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Development of a dry-reagent-based nucleic acid-sensing platform by coupling thermostabilised LATE-PCR assay to an oligonucleotide-modified lateral flow biosensor

Geik Yong Ang [a,b,*], Choo Yee Yu [a,b], Kok Gan Chan [a], Kirnpal Kaur Banga Singh [b], Yean Chan Yean [b,*]

[a] Division of Genetics & Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[b] School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

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ABSTRACT

In this study, we report for the first time the development of a dry-reagent-based nucleic acid-sensing platform by combining a thermostabilised linear-after-the-exponential (LATE)-PCR assay with a one-step, hybridisation-based nucleic acid lateral flow biosensor. The nucleic acid-sensing platform was designed to overcome the need for stringent temperature control during transportation or storage of reagents and reduces the dependency on skilled personnel by decreasing the overall assay complexity and hands-on time. The platform was developed using toxigenic *Vibrio cholerae* as the model organism due to the bacterium's propensity to cause epidemic and pandemic cholera. The biosensor generates result which can be visualised with the naked eyes and the limit of detection was found to be 1 pg of pure genomic DNA and 10 CFU/ml of toxigenic *V. cholerae*. The dry-reagent-based nucleic acid-sensing platform was challenged with 95 toxigenic *V. cholerae*, 7 non-toxigenic *V. cholerae* and 66 other bacterial strains in spiked stool sample and complete agreement was observed when the results were compared to that of monosialoganglioside (GM_1)-ELISA. Heat-stability of the thermostabilised LATE-PCR reaction mixes at different storage temperatures (4–56 °C) was investigated for up to 90 days. The dry-reagent-based genosensing platform with ready-to-use assay components provides an alternative method for sequence-specific detection of nucleic acid without any cold chain restriction that is associated with conventional molecular amplification techniques.

1. Introduction

Diagnosis is an integral component of the health care system whereby it enforces evidence-based treatment and curbs associated consequences of misdiagnosis, such as morbidity and mortality, due to inappropriate or inadequate treatment (Peeling and Mabey, 2010). Culture-based methods are usually the gold standard in bacteriological diagnosis; however, they are time consuming and stringent transport conditions are required to maintain specimen viability (Yager et al., 2008). Hence, in vitro amplification-based nucleic acid diagnostic test is gradually gaining popularity in the routine microbiology laboratory because of its simplicity and robustness as well as shorter turnaround time with improved sensitivity and specificity in detecting the organism of interest when compared to traditional methods (O'Connor and Glynn, 2010).

In the past decade, lateral flow or dipstick-type DNA biosensor has emerged as a promising post-amplification analysis tool for the detection of amplicons by circumventing the need for laborious agarose gel preparation and equipment-dependent electrophoresis (Tian et al., 2014). The advantages of lateral flow devices such as simple operational procedure, ability to generate rapid visual result, stable at ambient temperature and availability of relatively inexpensive off-the-shelf assay components have contributed to the success of lateral flow test as a diagnostic technology (Yager et al., 2006). Apart from clinical assays, applications of lateral flow biosensor have extended to various fields ranging from aquaculture, agriculture, veterinary, bio-defence, food and water quality assurance to environmental health and safety monitoring (O'Farrell, 2009).

Generally, nucleic acid-based lateral flow assays can be divided into two categories: nucleic acid lateral flow immunoassays (NALFIA) and nucleic acid lateral flow assays (NALF) (Posthuma-Trumpie et al., 2009). NALFIA, which detects labelled double-stranded amplicons by antigen–antibody (Ab) or affinity binding interactions (Blazkova et al., 2011; Chua et al., 2011), is favoured for its rapid response time but the platform is susceptible to false positive results. The coupling of NALFIA to primer extension (PEXT) reaction (Kalogianni et al., 2009; Moers

* Corresponding authors at: Division of Genetics & Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
E-mail addresses: geikyong@gmail.com (G.Y. Ang), yeancyn@yahoo.com (Y. Chan Yean).

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et al., 2014; Vlachou et al., 2010) is another alternative for sequence-specific detection but the PEXT reaction has to be set up upon the completion of a standard PCR assay followed by additional thermocycling steps before the PEXT amplicons were analysed.

In contrast to NALFIA, NALF utilises capture probes as the recognition element and the probes are immobilised on the detection zone of the lateral flow strip (Posthuma-Trumpie et al., 2009). NALF device has been developed for detection of NASBA (Mugasa et al., 2009) and linear-after-the-exponential (LATE)-PCR (Xu et al., 2014) amplicons but these lateral flow assays were performed on a heating block at 55 °C and 45 °C, respectively. Furthermore, the molecular amplification assays in the studies cited above were not dry-reagent-based and hence, the applicability of these nucleic acid tests would be restricted by cold chain requirement.

