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Development of diffractive optics technology-based immunoassay protocol and its application in the detection of *Entamoeba histolytica* infections

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ABSTRACT

Amebiasis due to infection with *Entamoeba histolytica* is a problematic parasitic disease in many countries. By means of a novel technology developed by Axela Biosensors, Inc., the dotLab™ system, a rapid immunoassay was developed to detect at least 5.45 cells/mL of *E. histolytica*, the causative agent of amebiasis, in spiked stool samples in 66 min. Regeneration of the dotLab™ sensor using 0.1 M glycine (pH 2.5) solution was established, enabling the assessment of multiple stool samples (up to 8 X) using a single sensor. This developed assay was applied to assess the health status of a community in relation to *E. histolytica* infections of relocated families in San Isidro, Rodriguez, Rizal, Philippines. The community was found to be 15.6% and 26.1% positive for *E. histolytica* using real-time polymerase chain reaction (real-time PCR) and dotLab™ methods, respectively. Compared to real-time PCR, the dotLab™ method is 94.74% sensitive and 74.79% specific. The agreement of the two methods was tested using Kappa coefficient test and it showed that dotLab™ is a reliable alternative to real-time PCR. The optimized dotLab™ assay did not cross-react with stool samples containing *Escherichia coli*, *Blastocystis* sp., *Cryptosporidium* sp. and *Giardia intestinalis*. The community had 17 X to 24 X higher infection rate than previous reports in the Philippines. Sex, age, and duration of settlement in the relocation area were not related to the rate of infection. This increase may be due to improper hygiene and sanitation in the community.

1. Introduction

Amebiasis is caused by the enteric protozoan *Entamoeba histolytica* which manifests most commonly as diarrheal disease and less frequently as amebic liver abscess (Stanley et al., 1991). The World Health Organization (WHO) estimated that *E. histolytica* infection caused more than 103 million cases of diarrhea, the complications of which led to around 5450 deaths worldwide (Kirk et al., 2015). Previous reports indicated that 90% of *E. histolytica* infected individuals are asymptomatic. However, for many years, there has been confusion in the identity of the causative pathogen. In 1993, a redescription of the organism was published separating the pathogenic *E. histolytica* from the morphologically-indistinguishable but nonpathogenic *E. dispar* on the basis of biochemical, immunological and genetic data (Clark and Diamond, 1993). Re-evaluation of the earlier data using a method that will detect and differentiate *E. histolytica* from the nonpathogenic *E. dispar* is needed. Because of the difficulty in detecting and differentiating *E. histolytica* infections through conventional microscopic observation, other technologies are being created to increase specificity to differentiate these species.

Recent technologies that provide increased specificity as well as sensitivity for the detection of *E. histolytica* include antigen detection by enzyme-linked immunosorbent assay (ELISA) and DNA-based detection by polymerase chain reaction (PCR) in stool and other clinical samples (Fotedar et al., 2007a). DNA-based assays like PCR are found to be the most sensitive and the method of detection that is strongly endorsed by the WHO (Fotedar et al., 2007b). Axela Biosensors, Inc. presents a technology that claims to detect any protein-protein interactions at low concentration, less reagent volumes, less sample preparation, label-free and real-time measurements. This technology, called the dotLab™ system, combined principles of grating-based light diffraction and immobilized capture surfaces. This study applied this technology in the detection of *E. histolytica* infections directly using stool samples and compared it with the real-time PCR method. This study also assessed the occurrence of *E. histolytica* infections in a community of relocated individuals in San Isidro, Rodriguez, Rizal, Philippines. The data

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generated suggest the application of dotLab™ in detecting *E. histolytica* infections, and contribute as baseline data for the community to estimate the burden of disease and to formulate policies in controlling amebiasis.

2. Materials and methods

2.1. Development of immunoassay protocol using diffractive optics technology (dotLab™) in detecting E. histolytica cells

2.1.1. Preparation of E. histolytica cells for experiments

The BI-S-33 medium (pH 6.8) described by Diamond (1968) was used to culture *E. histolytica* HK-9 cells. To ensure consistent log growth phase, the cells were subcultured every 3 to 4 days. Prior to runs in the dotLab™ system, the cells were pooled by emptying the culture tubes, centrifuged at 3500 rpm for 5 min, supernatant decanted and replaced with freshly-prepared phosphate buffered saline (PBS). This washing step was repeated twice. Quantification of the cells was performed using hemocytometer.

2.1.2. Preparation of reagents

The antibodies utilized in the immunoassays were: biotinylated goat anti-mouse antibody (Axela Biosensors Inc., Toronto, Ontario, Canada), monoclonal mouse anti-*E. histolytica* IgG (Genway Biotech Inc., San Diego, California, USA), and anti-mouse antibody conjugated with horseradish peroxidase (HRP)-goat anti-mouse IgG (Invitrogen, Carlsbad, California, USA). Prior to use, these were diluted with blocking solution of 5 mg/mL bovine serum albumin (BSA). PBS with 0.01% Tween-20 (0.01% PBS-T) was used as running buffer.

Stool samples which were PCR-negative for the presence of *E. histolytica* were spiked with *E. histolytica* cells and diluted with PBS to standard concentrations of 5.45 cells/mL, 54.5 cells/mL, 545 cells/mL, and 1000 cells/mL. A negative control containing no *E. histolytica* cells was also maintained. These were used to optimize the immunoassay protocol.

