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ENVIRONMENTAL BIOTECHNOLOGY

Rapid detection of *Yersinia pestis* recombinant fraction 1 capsular antigen

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Abstract *Yersinia pestis*, an infectious bacterium that is a causative agent of plague, a disease which has been shown to be one of the most feared in history and which has caused millions of deaths. The capsule-like fraction 1 (F1) antigen expressed by *Y. pestis* is a known specific marker for the identification of the bacteria; therefore, the detection of F1 is important for *Y. pestis* recognition. In this study, a rapid, sensitive, and specific technique, the lateral flow assay (LFA), was successfully developed to detect *Y. pestis* by the recombinant F1 antigen. The assay that utilized an anti-F1 polyclonal antibody (Pab) to identify the bacteria was based on a double-antibody sandwich format on a nitrocellulose membrane. With the LFA method, 50 ng/ml of recombinant F1 protein and 10^5 CFU/mL of *Y. pestis* could be detected in less than 10 min. This assay also showed no cross-reaction with other *Yersinia* spp. or with some selected capsule-producing *Enterobacteriaceae* strains. Furthermore, detection of *Y. pestis* in simulated samples has been evaluated. The detection sensitivity of *Y. pestis* in various matrices was 10^5 CFU/mL, which was identical to that in PBS buffer. The results obtained suggest that LFA is an excellent tool for detection of *Y. pestis* contamination in an environment and hence can be used to monitor plague diseases when they emerge.

Keywords *Yersinia pestis* · Capsule-like F1 antigen · Lateral flow assay · Immunogold detection · Mouse bioassay

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Introduction

Yersinia pestis, one of the pathogenic species of the *Yersinia* genus, plays a significant role in the pathogenesis of plague (a.k.a. "the Black Death," responsible for the oldest recorded flea-borne disease pandemic in human society (Chanteau et al. 2003; Girard 1955; Dennis and Chu 2003; Mengis 1962). Plague is a known zoonotic disease of rodents (Thullier et al. 2003; Butler 1984). However, humans can be infected either by direct contact with infected animals or by transmission though fleas where they have first fed on infected animal blood and then bite human hosts, in this case Bubonic plague typically results. Both of these infection methods have caused several million deaths (Gage et al. 2000; WHO 2000). Previous studies (Chanteau et al. 1998; Tomaso et al. 2007) have reported that the presence of F1 antigen in patient sera with bubonic plague ranged from as low as 4 ng/ml to 50 μg/ml. Agar et al. (2008) also mentioned that the majority of the mice die due to pneumonic infection with approximately 10^7 to 10^9 CFU of the plague bacterium in the peripheral organs.

Y. pestis is a rod-shaped, nonmotile, nonsporulating, gram-negative bacterium of the *Enterobacteriaceae* family. It grows within macrophages (Straley and Harmon 1984) and is invasive in epithelioid cells (Cowan et al. 2000). The three major clinical forms of plague are bubonic, primary septicemic, and the more lethal form, pneumonic (Zietz and Dunkelberg 2004). Although neither bubonic nor septicemic plague is contagious, a small number of these patients may go on to develop to pneumonic plague. Pneumonic plague is highly contagious between humans since the distance required for effective aerosol transmission is only 2 m (Alvarez and Cardineau 2010), and it is always lethal if antibiotic therapy is not provided within 24 h after the onset of symptoms (Butler 2000; Campbell and Dennis 1998).

After infection, *Y. pestis* excretes a capsule-like surface antigen called fraction 1 (F1) protein (Williams et al. 1984), which is encoded by the *caf1* gene that unique to *Y. pestis* (Walker 1962). This protein coding *caf1* gene cluster also plays roles in the regulation of the expression and secretion of F1 protein. Furthermore, F1 is not only a proficient marker for *Y. pestis* identification but is also useful for immunization to protect against wild-type variants of plague (Wang et al. 2008). In previous studies, animal studies have conclusively demonstrated that some specific antibodies can protect animals from *Y. pestis* infection (Williamson et al. 1999; Simpson et al. 1990). For example, F1 protein has been confirmed to prevent phagocytic properties of macrophages by interfering with complement-mediated opsonization (Du et al. 2002); in another study, immunization with F1 induced high levels of antibodies that can partially protect F1-immunized animals against plaque disease (Titball et al. 1997); F1 antibody administered by passive immunization has been shown to protect mice against pneumonic plague (Green et al. 1999); and monoclonal antibodies (MAbs) for F1 protein were evidenced to protect animals from bubonic and pneumonic plague (Anderson et al. 1997).

