Comparison of diagnostic sensitivity and specificity of seven *Cryptosporidium* assays used in the UK

Rachel M. Chalmers,¹ Brian M. Campbell,² Nigel Crouch,¹ André Charlett³ and Angharad P. Davies^{1,2}

Correspondence

Rachel M. Chalmers
Rachel.chalmers@wales.nhs.uk

¹UK *Cryptosporidium* Reference Unit, Public Health Wales Microbiology, Singleton Hospital, Swansea SA2 8QA, UK

²Institute of Life Sciences, School of Medicine, Swansea University, Swansea SA2 8PP, UK

³Statistics Unit, Health Protection Agency Centre for Infections, 61 Colindale Avenue, London NW9 5DF, UK

To compare the diagnostic sensitivity and specificity of seven *Cryptosporidium* diagnostic assays used in the UK, results from 259 stool samples from patients with acute gastrointestinal symptoms were compared against a nominated gold standard (real-time PCR and oocyst detection). Of the 152 'true positives', 80 were *Cryptosporidium hominis*, 68 *Cryptosporidium parvum*, two *Cryptosporidium felis*, one *Cryptosporidium ubiquitum* and one *Cryptosporidium meleagridis*. The *Cryptosporidium* spp. diagnostic sensitivities of three *Cryptosporidium* and *Giardia* combination enzyme immunoassays (EIA) coupled with confirmation of positive reactions were 91.4–93.4 %, whilst the sensitivity of auramine phenol microscopy was 92.1 % and that of immunofluorescence microscopy (IFM) was 97.4 %, all with overlapping 95 % confidence intervals. However, IFM was significantly more sensitive ($P=0.01$, paired test of proportions). The sensitivity of modified Ziehl–Neelsen microscopy was 75.4 %, significantly lower than those for the other tests investigated, including an immunochromatographic lateral flow assay (ICLF) (84.9 %) ($P=0.0016$). Specificities were 100 % when the ICLF and EIA test algorithms included confirmation of positive reactions; however, four positive EIA reactions were not confirmed for either parasite. There was no significant difference in the detection of *C. parvum* and *C. hominis* by each assay, but the detection of other *Cryptosporidium* spp. requires further investigation, as the numbers of samples were small. EIAs may be considered for diagnostic testing, subject to local validation, and diagnostic algorithms must include confirmation of positive reactions.

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INTRODUCTION

Symptoms of human cryptosporidiosis, caused by the protozoan parasite *Cryptosporidium*, include watery diarrhoea, abdominal pain, low-grade fever, nausea and/or vomiting. The duration may be up to 2 weeks, and occasionally longer. Cases can be sporadic or as part of outbreaks. In the UK, guidance is for *Cryptosporidium* to be sought in stools from all community cases of diarrhoea, and laboratory diagnosis became notifiable in 2010 (Chalmers *et al.*, 2010a). Routine diagnosis is mainly by recognition of oocysts in fresh, unconcentrated stools by microscopy of smears stained with either auramine phenol (AP) or modified Ziehl–Neelsen (mZN); these are the UK Standards for Microbiological Investigations (SMIs), for-

merly known as national standard methods (Health Protection Agency, 2007). To facilitate testing, especially in larger laboratories processing more samples, an emerging trend in the UK since 2009 is the use of commercially available immunoassays, targeting either *Cryptosporidium* antigen alone or in combination with *Giardia* (Chalmers *et al.*, 2010a). Although PCR-based assays provide sensitive and specific diagnosis of protozoan infections including *Cryptosporidium* (Bruijnesteijn van Coppenraet *et al.*, 2009), they are not yet in routine diagnostic use in the UK. Most diagnostic assays permit the reporting of the presence/absence of *Cryptosporidium* spp., but PCR-based methods can also be used to identify the infecting species. This is useful because there are differences in epidemiology, risk factors, clinical manifestation and sequelae between species (Xiao, 2010).

