Evaluation of the BD MAX Enteric Parasite Panel for the detection of Cryptosporidium parvum/hominis, Giardia duodenalis and Entamoeba histolytica

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INTRODUCTION

Intestinal parasite gastroenteritis is challenging to diagnose and differentiate clinically. Similarity of symptoms resulting from infection with many different parasites, including both protozoa and helminths, is further compounded by the plethora of bacterial, viral and non-infectious causes of diarrhoea [1].

Outside of resource-poor settings the most common protozoan parasites found in enteric specimens from patients with no travel history include Cryptosporidium spp. and Giardia duodenalis [2]. Infection with Entamoeba histolytica is also of particular concern in returning travellers from areas of endemicity [3]. Although microscopy allows for the recognition of the widest range of parasites, including Cryptosporidium spp., Entamoeba complex and G. duodenalis, it requires considerable technical expertise, is labour intensive and may suffer from poor analytical sensitivity, if more extensive concentration methods and staining procedures are not used. Microscopy is also unable to differentiate pathogenic and non-pathogenic members of the Entamoeba complex, E. histolytica and E. dispar [4]. Antigen detection tests are available for the diagnosis and differentiation of parasitic gastroenteritis, including Cryptosporidium spp., E. histolytica and G. duodenalis. These methods allow for greater standardization of testing with reduced levels of technical expertise but reported performance can be variable [5]. Nucleic acid amplification tests (NAATs) have also been developed for the detection and differentiation of parasites in stools [6–9].

The BD MAX Enteric Parasite Panel (BD MAX EPP) is a fully automated assay providing nucleic acid extraction and detection of this pathogen.

Abstract

Purpose. Conventional laboratory detection methods for gastrointestinal parasites are time consuming, require considerable technical expertise and may suffer from poor analytical sensitivity. This study sought to evaluate the automated BD MAX Enteric Parasite Panel (EPP) for the detection of Cryptosporidium parvum/hominis, Entamoeba histolytica and Giardia duodenalis.

Methodology. A total of 104 known positive samples (43 Cryptosporidium parvum/hominis and 61 G. duodenalis), 15 simulated samples (E. histolytica and other Entamoeba species) and 745 patient stool samples, submitted for enteric pathogen culture and microscopy, were inoculated into BD MAX EPP sample buffer tubes (SBTs). All specimens were blinded and tested within 7 days of SBT inoculation using the BD MAX EPP assay with results compared to those generated by microscopy.

Results/Key findings. Combining the results from the known positive samples and anonymously tested patient samples, the sensitivity of the BD MAX EPP assay was 100 % for both Cryptosporidium spp. and G. duodenalis. Specificities of 99.7 and 98.9 % were calculated for the detection of Cryptosporidium spp. and G. duodenalis respectively. Insufficient clinical specimen data was available to determine the performance of the assay for E. histolytica detection.

Conclusions. The findings of this study indicate that the BD MAX EPP is suitable for the detection of Cryptosporidium parvum/hominis and G. duodenalis from clinical specimens with reduced hands-on time and complexity compared to microscopy. Results for the detection of E. histolytica were promising although further work is required to evaluate the assay for the detection of this pathogen.
simultaneous real-time amplification for the detection of Cryptosporidium parvum/hominis, E. histolytica and G. duodenalis, using a single platform. The test targets the Cryptosporidium specific DNA fragment and small subunit (SSU) rRNA genes for the other parasites. Use of the BD MAX platform has recently been shown to be useful for the diagnosis of bacterial gastroenteritis [10] although reports on the performance of the EPP assay for G. duodenalis have been variable [11–13]. This paper describes an evaluation of the diagnostic accuracy of the BD MAX EPP using 104 positive archived specimens, 10 simulated positive samples and 734 consecutively collected routine stool samples submitted for culture and microscopy.

METHODS

Samples

A total of 104 unpreserved stool samples positive by microscopy for Cryptosporidium parvum/hominis (20 C. parvum, 20 C. hominis and three Cryptosporidium spp.) and G. duodenalis (20 assemblage A, 20 assemblage B and 21 assemblage unknown) and 10 simulated E. histolytica PCR positive stool samples (produced by the addition of two replicates each of 2 × 10^5 to 2 × 10^9 of E. histolytica trophozoites (Savyon Diagnostics, Ashdod, Israel) were available for retrospective validation using the BD MAX EPP (stored at 4°C for between 1 week and 6 months). In addition, 745 consecutive diarrhoeal stool samples sent for routine faecal investigations (culture and microscopy) were concurrently inoculated into BD sample buffer tubes (SBTs) and subsequently stored at 4°C for up to 7 days before testing using the BD MAX EPP. These samples were collected and tested during the months of November and December 2013.