Although *Y. pestis* no longer causes pandemics of disease, the World Health Organization (WHO) estimates that there are still up to 3000 cases of plague annually worldwide (Perry and Fetherston 1997). Because of high virulence and high mortality of plague, *Y. p*estis is thus currently categorized as a restricted agent (A-list) by the CDC (Centers for Disease Control and Prevention, Atlanta, USA) and is considered to be a significant threat as a potential biological aerosol weapon, even though *Y. pestis* is not stable when suspended in air (Pohanka and Skladal 2009). Therefore, it is of high importance to establish a highly sensitive and specific method for the detection of *Y. pestis*, or its antibody, in order to reduce the fatality rate and allow effective control of plague if a pandemic were to occur (Chanteau et al. 2003).

Some researchers have reported highly sensitive immunological and biochemical assays to detect F1 antigen, such as the indirect hemagglutination assay (IHA, the gold standard in detection of *Y. pestis* (Williams et al. 1982)), polymerase chain reaction (PCR) analysis (Loïez et al. 2003), enzyme-linked immunosorbent assays (ELISAs) (Rasoamanana et al. 1997; Splettstoesser et al. 2004) and the fiber optic biosensor measurement (Wei et al. 2007; Cao et al. 1995). These techniques all have high sensitivity and specificity; however, the high equipment cost (such as that for ELISA readers, laser power supplies and computers, etc.), requirement for skilled technicians, long assay times (minimum of several hours), and the complexity of operation procedures limit their use in the field. To reduce these inadequacies and extend the limits of detection, a low-cost, rapid, sensitive, and accessible method for detection of low levels of *Y. pestis* in humans and rodents, and for evaluation of environmental status and social security contexts is urgently required, and the lateral flow assay (LFA) seems to be an ideal candidate to meet these requirements.

The LFA, also called the immunochromatographic assay or the strip assay, has been well studied in various diagnostic fields (Wong 2002; Tsay et al. 2002; Shyu et al. 2010; Peng et al. 2007) and also in a real forensic case (Yeh et al. 2014). The strip assay is an immunochromatographic process accomplished by the antigen-antibody reaction through capillary forces on a nitrocellulose (NC) membrane (Al-Yousif et al. 2002; Qian and Bau 2003); results are indicated by a colored signal due to the attachment of colloidal gold beads (Shyu et al. 2010). This technique has several benefits, such as requiring no specific equipment or animals, an easy-to-use format, rapid results (less than 10 min), long-term stability over a wide range of weather conditions, and relatively low manufacturing costs. Moreover, the results of LFAs can be visualized directly with the naked eyes. These characters render the LFA suitable for on-site testing by untrained personnel. In this study, we used a polyclonal antibody (Pab) to develop a rapid and sensitive sandwich immunochromatographic assay that could detect *Y. pestis* in the environment and in simulated food samples.

Materials and methods

Ethics statement

All animal experiments were conducted in compliance with the IPM Institutional Animal Care and Use Committee (IACUC, ref. AN102-08) of the National Institute of Allergy and Infectious Diseases/National Institutes of Health.

