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Dry-reagent gold nanoparticle-based lateral flow biosensor for the simultaneous detection of *Vibrio cholerae* serogroups O1 and O139☆

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ABSTRACT

Cholera is a communicable disease caused by consumption of contaminated food and water. This potentially fatal intestinal infection is characterised by profuse secretion of rice watery stool that can rapidly lead to severe dehydration and shock, thus requiring treatment to be given immediately. Epidemic and pandemic cholera are exclusively associated with *Vibrio cholerae* serogroups O1 and O139. In light of the need for rapid diagnosis of cholera and to prevent spread of outbreaks, we have developed and evaluated a direct one-step lateral flow biosensor for the simultaneous detection of both *V. cholerae* O1 and O139 serogroups using alkaline peptone water culture. Serogroup specific monoclonal antibodies raised against lipopolysaccharides (LPS) were used to functionalize the colloidal gold nanoparticles for dual detection in the biosensor. The assay is based on immunochromatographic principle where antigen–antibody reaction would result in the accumulation of gold nanoparticles and thus, the appearance of a red line on the strip. The dry-reagent dipstick format of the biosensor ensure user-friendly application, rapid result that can be read with the naked eyes and cold-chain free storage that is well-suited to be performed at resource-limited settings.

1. Introduction

Cholera is an acute intestinal diarrhoea disease that manifests in the form of massive body water reflux which can rapidly lead to severe dehydration and death in the absence of prompt treatment (Sack et al., 2005). This food- and water-borne disease, caused by the gram negative bacterium *Vibrio cholerae*, is known for its propensity to explode into outbreaks especially in undeveloped countries and disaster-struck communities where clean water and sanitation is either compromised or unavailable (Zuckerman et al., 2007). Although more than 200 serogroups have been identified to date, only *V. cholerae* serogroups O1 and O139 are known to be capable of initiating epidemic and pandemic cholera (Faruque et al., 1998).

In 2009, a remarkable rise of 16% in the number of cholera cases has been reported to WHO in comparison to 2008 where 221 226 cases and 4946 deaths were reported worldwide (World Health Organization, 2010). In 2010, Haiti was struck by a devastating earthquake and soon followed by an explosive cholera outbreak which has recorded a total of 121 518 cases of cholera, resulting in 63 711 hospitalisations and 2591 deaths (Centers for Disease Control and Prevention, 2010). In such circumstances, diagnostic tests that can be easily performed and made available at low cost will not only combat the disease but also prevent its further spread.

The conventional method which is regarded as the gold standard for diagnosing cholera involves pre-enrichment in alkaline peptone water (APW), isolation by culture on thiosulfate citrate bile salt-sucrose agar (TCBS) and identification by slide agglutination test with specific antisera (Sakazaki, 1992). These laboratory methods are known to be time-consuming and result obtained after several days would have meant a delay in clinical diagnosis and patient treatment. Molecular method involving PCR amplification for the detection of *V. cholerae* (Yean et al., 2008) reduce the diagnosis time but such an assay requires skilled personnel, infrastructure and is difficult to perform in countries with low resource setting.

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Over the years, rapid diagnostic tests based on immunochromatography principle have been reported for the detection of *V. cholerae* serogroups O1 and O139, either discretely or simultaneously (Bhuiyan et al., 2003; Harris et al., 2009; Kalluri et al., 2006; Mukherjee et al., 2010; Nato et al., 2003; Wang et al., 2006). However, these studies only report on the evaluation of the immunochromatographic or dipstick tests. Therefore in this study, we present the in-house development and the evaluation of a gold nanoparticle-based lateral flow biosensor for the simultaneous detection of *V. cholerae* serogroups O1 and O139.

2. Materials and methods

2.1. Reagents and equipments

Sucrose, trehalose, Tween-20, polyvinyl alcohol (PVA) and sodium azide were products of Sigma (St. Louis, USA). Bovine serum albumin (BSA) was purchased from Amresco (Solon, USA) and western blocking reagent was obtained from Roche (Indianapolis, USA). Goat anti-mouse IgG (H & L) antibody (Ab) was obtained from Thermo Scientific (Rockford, USA) and colloidal gold nanoparticles (40 nm) was from Heron Diagnostics (Pathumthani, Thailand). All chemical and reagents used in this study were of high purity analytical grade.

