Multiattribute evaluation of two simple tests for the detection of *Cryptosporidium parvum* in calf faeces

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Abstract

There is a need for simple and inexpensive diagnostic and screening tests for the detection of *Cryptosporidium parvum* infection in calves. A sucrose wet mount test and a lateral immunochromatography test were evaluated for epidemiological sensitivity and specificity, cost per test, simplicity, test time and ease of batching. Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) of the *Cryptosporidium* oocyst wall protein (COWP) gene locus, with gel electrophoresis, was used as a gold standard. Cohen's kappa statistic of agreement (κ) between the Ontario Veterinary College (OVC) sucrose wet mount test and COWP PCR–RFLP was 0.82, and the sensitivity and specificity of the OVC sucrose wet mount test were 88.6% and 93.8%, respectively. The sensitivity and specificity of the lateral immunochromatography test were 78.3% and 93.3%, respectively, and agreement between this test and PCR–RFLP was good (κ = 0.73). There was substantial agreement between the OVC sucrose wet mount test and the lateral immunochromatography test (κ = 0.84). Both tests were inexpensive and easy to use; however, the lateral immunochromatography test was faster and simpler to perform than the sucrose wet mount test, and was generally more user-friendly. These tests provide practitioners and researchers with cheap, quick and accurate methods of detecting *C. parvum* infection in young calves.
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1. Introduction

Cryptosporidium parvum is a ubiquitous protozoan parasite that commonly infects neonatal calves, as well as other mammalian hosts (de Graaf et al., 1999; Fayer et al., 2000). Ingestion of infective oocysts excreted in the faeces of infected animals is followed by replication of the parasite in parasitophorous vacuoles within intestinal epithelial cells, resulting in diarrhoea and other signs of gastrointestinal disease. Cryptosporidiosis in dairy calves usually occurs in the

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first few weeks of life and, in most cases, is a self-limiting disease (O'Handley et al., 1999). However, the condition may represent a significant problem on farms where the prevalence of infection is high, with losses due to increased treatment costs and, occasionally, mortality (de Graaf et al., 1999). *C. parvum* in calves is also of zoonotic importance, with infections in people acquired either by direct contact with infected animals or by the ingestion of oocysts in contaminated water or food (Peng et al., 1997; McLauchlin et al., 2000).

C. parvum infection in calves may be diagnosed by several methods. At post-mortem, infection can be detected by histological examination of sections of the intestine. However, most diagnostic methods rely on the detection of oocysts in faeces by microscopy, with or without prior concentration of oocysts in the specimen. Such methods are less invasive, and enable ante mortem diagnosis of infection. Microscopy may be performed using stained or unstained preparations. Staining methods are most commonly used, and include acid-fast reagents, such as Ziehl-Neelsen, non-specific fluorescent stains, such as auramine-rhodamine, and immunofluorescent stains (Casemore et al., 1985; Quílez et al., 1996). These stains may be combined with concentration methods in order to improve the detection limit of the techniques. However, techniques using stains tend to be more time consuming than non-staining methods, and, where fluorescent reagents are used, require expensive equipment. Non-staining methods usually include concentration of the oocysts in the specimen before microscopic examination. This is most commonly achieved by the addition of an aqueous solution of sucrose to the sample (Abassi et al., 2000), most often followed by centrifugation (Sheather, 1922). Such techniques are simpler and cheaper to perform than methods employing stains.

Concentration methods (Bukhari and Smith, 1995) are commonly employed to improve the detection limit of diagnostic techniques. This is especially important where low numbers of oocysts are being excreted by an animal. Sheather's solution (Sheather, 1922), an aqueous solution of sugar, is often used, either in its original form or as a modified version, to concentrate oocysts in faecal samples using centrifugation. However, the use of concentration methods increases the time required for sample preparation.

Furthermore, as dairy calves usually excrete large numbers of oocysts (Nydam et al., 2001), microscopy without previous concentration may be adequately sensitive for routine diagnostic use.

Polymerase chain reaction (PCR) is becoming increasingly popular as a tool to detect *Cryptosporidium* DNA in faeces. When followed by restriction fragment length polymorphism (RFLP) analysis, PCR allows for differentiation between different genotypes of the organism (Spano et al., 1997; Pedraza-Díaz et al., 2001). This technique is usually very sensitive and specific. However, molecular analysis is relatively time-consuming and requires expensive equipment and reagents (Morgan et al., 1998); as such, it is used predominantly as a research tool.