Materials

Anti-F1 IgG was purified from anti-F1 serum by thiophilic gel (Pierce, Rockford, USA), an IgG-specific immunosorbent. The anti-F1 serum was obtained from F1 hyperimmunized rabbits. High flow NC membranes (AE 98), glass fiber conjugation pads (AccuFlow™ G), sample application pads (#12-S), and reagent adsorption pads (470 Zuschnitte) were purchased from Schleicher & Schuell GmbH (Dassel, Germany). Colloidal gold particles were obtained from Aurion (Wageningen, Netherlands). Goat anti-rabbit IgG were obtained from Sigma (St. Louis, MO, USA). The bacterial strains, killed-plague-vaccine immunized mouse serum and F1 expression plasmid pUC18F1 (Fig. 1a), were kindly supplied by Dr. Shih-Shiung Huang, institute of preventive medicine, national defense medical center, Taiwan. All the cultures, tests and treatment of *Y. pestis* were performedin a Bio-SafetyLevel3 (BSL-3) laboratory.

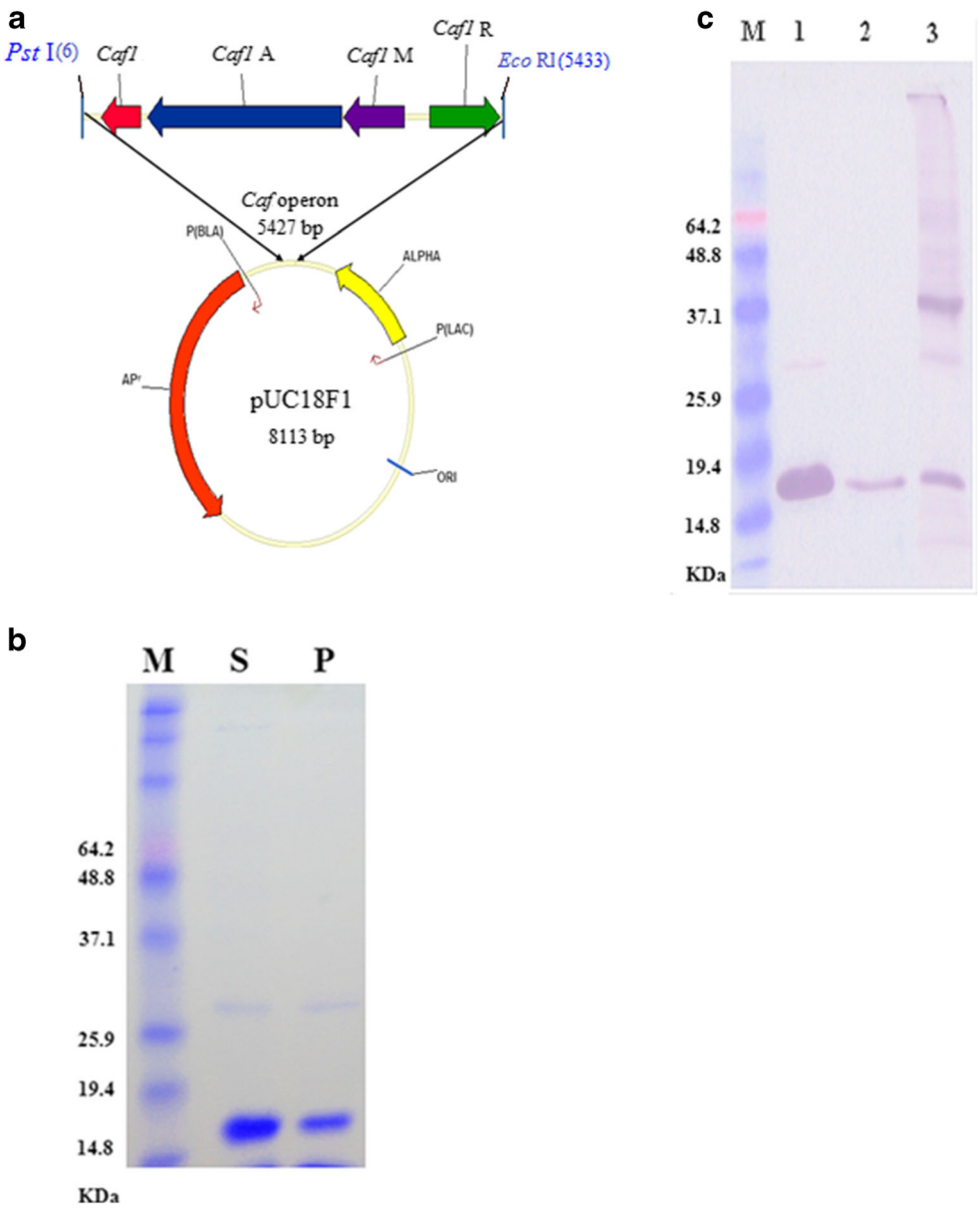

Fig. 1 Extraction of F1 recombinant protein. **a** Structure of *Caf1* recombinant protein expression vector (pUC18F1). Insert *caf* gene cluster into vector pUC18 for expression of F1 protein. The *caf* gene cluster thus can express F1 structural protein. **b** SDS-PAGE (12 % gel) of *E. coli* expressed recombinant F1. *Lane M*, molecular marker; *S*, culture supernatant precipitated with $(NH_4)_2SO_4$; *P*, bacteria pellet. **c** Western blot of *E. coli*-derived recombinant F1probed with anti-F1 mouse serum. *Lane M*, marker; *1*, culture supernatant; *2*, bacteria pellet; *3*, pellet of killed-plaque-vaccine

Construction of pUC18F1 and F1 extraction

The pUC18F1 containing the *caf1* gene cluster was constructed as previously described (Liu et al. 2006). Briefly, PCR products of DNA fragments, containing *caf1*A, *caf1*M, *caf1R*, *caf1*, and signal peptide, were cloned into thepUC18 plasmid (Promega) to generate a recombinant plasmid, pUC18F1 (Fig. 1a). Sequence analysis revealed that the *caf1* gene cluster has 99.9 % identity with *Y. pestis* A1122 (GenBank accession number cp002957).