Cellulose fibre sample pad (ref. CFSP173000), glass fibre conjugate pad (ref. GFCP083000) and laminated nitrocellulose membrane card (ref. HF135MC100) were products of Millipore. A VersaMax microplate reader (Sunnyvale, USA), a XYZ dispensing platform from BioDot (Irvine, USA) and an automatic cutter from Kinematic Automation (Twain Harte, USA) were used in this study.

2.2. Production of anti-O1 LPS and anti-O139 LPS monoclonal antibodies

Murine monoclonal antibodies (mAb) against *V. cholerae* LPS were produced by using conventional hybridoma procedures as described previously (Falero et al., 2003). Briefly, hybridomas were produced by fusion of SP_2/O myeloma cell line with splenocytes of BALB/c mice that were immunised with subcutaneous injections of crude outer membrane protein from *V. cholerae* O1 or O139. Two hybridomas, coded as 6G1E12F10 and 9A11D6, were selected and grown as mouse ascites in the peritoneal cavity of pristine-primed BALB/c mice. The mAbs were purified from the ascites fluid by affinity chromatography using Protein A Sepharose Fast Flow (GE Healthcare Life Sciences). The purified antibodies were diluted in a pH 7.4 buffer solution (137 mmol/l NaCl, 27 mmol/l KCl, 8 mmol/l Na_2HPO_4, 15 mmol/l KH_2PO_4, and 0.1% (w/v) sodium azide) and lyophilized. The classes and subclasses of mAbs secreted by 6G1E12F10 and 9A11D6 were determined with an ImmunoType Kit (Sigma) whilst the specificity of the mAbs was characterised by using immunoblot for its reactivity towards *V. cholerae* O1 and O139 cell lysates.

2.3. Optimisation of colloidal gold-mAb conjugation

Determination of the optimal pH and minimal amount of coating mAb for colloidal gold-mAb conjugation was carried out according to methods by Geoghegan (1988) and Guo et al. (2009) with slight modifications. The selection of optimal pH for colloidal gold-anti-O139 LPS conjugate preparation was performed by adding 40 μg/ml of anti-O139 LPS mAb into 1 ml of colloidal gold solution adjusted to pH 6.0, 7.0, 8.0, 9.0 and 10.0 respectively. After incubation for 15 min, 100 μl of 10% NaCl was added to the mixture. The OD_{525} (optical density at 525 nm) of the solution before and after the addition of NaCl were measured. The amount of decrease in absorbance after addition of NaCl was plotted versus pH of the colloidal gold solution. Any visual changes in the colour were also recorded after the addition of NaCl.

Likewise, estimation of the minimal amount of antibody required to stabilise the colloidal gold was carried out by adding increasing concentration of anti-O139 LPS mAb (1.25–40 μg/ml) into 1 ml of colloidal gold solution adjusted to pH 8.0. After 15 min, 100 μl of 10% NaCl was added to the mixture. OD_{525} measurement and graphical analysis of the solution absorbance before and after the addition of NaCl were performed as described above.

2.4. Preparation of colloidal gold-mAb conjugate

The colloidal gold-anti-O139 LPS conjugate was prepared by adding 20 μg/ml of anti-O139 LPS antibody to 30 ml of colloidal gold solution (pH 8.0), followed by incubation at room temperature with gentle mixing for 30 min. BSA (1%) was then added before mixing for another 30 min. Finally, the colloidal gold-mAb conjugate was centrifuged at $10000 \times g$ for 10 min and the loosely packed sediment was resuspended in suspension buffer [2 mM phosphate buffer (pH 7.2) containing 5% trehalose and 0.01% (w/v) sodium azide] for storage at 4 °C until use. The conjugation of anti-O1 LPS with colloidal gold was performed by Diagnostic Consulting Network (Carlsbad, USA).

