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CORRESPONDENCE

Evaluation of the EasyScreen Protozoan Detection Kit for the diagnosis of *Entamoeba histolytica*

Sir,

Entamoeba histolytica is the causative agent of amoebiasis and one of the most common parasitic infections worldwide.[1] Clinical manifestations range from asymptomatic carriage, amoebic dysentery and extraintestinal disease most commonly presenting as liver abscesses.[1] *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii* and *Entamoeba bangladeshi* are all morphologically indistinguishable; as such, polymerase chain reaction (PCR) has become the diagnostic method of choice in diagnosis of pathogenic *E. histolytica*.[2] It is clinically important to correctly diagnose amoebiasis to reduce morbidity and mortality in patients infected and to prevent undue antimicrobial therapy. This study evaluated the EasyScreen Protozoan Detection Kit (EP001, Genetic Signatures, Australia), a multiplex real time PCR (qPCR) assay for use with both faeces and liver aspirates for the detection of *E. histolytica*. This kit was evaluated previously under the same name, though it has since been updated to include a specific *E. histolytica* target as opposed to a generic *Entamoeba* complex target.[3]

All faeces specimens and liver aspirates submitted to the Department of Microbiology at St Vincent's Hospital, Sydney, Australia, from August 2015 to September 2016, were included in the evaluation. A total of 1,760 samples were processed with the EasyScreen Protozoan Detection Kit: 1,590 faecal samples and 170 liver aspirates. Included in the 1,590 faecal samples, 121 faecal samples were taken from previous studies that were positive for *E. dispar* (n=60), *E. moshkovskii* (n=50) and *E. histolytica* (n=11)[2,4–7] to test for specificity for *E. histolytica*. The EasyScreen Protozoan Detection Kit was also tested for specificity against non-*Entamoeba histolytica/dispar/moshkovskii* samples on 90 faecal samples containing one or more parasites (as identified by microscopy of modified iron haematoxylin stained smears[8]). This included specimens containing *Blastocystis* spp. (n=30), *E. coli* (n=4), *E. hartmanni* (n=3), *E. polecki* (n=1), *Giardia intestinalis* (n=30), *Endolimax nana* (n=12), *Iodamoeba butschlii* (n=3), *Cryptosporidium* spp. (n=15), and *Chilomastix mesnili* (n=2). DNA from *E. histolytica* strain HTH-56:MUTM, *E. dispar* strain SAW760 and the Laredo strain of *E. moshkovskii* were all used as controls. As no DNA or stool specimens containing *E. bangladeshi* were available for cross-reactivity testing, a 1500 bp gene block (Integrated DNA Technologies, USA) was designed based on the 18S ribosomal RNA gene from *E. bangladeshi* strain 8237 (Genebank accession number KR025412.1).

The faeces/liver aspirates were prepared for qPCR using the EasyScreen Sample Processing Kit (SP001, Genetic Signatures) as per the manufacturer's instructions. For end-point PCR, samples were prepared using the EZ1 BioRobot Automated Extraction System (Qiagen, Germany) combined with the EZ1 tissue DNA extraction kit (953034; Qiagen). Tissue lysis buffer (250 µL) and proteinase K (10 µL) from the EZ1 tissue kit was added to the sample then heated for 10 min at 95°C before being processed using the tissue card protocol according to the manufacturer's recommendations.

The EasyScreen Protozoan Detection Kit qPCR was performed on a CFX384 Touch Real-Time PCR Detection System (1855485; Bio-Rad, USA) according to the manufacturer's guidelines and visualised using the Bio-Rad CFX Manager program (Fig. 1), including both an internal positive control and an extraction control.