Different regeneration solutions which effectively dissociated the antibody-antigen complex while preserving the integrity of the sensor were tested. The following regeneration reagents were prepared: 50 mM NaOH with 0.5% SDS, 5% glacial acetic acid 20% $(NH_4)_2SO_4$, and 0.1 M glycine (pH 2.5).

2.1.3. Assembly of the dotLab™ system

Prior to use, the dot Avidin sensor (Axela Biosensors, Inc.) was brought to room temperature. This was then placed within the sensor block and connected to the fluidics panel by means of lidless tubing (Axela Biosensors, Inc.). The method for each run was determined by a user-controlled method interface, where various settings (e.g., reagent volume, reagent flow rate, incubation time, etc.) were manipulated to ensure maximum contact between complementary moieties. The needed volume of each reagent and sample was automatically calculated by the dotLab™ system. The reagents were then loaded into vessels and 96-well microtiter plate as specified by the system.

2.1.4. dotLab™ immunoassay protocol development

2.1.4.1. Optimization and calibration of dotLab™ immunoassay. The assay was optimized and calibrated using spiked stool samples with known concentrations of *E. histolytica* cells. Plate access steps were added to allow introduction of fresh antibodies and avoid denaturation or deactivation due to prolonged exposure to room temperature and light. Introduction of biotinylated goat anti-mouse IgG (Axela Biosensors, Inc.) was added to create a strong interaction with the avidin sensor surface and present a free antibody region with affinity to mouse IgG Fc molecule of monoclonal mouse anti-*E. histolytica* antibody (Genway Biotech Inc., San Diego California, USA). The monoclonal mouse anti-*E. histolytica* antibody was then introduced separately and sequentially to saturate the streptavidin sensor and prevent cross-reaction in the later stages where the same monoclonal anti-*E. histolytica* IgG was used. To increase the sensitivity of the detection of the assay, a pre-incubated sandwich antibodies of mouse monoclonal anti-*E. histolytica* IgG and HRP-goat anti-mouse IgG (Invitrogen, Carlsbad, California, USA) was added to the method and visualized by the addition of *o*-phenylenediamine (OPD) substrate at the end of the assay. The expected events in the assay were represented in Fig. 1.

The values generated in the dotLab™ using the spiked stool samples were used to generate a calibration curve to establish the cut-off value for the negative and positive samples.

2.1.4.2. Regeneration of the dotLab™ sensor. At the completion of every run, the sensor was subjected to a modified regeneration protocol in an attempt to dissociate the binding between the monoclonal mouse anti-*E. histolytica* antibodies and the *E. histolytica* cells. The effectivity of the protocol was judged according to three criteria: (1) the degree of dissociation of antigen-antibody complexes, exhibited as a drop in diffractive intensity (DI) to baseline or to a level of minimal baseline drift; (2) the preservation of binding specificity and sensitivity, measured as the rate of increase in DI after binding; or (3) the number of successful regeneration cycles achievable. The optimized method of the detection of *E. histolytica* and the regeneration of the sensor were then programmed in the system as shown Fig. 2.

2.2. Application of the optimized protocol in stool samples collected from the relocated community

2.2.1. Sample collection

One hundred nineteen (119) stool samples from volunteers were collected from a community of relocated families in San Isidro, Rodriguez, Rizal, Philippines where the statistically ideal sample size is at least 73 (calculation not shown). Families from this community came from different informal settling sites in the National Capital Region of the country. The randomly selected individuals were interviewed and given instructions on the nature of the study. Informed consent was obtained from each individual after the nature of the study had been fully explained. They were given instructions to label the sampling cups with name, age, and sex and to avoid sample contact with soil and with samples from other individuals. Ethics clearance was obtained from the Municipal Health Office of Rodriguez, Rizal. Sampling cups were collected not more than 12 h after the samples were obtained.

2.2.2. Detection of E. histolytica in stool samples and cross-reactivity test of the developed dotLab™ assay

Pea-sized stool samples diluted with PBS (to a total volume of 1 mL) were subjected to optimized and calibrated dotLab™ detection protocol (Fig. 1). Three replicates per sample (monitored as three spots on the biosensor) were performed to ensure data reliability. The presence of *E. histolytica* in the stool was determined by comparison with the established cut-off value in the ratio of deltas for positive and negative samples.

The possible cross-reactivity of the dotLab™ assay to other intestinal microorganisms was tested against stool samples containing *Escherichia coli*, *Blastocystis* sp., *Cryptosporidium* sp. and *Giardia intestinalis*. All the fecal samples were subjected to the same optimized protocol.