2.5. Preparation of the dry-reagent lateral flow biosensor

The dry-reagent dipstick (5 mm × 73 mm), assembled on a plastic adhesive backing, was composed of a sample pad, conjugate pad, laminated nitrocellulose membrane and an absorbent pad as shown in Fig. 1(a).

Anti-O1 LPS mAb (2 mg/ml), anti-O139 LPS mAb (2 mg/ml) and goat anti-mouse Ab (1 mg/ml) which served as capture antibodies were applied separately on the nitrocellulose membrane with the volume of 4 μl/cm using Biojet (XYZ frontline Dispensing Platform). The distance between each line was 5 mm. The nitrocellulose membrane was dried overnight in a desiccator at room temperature. The remaining protein binding sites of the membrane were blocked with 50 mM phosphate buffer (pH 7.4) containing 1% western blocking reagent and 0.05% Tween-20.

The conjugate pad was used to immobilise equal amount of colloidal gold-anti-O1 LPS and colloidal gold-anti-O139 LPS conjugates on the strip. The colloidal gold-antibody conjugates suspended in 0.1 M phosphate buffer containing 1% Tween-20, 0.5% (w/v) BSA and 5% (w/v) sucrose were dispensed on a glass fibre using Airjet (XYZ Dispensing Platform). It was dried and stored in a desiccator at room temperature until use.

The sample pad, conjugate pad, nitrocellulose membrane and absorbent pad were assembled with 2 mm overlapping between each component [Fig. 1(a)]. The assembled master card was then cut into strips of 5 mm wide and stored in a desiccator until use or sealed in aluminium pouches containing silica gels during transportation.

2.6. One-step dipstick assay procedure

The assay was performed by immersing the sample application region of the dipstick directly into 2 ml of APW enriched with *V. cholerae*. After 10 min, the dipstick was taken out of the sample and the result was read by naked eyes.

2.7. Diagnostic sensitivity and specificity of the dipstick assay

Two evaluations had been performed in order to determine the sensitivity and specificity of the assay. National evaluation was carried out at the Faculty of Food Science and Technology, Universiti Putra Malaysia (UPM), Malaysia and the international evaluation was conducted at the National Institute of Cholera and Enteric Diseases (NICED) which serves as the National Reference Center for cholera in India. An accumulative total of 278 archived clinical and