Several studies have been published that evaluate and compare various methods used for the diagnosis of *C. parvum* in humans and animals (Morgan et al., 1998; Abassi et al., 2000; Bialek et al., 2002), including cattle (Willson and Acres, 1982; McCluskey et al., 1995; Kváč et al., 2003). However, with a few exceptions (Muccio et al., 2004), published studies evaluating diagnostic techniques used in cattle have been concerned primarily with methods involving concentration of oocysts and staining. There is, therefore, a dearth of published information on the usefulness and performance of simple, non-staining screening and diagnostic techniques for *C. parvum* in cattle.

The objective of this study was to assess the performance and utility of a standardized sucrose wet mount method developed at the Ontario Veterinary College (OVC) and a lateral immunochromatography test stick method developed by BioX diagnostics, Belgium, as simple screening and diagnostic methods. Test performance was evaluated against *Cryptosporidium* oocyst wall protein (COWP) nested PCR–RFLP followed by gel electrophoresis. This technique produces a gel electrophoresis banding pattern specific to *C. parvum* (Sulaiman et al., 1999; Pedraza-Díaz et al., 2001). It has a lower limit of detection of ≤0.05 oocyst per PCR reaction, and was used in this study as a gold standard for evaluation of the microscopy and lateral immunochromatography tests. In addition to test accuracy, several other criteria are usually considered of importance by researchers and practitioners when contemplating the choice of available diagnostic methods. The utility of each test was

therefore also assessed in terms of cost, ease of use, time required for sample preparation and reading, and ease of batching.

2. Materials and methods

2.1. Collection and storage of faecal samples

Faecal samples were collected directly from the rectum of dairy calves under 30 days of age on southern Ontario farms, as part of a larger study on risk factors for *C. parvum* infection conducted at the OVC between June 2003 and September 2004. Samples were collected in clean plastic vials and stored at 4 °C without preservative for a maximum of 5 days, until microscopic examination and lateral immunochromatography testing could be performed. An aliquot of each sample was transferred to a 1.5 ml cryogen vial (Fisher Scientific) within 10 h of collection and frozen at −70 °C for subsequent PCR–RFLP analysis.

Faecal specimens used for the study described here were selected purposively from the larger collection acquired for the risk factor study, and included samples from calves with and without diarrhoea. In order to ensure an optimal proportion of positive and negative samples, only specimens from calves between 7 and 21 days of age were selected. Technicians performing the diagnostic tests were blinded to the *C. parvum* status, positive or negative, of individual samples.

2.2. Tests

The samples were analysed using three methods: microscopic slide flotation (sucrose wet mount), lateral immunochromatography and PCR–RFLP with gel electrophoresis.

2.2.1. Microscopic slide flotation (wet mount) examination

For microscopic examination of faecal samples, a modified Sheather's solution (specific gravity 1.32) was prepared by dissolving 2 kg of sugar in 1000 ml of tap water using gentle heat. To prepare slides for examination, 3 ml of the sugar solution was added to 1 g of faeces. The mixture was homogenised using an applicator stick. One drop of the suspension was then immediately placed on a clean microscope slide and covered with a 22 mm × 22 mm coverslip, using gentle pressure so that the mixture filled the entire area beneath the coverslip. Slides were examined for the presence of *C. parvum* oocysts under bright field microscopy at ×400 magnification using an optical microscope (Leica, Opti-Tech Scientific, Scarborough, Ont., Canada), and were read within 30 min of preparation. Oocysts were identified as spherical, pink, refractile structures 3–6 μm in diameter situated just beneath the coverslip. A single slide was prepared and examined for each faecal sample, and the sample was considered positive for *C. parvum* if at least one oocyst was detected. This method allowed for the estimation of the number of oocysts per gram of faeces by multiplying the average number of oocysts per ×400 microscope field by an appropriate factor. As there were 2304 fields per coverslip area, which covered approximately 0.04 g of faecal preparation, and the total weight of the preparation (1 g faeces and 3 ml sucrose) was approximately 5 g, the factor used in this study was 2.9×10^5 (=2304 × [5/0.04]).