F1 protein was extracted as previous described (Andrews et al. 1996). In brief, the pUC18F1 plasmid was first transformed into *E. coli* TOP10 (One Shot® TOP10 Chemically Competent *E. coli* Invitrogen (Life Technologies) #4040-06), and the transformed bacteria were grown in Luria-Bertani broth (LB; 37 °C overnight) containing ampicillin. Following centrifugation (12,000×*g*), ammonium sulfate (final concentration 30 % *w*/*v*) was added to the bacteria-free supernatant (4 °C for overnight) to acquire F1 precipitant. The F1 precipitant was then centrifuged and resuspended in phosphate-buffered saline (PBS, pH 7.2) at 4 °C (fraction A). On the other hand, the bacteria pellet was resuspended in a fourfold volume of acetone and air-dried, followed by adding toluene-saturated NaCl (2.5 %) with mild agitation. Then, the mixture was again precipitated by 30 % of ammonium sulfate. After centrifugation and dialysis against PBS (4 °C for overnight), the supernatant was further precipitated by 25 % ammonium sulfate. Through a final centrifugation, the pellets were discarded and the supernatants (altogether with fraction A) were dialyzed against PBS. The purity of the F1 was

assessed by the used of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the F1 recombinant protein was then used as the antigen in immune rabbits.

Production of Anti-F1 polyclonal antibody

For the production of anti-F1 antibodies, purified F1 protein (100 μg) was mixed with equal volumes of complete Freund's adjuvant (first injection) or incomplete Freund's adjuvant (subsequent injection) to immunize rabbits, via subcutaneous injection every 2 weeks. After three boosters, whole blood was collected from the rabbits' ear arteries, stored overnight at 4 °C, and then removed blood cells by further centrifugation. The specificity of the anti-F1 rabbit serum was evaluated by a Western blot assay (Burnette 1981; Matson 1965), and the anti-F1 IgG was purified from anti-F1 rabbit serum through the thiophilic gel.