2.2.3. Comparison of dotLab™ to real-time PCR

To compare the sensitivity of the optimized dotLab™ method, the results were compared to real-time PCR. DNA from the stool samples were extracted using QIAamp® DNA Stool Mini Kit (QIAGEN Inc.) Identification of *E. histolytica* by real-time PCR was done using specific primers for a 134-bp fragment in the 16S-like small subunit ribosomal RNA gene (Ehf: 5-AAC AGT AAT AGT TTC TTT GGT TAG TAA AA-3; and

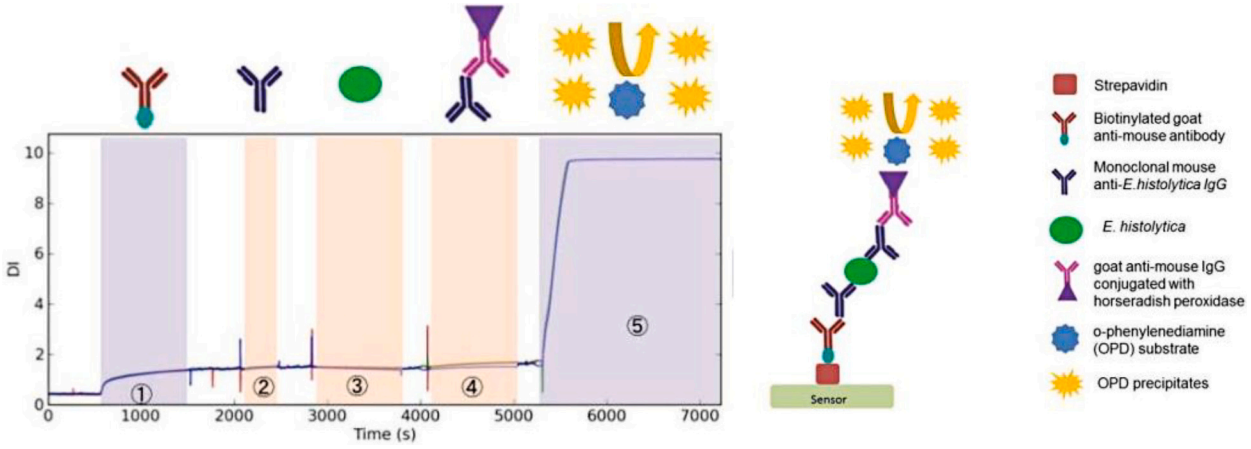

Fig. 1. Representation of the events in the dotLab™ method in the detection of *E. histolytica*. The sequential peaks in the graph represented as: (1) the association of biotinylated goat anti-mouse IgG to the streptavidin-lined sensor; (2) the binding of monoclonal mouse anti-*E. histolytica* IgG; (3) the capture of *E. histolytica* cells in spiked stool samples; (4) the attachment of a pre-incubated solution of monoclonal mouse anti-*E. histolytica* and a smaller concentration of HRP-goat anti-mouse IgG; and (5) signal amplification by precipitation of HRP.

Step Number	Reagent Name	Reagent Volume (µL)	Reagent FlowRate (µL/min)	Air Gap (µL)	Incubation Type	Incubation Volume (µL)	Incubation Duration	Incubation Flowrate (µL/min)
1: Wash/Condition	PBST	500	2000	-	Mix	25	00:01:00	2000
2: Wash/Condition	PBST	500	2000	-	Mix	25	00:01:00	2000
3: Plate Access	-	-	-	-	-	-	-	-
4: Load	BSA	60	500	5	Mix	10	10 cycle(s)	500
5: Tip Wash	PBST	500	2000	-	-	-	-	-
6: Wash/Condition	PBST	500	2000	-	Mix	25	00:01:00	2000
7: Load	Biotinylated goat anti-mouse	60	500	5	Mix	20	20 cycle(s)	500
8: Tip Wash	PBST	500	2000	-	-	-	-	-
9: Wash/Condition	PBST	500	2000	-	Mix	25	00:01:00	2000
10: Load	Monoclonal mouse anti-EH	60	500	5	Mix	20	30 cycle(s)	500
11: Tip Wash	PBST	500	2000	-	-	-	-	-
12: Wash/Condition	PBST	500	2000	-	Mix	25	00:01:00	2000
13: Load Sample		60	500	5	Mix	15	100 cycle(s)	500
14: Tip Wash	PBST	500	2000	-	-	-	-	-
15: Wash/Condition	PBST	500	2000	-	Mix	25	00:01:00	2000
16: Plate Access	-	-	-	-	-	-	-	-
17: Load	Preincubated: mAb anti-EH+ conj HRP	60	500	5	Mix	20	80 cycle(s)	500
18: Tip Wash	PBST	500	2000	-	-	-	-	-
19: Wash/Condition	PBST	500	2000	-	Mix	25	00:01:00	2000
20: Plate Access	-	-	-	-	-	-	-	-
21: Load	Substrate OPD	100	500	5	Static	-	00:10:00	-
22: Tip Wash	PBST	500	2000	-	-	-	-	-
23: Wash/Condition	PBST	500	2000	-	Mix	25	00:01:00	2000
24: Wash/Condition	0.1 M Glycine (pH2.5) regeneration solution	500	2000	-	-	-	-	-
25: Tip Wash	PBST	500	2000	-	-	-	-	-
26: Wash/Condition	PBST	500	2000	-	Mix	25	00:01:00	2000
27: Wash/Condition	PBST	500	2000	-	Mix	25	00:01:00	2000
28: Load	Monoclonal mouse anti-EH	60	500	5	Mix	20	30 cycle(s)	500
29: Tip Wash	PBST	500	2000	-	-	-	-	-
30: Wash/Condition	PBST	500	2000	-	Mix	25	00:01:00	2000

Fig. 2. Optimized method and the sequence of the steps in the detection of *E. histolytica* as programmed in the dotLab™ machine. The boxed area included the regeneration step (step 24) and was looped to accommodate for multiple samples per run.