2.2.2. Lateral immunochromatography testing

Testing for the presence of *C. parvum* antigen in the faecal samples was carried out using a lateral immunochromatography test kit for *C. parvum*, *E. coli* F5 (K99) attachment factor, rotavirus and coronavirus (BioX Diagnostics, Jemelle, Belgium). Testing was carried out in accordance with manufacturer's instructions. Briefly, approximately 0.1 g of fresh, unpreserved feces was mixed into the test reagent provided, to form a homogenous suspension. A test strip ('immunostrip') was inserted into the suspension and was observed after 3 min, and within 10 min, for the presence of control and test sample lines. The presence of both lines indicated a positive test result. Results were recorded as positive or negative; this test did not allow for quantification of shedding.

2.2.3. Polymerase chain reaction—restriction fragment length polymorphism (PCR–RFLP) analysis
2.2.3.1. DNA extraction.
DNA was extracted directly from frozen faecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN Inc., Mississauga, Ont., Canada). Extraction was performed according to

manufacturer's instructions, modified by the addition of three 10-min rounds of freezing in liquid nitrogen and thawing at 70 °C in a water bath, between the addition of lysis buffer (buffer ASL) and centrifugation of the buffer ASL-stool suspension. Eluted DNA was stored at −20 °C until PCR–RFLP analysis could be carried out.

2.2.3.2. PCR amplification. PCR amplification was performed using the nested primer set developed by Pedraza-Díaz et al. (2001): outer primers BCOWPF and BCOWPR, and nested primers Cry9 and Cry15. For the primary PCR reaction, the PCR master mix was prepared by mixing 10× QIAGEN PCR buffer (QIAGEN Inc.), dinucleotide triphosphate (dNTP) mix (QIAGEN Inc.) containing 10 µM of each dNTP, forward and reverse primer mix containing 10 mM of each of the primers BCOWPF and BCOWPR, and *Taq* DNA polymerase (QIAGEN Inc.; 5 units/µl) in the proportions 5:1:1:0.2 by volume, respectively. In the master mix for the nested reaction, the nested primer set Cry9 and Cry15 was substituted for the outer primers.

Primary PCR amplification was performed in 25 µl total volumes: 3.6 µl primary PCR mastermix, 20.4 µl nuclease-free water (Promega Corporation, Wisconsin, USA) and 1 µl eluted DNA. Positive and negative controls were included in each batch of reactions. The reaction mixture was covered with one drop of mineral oil (Promega Corporation) and, following an initial melt at 94 °C for 10 min, PCR amplification was run under 30 cycles of the following thermal profile: melting at 94 °C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 1 min. The reaction was completed by a final extension at 72 °C for 10 min.

Nested PCR reactions were prepared in 25 µl volumes (1 µl of primary PCR product, 3.6 µl of master mix and 20.4 µl of nuclease-free water). Following an initial melt at 94 °C for 10 min, amplification was performed under 35 cycles of the same thermal profile as that used for primary amplification, with the exception that an annealing temperature of 55 °C was used. The reaction was completed by a final extension at 72 °C for 10 min.

2.2.3.3. RFLP analysis. The master mix used for restriction digestion contained 2 µl of 10× reaction buffer (Buffer C, Promega Corporation), 7.8 µl of nuclease-free water (Promega Corporation) and 0.2 µl

of *Rsa*1 restriction enzyme (10 units/µl; Promega Corporation) per reaction. Each reaction mixture contained 10 µl of master mix and 10 µl of nested PCR product, and restriction digestion was carried out at 37 °C for 60 min.

Restriction products were separated on a 2% agarose gel in tris/borate/ethylene-diamine tetra-acetic acid (TBE) buffer with pGEM DNA molecular markers (Promega Corporation) in the first lane, and were visualized by staining with ethidium bromide (10 µg/ml) for 30 min followed by de-staining in distilled water and ultraviolet transillumination (Pedraza-Díaz et al., 2001).

2.3. Statistical analysis

Statistical analysis was performed using computer software (Stata 8.0, Stata Corporation, College Station, Texas). Comparisons were made by assessing epidemiologic sensitivity and specificity, using the "diagti" command in Stata. Agreement between tests was also evaluated using Cohen's kappa statistic.

