Analytica Chimica Acta 966 (2017) 71–80

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Development of a thermostabilised triplex LAMP assay with dry-reagent four target lateral flow dipstick for detection of *Entamoeba histolytica* and non-pathogenic *Entamoeba* spp.

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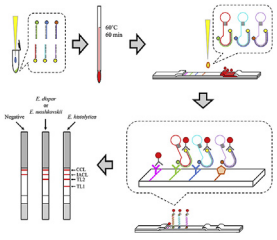
HIGHLIGHTS

- The developed triplex LAMP-NALFIA assay is ready-to-use and cold-chain-free.
- The triplex LAMP reagent mix is thermostabilized and lyophilised.
- The LAMP assay is designed to simultaneously amplified three specific DNA targets.
- The biosensor is developed to facilitate interpretation of post-LAMP analysis.
- The triplex LAMP-NALFIA assay could detect as low as 10 *Entamoeba* trophozoites.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 November 2016
Received in revised form
7 February 2017
Accepted 10 February 2017
Available online 20 February 2017

Keywords:
DNA lateral flow biosensor
Multiplex LAMP
Thermostabilization
Entamoeba histolytica
Isothermal amplification

ABSTRACT

This study highlighted the development of a four target nitrocellulose-based nucleic acid lateral flow immunoassay biosensor in a dry-reagent strip format for interpretation of double-labelled double-stranded amplicons from thermostabilised triplex loop-mediated isothermal amplification assay. The DNA biosensor contained two test lines which captured biotin and texas red labelled amplicons; a LAMP internal amplification control line that captured digoxigenin labelled amplicon; and a chromatography control line that validated the functionality of the conjugated gold nanoparticles and membrane. The red lines on detection pad were generated when the gold nanoparticles conjugated antibody bound to the fluorescein labelled amplicons, and the capture agents bound to their specific hapten on the other 5′ end of the double-stranded amplicon. The applicability of this DNA biosensor was demonstrated using amoebiasis-causing *Entamoeba histolytica* simultaneously with the non-pathogenic but morphologically identical *Entamoeba dispar* and *Entamoeba moshkovskii*. The biosensor detection limit was 10 *E. histolytica* trophozoites, and revealed 100% specificity when it was evaluated against 3 medically important *Entamoeba* species and 75 other pathogenic microorganisms. Heat stability test showed that the biosensor was stable for at least 181 days at ambient temperature. This ready-to-use and cold-chain-free

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http://dx.doi.org/10.1016/j.aca.2017.02.019

biosensor facilitated the post-LAMP analysis based on visualisation of lines on strip instead of observation of amplicon patterns in agarose gel.

1. Introduction

The introduction of nucleic acid amplification technology had revolutionised disease diagnostics. The ability of polymerase chain reaction (PCR) to amplify as little as a single gene copy had established it as a highly sensitive diagnostic assay [1]. However, PCR relied on repetition of several temperatures in a continuous chain reaction which made it dependent on thermocycler. The advent of several isothermal amplification methods as alternatives to PCR had facilitated molecular diagnostics. In particular, loop-mediated isothermal amplification (LAMP) introduced by Notomi, Okayama, Masubuchi, Yonekawa, Watanabe, Amino and Hase [2] had innovated the DNA amplification platform by circumventing thermal cycling to amplify its target. LAMP is basically an amplification method that uses either two or three sets of primers and a high strand-displacement activity DNA polymerase to amplify DNA target at a constant temperature ranging from 60 °C to 65 °C. It also provides highly efficient DNA amplification of up to 10^9-10^{10} times in 15–60 min, and the concentration of the LAMP product is much higher than that generated by conventional PCR [3]. Since then, LAMP had been widely applied in various studies for detection of medically important pathogens such as *Salmonella* Typhi, *Leptospira* spp. and *Burkholderia pseudomallei* [4–6].