Conjugation of anti-F1 antibody to colloidal gold particles

Colloid gold particles (25 nm in diameter) were used for conjugation of anti-F1IgG. The colloid gold solution (1 % *w*/*v*) was adjusted to pH 8.5 with 0.2 N NaOH before use. The anti-F1 IgG (1 mg/ml, 0.1 ml) was added to 40 ml pH-adjusted colloid gold solution with gently mixed (30 min, room temperature). Followed by centrifuged for 30 min (4 °C, 1550×*g*, 8178 swing-out rotor, Labofuge 400R, Heraus Instrument, USA), the conjugated colloid gold pellets were suspended in 4-ml dilution buffer (20 mM Tris/HCI buffer (pH 8.2) containing 1 % *w*/*v* BSA) and the optical density of the suspension was adjusted to 5.0 at 520 nm. The prepared anti-F1 IgG-coated colloidal gold probes were stored at 4 °C until use (20 μl/cm on the conjugation pad).

Preparation of immunochromatographic test strips

A schematic description and the composition of the immunochromatographic test device have been described previously (Shyu et al. 2010; Yeh et al. 2014). Briefly, 2 μl of goat anti-rabbit IgG (0.5 mg/ml) and rabbit anti-F1 IgG (0.5 mg/ml) were separately sprayed onto a NC membrane using a BioDot dispensing apparatus (BioDot XYZ 3000 1414) to form a control region (C) and a test region (T). Any remaining active sites on the membrane were blocked by incubation with 1 % *w*/*v* polyvinyl alcohol for 30 min at room temperature, followed by washing of the strip once with water and then drying. The membrane was then adhered to an adhesive paper plate (2.44×11.81 in., Adhesive Research Inc., Taiwan) with an additional reagent adsorbent pad, a colloidal gold conjugate pad, and a sample application pad. The plate was then cut into 5-mm-wide strips (CM4000 cutter, BioDot) and mounted in a plastic cassette to render the device ready for use.

Sensitivity and specificity of the F1 test strip

The sensitivity of the F1 test strip was evaluated by applying 100 μl of various concentrations of F1 proteins (or *Y. pestis* only) to the device. The sample rose up along the membrane, and colloidal gold was deposited at the site of the solid-phase antibody. In addition, samples containing *Yersinia* spp. (above 10^7 CFU/ml) and other capsule-producing *Enterobacteriaceae* strains were also assayed by F1 test strip for cross reactivity evaluation. On the other hand, with the aim of successfully detecting very low levels of F1 protein (or *Y. pestis*), a silver enhancement process was applied to intensify the colloidal gold-binding signal, as previously described (Shyu et al. 2002, 2010). Briefly, the tested strips were washed once with PBS containing 0.1 % *w*/*v* Tween 20 and twice with distilled water when the strip assay was complete. Then, the washed strips were soaked in the silver enhancer reagent (Sigma, St. Louis, MO, USA) for 5 min and fixed with sodium thiosulfate at room temperature.

Evaluation of the F1 test strip with simulated samples

To evaluate the F1 test strip in various, naturally occurring conditions, three different matrices were used as *Y. pestis* diluents to mimic (simulate) these naturally occurring situations. The samples were prepared by the addition of 10^5–10^7 CFU of *Y. pestis* with the appropriate volume of human serum (1:5 in PBS), skimmed milk powder (5 % *w*/*v* in PBS), and homogenized flea slurry (20 fleas in 1 ml PBS). The serum required dilution as it was too viscous in the undiluted state to flow through the membrane. Control samples were prepared by direct dilution of the various matrices with PBS. All simulated samples (100 μl) were assayed on F1 test strips.

Results

Identification and Western blot assay of the expressed F1 recombinant protein

After purified and dialyzed against PBS, the expressed F1 recombinant protein was then assayed by SDS-PAGE (Fig. 1b), and the results were consistent with the predicted molecular weight (15.5 kDa) of F1 protein. Furthermore, the Western blot also showed that this expressed protein could be recognized by killed-plague-vaccine immunized mouse serum (Fig. 1c).

