTG	
	CPB-DIAGR(Inner) rev	TAA-GGT-GCT-GAA-GGA-GTA-AGG	

Table-2 Percentage of positive results observed from each test

	Sheep	Cattle	Horse
Kinyoun's	48.4%(32/66)	38.5%(31/80)	19.4%(7/36)
DFAT	15.1%(10/66)	22.5%(18/80)	27.7%(10/36)
ELISA	15.1%(10/66)	18.7%(15/80)	8.3% (3/36)
PCR	16.6% (11/66)	35% (28/80)	52.7% (19/36)

Table-3 Specificity and sensitivity (%) of each test based on Latent Class Analysis

	DFAT	Kinyoun's	ELISA	PCR
Cattle				
Specificity	93	78	82	84
Sensitivity	58	76	22	78
Sheep				
Specificity	80	84	24	10
Sensitivity	90	54	85	90
Horse				
Specificity	31	18	0	56
Sensitivity	100	66	0	100

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Horse	*Sheep*	*Cattle*

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Chi-square test* (P-value)	Kinyoun's	ELISA	PCR	Kinyoun's	ELISA	PCR	Kinyoun's	ELISA	PCR
DFAT	0.958	0.26	0.34	0.917	0.64	0.02	0.06	0.668	0.01
Kinyoun's		0.52	0.55		0.42	0.27		0.912	0.03
ELISA			0.05			0.75			0.65
Kappa test ** (κ) value									
DFAT	0.008	-0.147	-0.13	0.009	0.05	0.37	0.287	0.04	0.34
Kinyoun's		0.09	-0.07		0.07	0.10		0.01	0.33
ELISA			-0.16			-0.03			0.04
P_O **									
DFAT	0.63	0.63	0.41	0.51	0.75	0.83	0.68	0.68	0.72
Kinyoun's		0.77	0.44		0.54	0.56		0.57	0.68
ELISA			0.61			0.74			0.61
P_A **									
DFAT	0.23	0	0.27	0.23	0.2	0.47	0.48	0.24	0.52
Kinyoun's		0.2	0.23		0.28	0.32		0.26	0.57
ELISA			0			0.19			0.27
P_N **	0.76	0.77	0.51						
DFAT	0.76	0.77	0.51	0.64	0.85	0.9	0.77	0.8	0.8
Kinyoun's		0.87	0.56		0.66	0.67		0.7	0.75
ELISA			0.56			0.84			0.73

Table 4- Raw measures of agreement between each two tests to detect *Cryptosporidium* in horse, sheep, and cattle samples

****Values close to 1 indicate there is a significant relationship between tests**

***p-value < 0.05 indicates there is a significant relationship between tests**

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521 **Table 5- Sequencing results of the 49 animal samples selected for comparison of the three PCR**
522 **protocols**
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Animal	Protocol 1	Protocol 2	Protocol 3
Horse	-	*Cryptosporidium* spp.	-
Horse	-	*Cryptosporidium* spp.	-
Horse	-	*Cryptosporidium* spp.	-
Horse	-	*Cryptosporidium* spp.	-
Horse	-	*C. parvum*	N.S
Horse	*C. andersoni*	*C. bovis*	-
Horse	-	*C. parvum*	N.S
Horse	-	0	-
Horse	-	0	-
Horse	-	0	-
Horse	-	0	-
Horse	-	*Cryptosporidium* spp.	-
Horse	-	0	-
Horse	-	*C. ryanae*	-
Horse	N.S	*C. ryanae*	*Cryptosporidium* spp.
Horse	*C. ryanae*	*C. ryanae*	*Cryptosporidium* spp.
Horse	N.S	*C. ryanae*	*C. parvum*
Cattle	*C. parvum*	*C. parvum/hominis*	N.S
Cattle	-	*Cryptosporidium* spp.	-
Cattle	*C. parvum*	*C. parvum*	*C. parvum*
Cattle	-	*C. parvum*	-
Cattle	*C. parvum*	*C. parvum*	N.S
Cattle	-	*C. ryanae*	-
Cattle	-	N.S	-
Cattle	-	-	-

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Table 5 to be continued

Animal	Protocol 1	Protocol 2	Protocol 3
Cattle	-	-	-
Cattle	-	-	-
Cattle	*C. bovis*	*C. bovis*	-
Cattle	*C. bovis*	N.S	-
Cattle	-	-	-
Cattle	*C. ryanae*	*Cryptosporidium* spp.	-
Cattle	-	-	-
Cattle	-	-	-
Cattle	-	-	-
Cattle	-	*C. ryanae*	*C. parvum*
Cattle	-	-	-
Cattle	-	*C. ryanae*	-
Cattle	-	*Cryptosporidium* spp.	-
Cattle	-	-	-
Sheep	*C. parvum*	*C. xiaoi/bovis*	*C. parvum*
Sheep	-	-	-
Sheep	-	*Cryptosporidium* spp.	-
Sheep	-	*Cryptosporidium* spp.	-
Sheep	-	*C. parvum*	-
Sheep	-	*C. parvum*	-
Sheep	-	*C. parvum*	-
Sheep	-	*C. parvum*	-
Sheep	-	*C. parvum*	-
Sheep	-	*C. ubiquitum*	-
Sheep	-	-	-
Sheep	-	-	-

N.S Cryptosporidial DNA was amplified but the sequencing failed to provide information on *Cryptosporidium* genotype present in the sample
- No DNA was amplified

In some instances incomplete sequences were generated and it was not possible to identify some species. If less than 98% similarity match was found between the query sequence and reference sequences, the query sequence was identified as '*Cryptosporidium* spp.'. In some cases, it was not possible to distinguish between *C. bovis* and *C. xiaoi* or *C. parvum* and *C. hominis* due to the high sequence similarity in the 18S rRNA gene locus.