All specimens consisted of residual anonymized sample material collected for the performance evaluation, with no impact on patient management and thus not requiring ethical approval. Microscopy results were blinded to the scientist performing the BD MAX EPP testing.

Subsequent to the initial simulated sample and clinical specimen test period further testing was undertaken with differing inoculums of Cryptosporidium and Giardia positive and negative samples. Duplicate samples (n=24) were prepared in SBTs containing 1.5 ml of sample buffer inoculated with 1, 5, 10, 50, 100 or 200 µl of positive stool prior to introduction into the recommended processing protocol described above. Additional negative samples (n=27) inoculated with 10–200 µl of the sample were also diluted with 1.5 ml additional sample buffer prior to testing.

Confirmatory PCR assays

All BD MAX results that were discordant with the standard investigations were confirmed by secondary molecular testing. Nucleic acid extraction for confirmatory test samples was undertaken using a NucliSENS easyMag (bioMérieux, Basingstoke, UK) extractor with the Generic 2.01 protocol using 0.75 ml of residual inoculated BD MAX EPP sample buffer with an elution volume of 25 µl.

Cryptosporidium spp. confirmatory testing was undertaken at the UK Cryptosporidium Reference Unit (CRU) using a

**BD MAX EPP assay**

An overview of the workflow and timings for the BD MAX EPP is shown in Fig. 1. Sample preparation and testing was accomplished according to the manufacturer’s instructions [14] with the exception that samples were vortexed prior to pre-warming rather than after.

In brief, a 10 µl loopful of diarrhoeal stool was inoculated into a SBT containing 1.5 ml of sample buffer prior to capping with a septum cap. Inoculated SBTs were then vortexed for 30 s before heating on a BD MAX Pre-Warm Station using the BD MAX EPP profile. Upon completion of the pre-warm step, the system was set up as per the manufacturer’s instructions [14]. Results were generated automatically by the BD MAX software at the completion of each run after which they were compared with microscopy results.

Subsequent to the initial simulated sample and clinical specimen test period further testing was undertaken with differing inoculums of Cryptosporidium and Giardia positive and negative samples. Duplicate samples (n=24) were prepared in SBTs containing 1.5 ml of sample buffer inoculated with 1, 5, 10, 50, 100 or 200 µl of positive stool prior to introduction into the recommended processing protocol described above. Additional negative samples (n=27) inoculated with 10–200 µl of the sample were also diluted with 1.5 ml additional sample buffer prior to testing.

**Microscopy**

Examination for ova, cysts and parasites was undertaken using both saline wet mount and modified Ziehl-Neelsen microscopy for all of the 104 retrospective clinical samples, for 734 of the 745 prospective samples collected and for the five non-histolytica Entamoeba positive stool samples. Where a clinical history of travel to endemic countries was given (23 samples) a formol-ether concentration method was employed in addition to the aforementioned techniques. Ova, cysts and parasites were identified according to morphology and size.

**Fig. 1.** BD MAX EPP (left) and conventional microscopy (right) workflow and timings for 24 samples. Conventional microscopy timings are based on 5 min per sample for preparation and examination time although it is acknowledged that this will vary depending on the experience of the operator.
species-specific real-time PCR assay based on the *Lib13* locus targeting *C. parvum* [15] and the A135 locus targeting *C. hominis* (manuscript in preparation). All BD MAX EPP *E. histolytica* or *G. duodenalis* positive/microscopy negative samples were confirmed using previously published in-house assays [9] in monoplex format, to ensure maximum sensitivity of detection, on an ABI 7500 fast (Life Technologies, Paisley, UK). If required, the Luminex xTAG Gastrointestinal Pathogen Panel (GPP) assay was utilized as a secondary confirmatory assay [6]. For the in-house assays 5 µl of eluate was used. For the Luminex GPP assay 10 µl of sample extract (extracted as described above and not according to the Luminex GPP protocol) was used according to the manufacturer’s instructions for PCR and detection [16].

**Statistical analysis**

Performance (sensitivity, specificity, positive and negative likelihood ratio) was determined using 2×2 contingency tables where a ‘true-positive’ was defined as a sample where a parasite was detected by microscopy. Agreement (Kappa statistic) was also calculated [17]. For all proportional values Wilson 95% confidence intervals were calculated [18].