On the other hand, another Western blotting assay was carried out to analyze the specificity of anti-F1 rabbit serum (as shown in Fig. 2). Three rabbits were immunized in

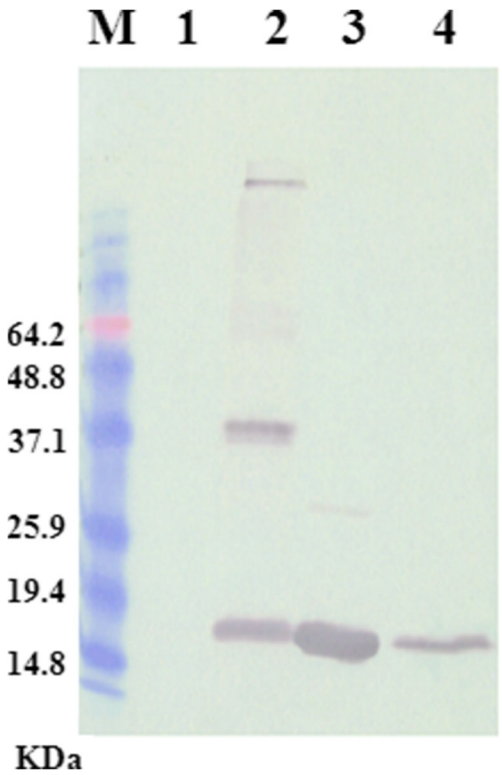

Fig. 2 The specificity of rabbit anti-recombinant F1 serum. Various samples were assayed by SDS-PAGE (12 % gel) and probed with rabbit anti-recombinant F1serum by Western blotting. *Lane M*, marker; *1*, pellet of *E. coli* host (TOP10); *2*, pellet of killed-plaque-vaccine; *3*, *E. coli* (pUC18F1) culture supernatant; *4*, *E. coli* (pUC18F1) pellet

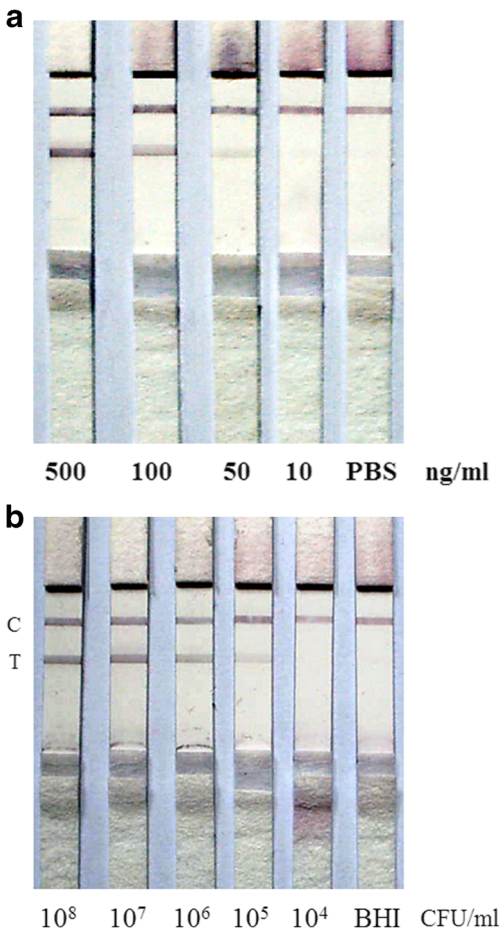

Fig. 3 Sensitivity test of *Y. pestis* F1 strips. **a** A series of dilutions (10–500 ng/ml) of F1was prepared in PBS. The detection limit of F1 was 50 ng/ml. **b** A series of dilutions (10^5–10^9 CFU/ml) of *Y. pestis* strain was prepared in PBS. The detection limit of *Y. pestis* was 10^5 CFU/ml

advance with expressed F1protein, and the serum (anti-F1 rabbit serum) titers obtained were all higher than 120,000 (data not shown). In Fig. 2, the pellet of *E. coli* (TOP 10), the pellet of killed-plague-vaccine, both the culture supernatant and pellet of *E. coli* (pUC18F1), were all probed with the anti-F1 rabbit serum. The results showed that both *E. coli* (pUC18F1) culture broth (lane 3) and pellet (lane 4) could be recognized by anti-F1 rabbit serum. These results suggest that high titer anti-F1 recombinant Pab might be a good candidate for LFA use in the detection of *Y. pestis*.