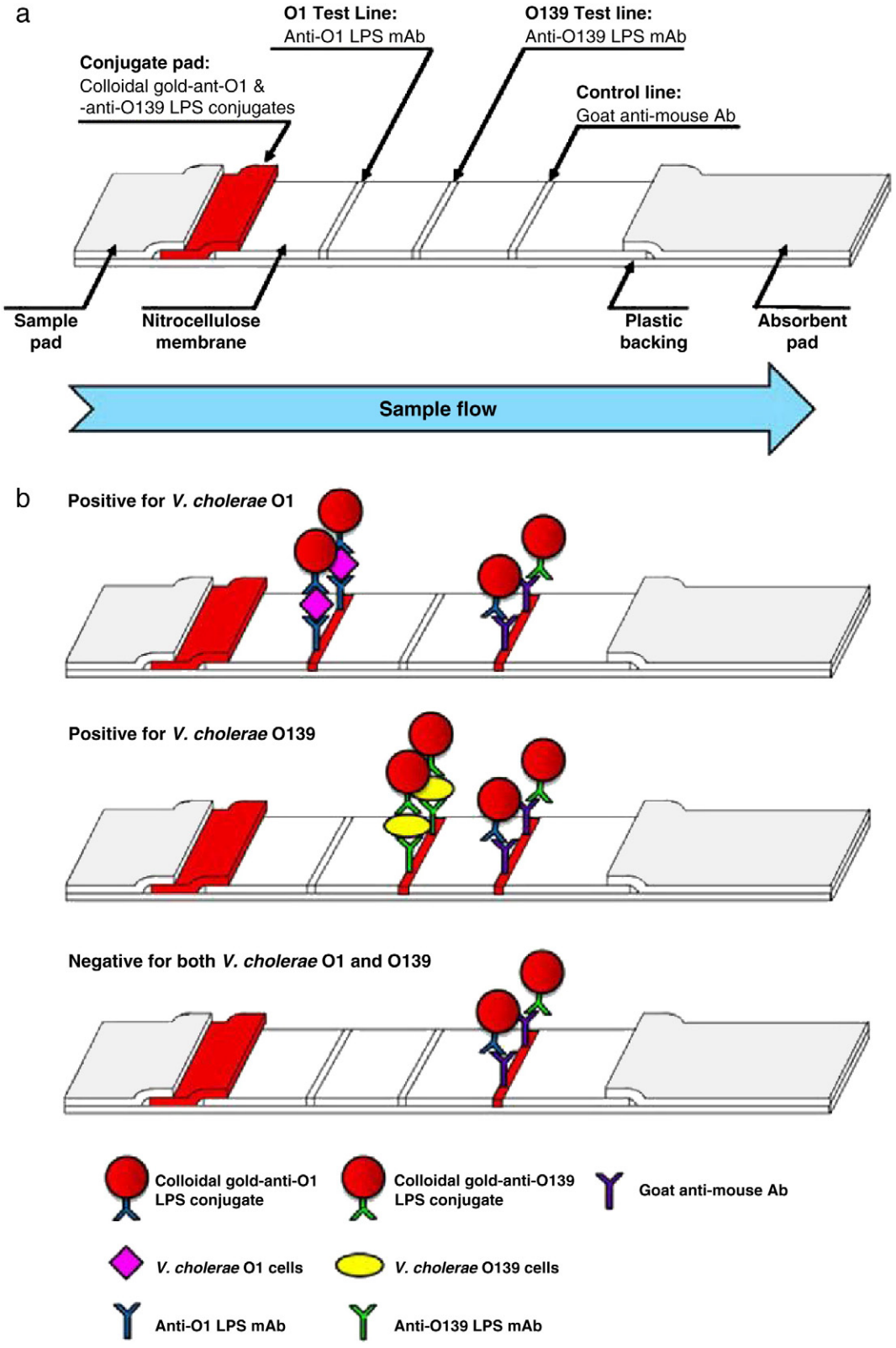

Fig. 1. (a) Cross-section of the dipstick showing its assembled components. (b) Schematic diagram of the immunochromatographic principle for the dipstick assay.

environmental bacterial strains were used for the evaluation of the dipstick assay; 158 strains were tested in UPM whilst another 120 strains were tested in NICED. All the strains were grown in APW at 37 °C for 6 h before testing with the dipstick assay.

3. Results and discussion

3.1. Detection principle

The lateral flow biosensor is based on the detection of serogroup specific LPS of *V. cholerae* using monoclonal antibodies coupled to colloidal gold nanoparticles. As illustrated in Fig. 1(b), the solution migrates towards the absorbent pad via capillary action once the dipstick is immersed in the culture solution which also helps to reconstitute the colloidal gold conjugates immobilised on the conjugate pad and thus, allowing antigen–antibody bound complex to travel during the migration process. In the presence of *V. cholerae* O1 LPS antigen, it complexes with the colloidal gold-anti-O1 LPS conjugate which is then captured by the anti-O1 LPS mAb immobilised at the O1 test line. Similarly, in the presence of *V. cholerae* O139 LPS, it complexes with the colloidal gold-anti-O139 LPS conjugate which is then captured by the anti-O139 LPS mAb immobilised at the O139 test line. The excess colloidal gold-mAb conjugates would be captured by the goat anti-mouse IgG Ab at the control line.

Gold nanoparticles are reported to be inert, non-toxic, and have long retention of their optical properties (Thobhani et al., 2010) which makes them an excellent choice as a signal generator. The accumulation of gold nanoparticles produces a characteristic red coloured line as a result of surface plasmon resonance. A positive result for *V. cholerae* serogroup O1 was indicated by the presence of a red line at O1 test line whereas a red line at O139 test line indicates a positive result for *V. cholerae* serogroup O139. In both cases, the presence of a red line at the control line validates the result as a true positive.

The dipstick also has the capability of detecting both *V. cholerae* serogroup O1 and O139 in a single sample wherein all three red lines were visible (data not shown). The control line served as a procedural control in each assay and should appear regardless of the presence of *V. cholerae* serogroup O1 and/or O139 cells in the sample. Absence of the control line will render the result invalid.