**RESULTS**

**BD MAX EPP assay validation**

The BD MAX EPP correctly detected *Cryptosporidium parvum/hominis* (43) and *G. duodenalis* (61) in the previously characterized positive stool samples. It was also able to detect *E. histolytica* in the 10 simulated samples spiked with *E. histolytica*.

**BD MAX EPP assay blind evaluation**

In total 354 samples were collected from male patients and 391 from females. Of the specimens 378 originated from hospital in-patients with 367 from out-patient/community settings with an overall median age of 59 years (range 0–101 years). Of the 745 samples tested only 734 were included on 11 samples (five male and six female patients). Microscopy was not undertaken on eight samples from in-patients on 101 years). Of the 745 samples tested only 734 were included on 11 samples (five male and six female patients). Microscopy was not undertaken on eight samples from in-patients as only *C. difficile* testing had been requested. It was not clear why microscopy had not been performed on three patient samples originating from community settings. No parasite DNA was detected in any of these samples although one sample was reported as ‘unresolved’ due to either specimen-associated inhibition or reagent failure.

The results of microscopic and BD MAX EPP analysis of 734 samples are shown in Table 1. BD MAX EPP testing was not repeated for any of the samples yielding unresolved or indeterminate results.

All BD MAX EPP *Cryptosporidium* and *Giardia* positive samples were also positive using confirmatory molecular assays. The single *Cryptosporidium* spp. microscopy positive sample that did not give a BD MAX EPP positive result was reported as ‘unresolved’ due to inhibition. Testing of the solitary BD MAX EPP *E. histolytica* positive sample using the specific real-time assay was unsuccessful in confirming the presence of *E. histolytica* DNA, but was confirmed using the Luminex GPP assay.

**BD MAX EPP additional testing**

All five non-histolytica *Entamoeba* positive samples (*E. dispar*, *E. gingivalis, E. invadens, E. moshkovskii* and *E. rana-rum*) were negative using the BD MAX EPP assay. Over-inoculation of SBTs with 100 and 200 µl of positive *Cryptosporidium/Giardia* stool sample resulted in unresolved reports for the pathogens not present in the sample in three out of six tests. Positive results were nevertheless obtained for the infecting organism in all samples (6/6) regardless.

Over inoculation of SBTs with negative stool resulted in unresolved reports for 75, 50 and 25 % of samples inoculated with 200, 100 and 50 µl of faeces respectively (*n*=4). Dilution (1:2) of unresolved samples was unsuccessful in yielding a negative result for all 200 µl simulated samples and only one 100 µl over-inoculated simulated sample respectively. It was successful however for the over-inoculated 50 µl simulated samples.

**BD MAX EPP workflow and timings**

Fig. 1 shows an overview of the workflow and timings for the BD MAX EPP assay for 24 samples. Approximately 30 min of hands-on time was devoted to sample preparation (inoculation of SBTs and vortexing). The remainder was taken up by loading and unloading SBTs and BD MAX consumables and
by entering details onto the BD MAX software. Sample pre-warm and automated extraction, PCR cartridge loading and PCR took 50, 66, 18 and 81 min respectively.

**Statistical performance**

Table 2 shows the performance of the BD MAX EPP assay for the detection of Cryptosporidium and Giardia using microscopy as the gold standard, excluding samples from which invalid and unresolved results were produced. Data is shown for the blind evaluation consecutive cohort alone, in addition to combined performance (which included the results from the blind evaluation and previously characterized validation samples) due to the low number of positive samples seen during the study. Performance for the detection of E. histolytica is not included due to only one positive clinical sample being available for analysis.

Sensitivity and specificity were greater than 98 % for the detection of both Cryptosporidium and Giardia in both analyses. Positive likelihood ratio results showed in both analyses that BD MAX EPP Cryptosporidium and Giardia detections were related to a large increase in the likelihood of disease. Negative likelihood ratios were calculated using sensitivity of 99.9 %. The Kappa statistics demonstrate substantial and fair agreement in the blind evaluation and almost perfect agreement for the combined analysis for the detection of Cryptosporidium and Giardia respectively. Accuracy was high in all analyses (see Table 2).