2.4. Assessment of test attributes

The three tests carried out were compared by qualitative assessment of agreement, epidemiologic sensitivity and epidemiologic specificity. The cost of consumables required per sample in US dollars (conversion rates: Can $ 1.20: US$ 1.00; €1.00: US$ 1.30), testing time per sample and ease of batching were also compared.

3. Results

In the evaluation of the sucrose wet mount and lateral immunochromatography tests, COWP PCR–RFLP with gel electrophoresis was used as a gold standard.

3.1. Test performance: agreement between tests, sensitivity and specificity

A total of 199 faecal samples from calves 7–21 days of age were used for this study, of which 84 were positive by the sucrose wet mount test performed at the OVC. Of the 199 samples, 166 (55 positive and 111

negative by microscopy) were tested using the Bio-X lateral immunochromatography kit, and 168 (83 positive and 85 negative by microscopy) of the 199 samples were tested by PCR–RFLP. One hundred and thirty-five samples (54 positive and 81 negative by microscopy) were tested by all three diagnostic methods.

3.1.1. Sucrose wet mount versus COWP PCR–RFLP

Of the 168 samples tested by both of these methods, 15 samples gave discordant results (Table 1). Very low numbers of oocysts (six oocysts and one oocyst per slide) were detected in two of the five 'false positive' samples, while the average number of oocysts detected per field in the other three 'false positive' samples ranged from 0.1 to 12.

For 8 of the 10 'false negative' samples, microscopy test results were available for fecal samples taken from the same calves 1 week after collection of the samples used for this study. For five of those eight calves, oocysts were detected by microscopy in fecal samples collected 1 week after the samples used in this study, while samples taken a week later from three calves tested negative.

Assuming that the gold standard, COWP PCR–RFLP analysis, was 100% sensitive and specific, the sensitivity and specificity of the OVC sucrose wet mount test were 88.6% (95% confidence interval (CI) 80.1%, 94.4%) and 93.8% (95% CI 86.0%, 97.9%), respectively. Cohen's kappa statistic of agreement between the OVC sucrose wet mount test and COWP PCR–RFLP was 0.82 (95% CI 0.74, 0.91).

3.1.2. Sucrose wet mount versus lateral immunochromatography

Of the 166 samples tested by both of these methods, 12 samples gave discordant results (Table 2), five of

Table 1
OVC sucrose wet mount test results compared to *Cryptosporidium* oocyst wall protein (COWP) PCR–RFLP

Sucrose wet mount	COWP PCR–RFLP		Total
	Positive	Negative	
Positive	78	5	83
Negative	10	75	85
Total	88	80	168

Table 2
OVC sucrose wet mount test results compared to Bio-X lateral immunochromatography test

Sucrose wet mount	Lateral immunochromatography		Total
	Positive	Negative	
Positive	48	7	55
Negative	5	106	111
Total	53	113	166

which were positive by lateral immunochromatography. For two of the five discordant samples that were positive by lateral immunochromatography, the lateral chromatography results were supported by positive PCR–RFLP results, and a faecal sample taken a week later from one of those two animals tested positive by microscopy. No follow-up sample was obtained from the other calf. Three of the five samples were negative by PCR–RFLP.

For two of the seven discordant samples negative by lateral immunochromatography, the results of the test were consistent with PCR–RFLP results, which were also negative. However, five of the seven were positive by PCR.

Cohen's kappa statistic of agreement between the OVC sucrose wet mount test and the lateral immunochromatography test was 0.84 (95% CI 0.75, 0.92).

3.1.3. Lateral immunochromatography versus COWP PCR–RFLP

Of the 135 samples tested by lateral immunochromatography as well as by COWP PCR–RFLP, 18 samples gave discordant results (Table 3), of which 13 were 'false negatives' and 5 were 'false positives'. Eight of the 13 'false negatives' were also negative by microscopy; samples taken the following week from 4 of the 8 calves tested positive by microscopy. The remaining 5 of the 13 'false negative' samples tested positive by microscopy. Of the five 'false positives', two were positive and three were negative by microscopy.

The sensitivity and specificity of the lateral immunochromatography test were 78.3% (95% CI 65.8%, 87.9%) and 93.3% (95% CI 85.1%, 97.8%), respectively. Cohen's kappa statistic of agreement between the lateral immunochromatography test and COWP PCR–RFLP was 0.73 (95% CI 0.61, 0.84).