There have been many publications comparing diagnostic tests for *Cryptosporidium* and, whilst enzyme immunoassays (EIAs) require reduced labour time and microscopy

Abbreviations: AP, auramine phenol; CI, confidence interval; C_T , cycle threshold; EIA, enzyme immunoassay; ICLF, immunochromatographic lateral flow; IFM, immunofluorescence microscopy; mZN, modified Ziehl–Neelsen; SMI, Standard for Microbiological Investigations.

skills (Garcia & Shimizu, 1997), the sensitivity and specificity when measured against immunofluorescence microscopy (IFM) varies among studies (Garcia & Shimizu, 1997; Weitzel *et al.*, 2006). However, there has been no direct evaluation of all assays used routinely in the UK, where testing is common in clinical laboratories (Chalmers *et al.*, 2010a). Comparison of assay performance among *Cryptosporidium* spp. has so far been limited to immunochromatographic assays, and the ability of immunology-based diagnostic assays to detect different species is uncertain (Agnomey *et al.*, 2011). The aim of this study was to compare the diagnostic sensitivity and specificity of the assays used in the UK and their ability to detect different *Cryptosporidium* spp.

METHODS

Stool samples and *Cryptosporidium* spp. In July 2009, a panel of 259 anonymized stools was made from those submitted for diagnosis by patients with gastrointestinal symptoms to the local hospital diagnostic microbiology laboratory ($n=109$, mostly *Cryptosporidium*-negative) and those referred to the *Cryptosporidium* Reference Unit ($n=150$, mostly *Cryptosporidium*-positive), collected within the previous 4 weeks. The only selection criterion from each laboratory was sufficient material for the study. All stools were stored without preservative in a refrigerator (2–8 °C). Stools were categorized as true positives ($n=152$) if *Cryptosporidium* was confirmed by real-time PCR of the small-subunit rRNA gene (Hadfield *et al.*, 2011). Stools were categorized as true negatives ($n=107$) if *Cryptosporidium* was not detected by real-time PCR. To confirm that oocysts as well as *Cryptosporidium* DNA were present in the positive panel, these were retrieved using immunomagnetic separation (Isolate; TCS Biosciences) and visualized by direct IFM (Crypto-Cel; Cellabs) (Robinson *et al.*, 2008). This is because oocysts or their antigens are the target for all of the routine assays investigated in this study.

Testing of at least 250 samples ensured that differences in sensitivity and specificity >10% had an 80% chance of being detected at a 5% significance level. To compare assay performance among species, the identity of the *Cryptosporidium* spp. in positive samples was established by real-time PCR of the small-subunit rRNA and *Lib13* genes and sequencing of products where required (Hadfield *et al.*, 2011). The panel was blind-coded, prior to testing by two experienced laboratory personnel.

Diagnostic assays. The assays to be compared were identified as those found to be in greatest use in the UK from a survey of contemporaneous diagnostic practice (Chalmers *et al.*, 2010a) and a UK market survey. The assays were: mZN staining and microscopic examination of 50 fields at $\times 500$ magnification (an SMI) (Health Protection Agency, 2007); AP staining and microscopic examination of 50 fields at $\times 200$ magnification (an SMI) (Health Protection Agency, 2007); direct IFM by examination of a 10 mm diameter well at $\times 200$ magnification (Crypto-Cel; Cellabs) (Robinson *et al.*, 2008); three *Cryptosporidium*/*Giardia* combination EIA kits: Remel ProSpecT *Giardia*/*Cryptosporidium* Microplate Assay (Thermo Fisher Scientific), IVD Research *Giardia*/*Crypto* Combo EIA (Launch Diagnostics) and *Giardia*/*Cryptosporidium* CHEK (Alere); and an immunochromatographic lateral flow (ICLF) *Cryptosporidium* assay (RIDAQUICK *Cryptosporidium* cassette; R Biopharm Rhône). Another EIA (RIDASCREEN *Cryptosporidium*; R Biopharm Rhône) used in one UK laboratory was not evaluated because of limited stool volumes.