Ehr: 5-CTT AGA ATG TCA TTT CTC AAT TCA T-3) (Roy et al., 2005). Reactions were performed using 10 μL of SYBR Green Supermix (Bio-Rad, Jurong, Singapore), 5 μM of each primer, 100 ng of template DNA, and sterile milli-Q water to a final volume of 20 μL. Cycling conditions began with an initial hold at 95 °C for 10 min, followed by 40 cycles consisting of 95 °C for 15 s, 63 °C for 30 s, and 72 °C for 30 s. Reactions were run in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Corp., CA, USA) enabling a melting curve to obtain the melting temperature (Tm) of the amplicon. Amplicon length was confirmed with electrophoresis in 2% agarose gel stained with GelRed (Biotium, Fremont, USA).

3. Theory

3.1. Diffractive optics technology (dotLab™)

The novel diffractive optics technology (dotLab™) pioneered by Axela Biosensors, Inc. is based on two well-known scientific principles: the law of diffraction of light, and the immobilization of molecules on a surface by high-affinity binding. When coherent light penetrates the well-defined pattern on a diffraction grating, this causes the formation of constructive and destructive interferences that in turn create a distinct diffraction image (Cleverley et al., 2010). The grating is composed of a fixed pattern of streptavidin molecules that are captured onto eight 2-mm assay spots on a single dot-Avidin sensor. When a biotinylated molecule passes through the linear flow channel above the assay spots, a high-affinity non-covalent bond occurs between the biotin and streptavidin, effectively capturing the entity. This molecule may be any biotinylated species, from an antibody or antigen, to an oligonucleotide. Highly specific binding is then facilitated by these fixed capture molecules. As a new species is bound on the surface of the biosensor, a focused laser strikes the prism-like bottom of the flow channel, subsequently penetrating the streptavidin-biotin grating by total internal reflection. This technique ensures maximum intensity (about 95%) with minimum loss of light, and forgoes the need for the light wave to directly pass through the channel which contains complex biological samples. Each binding event causes an improved DI which is manifested as an increase in the diffraction signal measured by a photodiode. It is therefore expected that subsequent bindings would yield a further increase in the diffractive signal, with the amplification directly correlated to the concentration of the analyte (Borinseko et al., 2006).

Unlike other optical biosensors which are based on refractive index or surface plasmon resonance, diffraction is inherently self-referencing. This implies that the increases in DI of subsequent binding events are based on the initially established biotin-streptavidin pattern, and is therefore solely dependent on exclusive binding between complementary molecules. Any non-specific binding event would not have any effect on the diffractive signal, eliminating errors caused by background interference from unpurified biological samples, such as stool (Borinseko et al., 2006). Furthermore, as this detection method is highly sensitive, only relatively small volumes of reagents and samples are required for a palpable signal to be detected (Cleverley et al., 2010).

With its high sensitivity, specificity, and flexibility, dotLab™ shows great potential in the rapid detection and quantitation of up to picomolar concentrations of analytes in complex solutions (Borinseko et al., 2006). The development of such immunoassays may pave the way for accurate point-of-care diagnosis of patients suspected of amebiasis or any parasitic illness, among others. This novel technology has already been applied to the rapid detection of the nematode parasite, *Strongyloides stercoralis*. Similar to *E. histolytica*, this parasite is transmitted though contact with infected feces which causes mostly asymptomatic strongylodiasis in immunocompetent individuals. By employing an ELISA-based immunoassay which utilizes a recombinant *Strongyloides* antigen to detect the host antibodies in only 10 μL of serum, Ndao et al. (2008) were able to reduce the diagnostic time to approximately 30 min. The sensitivity of the developed assay was at 75% and its specificity at 96.4%. This proves that the utility of the dotLab™ system is capable of being extended past research and into effective clinical diagnoses.

3.2. Sensor regeneration

Although the dotAvidin sensor is primarily a plastic consumable, sensor regeneration can be employed to minimize reagent and material waste on successive assays. This method exploits the reversibility of antigen-antibody associations to reactivate the capture surface of the sensor, such that, it can be reused in successive quantification and detection steps (Morgan et al., 1996). Two applicable strategies in sensor regeneration require either: (1) the release of the biotinylated capture molecule from the fixed streptavidin sensor, or (2) the dissociation of the antigen-antibody bond. The latter was considered in this study.

In natural conditions, both *in vivo* and *in vitro*, the formation of an antigen-antibody complex is a favorable occurrence (Katakura et al., 2000). The rate of association, however, falls within a relatively narrower range of 10^{-6} to 10^{-7} M-1 s-1 in comparison to the biotin-streptavidin interaction (Asanov et al., 1998). Thus, the dissociation of such interactions seems more plausible in the context of real-time immunoassays. To reverse the reaction, the reduction of reactive antigen or antibody species via addition of competitive analogues seem to be an effective strategy. However, the dissociation of such robust interactions between analogue and antibody pose yet another problem (Katakura et al., 2000). Several conditions such as extremes in pH, temperatures, and the presence of strong chaotropic ions have been known to compromise antigen-antibody associations. However, such harsh treatments have also been known to lower biospecificity (Asanov et al., 1998). As the dotLab™ system has no means to control temperature within the biosensor, regeneration by means of pH and chaotropic ions were investigated.