Here, we describe the development of a thermostabilised LATE-PCR assay and the combination with a sequence-specific NALF biosensor which detects single-stranded amplicons via in situ hybridisation at ambient temperature (20–25 °C). The thermostabilised LATE-PCR assay mitigates the dependency on skill personnel, minimises the risk of extrinsic contamination and reduces errors in reaction mix preparation as the assay procedure only requires two pipetting steps. The dry-reagent-based LATE-PCR-NALF assay targeting the *ctxA* gene is presented as a novel nucleic acid-sensing platform for the detection of toxigenic *V. cholerae* because the bacterium continues to pose a major threat to public health even in the 21st century due to its propensity to cause transboundary cholera pandemics (Charles and Ryan, 2011). The proposed nucleic acid-sensing platform has potential applications in clinical diagnostics as well as in routine public health surveillances and outbreak investigations.

2. Materials and methods

2.1. Reagents and apparatus

Neutravidin, mouse anti-fluorescein (FITC) Ab, goat anti-mouse IgG Ab and all PCR reagents were purchased from Thermo Scientific (Waltham, USA). Colloidal gold solution (40 nm) was procured from Kestrel BioSciences (Pathumthani, Thailand) and mung bean nuclease was from Promega (Wisconsin, USA). Western blocking reagent was purchased from Roche (Indianapolis, USA). Methanol and bovine serum albumin (BSA) were purchased from Amresco (Solon, USA). All other common chemicals were obtained from Sigma-Aldrich (St. Louis, USA). All reagents used in this study were of analytical reagent grade and all solutions were prepared using ultrapure water (> 18 MΩ) from a Millipore Milli-Q water purification system (Billerica, USA).

PCR primers, capture probes and synthetic targets were synthesised by Integrated DNA Technologies (Coralville, USA) and the nucleotide sequences are given in Table S1. Laminated nitrocellulose membrane card, cellulose fibre pads, glass fibre pads and Amicon Ultra 30 K were Millipore products. A Heto Lyolab 3000 from Thermo Scientific, XYZ dispensing platform from BioDot (Irvine, USA) and Matrix 2360 programmable shear from Kinematic Automation (Twain Harte, USA) were used in this study.

2.2. Preparation of thermostabilised LATE-PCR mix

Deglycerolisation of *Taq* DNA polymerase by centrifugal filtration was performed using Amicon Ultra 30 K. Recombinant *Taq* DNA polymerase (100 U) in 10 mM Tris hydrochloride (Tris–HCl), pH 8, was added into a centrifugal filter unit to a final volume of 500 μl. The centrifugal device was centrifuged at 14,000 × *g* for 10 min at 4 °C and the flow-through was discarded. A washing step was performed with 10 mM Tris–HCl (pH 8) before the centrifugal filter unit was inverted and the retentate was retrieved by centrifugation at 1000 × *g* for 2 min at 4 °C. Deglycerolised *Taq* DNA polymerase was kept on ice and used immediately for preparation of thermostabilised LATE-PCR mix.

Different combinations of stabilising sugars and bulking agents were tested to convert the LATE-PCR mix into a dry-reagent format. The optimised dry-reagent LATE-PCR mix (20 μl) contained PCR buffer, 320 μM dNTP mix, 2.5 mM $MgCl_2$, 0.5 μM ctxA_RX, 0.1 μM ctxA_FL, 0.5 μM IAC_FX, 0.05 μM IAC_RL, 5% (*w*/*v*) raffinose, 6.25% (*w*/*v*) Ficoll, 0.25 mg/ml BSA, 0.01% (*w*/*v*) orange G, 1.5 U deglycerolised *Taq* DNA polymerase, 10 pg of internal amplification control (IAC) template and ultrapure water. The aqueous LATE-PCR mix was lyophilised using Heto Lyolab 3000 as described by Yu et al. (2015) until an amorphous glassy matrix was formed. The thermostabilised LATE-PCR mixes were stored in a desiccator or sealed in aluminium pouches containing silica gel.

2.3. Construction of NALF biosensor

The signal generator and bio-recognition elements on the lateral flow biosensor were constructed as described previously (Ang et al., 2012). Briefly, 10 ml of colloidal gold nanoparticles (pH 8.0) were conjugated with 22 μg of anti-FITC Ab at room temperature for 1 h before passivation of gold surface was conducted with 1% (*w*/*v*) BSA for another 1 h. The pellet of colloidal gold-anti-FITC Ab conjugates was collected by centrifugation at 10,000 × *g* for 30 min at 4 °C and resuspended in 10 mM phosphate buffer (PB) containing 1% (*w*/*v*) BSA and 0.05% (*w*/*v*) sodium azide.

Biotinylated capture probes (CP_ctxA and CP_IAC) were bound to neutravidin by adding 1.53 nmol of each capture probe to 2.04 mg of neutravidin followed by 1 h of incubation at room temperature. Unbound biotinylated capture probes were removed by centrifugal filteration using Amicon Ultra 30 K. The retentate was washed twice with 10 mM PB (pH 7.4) and reconstituted with 10 mM PB (pH 7.4) containing 1% (*w*/*v*) BSA and 1% (*v*/*v*) methanol after retrieval. Goat anti-mouse Ab (0.25 mg/ml) in 10 mM PB (pH 7.4) containing 1% (*w*/*v*) BSA and 1% (*v*/*v*) methanol was prepared for the chromatography control line.