To reflect routine diagnostic practice, each test was performed once on each sample. *Cryptosporidium* direct IFM results were recorded using an arbitrary scoring system for the number of oocysts seen in each well (Table 1). Commercially available assays were conducted following the manufacturers' instructions, and the EIAs were carried out using an automated processing system (Dynex DS2; Alere). Positive ICLF and EIA reactions were confirmed, and EIAs were resolved for *Cryptosporidium* or *Giardia* using individual genus-specific IFM reagents (Cellabs) applied to air-dried and methanol-fixed test suspensions on multiwell slides. The confirmed results were used in statistical analyses.

Statistical analysis. The sensitivity and specificity [with 95% confidence intervals (CIs)] for *Cryptosporidium* were calculated using CI Analysis (CIA) software, version 2 (University of Southampton, UK). Overlapping CIs were subjected to tests of paired proportions. The differences in median real-time PCR threshold cycle (C_T) values in true-positive samples between diagnostic assay-positive and -negative results were calculated for each assay using a Mann–Whitney *U* test. The results for samples containing *Cryptosporidium hominis* and *Cryptosporidium parvum* were compared for each assay by Yates' corrected χ^2 test. $P<0.05$ was considered statistically significant. *Giardia* data were considered in this study only to assess whether there were false positives in the combination assays; the collection of sufficient samples for diagnostic sensitivity and specificity assessment was outside the scope of this work.

RESULTS AND DISCUSSION

Diagnostic sensitivity and specificity for *Cryptosporidium*

Varying numbers of samples were positive by each of the diagnostic *Cryptosporidium* assays (Table 1). Comparing the 95% CI for sensitivity, there was overlap between the three EIA kits (range 91.4–93.4%), AP (92.1%) and Crypto-Cel IFM (97.4%) (Table 1). However, greater sensitivity of Crypto-Cel IFM was demonstrated because the paired test of proportions performed with the next closest test, the Techlab EIA, was significantly different ($P=0.01$). The sensitivity of the RIDAQUICK ICLF assay (84.9%) was lower than those of the EIAs and AP microscopy, but the CIs overlapped; however, this test was significantly less sensitive than IFM (97.4%) (Table 1). The sensitivity of the RIDAQUICK ICLF reported here is similar to that found by Weitzel *et al.* (2006). mZN microscopy was significantly less sensitive than all other tests (Table 1); when mZN and ICLF were compared by a paired test of proportions, the difference was significant ($P=0.0016$). A large percentage of cryptosporidiosis cases (24% for mZN and 15% for ICLF) would not have been diagnosed using these assays.

Of the 152 true-positive samples, 102 were positive by all seven *Cryptosporidium* assays and 50 were negative by one or more assays; only one sample was negative by all seven assays. This sample had a high C_T value (39.80), indicating that only a small amount of *Cryptosporidium* DNA was present. Discordant results were associated mainly with false negatives by mZN microscopy: 22 samples were negative by this method compared with only three by the

Table 1. Sensitivity and specificity of *Cryptosporidium* assays in diagnostic stools and comparison of *Cryptosporidium* quantification

NA, Not applicable.