Table 3
Bio-X lateral immunochromatography test results compared to *Cryptosporidium* oocyst wall protein (COWP) PCR–RFLP

Lateral immunochromatography	COWP PCR–RFLP		Total
	Positive	Negative	
Positive	47	5	52
Negative	13	70	83
Total	60	75	135

3.1.4. Results for samples tested by all three methods

Of the 135 samples tested by all three methods, 45 samples were positive by all three tests and 68 were negative by all three tests. Test results for at least one test were discordant for 22 samples.

3.1.5. Results for samples from diarrhoeic calves

Calculation of test performance parameters for a subset of 43 samples collected from calves that were known to have been diarrhoeic at the time of sample collection gave values for sensitivity and specificity of 89.7% (95% CI 72.6%, 97.8%) and 100.0% (95% CI 76.8%, 100.0%), respectively, for the sucrose wet mount test. The sensitivity and specificity of the lateral immunochromatography test as calculated for this subset of samples were 86.2% (95% CI 68.3%, 96.1%) and 100.0% (95% CI 76.8%, 100.0%), respectively. Cohen's kappa statistic of agreement between the PCR and sucrose wet mount test was 0.85 (95% CI 0.69, 1.00), between the PCR and lateral immunochromatography tests was 0.80 (95% CI 0.62, 0.98) and between the sucrose wet mount and the lateral immunochromatography tests was 0.85 (95% CI 0.70, 1.00).

3.2. Other test attributes

3.2.1. Cost

The cost of consumables required for the OVC sucrose wet mount test was US$ 0.36 per sample, while the BioX test cost US$ 2.70–3.10 per sample. Consumables required for COWP PCR–RFLP analysis were considerably more expensive, at US$ 8.40 per sample.

No additional equipment was required to use the BioX test kit according to the manufacturer's instructions. However, the sucrose wet mount test required the use of an optical microscope and, for quantification of oocysts, a balance, representing a total capital invest-ment in the region of US$ 4170. PCR–RFLP necessitated the largest capital investment as it required the use of relatively expensive equipment, such as a thermocycler and microcentrifuge (approximate total price US$ 10,000).

3.2.2. Test time and ease of batching

The Bio-X lateral immunochromatography test required the least time for preparation and testing of samples; a single sample could be tested in 3 min. The sucrose wet mount test required approximately 10 min for preparation and reading of a single slide. DNA extraction, preparation and running of PCR reactions, RFLP and gel electrophoresis took the longest time, with a minimum of 8 h required for the processing of a single sample. However, the time required per sample could be greatly reduced by batching of samples for each stage of the procedure. Batching of samples also reduced the time required per sample for the lateral immunochromatography test, but because of the need to read each slide individually in the sucrose wet mount test, batching had little effect on the time required per sample for this test. The ability to process samples in batches using the sucrose wet mount test was also restricted by the degeneration of *C. parvum* oocysts in sugar solution after approximately 40 min; batch size had to be restricted to the number of slides that could be examined in this time period. Overall, PCR–RFLP was most suitable, and the sucrose wet mount method was least suitable, for batching of samples.

3.2.3. Simplicity of technique

The Bio-X lateral immunochromatography test was the easiest test to perform, as it could be carried out in three simple steps and required minimal technician training. The sucrose wet mount test was also simple to perform, in that it involved no staining and could be carried out in four steps. However, recognition of the oocysts required moderate training and practice. PCR–RFLP was the least simple to perform, involving many steps during the various stages of DNA extraction, PCR, RFLP and gel electrophoresis.

4. Discussion

In this study, a COWP nested PCR–RFLP assay was used as a gold standard. Though the use of this

assay may not be sufficiently established to allow it to conform to the definition of a widely accepted gold standard, the sensitivity and specificity of the method were assumed to be high enough to justify its use as a gold standard for the purposes of this work. Bialek et al. (2002) reported that the sensitivity of a similar COWP nested PCR assay, calculated by the maximum likelihood method, was 96.5%. Likewise, Kato et al. (2003) calculated a sensitivity of 85.7% and a specificity of 100%, using 16 samples inoculated with 1, 10 and 100 oocysts per 1 ml of faeces. Unpublished work performed in our laboratory, using inoculated samples and the protocol used in this study, estimated a minimum detection limit of the equivalent of less than 0.05 oocyst per PCR reaction. The assumption of a sensitivity and specificity of 100% for the PCR–RFLP protocol when assessing the performance of the two tests evaluated here should, therefore, be sufficiently accurate for this purpose.