Neutravidin-bound biotinylated capture probes for target DNA (CP_ctxA) and IAC (CP_IAC) as well as goat anti-mouse Ab were applied on the test, IAC and chromatography control lines of the nitrocellulose membrane, respectively using a Front Line dispenser at a rate of 1 μl/cm. The remaining unreacted sites of the membrane were blocked with 1% (*v*/*v*) western blocking reagent and 0.1% (*v*/*v*) Triton X-100 in 2 mM PB (pH 7.4). A suspension of gold conjugates at an optical density at 525 nm (OD_{525}) of 4 in 2 mM PB supplemented with 0.01% (*v*/*v*) Tween-20, 0.01% (*v*/*v*) PVA and 20% (*w*/*v*) sucrose was dispensed onto a glass fibre conjugate pad and dried overnight in a desiccator. The buffer application pad, conjugate pad, nitrocellulose membrane and absorbent pad were assembled with 2 mm overlapping between each component and a programmable shear was used to cut the assembled master card into 4 mm wide strips. The strips were stored in a desiccator or sealed in aluminium pouches containing silica gel.

2.4. Dry-reagent-based LATE-PCE-NALF assay protocol

The thermostabilised LATE-PCR mix was reconstituted by adding 18 μl of ultrapure water and 2 μl of DNA template. The thermocycling conditions for the amplification reaction were as follows: initial denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s followed by a final extension step at 72 °C for 5 min. The reaction was terminated at 95 °C for 5 min and LATE-PCR amplicons were snap-cooled on ice for 30 s before analysis. Test sample for the NALF assay was prepared by adding an equal volume of 1.5 M $MgCl_2$ hybridisation buffer to the LATE-PCR product. A 10 μl aliquot of the mixture was applied onto the sample application region and the NALF biosensor was then placed in contact with 250 μl of running buffer

[2 mM PB, 0.01% (*v*/*v*) Triton X-100]. The result was visualised in the form of red/pinkish lines within 20 min.

2.5. Evaluation of nucleic acid-sensing platform

Purified genomic DNA from 30 bacterial strains was used to evaluate the analytical specificity of the dry-reagent-based LATE-PCR-NALF assay. The bacterial strains used for inclusitivity testing were toxigenic *V. cholerae* serogroup O1 (5 strains) and toxigenic *V. cholerae* serogroup O139 (5 strains) whereas non-target bacterial strains used for cross-reactivity testing were non-toxigenic *V. cholerae* non-O1/non-O139 (5 strains), other *Vibrio* spp. (5 strains), other Gram-negative strains (5 strains) and Gram-positive strains (5 strains). The purified genomic DNA was used as a template at 100 ng per LATE-PCR reaction. Limit of detection was determined using synthetic targets (ST_ctxA and ST_IAC) as well as purified genomic DNA and bacterial cell suspensions of toxigenic *V. cholerae* O1 classical, toxigenic *V. cholerae* O1 El Tor and toxigenic *V. cholerae* O139.

Performance of the nucleic acid-sensing platform was also evaluated with 168 archived clinical and environmental strains which consisted of 95 toxigenic *V. cholerae*, 7 non-toxigenic *V. cholerae* and 66 other bacterial strains (Table S2). The spiked stool samples were prepared as described by McOrist et al. (2002) and each aliquot of the suspension was spiked with a single colony of bacteria before inoculated into 5 ml of alkaline peptone water. After incubation at 37 °C for 6 h, 1 ml of each culture was transferred into a microcentrifuge tube and cell lysates obtained by boiling-lysis method were used as DNA template for LATE-PCR amplification.

3. Results and discussion

3.1. Assay principle

A schematic diagram illustrating the workflow and principle of the nucleic acid-sensing platform is presented in Fig. 1. Alkaline peptone water was used as the enrichment broth for *V. cholerae* isolation because the bacterium is able to multiply in alkaline pH and the DNA template for LATE-PCR amplification reaction was prepared from the broth culture with a simple boiling-lysis method. LATE-PCR was selected as the molecular amplification scheme in this study because it is an advanced form of asymmetric PCR that is highly robust and efficient in producing single-stranded DNA amplicons (Sanchez et al., 2004). Furthermore, specific nucleic acid sequences could be simultaneously amplified and labelled during LATE-PCR and interference from various non-target sequences is reduced as the signal-to-noise ratio is increased. The multiplexing capability of LATE-PCR also allowed the incorporation of a non-target IAC which is crucial in molecular diagnostic tests. IAC amplifies simultaneously with the target DNA under the same amplification condition and hence, demonstrates the integrity of a reaction mix.