3.2. Characterisation of mAbs

The hybridoma producing anti-O1 LPS mAbs, coded as 6G1E12F10, was found to secrete antibodies of the IgG_3 isotype whilst the anti-O139 LPS mAbs-producing hybridoma, coded as 9A11D6, secreted antibodies of the IgG_1 isotype. The approximate quantities of MAbs secreted by 6G1E12F10 and 9A11D6 hybridomas as estimated from the yield of the purification process were 1.508 mg/ml and 2.198 mg/ml, respectively. The immunoblot analysis revealed that the anti-O1 LPS mAbs reacted with LPS from both *V. cholerae* O1 serotypes (Ogawa and Inaba) but not with the O139 strains of *V. cholerae* analysed. The anti-O139 LPS mAbs were found to react with LPS of O139 serogroup but not with the O1 strains of *V. cholerae* analysed.

3.3. Preparation of colloidal gold-mAb conjugate

The conjugation process was assessed by the changes in surface plasmon resonance of the colloidal gold particles after exposure to high salt concentration. Particle flocculation, which is characterised by a blue grey colour, occurs when particles aggregate together as a result of reduction in electrostatic repulsive charges between the particles at high concentration of electrolyte. Therefore, optimum amount of adsorbed antibodies is able to confer stability to the colloidal gold by preventing flocculation in the presence of high salt concentration (Chun, 2009).

Flocculation assay was used to determine the stability of the gold conjugates and the optimum condition was selected based on the minimal amount of decrease in absorbance at OD_{525} and no apparent colour change from red to greyish blue (Fig. 2). The optimum pH and concentration of mAb for colloidal gold-anti-O139 LPS conjugation was found to be pH 8 and 20 μg/ml of anti-O139 LPS mAb.

3.4. Development of the dipstick assay

The hydrophobic nature of NC membrane tends to cause non-specific protein adsorption which can significantly affect the performance of the assay. An array of blocking reagents (BSA, polyvinyl alcohol, foetal calf serum and western blocking reagent) was tested by trial and error for their effectiveness in blocking non-specific binding on the dipstick assay. The best blocking reagent out of the various types tested was found to be western blocking reagent.