4.1. Sucrose wet mount test

The value of kappa (0.82), given by the data comparing the sucrose wet mount test results with the results of PCR–RFLP analysis as a gold standard, indicated substantial agreement between the two tests (Dohoo et al., 2003). This agreement, and the fact that the test is easy to perform and requires only a low level of capital investment and inexpensive consumables, makes the test suitable for quick screening or diagnostic use in young calves. The test has the added advantage of allowing for the assessment of the intensity of oocyst shedding by infected animals, which can be used as an indication of the level of *C. parvum* infection. However, unlike staining methods that are commonly used for the diagnosis of *C. parvum* infection (McCluskey et al., 1995; Kváč et al., 2003), slides prepared for sucrose wet mount examination cannot be preserved for future reference. The ability to batch samples is also restricted by the need to read slides before degeneration of oocysts in the sucrose solution has occurred.

In 8 of the 10 cases where the results of the sucrose wet mount test were recorded as false negatives, the calves tested positive by microscopy 1 week after collection of the negative sample. At the time of collection of the first sample, these calves may have been in the early stages of infection, with levels of oocyst shedding that were below the limit of detection of the microscopy test. On the other hand, the five false positive results reported here may have been due to the detection of *Cryptosporidium* oocysts similar to those of *C. parvum* that cannot be detected by COWP PCR–RFLP (Xiao et al., 2000), or structures of a similar size and refractile appearance as *C. parvum* oocysts. *Cryptosporidium muris* is the only other species of *Cryptosporidium* known to infect cattle. The oocysts of this species are larger than those of *C. parvum*, and are usually found only in cattle older than those sampled in this study (de Graaf et al., 1999). Furthermore, COWP PCR–RFLP would be expected to amplify DNA of *C. muris* (Sulaiman et al., 1999; Xiao et al., 2000). A few genotypes of *C. parvum* are not readily detectable using COWP-based genotyping techniques (Xiao et al., 2000); however, such genotypes are not commonly found in young calves. It is therefore more likely that the structures detected in the false positive samples were non-*Cryptosporidium* structures.

4.2. Bio-X lateral immunochromatography test

This test, like the sucrose wet mount test, showed good agreement with the results of PCR–RFLP analysis, though the level of agreement between the two tests ($\kappa = 0.73$) was lower than that between the sucrose wet mount test and PCR. Agreement between the lateral immunochromatography test and the sucrose wet mount test was very good, with a kappa statistic of 0.84 (Dohoo et al., 2003).

Consumables required for the lateral chromatography test cost less than those needed for PCR–RFLP, but more than those used for the sucrose wet mount test. However, the lateral immunochromatography test required no investment in expensive equipment. This test was also easy to read, required little or no training, and could be performed in only a few minutes. In comparison to the sucrose wet mount test, however, lateral immunochromatography was less sensitive and specific for the diagnosis of *C. parvum*. As in the case of the wet mount test, results cannot be preserved for future reference. This test has the added disadvantage of giving only a positive or negative result; the level of shedding cannot be quantified.

The results of this study indicate that both of the simple diagnostic and screening techniques assessed are suitable for quick and inexpensive testing for *C.*

parvum infection in young calves. The sucrose wet mount test requires moderate training for detection of the oocysts, and enables quantification of oocyst shedding, while the lateral immunochromatography test requires no additional equipment and little training for reading of the test results. The sensitivity and specificity of both tests were higher when used on samples from diarrhoeic calves, further confirming the suitability of the tests for use in a clinical setting.

This study is the first published multiattribute evaluation of diagnostic and screening methods for *C. parvum* infection in calves that are both simple and non-costly. The use of either of these tests for diagnosis of *C. parvum* infection in individual animals or for the screening of groups of animals should provide practitioners and researchers with a cheap, quick and accurate method of detecting infection in young calves.

Acknowledgements

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