Sequence-specific detection of LATE-PCR amplicon was achieved with a one-step NALF biosensor without any prior PCR purification step. LATE-PCR amplification product added with hybridisation buffer was directly loaded on the lateral flow strip for analysis. The hybridisation reaction between complementary fluorescein-labelled-single-stranded LATE-PCR amplicons and immobilised capture probes on the lateral flow biosensor served to anchor the target DNA at the detection region. Introduction of a running buffer at the proximal end of the strip enabled dried gold conjugates to be rehydrated and transferred from the conjugate pad to the nitrocellulose membrane by capillary migration. The aggregation of gold nanoparticles due to the antigen–Ab reaction between gold conjugates and universal fluorescein tags at the 5′ termini of captured LATE-PCR amplicons allowed the presence of target-probe hybrids to be visualised in the form of a red/pinkish line with naked eyes.

A positive result is indicated by the presence of a test line regardless of the presence or absence of an IAC line. A negative result is indicated by the absence of a test line but the IAC line must be present to validate the negative result. The absence of both test and IAC lines indicate a false-negative result because no amplification reaction took place. Presence of the chromatography control line is mandatory to ensure that proper lateral flow assay procedure was carried out.

3.2. Optimization of thermostabilised LATE-PCR assay

The aqueous LATE-PCR mix was converted into a dry-reagent form by lyophilisation whereby the solution fractionates into ice crystals and freeze-concentrated solutes before the dehydration step (Allison et al., 1998). In order to inhibit lyophilisation-induced denaturation of protein, the use of trehalose as a stabilising sugar in thermostabilised PCR mix has been reported in several published literatures (Chua et al., 2010; Klatser et al., 1998; Low et al., 2013) but the present study found that dry-reagent LATE-PCR mixes containing different

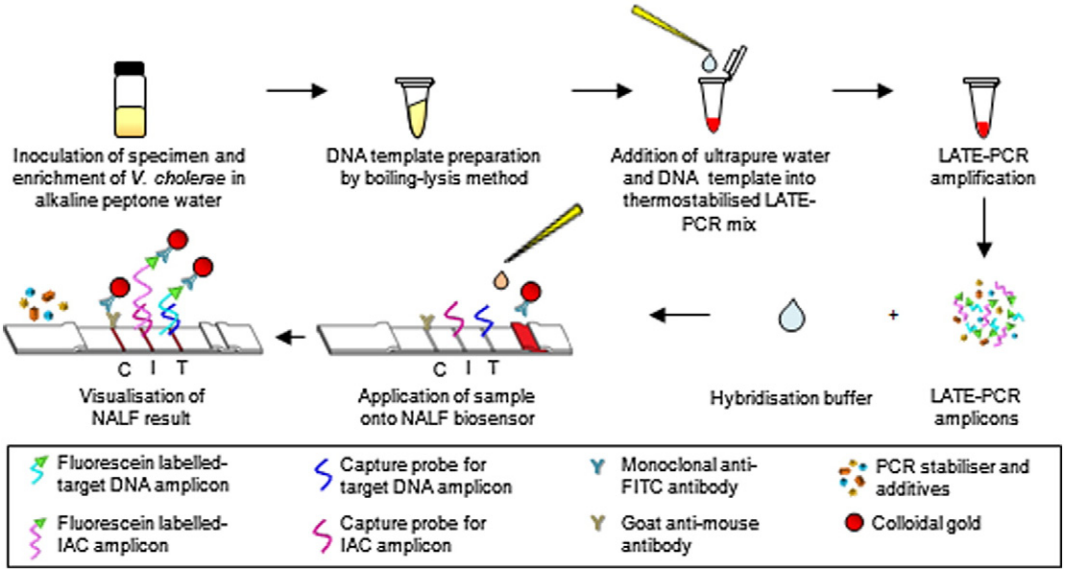

Fig. 1. An illustration of the proposed nucleic acid-sensing platform for sequence-specific detection of labelled single-stranded amplicons. T, test line; I, internal amplification control line; C, chromatography control line; IAC, internal amplification control.