Diagnostic assay	<i>Cryptosporidium</i> true-positive (<i>n</i> =152) and true-negative (<i>n</i> =107) samples	Comparison with nominated gold standard in 259 samples		Direct IFM scores in samples negative by each assay*	Median real-time PCR C _T values in true-positive samples (range) compared by Mann–Whitney <i>U</i> test		
		Confirmed assay-positive; -negative	Percentage sensitivity (95 % CI)		Percentage specificity (95 % CI)	Range (mode; median)	Assay-positive
mZN microscopy	115; 107	75.7 (68.3–81.8)	100 (96.5–100)	1–6 (6; 5)	29.03 (18.95–38.96)	29.95 (19.08–39.95)	0.1212
RIDAQUICK	129; 107	84.9 (78.3–89.7)	100 (96.5–100)	1–6 (2; 2)	28.64 (19.00–40.00)	32.65 (22.84–39.80)	0.0002
Cryptosporidium ICLF†							
Remel ProSpecT Giardia/ Cryptosporidium Microplate EIA†	139; 107	91.4 (85.9–94.9)	100 (96.5–100)	1–5 (2; 2)	29.04 (18.95–39.95)	33.38 (22.84–39.80)	0.0014
AP microscopy	140; 107	92.1 (86.7–95.4)	100 (96.5–100)	1–5 (2; 2)	28.94 (18.95–39.95)	32.56 (29.05–39.80)	0.0029
IVD Research Giardia/ Crypto Combo EIA†	141; 107	92.8 (87.5–95.9)	100 (96.5–100)	1–4 (2; 2)	29.04 (18.95–39.95)	33.38 (27.94–39.80)	0.0030
Techlab Giardia/ Cryptosporidium CHEK EIA†	142; 107	93.4 (88.3–96.4)	100 (96.5–100)	1–4 (2; 2)	29.05 (18.95–39.95)	32.96 (27.94–39.80)	0.0086
Crypto-Cel IFM	148; 107	97.4 (93.4–99.0)	100 (96.5–100)	NA	29.40 (18.95–39.95)	31.98 (29.52–39.80)	0.0822

*IFM score: 1, oocysts not seen; 2, one oocyst seen in each 10 mm microscopy slide well; 3, two to five oocysts; 4, six to ten oocysts; 5, 11–50 oocysts; 6, >50 oocysts.

†Positive reactions confirmed for *Cryptosporidium* by IFM of the faecal suspension used in the assay; four false-positive EIA reactions were categorized as negative as the test algorithm included confirmation of positive reactions.

RIDAQUICK ICLF assay and one by AP microscopy. Neither IFM nor any of the EIAs was negative when all other assays (ICLF, and mZN and AP microscopy) were positive. The other 23 samples with discordant results showed no association with any one assay. Poor sensitivity of acid-fast microscopy has been reported previously (Johnston *et al.*, 2003); slide preparation, parasite density and variable oocyst staining, as well as time spent examining the slide, contribute to the lower detection rates in routine testing. We found higher IFM scores in samples remaining undiagnosed by this method compared with other methods (Table 1), indicating that slide examination is a particular problem for mZN microscopy where parasites are less readily observed under routine diagnostic conditions.

There was a linear relationship between increasing IFM scores (indicating more oocysts present) and decreasing real-time PCR C_T values (indicating more *Cryptosporidium* DNA present) (data not shown). For the true-positive samples, Mann–Whitney U tests showed significantly lower real-time PCR C_T values in those positive by AP microscopy, the three EIAs and the ICLF assay, compared with samples negative by these assays ($P < 0.05$) (Table 1). There was no significant difference in C_T values between true-positive samples that were positive and those that were negative by mZN microscopy ($P = 0.1212$) or by IFM ($P = 0.0822$) (Table 1). Oocysts were not detected in only four true-positive samples by IFM. For mZN microscopy, the lack of difference between test outcome and C_T values probably supports the general difficulties in observing parasites by mZN microscopy under routine diagnostic conditions that are not solely due to parasite density.