**DISCUSSION**

The results presented in this report demonstrate, as shown by other authors, the increased detection rate for intestinal parasites that can be achieved if NAATs are employed for enteric specimen processing [6, 7, 12, 13, 19]. The sensitivity of microscopy for Giardia [6] detection (20 %) was particularly low, with 40 % of Cryptosporidium positives also being missed, highlighting why NAATs are becoming the method of choice in many laboratories where workload prohibits following guidance in the use of concentration methods for the detection of ova, cysts and parasites from all samples [20]. The fact that these results were accomplished on the BD MAX with fully automated extraction, amplification, detection and result analysis is a significant technological improvement making NAATs readily accessible to laboratories with limited molecular expertise.

Microscopy, unlike PCR, is unable to differentiate between pathogenic and non-pathogenic Entamoeba species [4]. The solitary BD MAX E. histolytica detection from this study that could not be confirmed using the in-house PCR assay [9] may represent a true low-level positive or false positivity [6, 21, 22]. Testing other closely related Entamoeba species (E. dispers in particular which cannot be distinguished from E. histolytica by microscopy) did not yield false positive E. histolytica results using the BD MAX assay. In addition, serial dilutions of simulated E. histolytica positive samples (unpublished data) showed that the BD MAX EPP assay had superior analytical sensitivity for this target than the in-house assay. These findings, the confirmatory Luminex GPP E. histolytica detection from the same sample and the excellent specificity demonstrated for this target in other studies [11–13] support the possibility that the result represented a low-level positive. Further work is required, ideally in an area of substantial E. histolytica prevalence, to assess the diagnostic accuracy of the BD MAX EPP for the detection of E. histolytica.

The results presented for G. duodenalis reflect the findings of other studies when using a molecular assay for its detection [6, 19, 23] but are significantly improved in comparison to previously published reports for the BD MAX EPP, in spite of the use of a significant number of stored samples [12, 13]. The cause of this encouraging discrepancy cannot be explained although it has been seen when using other molecular G. duodenalis assays by the authors (unpublished data).

The fact that the BD MAX EPP was not designed to detect Taenia spp. and one case of Taenia was detected by microscopy demonstrates a possible advantage of microscopy over targeted PCR and similar findings have been seen by other authors [11]. However, this sample was tested using a faecal concentration method. The exact reason for undertaking the faecal concentration is unknown, due to the blinded nature of this study, but this method is only performed as a result of relevant clinical information accompanying a sample request. In agreement with other study conclusions [11], the authors recommend that the BD MAX and similar assays should be used

**Table 2. Performance of the BD MAX EPP for the detection of Cryptosporidium parvum/hominis, Giardia duodenalis and Entamoeba histolytica**

<table>
<thead>
<tr>
<th></th>
<th>Prospective Cryptosporidium</th>
<th>Prospective Giardia</th>
<th>Combined Cryptosporidium*</th>
<th>Combined Giardia*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity (%)</strong></td>
<td>100 (13.9–100)</td>
<td>100 (13.9–100)</td>
<td>100 (85.9–100)</td>
<td>100 (89.6–100)</td>
</tr>
<tr>
<td><strong>Specificity (%)</strong></td>
<td>99.7 (98.4–100)</td>
<td>98.8 (97.1–99.5)</td>
<td>99.7 (98.6–100)</td>
<td>98.9 (97.3–99.6)</td>
</tr>
<tr>
<td><strong>LR+ve</strong></td>
<td>339 (58–1978)</td>
<td>85 (35–204)</td>
<td>369 (63–2156)</td>
<td>90 (37–217)</td>
</tr>
<tr>
<td><strong>LR–ve†</strong></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Kappa</strong></td>
<td>0.67 (0.58–0.75)</td>
<td>0.33 (0.26–0.40)</td>
<td>0.98 (0.89–1.00)</td>
<td>0.94 (0.85–1.00)</td>
</tr>
<tr>
<td><strong>Accuracy</strong></td>
<td>0.99 (0.99–1.00)</td>
<td>0.98 (0.97–1.00)</td>
<td>0.99 (0.99–1.00)</td>
<td>0.99 (0.98–1.00)</td>
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*Combined results are derived from calculating statistics using the blinded sample and previously characterized positive sample results; LR+ve, positive likelihood ratio; LR–ve, negative likelihood ratio.
†Calculated using sensitivity of 99.9 %.
for routine screening of enteric samples in non-endemic areas, but where there is relevant clinical information, such as foreign travel, faecal concentration microscopy should be performed to complement the service. Following such an algorithm would mean that this *Taenia* species would still have been reported if the BD MAX, or a similar assay, was being used as the frontline screening tool. Whilst the recommended faecal concentration microscopy allows for more sensitive and broad-range detection of parasites, which would have almost certainly improved the microscopy results herein had it been used, its routine diagnostic use outside of specialist centres is compromised by the time-consuming and technically demanding nature of the method.