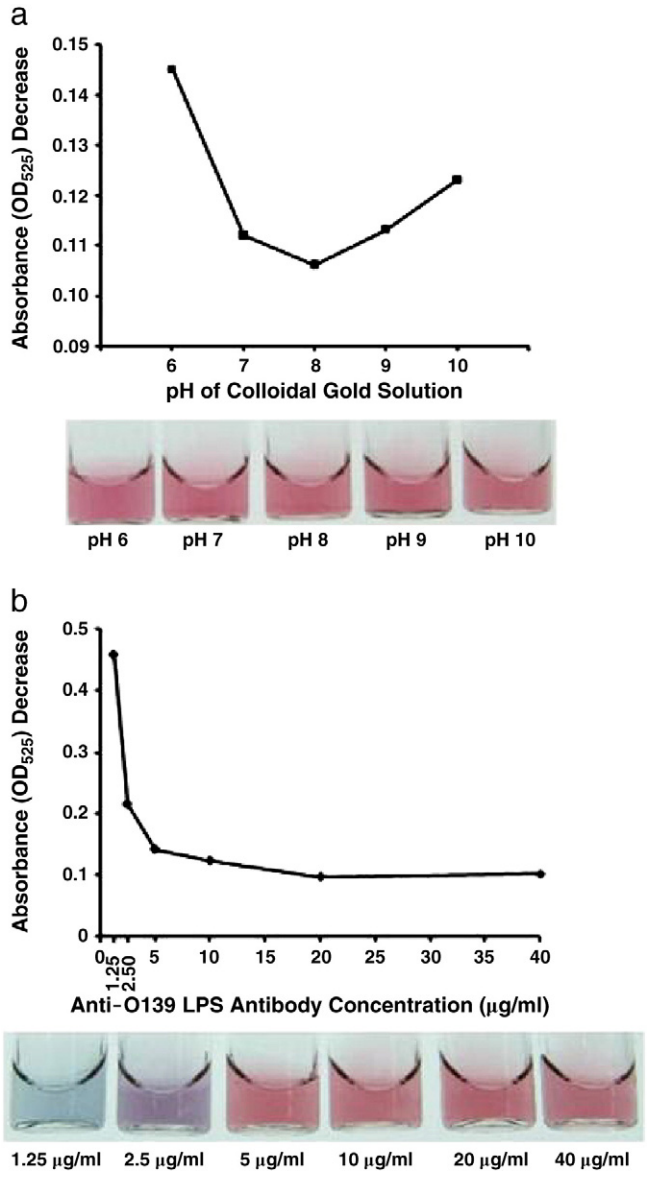

Fig. 2. (a) A graph showing the change in OD_{525} observed for the colloidal gold-mAb complex after the addition of NaCl at various pH of colloidal gold solution. (b) A graph showing the change in OD_{525} observed for the colloidal gold-mAb complex after the addition of NaCl at various amount of coating anti-O139 LPS antibody. Below the graph are the visual colour observed in the respective tubes after the addition of NaCl.

The composition of buffer solution for immobilisation of colloidal gold-mAb conjugates is crucial to ensure that the activity of the conjugates is preserved whilst dried on the conjugate pad and retained after rehydration during assay. The most suitable buffer was found to be 0.1 M phosphate buffer containing 1% Tween-20, 0.5% BSA and 5% sucrose.

3.5. Performance of the assay

Based on optimal assay conditions, the detection limit of the dipstick assay with enriched culture was determined with a serially diluted *V. cholerae* serogroup O1 or O139 cultures in duplicate. The *V. cholerae* serogroups O1 and O139 strains were prepared from 10^9 CFU/ml *V. cholerae* APW culture and serially diluted with sterile saline. The visual detection limit of the assay was defined as the minimum concentration of cells capable of producing red colour line on the test line and control line when visualised by the naked eye. The detection limit was found to be 10^8 and 10^7 CFU/ml for *V. cholerae* serogroup O1 and O139, respectively. A separate set of serially diluted cultures were also incubated at 37 °C for 6 h before the assay was performed. The minimum amount of inoculum in a 2 ml APW culture that was able to give a positive result after 6 h of enrichment was found to be 10^2 CFU/ml for both *V. cholerae* serogroup O1 [Fig. 3(a)] and O139 [Fig. 3(b)].

3.6. Diagnostic sensitivity and specificity of the dipstick assay

National evaluation result showed 100% sensitivity and 100% specificity for both O1 and O139 test lines for all the strains tested as shown in Table 1. International evaluation showed that for the O1 test line evaluation, 66 of the 71 *V. cholerae* serogroup O1 strains tested were dipstick positive. The remaining 5 strains were dipstick negative, indicating false negative results by the dipstick assay. No cross-reactivity was seen with other strains tested. The overall sensitivity and specificity of the dipstick assay for O1 test line were 93–100% and 100%, respectively.

For the O139 test line evaluation, all of the 30 *V. cholerae* serogroup O139 strains tested were dipstick positive and no false negative was observed. In two cases, the non-*V. cholerae* serogroup O139 strains gave positive results for O139 test line, indicating false positive results. The overall sensitivity and specificity of the dipstick assay for O139 test line were 100% and 98–100%, respectively.

Representative results of the dipstick assay are shown in Fig. 3(c). The overall sensitivity and specificity of the dipstick assay were excellent and no invalid results were found.

Table 1
Bacteria strains tested.