In our test algorithm, which included resolution and confirmation of positive combined *Cryptosporidium* and *Giardia* EIA reactions using genus-specific IFM reagents, observed diagnostic specificities for all assays were 100% (Table 1), and the positive predictive value of the assays would be high where high specificity is achieved. Specificity may be lower for microscopy testing in laboratories with less experience at observing oocysts and differentiating other organisms and staining artefacts. The choice of test used to confirm positive ICLF and EIA reactions and resolve the organism(s) present in combined parasite assays is important

and should have high sensitivity and specificity; we used IFM. Two samples that gave positive reactions in the Techlab Giardia/Cryptosporidium CHEK EIA, one other sample giving a positive reaction in the IVD Research Giardia/Crypto Combo EIA and one other sample giving a positive reaction in the Remel ProSpecT Giardia/Cryptosporidium Microplate EIA remained unconfirmed for either parasite by IFM and were negative in all other assays. These must be considered false-positive reactions and highlight the importance of confirmatory testing. *Giardia* spp. were not seen in any samples by the SMIs, but were confirmed by IFM in up to two additional samples detected by each of the combination EIA kits, which concurs with improved diagnosis of *Giardia* infections facilitated by EIA, reported by Ellam *et al.* (2008). Technical problems have been reported previously with first-generation *Cryptosporidium* EIAs (Gradus *et al.*, 1996) leading to false-positive results, the occurrence of which was reduced following product correction (Doing *et al.*, 1999). Using automated plate-washing and -reading equipment and highly specific assays to confirm positive results, our results showed increased sensitivity and specificity for the ProSpecT EIA compared with those reported by Johnston *et al.* (2003). However, as highlighted previously by Weitzel *et al.* (2006), comparisons may also be influenced by the study population from which the samples are drawn, the study design – especially in the size and power of the study – and in the nomination of a gold standard, as a true standard is lacking. The requirement to confirm and resolve the genus (*Cryptosporidium* and/or *Giardia*) in combination assays adds assurance, and single assays should likewise be confirmed. This is especially important to eliminate false positives (Doing *et al.*, 1999; Weitzel *et al.*, 2006).

Detection of *Cryptosporidium* spp.

Of the 152 ‘true positives’, 80 were *C. hominis*, 68 *C. parvum*, two *Cryptosporidium felis*, one *Cryptosporidium ubiquitum* and one *Cryptosporidium meleagridis*, a distribution reflecting that found contemporaneously in the UK (Elwin *et al.*, 2011), where nearly all primary diagnostic testing is undertaken by AP or mZN microscopy (Chalmers *et al.*, 2010a). There was no significant difference between detection rates of *C. parvum* and *C. hominis* by the seven

Table 2. *Cryptosporidium* detection by the seven diagnostic assays in stools containing *C. parvum* and *C. hominis*

Diagnostic assay	<i>C. parvum</i> (<i>n</i> =68)	<i>C. hominis</i> (<i>n</i> =80)	Comparison of species detection (Yates' corrected χ^2)	<i>P</i> value
mZN microscopy	48 (70.6 %)	65 (81.3 %)	1.76	0.1845
RIDAQUICK <i>Cryptosporidium</i> ICLF	58 (85.3 %)	69 (86.3 %)	0.00	0.9440
Remel ProSpecT Giardia/Cryptosporidium EIA	65 (95.6 %)	72 (90.0 %)	0.96	0.3284
AP microscopy	62 (91.2 %)	76 (95.0 %)	0.35	0.5519
IVD Research Giardia/Crypto Combo EIA	66 (97.1 %)	73 (91.3 %)	1.27	0.2591
Techlab Giardia/Cryptosporidium CHEK EIA	66 (97.1 %)	74 (92.5 %)	1.37	0.2422
Crypto-Cel IFM	67 (98.5 %)	78 (97.5 %)	0.02	0.8868

Table 3. Relative attributes of the seven *Cryptosporidium* diagnostic assays

Assay or format	Diagnostic sensitivity	Diagnostic specificity	Technical expertise	Speed of turnaround time	Performed in batch mode	Hands-on time	Consumables cost per test	Specialist laboratory equipment required
mZN microscopy	Moderate	Moderate – need to differentiate staining artefacts and other organisms	High degree of interpretation and continued practice required	Good	Only for staining slides	Highly labour-intensive	Low	Bright-field microscope with good optics
ICLF	Moderate	Good	Simple to perform	Excellent (<10 min)	No	Not labour-intensive	High	None
EIA	Good	Good when used with confirmatory test	Moderately simple to perform	Good	Yes	Not labour-intensive	High	Plate washer and reader
AP microscopy	Good	Good	Moderate degree of interpretation and continued practice required	Very good	Only for staining slides	Moderately labour-intensive	Low	Fluorescence microscope with good optics
Immunofluorescence microscopy	Excellent	Excellent	Moderate degree of interpretation and continued practice required	Very good	No	Moderately labour-intensive	Moderate	Fluorescence microscope with good optics