End-point PCR was performed using Illustra PuReTaq Ready-To-Go PCR beads (27-9557-02; GE Healthcare, USA) in a Thermal Cycler (TC020A-230V; LabNet, USA). The beads were dissolved in 18 µL of DNAse free water and 0.5 µM (2.5 µL) of both the forward and reverse primer (Table 1). As template, 2 µL of extracted DNA/positive control DNA were added. DNAse free water (2 µL) was used as the negative control reaction. The reactions were subjected to the following temperature cycling conditions: (1) an initial denaturation of DNA at 94°C for 3 min, (2) denaturation at 94°C for 1 min, (3) primer annealing at 60°C for 1 min, (4) primer extension at 72°C for 1 min, (5) repeat steps 2–4 for 40 cycles, and lastly, (6) a final extension step at 72°C for 10 min. Results were visualised on E-Gel EX 2% Agarose Gels (G401002; Invitrogen, USA) using a 50 bp ladder (10416014; Invitrogen, USA) (Fig. 2).

In order to compare the end-point PCR to the EasyScreen Protozoan Detection Kit qPCR, the sensitivity of each assay was calculated as [number of true positives/(number of true positives + number of false negative)] × 100. Specificity was calculated as: [number of true negatives/(number of true negatives + number of false positives)] × 100. Comparison of the limit of detection was performed using cloned *E. histolytica* target DNA (*E. histolytica* strain HTH-56:MUTM). Ten-fold dilutions of the DNA were spiked onto clinical samples negative for parasitic cysts and ova by microscopy and PCR with *E. histolytica*, *E. dispar*, and *E. moshkovskii*-specific primers. The DNA was then extracted and processed with the Genetic Signatures qPCR and end-point PCR. Samples were tested in duplicate.

Our testing found that the EasyScreen kit has excellent sensitivity (100%) and specificity (100%) for detection of *E. histolytica* in faecal samples. In the previously untested stool specimens (n=1,379), the EasyScreen kit successfully detected 13 new *E. histolytica* infections which were subsequently confirmed by end-point PCR. The EasyScreen kit also detected *E. histolytica* in all 11 previously characterised positive stool specimens. All 1,366 negative specimens were also tested using end-point PCR and the results showed complete concordance with the EasyScreen kit results. When evaluated using the 90 faecal samples known to contain one or more protozoan parasites, as well as against the 121 *E. histolytica/dispar/moshkovskii* complex positive specimens, the new targets showed no cross reactivity with other protozoan pathogens in faecal samples, consistent with results from previous evaluations of the kit.[3] While no *E. bangladeshi* DNA was available to test, a gene block was tested and also showed no cross reactivity. As compared to end-point PCR, the EasyScreen kit provided identical results; however, its ability to be combined with other targets

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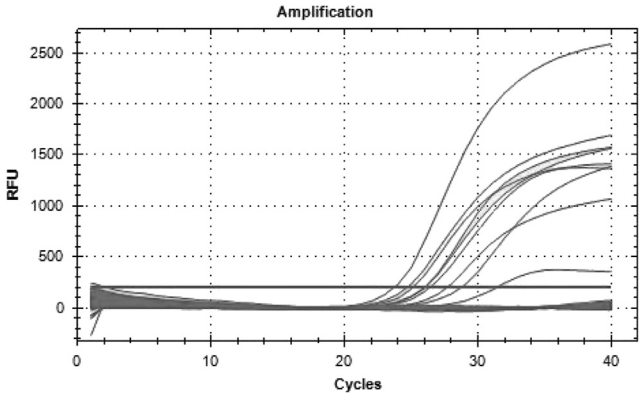

Fig. 1 qPCR amplification curves obtained from specimens tested with the EasyScreen assay.

including bacterial and viral EasyScreen kits makes it a more effective clinical test. Further, the EasyScreen kit limit of detection was 1 pg of *E. histolytica* DNA, a value approximately ten times more sensitive than the utilised end-point PCR (10−100 pg).