Despite its potential, LAMP had not been well studied for multitarget detection due to its ladder-like products pattern that restricted the differentiation among the targets on agarose gel after electrophoresis. Attempts to use signal quantification method in detecting the multiplex LAMP products had been reported to be promising [7–9]. However, requirement of trained personnel and expensive real-time PCR thermal cycler were deterrents to its application in resource-limited laboratories. Therefore, innovation on improving the visualisation and interpretation of amplified LAMP products was still pertinent when it involved two or more targets in a single reaction. Lateral flow platform technology had been reported to be promising in enhancing the visualisation and analysis of amplified LAMP products [10,11]. Preference of lateral flow platform to colorimetric agents, fluorescent agents, lab-on-a-chip devices and turbidimeter was due to its rapid display, disposable format and ease-of-use as well as its potential to be developed into point-of care biosensor [12].

Molecular detection of *Entamoeba histolytica* was important for diagnosis of amoebiasis as it was morphologically indistinguishable from two other non-pathogenic *Entamoeba* species, namely *Entamoeba dispar* and *Entamoeba moshkovskii* [13–15]. Several highly sensitive and specific LAMP assays for detection of *E. histolytica* had been successfully developed [16–18]. To date, all these assays were cold-chain dependent and the post-amplification analysis required agarose gel electrophoresis and UV transilluminator for result visualisation. Besides, these reported LAMP assays were unable to verify the validity of negative results as internal amplification control (IAC) was not incorporated. Validation of amplification process was pertinent in this context, as diagnosis of enteric pathogens such as *E. histolytica* involved extraction of DNA from stool samples which may contain amplification inhibitors; hence inclusion of IAC was important to verify false negative results [19,20].

Nurul Najian et al. [21] had previously described a duplex LAMP assay with label-based lateral flow dipstick for detection of pathogenic *Leptospira*. However, application of the previous study was not convenient at remote areas because the LAMP reagent mix and the lateral flow were dependent on cold-chain storage. Moreover, in addition to IAC, the system had only one test line which was not applicable for detection that required two test lines. The objective of this study was to develop a thermostabilised triplex LAMP assay coupled with a dry-reagent nucleic acid lateral flow immunoassay (NALFIA) for simultaneous detection of *E. histolytica* and *Entamoeba* spp. with incorporation of IAC. A nitrocellulose membrane-based NALFIA was developed prior to the triplex LAMP assay development in order to facilitate the development and interpretation of the LAMP double-labelled double-stranded amplicons. Then, a thermostabilised triplex LAMP assay was developed for detection and differentiation of *E. histolytica* from *E. dispar* and *E. moshkovskii* as well as simultaneous validation of the assay using IAC.

2. Experimental

2.1. Reagents and apparatus

Rabbit polyclonal IgG texas red antibody was purchased from Invitrogen, Thermo Fisher Scientific (Massachusetts, USA); while streptavidin, sheep polyclonal IgG digoxigenin antibody, goat anti-mouse IgG secondary antibody and fluorescein isothiocyanate (FITC) IgG1 monoclonal antibody were purchased from Pierce Thermo Fisher Scientific (Massachusetts, USA). The colloidal gold solution (40 nm) was purchased from Kestrel Bio Sciences (Thailand). Bovine serum albumin (BSA) was purchased from Amresco (Solon, USA) and western blocking reagent (WBR) was purchased from Roche (Indianapolis, USA). Mineral oil, betaine, sodium azide (NaN_3), polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), Tween-20, Triton X-100, sucrose, and other common chemicals were from Sigma (St. Louis, USA). All reagents and chemicals in this study were prepared using ultrapure water (>18MΩ) from a Millipore Milli-Q water purification system (Billerica, USA). Nitrocellulose membrane card HF135, cellulose fiber pads, glass fiber pads and Amicon Ultra 50 K were also Millipore products.

All labelled and non-labelled oligonucleotides were synthesised by Integrated DNA Technologies (Singapore). The LAMP isothermal amplification *Bst* DNA polymerase was purchased from New England Biolabs (Massachusetts, USA) and amplification reactions were performed using Cole-Parmer chilling heating block (Illinois, USA). The LAMP amplicons were analysed using Alpha Innotech ChemiImager 5500 UV illuminator and image capturing unit (California, USA). The NALFIA was lined with streptavidin and antibodies using BioDot XYZ3050 dispensing platform (Irvine, USA) and were cut into strips using Matrix 2360 programmable strip cutter from Kinematic Automation (Twain Harte, USA).

2.2. Entamoeba species and other microorganisms

Microorganism isolates used in this study were listed in Table 1 and were acquired from the Departamento de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico; the London School of Hygiene and Tropical