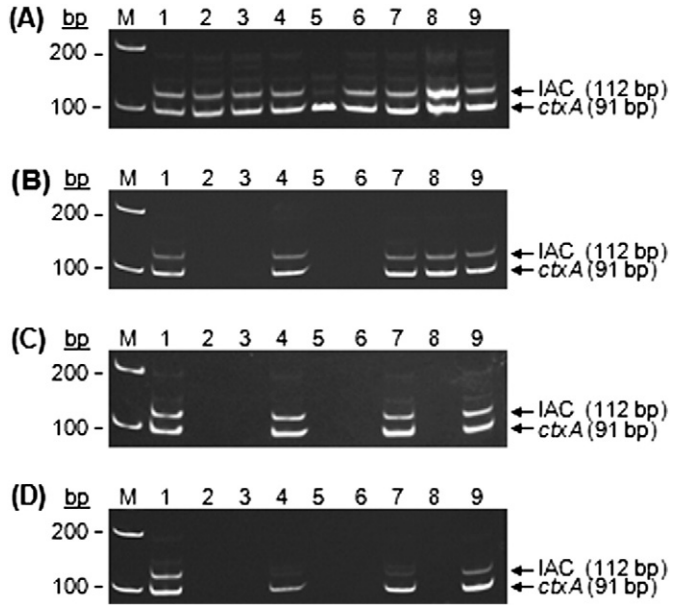

Fig. 2. Analysis of dry-reagent LATE-PCR mixes after (A) lyophilisation and 30 days of storage at (B) 25 °C, (C) 37 °C and (D) 56 °C. Lane M, GeneRuler 100 bp DNA ladder; lane 1, positive control; lane 2, 3% trehalose; lane 3, 5% raffinose; lane 4, 3% trehalose, 0.25 mg/ml BSA, 1% PEG 10,000 and 0.006% gelatin; lane 5, 100 mM sorbitol; lane 6, 5% raffinose, 12.5% ficoll, 0.05% Tween-20 and 0.05% nonidet P-40; lane 7, 5% raffinose, 0.25 mg/ml BSA and 1% PEG 10,000; lane 8, 5% dextran and 3% trehalose; lane 9, 5% raffinose, 0.25 mg/ml BSA and 6.25% Ficoll.

concentrations of trehalose were not stable when stored at 37 °C for 30 days (Fig. S1). Furthermore, a two-fold increment in the amount of *Taq* DNA polymerase from 0.75 U to 1.5 U did not yield any improvement. The removal of hygroscopic glycerol from the *Taq* DNA polymerase storage buffer was found to improve the stability of dry-reagent LATE-PCR mix that were stored at 25 °C but the presence of trehalose alone was unable to confer the needed thermotolerance against thermal stress at higher storage temperatures. Therefore, other stabilisers and additives such as Ficoll, raffinose and sorbitol were investigated by trial and error and the thermal stability of these dry-reagent LATE-PCR mixes were tested after 30 days of storage at 25 °C, 37 °C, 45 °C and 56 °C (Fig. 2). It was found that 3 out of 8 formulations tested were able to generate the 91 bp target amplicon after 30 days of storage at 56 °C but the formulation containing 5% (*w*/*v*) raffinose, 6.25% (*w*/*v*) Ficoll, and 0.25 mg/ml BSA was selected because the yield IAC amplicon was observed to be higher.

The production of single-stranded LATE-PCR amplicons was demonstrated by Southern blot analysis whereby capture probes complementary to the sense and anti-sense strands of *ctxA* and IAC amplicons were utilised to differentiate between single-stranded and double-stranded amplicon (Fig. S2). Single-stranded amplicon located slightly above the double-stranded amplicon was only visible when its complementary capture probe was used. The presence of both single-stranded and double-stranded amplicons in LATE-PCR product was further verified by mung bean nuclease digestion. The absence of an additional band above the double-stranded amplicon in mung bean-digested LATE-PCR products corresponded to the digestion of single-stranded DNA by mung bean nuclease.

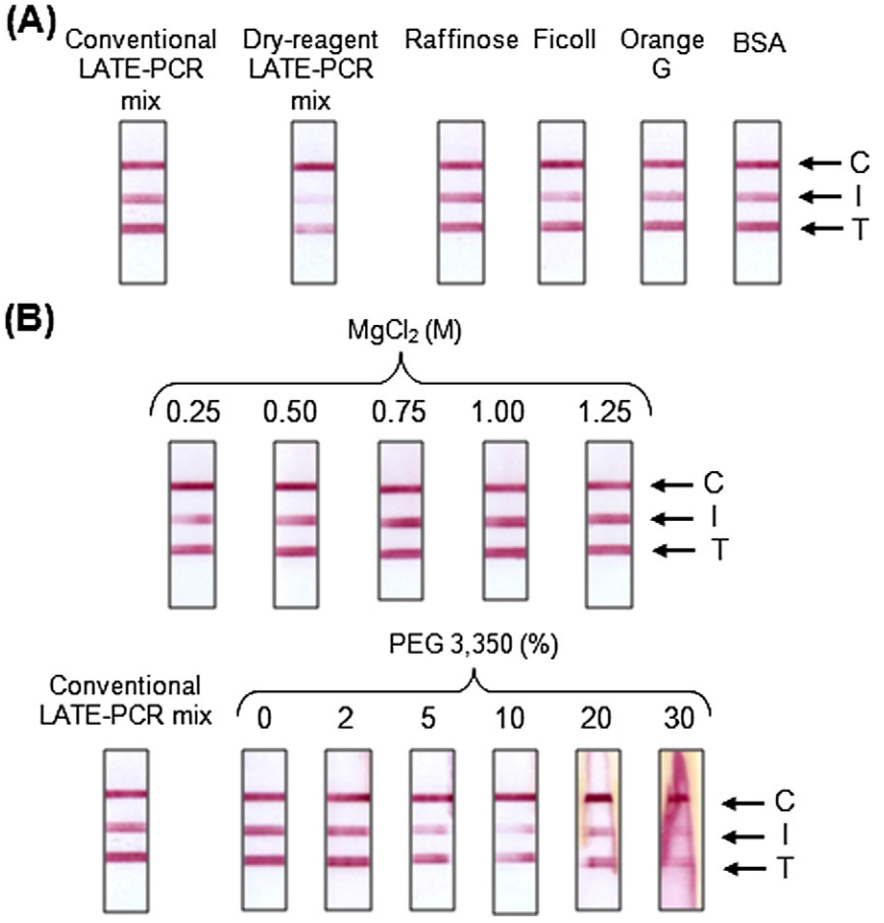

Fig. 3. Analysis of (A) thermostabilised LATE-PCR formulation and (B) optimization of $MgCl_2$ concentration and percentage of PEG in hybridisation buffer. C, chromatography control line; I, internal amplification control line; T, test line.