Similarly, the EasyScreen kit showed the same sensitivity (100%) and specificity (100%) with liver aspirate samples, which had not been previously tested. The kit successfully detected *E. histolytica* DNA in 25 specimens, confirmed by end-point PCR methods. The EasyScreen kit has demonstrated that it can be used effectively for these sample types in addition to stool samples, to detect the presence of pathogenic *E. histolytica.* This is beneficial diagnostically for medical teams and diagnostic laboratories where timely and reliable detection of *E. histolytica* may have significant effects on the treatment and prognosis for a patient. Similar to the testing of *E. histolytica* in faecal specimens, there exists ELISA tests that are capable of detecting amoebiasis in liver abscess pus but these tests are reportedly less sensitive and/or specific than PCR alternatives.[9,10]

The current study presents evidence for the effective use of new targets for differentiating *E. histolytica* from the non-pathogenic *Entamoeba* complex members. Given the inability for conventional microscopy to differentiate between pathogenic *E. histolytica* and the non-pathogenic members of the *Entamoeba* complex, it is vital that there is a highly specific and sensitive assay available for diagnostic laboratories. As such, molecular detection of parasites using qPCR is now considered the gold standard for diagnosis given its high sensitivity, specificity and cost/time sensitive capabilities when compared to conventional techniques.[11,12] Multiplex qPCR assays allow for the detection of numerous targets in a single sample; the EasyScreen Protozoan Detection kit in particular can also detect *Cryptosporidium* spp., *Giardia intestinalis*, *Dientamoeba fragilis* and *Blastocystis hominis* alongside *Entamoeba histolytica*. It should be noted, however, that currently the EasyScreen Kit is not a complete

Table 1 Primers used for end-point PCR for *Entamoeba histolytica*

Target	Pair	Sequence (5′ −> 3′)
E. histolytica SSU rDNA	Eh5′	GTAACTTACTTAACCGGTAAA ACATG
	Eh3′	TCTCTTCGTAACAAAGATCTAG ACTC

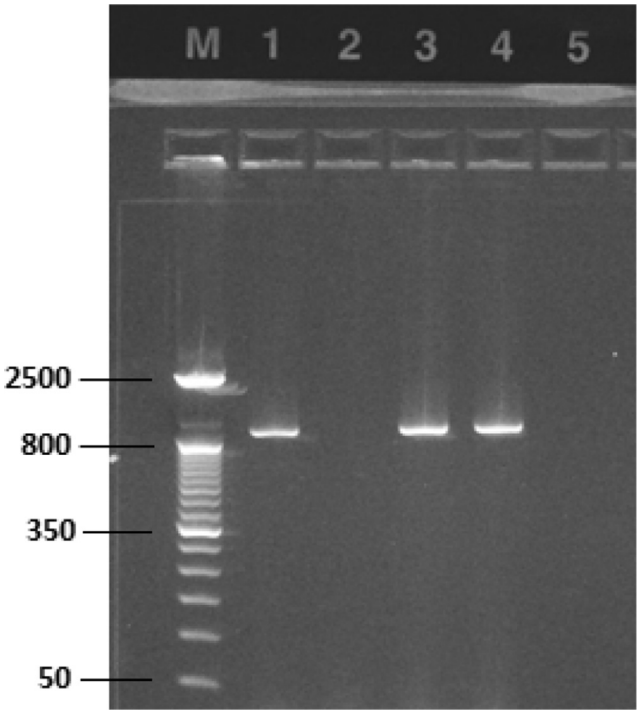

Fig. 2 Example Invitrogen E-Gel EX 2% Agarose Gel image of a positive *Entamoeba histolytica* end-point PCR product. Lanes 1 and 2 are the positive and negative controls, respectively, and lanes 3 and 4 are positive specimen samples. Lane M contains a 50 bp ladder.

substitute for microscopy or additional PCRs as it does not detect some pathogenic protozoa such as *Coccidia* spp., *Cystoisospora belli* or *Cyclospora cayetanensis*. The EasyScreen Protozoan Detection kit also benefits from being run in tandem with other Genetic Signatures EasyScreen kits to detect viral and bacterial pathogens.

The full EasyScreen sample process is mostly an automated procedure in comparison to labour intensive microscopy and staining techniques, thereby removing the variables of human error and the need for adequate training of laboratory personnel in recognising protozoa visible by staining. For the *Entamoeba* complex, the availability of this multiplex EasyScreen kit removes the need for further reflex testing of positive samples. Differentiation between pathogenic and non-pathogenic members of the complex occurs within the process allowing for rapid detection and diagnosis.