4. Results

4.1. Diffraction-based immunoassay protocol development and sensor regeneration

A sandwich ELISA-based approach was employed to ensure maximum signal generation. A saturating concentration of the capture monoclonal mouse anti-*E.histolytica* IgG was applied onto the underlying biotinylated goat anti-mouse IgG in order to prevent cross-reactivity in the later steps, where the sandwich monoclonal anti-*E. histolytica* IgG may bind onto the free sites on the biotinylated layer, thereby causing a peak at the amplification step, even in the absence of *E. histolytica* cells. The saturating concentration of the monoclonal anti-*E. histolytica* IgG was determined to be 50 μg/mL. The sandwich antibodies were also pre-incubated outside the system, with the HRP-goat anti-mouse IgG in limiting concentrations in order to prevent cross-reactivity with the capture layer.

Three spots on the dotAvidin sensor were monitored to ensure a statistically-significant number of replicates. The results of the calibration runs utilizing the optimized method are presented in Fig. 3 and the quantified ratio of deltas are summarized in Table 1.

Using the data, a calibration curve was established and a cut-off value of 0.150 for the ratio of deltas was applied for the negative samples. Samples with more than 0.150 ratio of delta were considered positive.

Various regeneration solutions were applied to the dotAvidin sensor following an initial run using the developed sandwich-based assay (specific *E. histolytica* concentrations are indicated). The regeneration result using 0.1 M glycine (pH 2.5) is the most promising (Fig. 4). The highlighted regions represent significant binding events: the green region labeled with an ® indicate the point of addition and incubation of the regeneration solution. Using 0.1 M glycine (pH 2.5), regenerating the sensors up to 8 x still shows stable results as shown in Fig. 5 and Table 2.

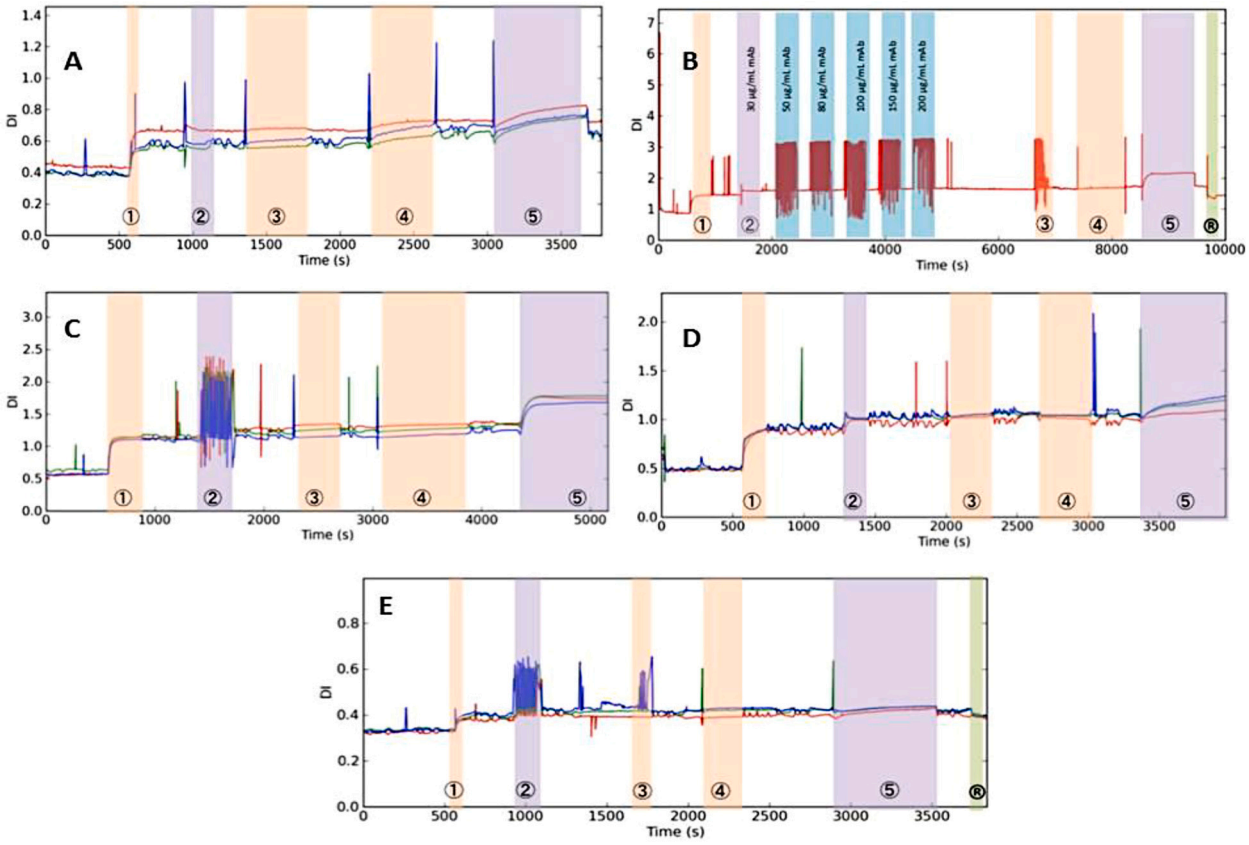

Fig. 3. Calibration of the dotLab™ system using spiked stool with defined concentration of *E. histolytica* cells: (A) dotLab® run on stool with 1000 cells/ml; (B) dotLab™ run on stool with 545 cells/ml; (C) dotLab™ run on stool with 54.5 cells/ml; (D) dotLab™ run on stool with 5.45 cells/ml; (E) dotLab™ run on stool with 0 cells/ml.