3.3. Optimisation of LATE-PCR-NALF assay

The yield of single-stranded amplicons is dependent on the number of amplification cycles (Salk et al., 2006) and hence, the number of LATE-PCR cycles was first optimised. The limit of detection (LOD) was observed to be 100 pg of genomic DNA after 30 cycles of amplification and a ten-fold decrease in LOD was obtained when the number of cycles was increased to 35 and 40 (Fig. S3A). Intensity of the test line at the LOD was not significantly different between 35 and 40 amplification cycles and in view of a shorter assay time, 35 cycles was selected as optimum. Termination of the LATE-PCR assay with a 5 min denaturation step led to a further decrease in the LOD to 1 pg of genomic DNA (Fig. S3B).

The location for sample application was investigated by testing all plausible loading sites on the lateral flow strip. It was found that sample loaded on the nitrocellulose membrane was able to generate a strong signal intensity at the test and IAC lines but sample loaded on buffer application pad and gold conjugate pad only generated weak and faint bands (Fig. S4A). This observation could be attributed to the increased proportion of gold conjugates being bound to labelled excess primers and double-stranded amplicons that do not contribute to signal generation when the sample was loaded on the buffer application and gold conjugate pads. Application of sample on the nitrocellulose membrane allowed specific amplicons to be captured at the detection region whilst unbound LATE-PCR constituents were removed by capillary migration to the absorbent pad. Different volumes of LATE-PCR product (5–20 μl) were also tested to determine the loading capacity of the NALF biosensor. Faint signals were observed when volumes of LATE-PCR product exceeding 5 μl were tested because target analytes were able to bypass the capture probes when the device was flooded (Fig. S4B).

3.4. Optimisation of dry-reagent-based LATE-PCR-NALF assay

The NALF biosensor was further optimised to enable the direct detection of amplicons generated from the thermostabilised LATE-PCR mix. Previously, we have described the use of a hybridisation buffer for NALF assay containing Mg^{2+} cations to counteract the electrostatic repulsion between capture probes and target DNA as well as PEG to improve the hybridisation efficiency through volume exclusion (Ang et al., 2012). In the present study, amplification product generated from thermostabilised LATE-PCR mix was incompatible with the hybridisation buffer containing $MgCl_2$ and PEG as a decrease in signal intensity was observed when compared to that obtained with conventional LATE-PCR mix prepared without any stabilisers (Fig. 3A). Deterioration in the signal intensity only occurred when the thermostabilised LATE-PCR formulation was used as a whole and not when each component of the formulation was added individually into a conventional LATE-PCR mix. Further investigation revealed that the dry-reagent LATE-PCR mix required only $MgCl_2$ in the hybridisation buffer because macromolecules in the LATE-PCR stabiliser formulation were able to promote amplicon–probe hybrid formation (Fig. 3B). Therefore, the function of PEG as a hybridisation accelerator was effectively replaced by the developed LATE-PCR stabiliser formulation.

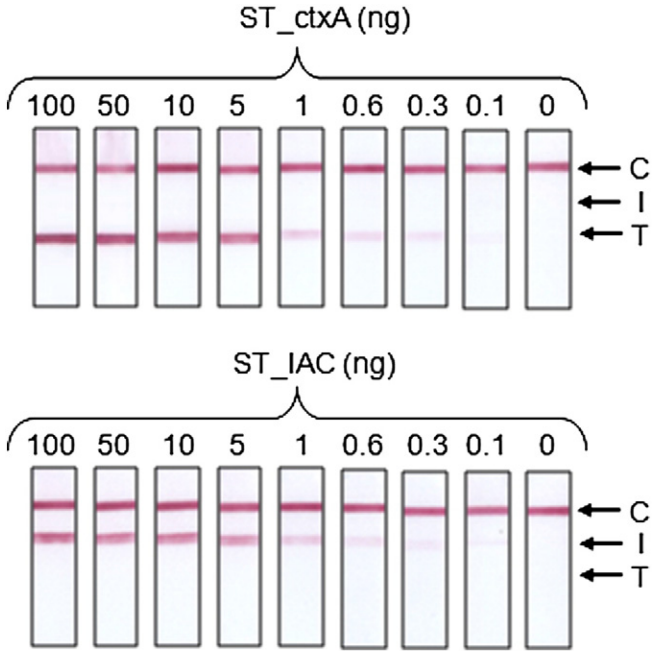

Fig. 4. Determination of detection limit with ST_ctxA (top) and ST_IAC (bottom) synthetic targets. C, chromatography control line; I, internal amplification control line; T, test line.