Standardization and effective use of technician time are increasingly important in today's diagnostic microbiology laboratories. Faecal parasite microscopy has been shown to suffer from high variability between operators of varying experience [24] with historic recommended minimal examination times of at least 15 min per sample [25, 26] being impractical for most institutions. Use of the BD MAX EPP affords a service that is standardized and allows more efficient use of laboratory personnel even when realistic examination time data is included for comparison (Fig. 1). The increased overall turnaround time for the BD MAX EPP may be offset by the use of more than one platform concurrently (allowing processing of 48 samples in approximately 5 h) and by the automated nature of the assay allowing processing without breaks and assays to be set up at the end of a shift and run overnight.

The 55 unresolved/indeterminate results (~7%) would appear to be a limitation for the use of this assay. However, the majority were produced at the beginning of the study potentially due to over-inoculation of SBTs. On resolving this, unresolved results reduced to acceptable levels (~3%). A limitation of this study was that repeat testing of unresolved samples, in particular the sample that was *Cryptosporidium* spp. microscopy positive, was not possible. The further work undertaken to test the effect of over-inoculation of SBTs demonstrated that over-inoculating SBTs did result in the production of unresolved reports for negative samples. However, when positive samples were over-inoculated the positive result was not affected even when up to 20× (200 µl) the recommended amount of sample was introduced into the buffer. Introduction of up to 10× less (1 µl) of several positive clinical samples (unpublished data) did not affect the sensitivity although it is likely that for very low level positive samples the sensitivity of the assay would be adversely affected. It is possible that, despite the inhibition seen, the BD MAX false negativity was due to the presence of a non-*parvum/hominis* *Cryptosporidium* spp. A pan-*Cryptosporidium* genus probe and a more objective description for the correct inoculation level for the BD MAX EPP would be desirable [14].

Recent publications document the use of the BD MAX Enteric Bacterial Panel (BD MAX EBP) for the sensitive and accurate detection of enteric bacterial pathogens [10, 27] Combining the information from this and other studies shows the potential for utilizing the BD MAX to replace routine faecal culture and microscopy. Although cost is a barrier to the introduction of such technologies, the increased sensitivity, reduced hands-on time and assay complexity of the BD MAX and similar assays may ease the transition as a result of staff cost efficiency savings and improved demand management [10, 23].

As yet, there is no published data comparing the BD MAX EPP with other commercial NAATs, although comparison with in-house assays is promising [12, 13]. Unfortunately this work, similar to that of Batra and colleagues [11], was limited to comparison with current routine diagnostic methods. An additional limitation of this study was the use of direct wet mount microscopy as opposed to the recommended concentration microscopy methods. However, this merely reflects practice in many laboratories in the UK and further afield and highlights the need for more sensitive and efficient diagnostic methods that can be undertaken in high-throughput laboratories [3].

In conclusion, this study has shown that the BD MAX EPP can accurately detect *Cryptosporidium parvum/hominis* and *Giardia duodenalis* in fresh and stored clinical stool samples. It also indicates, as other studies have [12, 13], that the assay may be of use in the detection of *E. histolytica* from such samples although further work will be required to confirm this. Limited hands-on time and simple and standardized workflows allow for efficient use of laboratory personnel and reduced inter- and intra-operator variability in the detection of some of the most commonly detected protozoan parasites. Used in tandem with the BD MAX EBP it may be able to replace most of the culture and microscopy of enteric pathogens currently undertaken by routine microbiology laboratories.

**Funding information**

BD funded the BD MAX Enteric Parasite Panel platform, consumables and staff time during the period of the study. PLW received project funding from Myconostica, Luminex and Renishaw diagnostics, was sponsored by Myconostica, MSD and Gilead Sciences to attend international meetings, on a speaker's bureau for Gilead Sciences, and provided consultancy for Renishaw Diagnostics Limited.

**Acknowledgements**

The authors would like to acknowledge the contribution of colleagues from the UK Cryptosporidium Reference Unit in performing confirmatory *Cryptosporidium* spp. PCR testing.

**Conflicts of interest**

MP has received travel grants, honoraria and project funding from BD, Luminex, Roche and Savion. SC has received travel grants and honoraria from Luminex.

**References**

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