Table 1
Summary of quantified ratios of delta for stool spiked with known number of *E. histolytica* cells.

E. histolytica concentration (cells/ml)	Ratio of delta (average)
1000	10.206 ± 3.948
545	3.456
54.5	2.143 ± 1.457
5.45	1.427 ± 0.380
0 (negative control)	0.114 ± 0.037

4.2. Analysis of E. histolytica infections in a relocated community and cross-reactivity test of the developed dotLab™ assay

The established dotLab™ method, regeneration protocol, and calibrated cut-off value for the ratio of deltas (0.150) were applied using 119 stool samples collected from volunteers in the relocation area in Rodriguez, Rizal. The study population consisted of 45 (37.8%) males and 74 (62.2%) females, with ages ranging from less than 1 to 68 years old (median of 12 and mean of 20). The years of stay of the individuals in the relocation area ranged from less than 1 to 11 years (median of 4 and mean of 4.52).

Of the 119 samples, 31 were positive for *E. histolytica* infections. Nineteen (19) of the positive samples came from females (64.5%) and eleven (11) from males (35.5%). The median age of the positives is 8 with mean age of 16.9 ± 3.1 (SEM). The median of the positives for the years of stay in the relocation site is 4 with mean of 4 ± 0.4 years (SEM).

The optimized dotlab™ assay did not cross-react with stool samples containing *Escherichia coli, Blastocystis* sp., *Cryptosporidium* sp., and *Giardia intestinalis*.

4.2.1. Real-time PCR detection

The developed dotLab™-based immunoassay was compared with real-time PCR. Samples yielded the expected 134-bp PCR product using Ehf and Ehr primers for *E.histolytica*-positive samples. A Tm of 76.62 °C was obtained in the melt curve (data not shown). Nineteen (19) of the 119 samples gave positive results. Fourteen (14) of the positive samples came from females (73.7%) and 5 from males (26.3%). The median age of the positives is 12 with mean age of 19.5 ± 4.4 (SEM). The median of the positives for the years of stay in the relocation site is 3 with mean of 3.7 ± 0.5 years (SEM).

4.2.2. Comparison of the real-time PCR and dotLab™ methods

The occurrence of *E. histolytica* infections using real-time PCR was 15.97% while 26.05% using the developed dotLab™-based assay. The dotLab™ method has a sensitivity of 94.74% and specificity of 74.79% with reference to real-time PCR. Kappa coefficient agreement of the two methods was 0.651 and p value of <0.0001. The p value indicates a good correlation between the two methods (Morgan and Griego, 1998). The Kappa coefficient indicates that the dotLab™ method can be used as an alternative to PCR in *E. histolytica* detection.

4.3. Association of the occurrence of E. histolytica infections with sex, age and duration of stay in the relocation area

The samples obtained were not normally distributed with reference to the sex, age, and duration of stay in the relocation site, thus, nonparametric tests were used to analyze any associations within these parameters. Mann-Whitney rank-sum test, was used for the association of the infection in relation to the sex of the individual. Both the real-time PCR and dotLab™ results showed no significant association with sex:

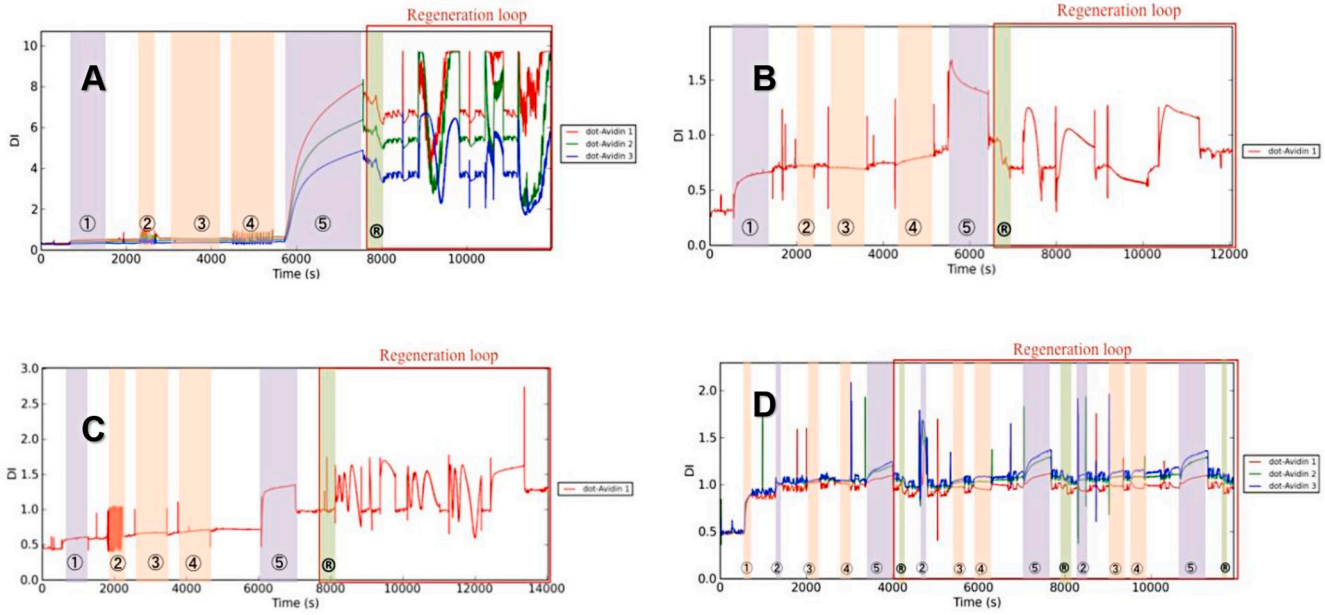

Fig. 4. Sensor regeneration trials using 50 mM NaOH +0.5% SDS (A), 5% glacial acetic acid (B), 20% $(NH_4)_2SO_4$ (C), and 0.1 M glycine (pH 2.5) (D).

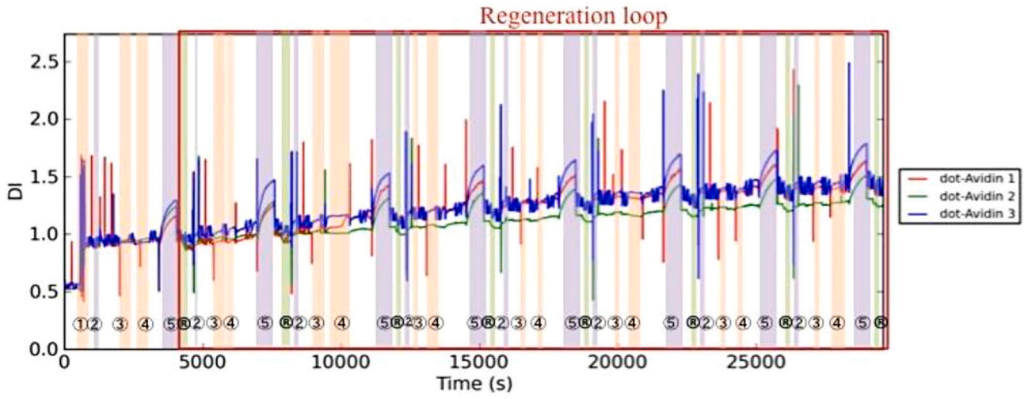

Fig. 5. A regeneration trial with 0.1 M glycine (pH 2.5) after an initial assay utilizing a 5450 cells/mL *E. histolytica*-spiked stool sample (Method B). This sensor was regenerated 8 x.

Table 2
Summary of quantified ratios of delta per regeneration cycle based on Fig. 5.

Regeneration cycle	Ratio of delta (average)
1	4.505
2	4.536
3	3.446
4	3.176
5	2.919
6	2.712
7	2.547
8	3.476

where $p = 0.102$ and $p = 0.757$, respectively. Kruskal-Wallis test was used to measure the association of infection with age and duration of stay in the relocation area. It was found that there was no significant correlation between the age and duration of stay in the relocation area in relation to frequency of infection with *E. histolytica*.

5. Discussion

5.1. Diffraction-based immunoassay

The developed dotLab™-based immunoassay protocol ensured specificity by using a saturating concentration of monoclonal mouse anti-*E. histolytica* IgG in the bound biotinylated goat anti-mouse IgG onto the streptavidin-lined sensor. The pre-incubation of HRP-goat anti-mouse IgG with small concentration of monoclonal mouse anti-*E. histolytica* outside the dotLab™ system also ensured that it would only recognize and associate with the anti-*E. histolytica* antibodies it was pre-incubated with, and would not cross-react with the anti-*E. histolytica* antibodies in the pre-established pattern. The addition of the substrate, OPD at the end of the assay yields a significant increase in the detected DI by the formation of a yellow-orange precipitate that further raises the diffractive grating and changes the diffraction image generated. The amplification of the signal is essential since *E. histolytica* cells are not always present in high concentrations in stool (Sheehan et al., 1979). These modifications resulted in a more concentration-sensitive DI peak at the amplification step (Fig. 3), which was still correlated to the concentration of *E. histolytica* in the stool samples (Table 1). Furthermore, by virtue of using monoclonal antibodies specific only for *E. histolytica*,

the presence of any other pathogen, whether morphologically identical or not, will not affect the outcome of the assay. The monoclonal mouse anti-*E. histolytica* IgG is also capable of identifying both cyst and trophozoite stages of the pathogen, ensuring a wider range of detection. The calibration curve generated using the averaged ratios of deltas of known concentrations of *E. histolytica* quantified the resulting data of the dotLab™ assay. From the generated calibration curve, a cut-off value of 0.150 was established for negative samples; beyond this value, samples should be considered positive in reference to the calibration curve.

5.2. Sensor regeneration

The choice of a regeneration solution is critical. It should be strong enough to dissociate *E. histolytica* cells from the antibody pattern, while at the same time preserving the binding capability of the sensor. Of all the regeneration solutions considered, only 0.1 M glycine (pH 2.5) satisfied the criteria of a good regeneration solution since it was able to drop to near baseline with minimal drift, exhibited as a ladder-like increase in DI per binding event even after sensor regeneration, and was able to regenerate the sensor up to 8 x. Other solutions used (50 mM NaOH +0.5% SDS, 5% glacial acetic acid, and 20% $(NH_4)_2SO_4$ failed to cause a drop to baseline. These solutions may have affected the integrity of the sensor, as well as its ability to strongly bind to associated antibodies — both seen as a fluctuating DI signal after the regeneration step. The ratio of deltas after successive regeneration cycles are relatively close to each other and any minor discrepancies may be attributed to the natural decrease in affinity of the biotinylated goat anti-mouse IgG to the monoclonal mouse anti-*E. histolytica* IgG per regeneration cycle.