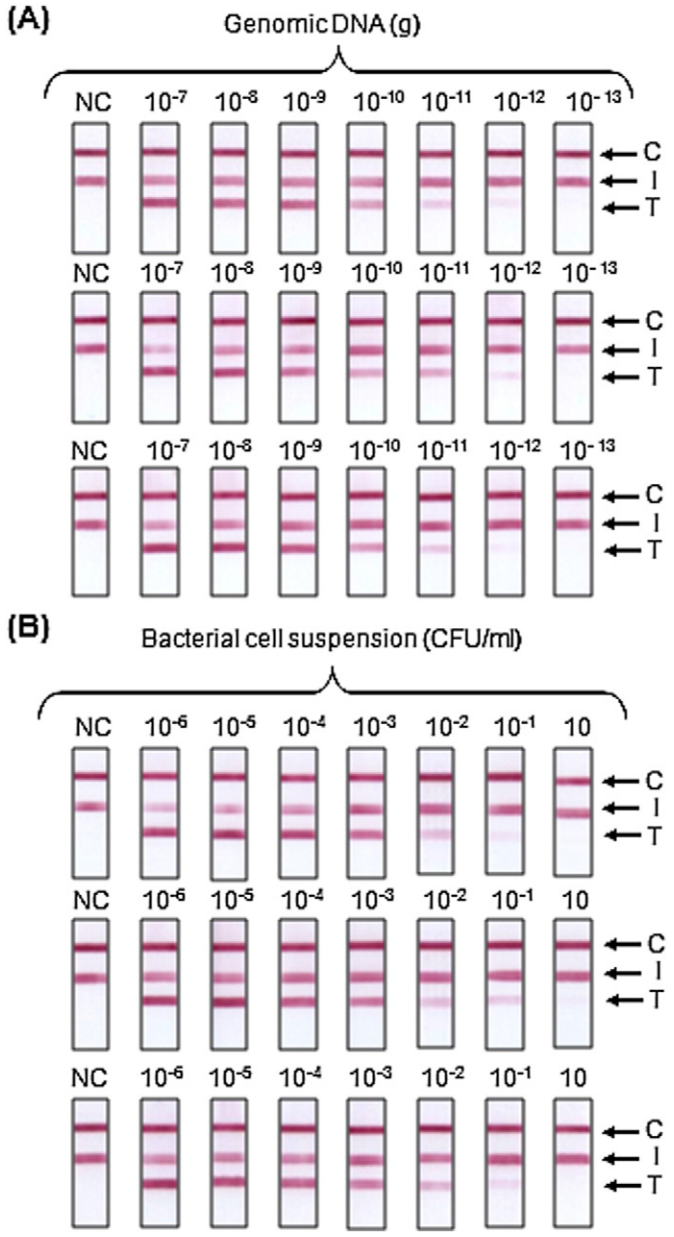

Fig. 5. Determination of detection limit with serial dilutions of (A) genomic DNA and (B) bacterial cell suspensions of toxigenic *V. cholerae* O1 El Tor (top), toxigenic *V. cholerae* O1 classical (middle) and toxigenic *V. cholerae* O139 (bottom). NC, negative control; C, chromatography control line; I, internal amplification control line; T, test line.

3.5. Evaluation of nucleic acid sensing platform

Fig. 4 presents the typical photo images of the NALF biosensor when tested with increasing amounts of synthetic target DNA (0 ng (blank), 0.1, 0.3, 0.6, 1, 5, 10, 50 and 100 ng). The presence of ST_ctxA and ST_IAC synthetic targets were indicated by the formation of a red/pink band at the test and IAC lines, respectively. No band was observed in the absence of target DNA as well as in the presence of non-target DNA. The detection limit for both synthetic targets were 0.3 ng when inspected visually. Serial dilutions of purified genomic DNA and bacterial cell suspensions of toxigenic *V. cholerae* O1 El Tor, *V. cholerae* O1 classical and *V. cholerae* O139 were used as DNA templates to assess the NALF performance in combination with the developed thermostabilised LATE-PCR mix. The limit of detection based on the presence of a visible red/pinkish line was found to be 1 pg genomic DNA and 10 CFU/ml for both serogroups of *V. cholerae* (Fig. 5A and B).

The dry-reagent-based LATE-PCR-NALF assay was shown to be reproducible when different concentrations of genomic DNA and dilutions of bacterial cell suspension of toxigenic *V. cholerae* O1 El Tor, *V. cholerae* O1 classical and *V. cholerae* O139 were tested thrice (Fig. S5). The detection limit reported in this study which is 1 pg of genomic DNA is lower than that of a LAMP-NALFIA reported by Nimitphak et al. (2008) (1 ng), a PCR-NALFIA by Blazkova et al. (2009) (50 pg) and a LAMP-NALFIA by Kaewphinit et al. (2013) (5 pg) whereas a PCR-NALFIA developed by Soo et al. (2006) reported a similar LOD of 10 CFU/ml. An amplification-free NALFIA assay described by Pohlmann et al. (2014) reported a higher detection limit of 10^4 CFU/ml.