Overall, the EasyScreen Protozoan Detection Kit shows excellent sensitivity (100%) and specificity (100%) for *Entamoeba histolytica* detection using both faecal and liver aspirate sample types. Having taken into consideration several factors impacting the effectiveness of the EasyScreen Protozoan Detection kit as a diagnostic tool, especially when used alongside other Genetic Signatures EasyScreen Enteric Pathogen detection kits, we would still consider it a suitable diagnostic tool for routine testing of patient specimens.

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Increasing prevalence of methicillin-resistant *Staphylococcus aureus* in remote Australian communities: implications for patients and clinicians

Sir,

Staphylococcus aureus is a leading cause of life-threatening community and hospital-acquired infection. Methicillin-resistant *S. aureus* (MRSA) was initially limited to the hospital environment [healthcare-associated MRSA (HA-MRSA)]; however, in Australia, community-acquired MRSA (CA-MRSA) is now commonly isolated, particularly in Indigenous Australians.[1,2] In the Northern Territory, Western Australia and New South Wales, residence in a remote setting is also an independent predictor of CA-MRSA isolation.[1–3] This has important implications for empirical antibiotic regimens in these locations, where clinicians usually have limited access to microbiology laboratory services and are often a long way from sophisticated critical care support.

Far North Queensland (FNQ) covers an area of 380,000 km^2 in tropical Australia and has a population of approximately 280,000 people, 12% of whom identify as Indigenous Australians. The AUSLAB database records the pathology results of all the hospitals and community clinics in Queensland's public health system. To determine the changing local antibiotic susceptibility of *S. aureus*, AUSLAB was interrogated to identify all clinical isolates collected in FNQ between 1 January 1997 and 31 December 2016 and their patterns of antibiotic resistance. In the absence of genetic testing, MRSA was defined as *in vitro* resistance to flucloxacillin; while CA-MRSA was defined as *in vitro* resistance to flucloxacillin but susceptibility to non-beta-lactam antibiotics.[4] The geographical location of each isolate and basic demographic data including patient age, gender, residential address and Indigenous status were recorded. Groups were compared using the chi-squared test; logistic regression analysis was performed using statistical software (Stata version 14.2; StataCorp, USA). Maps were generated using geographic information system software (MapInfo Pro version 15.0; Pitney Bowes, USA) with FNQ divided into eight areas based on key clinical hubs. The Far North Queensland Human Research Ethics Committee provided ethical approval for the study (HREC/16/QCH/112–1085) and waived the requirement for informed consent as the data were retrospective and de-identified.

After excluding non-FNQ residents and repeated isolates from the same patient within a 12-month period, *S. aureus* was isolated on 46,304 separate occasions; 36,802 (79%) were methicillin-sensitive, 8766 (19%) were MRSA, while in 736 (2%) incomplete resistance data precluded classification. Of the 8766 MRSA isolates, 8038 (92%) had antibiograms consistent with CA-MRSA. There was an increase in the prevalence of MRSA over the study period, from 187/786 (24%) in 1997 to 1388/4373 (32%) in 2016 (p for trend <0.001). In some regions this was particularly notable: in the area around Cooktown, MRSA isolates increased from 2/5 (40%) [95% confidence interval (CI) 5–85%] in 1997 to 24/33 (73%) in 2016 (p for trend <0.001) (95% CI 55–87%) (Fig 1). In 2016, 68/3729 (2%) *S. aureus* isolates were resistant to sulfamethoxazole-trimethoprim; inducible resistance to clindamycin was reported in 368/4350 (9%). There were no cases of vancomycin resistance during the study period.

In univariate analysis, MRSA was more commonly isolated in Indigenous patients than non-Indigenous patients [odds ratio (OR) 1.59; 95% CI 1.51–1.68; $p<0.001$]; in patients aged $\geq$40 than aged <40 years (OR 1.19; 95% CI 1.12–1.24; $p<0.001$); and in patients living in metropolitan Cairns than those from a remote setting (OR 1.28; 95% CI 1.22–1.34; $p<0.001$). There was no difference in univariate analysis between the prevalence among men and women [5296/25972 (21%) versus 4170/20295 (21%), p=0.97; in 217 cases the patient's gender was not available].