assays investigated (Table 2) ($P>0.05$), although, as far as we are aware, the antibodies in commercially available EIA and ICLF assays were raised against *C. parvum*. Agnamey *et al.* (2011) also found no difference in the detection rates of *C. parvum* and *C. hominis* when they compared three commercial immunochromatographic assays, including the RIDAQUICK assay that was also used in our study. The detection of other *Cryptosporidium* spp. in our study must be interpreted with caution as the number of samples was small. Very little information is available about the antibodies that are incorporated into each assay, and it is not known how strongly they react with the exposed epitopes on the antigens of species genetically distant from *C. parvum*. Furthermore, results may also be influenced by parasite density. The *C. meleagridis* isolate was detected by all seven assays and had a high IFM score of 6. The *C. ubiquitum* isolate was not detected by mZN microscopy, even though the IFM score was high (score of 5), but it was detected by all other assays. Of the two *C. felis* isolates, one was detected by IFM only (score of 2) and the other by mZN microscopy only, but they were not detected by the six other assays. This is in agreement with the results of Agnamey *et al.* (2011) who found imperfect detection of *C. felis* by immunochromatographic assays. They also reported a lack of detection of *C. meleagridis*, *Cryptosporidium canis* and an undefined *Cryptosporidium* sp. using these assays, but again the numbers of samples tested were small and the parasite density was not reported (Agnamey *et al.*, 2011). However, since our study was undertaken, more laboratories in the UK are now using EIA and ICLF assays as their primary test, and we continue to identify 'unusual' *Cryptosporidium* spp. in positive stools submitted from these laboratories as well as from those using conventional microscopy (*Cryptosporidium* Reference Unit, unpublished data). We have also confirmed, using molecular methods, a variety of *Cryptosporidium* spp. in water samples, originally isolated using immunomagnetic separation and detected by IFM (Chalmers *et al.*, 2010b; Robinson *et al.*, 2011), suggesting that the antibodies in these tests have broad species specificity. Although cross-reactivity of several antibody-based tests has been demonstrated (Barugahare *et al.*, 2011), the species specificity of antibody-based *Cryptosporidium* assays requires further investigation and this is under way at the *Cryptosporidium* Reference Unit.

In conclusion, whilst IFM offered the best diagnostic sensitivity compared with our nominated gold standard, this microscopy-based test is more expensive than mZN and AP microscopy and requires a fluorescence microscope, trained staff and more hands-on time per sample than non-microscopy-based methods (Table 3). The ICLF format eliminates the need for skilled technical staff and specialized equipment and offers a rapid diagnosis (Table 3), but is less sensitive than EIAs (Table 1). The EIAs tested here offer an alternative to traditional diagnostic tests and may expand or enable continued testing of a high proportion of acute diarrhoea cases for *Cryptosporidium*, as the tests can be undertaken in batches (Table 3) (Chalmers *et al.*, 2010a).

They have been used to expand patient testing for *Giardia*, which is often restricted to patients with foreign travel history, with beneficial results (Ellam *et al.*, 2008). Although specialist equipment (plate washers and readers) is required to automate the process (Table 3), this must be validated locally, and diagnostic algorithms must include confirmation of positive reactions. The higher cost of the immunoassay kits may be offset by reduced labour costs, but a skill base needs to be maintained for confirmation. Accurate and prompt diagnosis of diarrhoeal illness is essential for individual patient management and specific control interventions.

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