A specificity test performed using 100 ng of genomic DNA from a panel of 30 bacterial strains showed that the lyophilisation process and/or the presence of LATE-PCR stabilisers did not affect the binding selectivity of the LATE-PCR primer pairs and capture probes that were immobilised on the NALF biosensor (Fig. S6). The specificity test also demonstrated that the developed nucleic acid-sensing platform was able to detect toxigenic *V. cholerae* of different serogroups, biotypes and serotypes. Cross-reactivity with non-toxigenic *V. cholerae* and non-*V. cholerae* strains were not observed. The dry-reagent-based LATE-PCR-NALF assay exhibited 100% sensitivity and specificity when tested with 168 archived clinical and environmental strains that were spiked into stool sample. Toxigenicity of *V. cholerae* O1 and O139 isolates as well as the absence of cholera toxin production in other bacterial strains were verified by GM_1-ELISA. Inhibition of the LATE-PCR assay was not observed as all negative results were validated by the IAC line and the presence of chromatography control line in each strip indicated that proper lateral flow protocol was followed.

Heat stability of the thermostabilised LATE-PCR mixes that were stored at different temperatures was evaluated after 1, 14, 30 and 90 days of storage. Thermostabilised LATE-PCR mixes were found to be stable at 56 °C for at least 30 days (Fig. 6). At lower storage temperatures ranging from 4 °C to 37 °C, the thermostabilised LATE-PCR mixes were demonstrated to be stable for 90 days as no significant decrease in signal intensity was observed. Therefore, the developed dry-reagent-based LATE-PCR formulation was shown to be robust against a wide-range of temperatures.

4. Conclusion

In summary, a dry-reagent-based nucleic acid-sensing platform was developed by coupling a two-step thermostabilised LATE-PCR assay with a hybridisation-based lateral flow biosensor. The sequence-specific nucleic acid-sensing platform is DNA extraction-free and has multiplexing capability which allows more information to be retrieved from a single reaction. The risk of carry-over contamination is also minimised as the thermostabilised LATE-PCR mix and lateral flow strip were designed for single-use. The incorporation of dry-reagent technology in the assay components enabled the whole nucleic acid-sensing platform to be cold chain-free and resistant against temperature fluctuations during transportation or storage; hence, greater access to molecular diagnostics would be possible by enabling a minimally equipped laboratory to perform nucleic acid testing.

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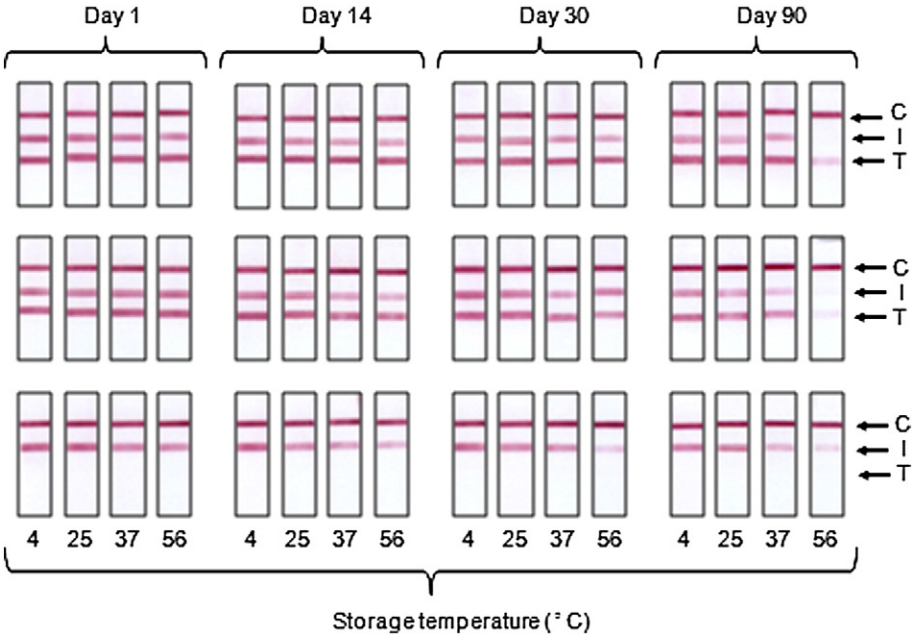

Fig. 6. Heat stability test result of thermostabilised LATE-PCR mixes after various intervals at different storage temperatures. Thermostabilised LATE-PCR mixes were tested with genomic DNA at a concentration of 100 ng (top), 1 ng (middle) and 0 ng (bottom). C, chromatography control line; I, internal amplification control line; T, test line.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2015.08.024.

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