5.3. Usefulness of dotLab™ immunoassay in detection of E. histolytica in stool samples

This study discovered the usefulness of dotLab™ of Axela Biosensors, Inc. as diagnostic tool for *E. histolytica* detection. The optimized dotLab™ method detected 31 samples positive for *E. histolytica* compared to 19 positive samples detected by real-time PCR. Despite the difference in the result, Kappa reliability test of dotLab™ method against real-time PCR showed that dotLab™ is a reliable alternative for PCR. All positive samples detected by real-time PCR were also detected positive using the dotLab™ method except for one sample. PCR methods were reported to be prone to false-positive results due to possible cross-contamination (Blessmann et al., 2002). It was observed that 12 samples were detected positive for *E. histolytica* by dotLab™ that were not detected by real-time PCR. In multiplex nested PCR, at least 15 cells/mL (if from urine or pus) or 25 cells/mL (if from stool) of *E. histolytica* must be present in the sample to be detected positive (Khairnar and Parija, 2007; Parija and Khairnar, 2007). The dotLab™, however, was able to detect as little as 5.45 cells/ml of *E. histolytica* in stool sample (Table 1). The technical brief of Axela Inc (2007) reported that dotLab™ is able to detect picomolar concentration of natriuretic peptides specifically NT-proBNP in plasma. The result of this dotLab™ assay for *E. histolytica* can claim that dotLab™ is more sensitive than PCR detection method. With regard to the specificity in differentiating *E. histolytica* from *E. dispar*, tests should be done using stool-spiked with *E. dispar*. Should the specificity of this assay be found low in the future assays, it is important to note that specificity of the assay is also dependent on the purity and specificity of the antibodies used in the assay.

The dotLab™ method was also proven to be more rapid than PCR detection. Sample preparation for dotLab™ consisted of simple filtering and dilution which can be completed in few minutes as compared to 6 h (QIAamp DNA Stool Mini Kit of QIAGEN, Inc.) or 1 day (formol-ether concentration technique and organic extraction) DNA extraction procedures. The procedure itself only requires the dotLab™ at least 66 min to finish the assay with real-time viewing of the progress of the test. The PCR requires at least 1.5 h for the amplification procedure and additional 30–40 min for agarose gel electrophoresis for viewing the amplicons.

The disposable sensor of the dotLab™ assay, as it was successfully regenerated using 0.1 M glycine (pH 2.5), has enabled the reuse of the sensor up to 8 x, thus, lowering the cost of the detection assay.

5.4. Assessment of E. histolytica infections in the relocation site

Based on this study, sex, age, or the duration in the settlement of the people are not significant factors in the occurrence of *E. histolytica* infections in the relocation site. It was observed that compared to the community in the northern Philippines as reported by Rivera et al. (1998), the relocation site has 17 X (real-time PCR) or 27 x (dotLab™) more infection rate. It should be noted that the population of the relocation area is a pool of families from informal settling areas of the National Capital Region. Previous reports from the slum community in Brazil (Braga et al., 1998), a rural community in Mexico (Ramos et al., 2005) and the continent of Africa (Stauffer et al., 2006) have comparable incidence rates (10.6%, 11.4% and > 21%, respectively) to the relocation site in this study (15.9% using PCR and 26.1% using dotLab™). Slum is defined by UN-HABITAT (2007) as a wide range of low-income settlements and poor human living conditions or a heavily populated urban area characterized by substandard housing and squalor. Although these people were resettled with better housing, UNICEF reported that slum dwellers typically have low hygiene awareness and knowledge (UNICEF, 2007). A good housing program may not be enough to lessen the burden of amebiasis or any sanitation-related disease in a community. Hygiene and sanitation education and promotion activities should also be intensively promoted and observed in this kind of community.

6. Conclusion

With the continuous search for better diagnostic tool for the detection of *E. histolytica*, the Axela's dotLab™ was found to be a promising tool. It is a reliable alternative to PCR. The dotLab™ method is more rapid than real-time PCR detection with approximately 66 min completion in dotLab™ method compared to 1.5 h of PCR run excluding 6 h to 1 day of DNA extraction. It was found that dotLab™ is even more sensitive since it was able to detect as low as 5.45 cells/mL of *E.histolytica* cells compared to the 25 cells/mL in real-time PCR assay. The dotLab™ method was able to detect 1.6 X more positive stool compared to real-time PCR method. The regeneration of the disposable sensors can make dotLab™ a low-cost diagnostic method. Thus, it can be concluded that dotLab™ is a rapid, sensitive, and low-cost assay for *E. histolytica* detection in stool samples.

It is recommended to conduct a separate study to test the assay's capability to differentiate *E. histolytica* from *E. dispar* by testing stool samples spiked with *E. dispar* cells. It is important to note that the sensitivity and selectivity of this assay is highly dependent on the quality of reagents and antibody to be used.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest and ethical issues exist.

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