Strains	UPM[a]		NICED[b]	
	Positive dipstick test line		Positive dipstick test line	
	O1	O139	O1	O139
Vibrio cholerae O1 (n = 93)	22/22	0/22	66/71	0/71
Vibrio cholerae O139 (n = 42)	0/12	12/12	0/30	30/30
Vibrio cholerae non-O1/non-O139 (n = 70)	0/61	0/61	0/9	0/9
Vibrio parahaemolyticus (n = 16)	0/14	0/14	0/2	0/2
Vibrio vulnificus (n = 11)	0/11	0/11	–	–
Escherichia coli (n = 12)	0/10	0/10	0/2	0/2
Listeria monocytogenes (n = 16)	0/16	0/16	–	–
Salmonella sp. (n = 14)	0/12	0/12	0/2	2/2
Aeromonas hydrophilia (n = 2)	–	–	0/2	0/2
Shigella boydii (n = 1)	–	–	0/1	0/1
Shigella flexneri (n = 1)	–	–	0/1	0/1

[a] Evaluation performed at the Universiti Putra Malaysia, Malaysia.
[b] Evaluation performed at the National Institute of Cholera and Enteric Diseases, India.

3.7. Stability of the assay

The stability of the dipstick was evaluated by performing the assay after storage in sealed aluminium pouches for 15 days, 4 weeks, 8 weeks, and 21 weeks at room temperature. The same batch of strips was tested against *V. cholerae* serogroup O1, O139, non-O1/non-O139 and also a negative control consisting of APW broth only. The interpretation of the result can still be made by the naked eye after 21 weeks of storage. The intensity of the test line appeared slightly weaker at 21 weeks compared to 4 weeks but loss of intensity was not significant. This showed that the dipstick remain stable when stored dry at ambient temperature for at least 3 months.

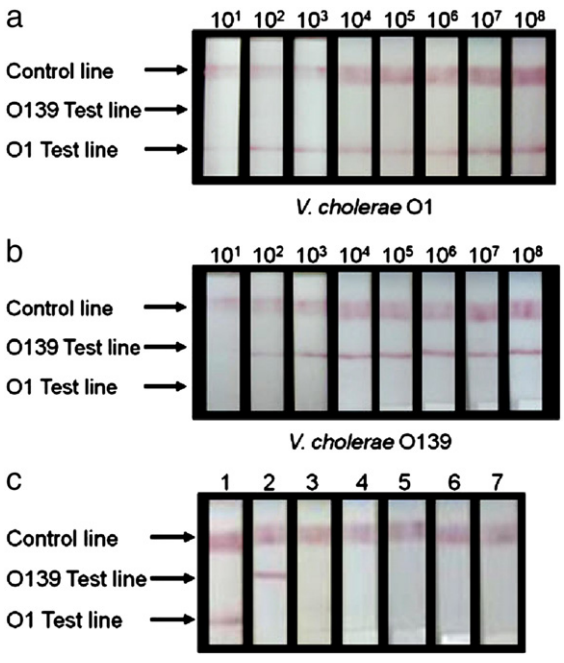

Fig. 3. Direct one-step lateral flow biosensor. (a) Dipstick assay results for *V. cholerae* O1 culture with different inoculum concentration and (b) dipstick assay results for *V. cholerae* O139. The numbers across the top of each dipstick shows the initial inoculum concentration (CFU/ml) prior to 6 h enrichment in APW. (c) Representative dipstick assay result of bacterial samples enriched in APW. Sample 1: *V. cholerae* O1; 2: *V. cholerae* O139; 3: *V. cholerae* non-O1/non-O139; 4: *Vibrio parahemolyticus*; 5: *Shigella flexneri*; 6: *Escherichia coli*; 7: *Salmonella typhi*.

4. Conclusion

In this study, *V. cholerae* O1 and O139 serogroup specific mAbs have been successfully produced and utilised as both capture and detection probes in a gold nanoparticle-based lateral flow biosensor for the simultaneous detection of epidemic and pandemic cholera causative agents. This antigen–antibody immunoassay capitalise on the accumulation of gold nanoparticles at the test and/or control regions to produce a visible result that can be read by the naked eye, thus eliminating any dependency on specific equipment or software for the result interpretation.

Assays in dipstick based format are generally more appealing to the end users because of their simplicity, user-friendly application, cost effectiveness and point-of-care approach. The dry reagent dipstick which is cold-chain free during transportation and storage will potentially be of great value in cholera outbreaks occurring in poor resource settings. This dipstick assay represents both a rapid test for screening of cholera that will enable treatment to be initiated as soon as possible and also as an epidemiological surveillance tool that will aid in controlling the outbreak from spreading.

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