Sensitivity of the *Y. pestis* test strips

Various samples containing different concentrations of *Y. pestis* and F1 protein were applied to *Y. pestis* test strips (Fig. 3). A positive result was indicated by the appearance of two red lines in the test and control region, whereas a negative result was indicated by the appearance of only one red line in the control region. The analysis was complete in less than 10 min, and the detection limit of F1 recombinant protein was 50 ng/ml (Fig. 3a) and 10^5 CFU/mL of *Y. pestis* (Fig. 3b). The red color intensity in the test region was proportional to pathogen concentrations between the ranges of 20–500 ng/ml of F1 and 10^5–10^7 CFU/ml of *Y. pestis*. In the absence of F1 (PBS only) and *Y. pestis* (BHI only), no immunogold particles were bound to the line of the test region; hence, no red signal could be visualized in this region. In the case of silver enhancement, the strong binding between silver molecules and colloidal gold particles promoted the development of a dark line at the location of the colloidal gold particles. By using silver enhancement, 10^2–10^4 CFU/ml of *Y. pestis* could be detected (Fig. 4). False-positive results were not obtained in any of the assays performed.

Cross-reactivity of the *Y. pestis* test strips

Y. pestis, *Y. pseudotuberculosis*, and *Y. enterocolitica*, as well as other species of the *Yersinia* genus are categorized as zoonotic pathogens and can cause human disease, including plague and the gastroenteritis. Therefore, some species of *Yersinia* were applied onto *Y. pestis* test strips in order to evaluate the cross-reactivity of the strips. Nine species of the *Yersinia* genus, including *Y. pestis*, *Y. mollaretii*, *Y. frederiksenii*, *Y.enterocolitica*, *Y. rucheri*, *Y. intermedia*,

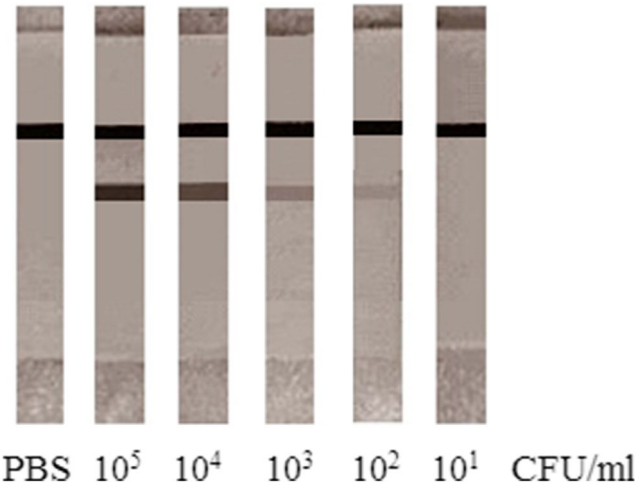

Fig. 4 Silver enhancement of immunochromatographic detection of *Y. pestis*. The tested membranes were immersed in silver enhancer solution to amplify detection limitation. Sensitivity of the F1 strip can be intensified to 10^2 CFU/ml of *Y. pestis* after the silver enhancement

Y. aldovae, *Y. pseudotuberculosis*, and *Y. rohdei* were applied to the strips. For each individual sample, 10^7 CFU/ml of culture supernatant was analyzed on the strip. As shown in Fig. 5a, only the strain *Y. pestis* obtained a positive result, whereas all other *Yersinia* strains did not present with any red signals in the test region of the strip (i.e., negative results were obtained). The cross-reactivity of some capsule-producing *Enteobacteriaceae* on the F1 strip was also examined. Samples from four different *Enteobacteriaceae* strains were applied to the strip individually, and the results demonstrated that only *Y. pestis* yielded positive results in the form of a red band in the test region (Fig. 5b).These results suggest that, although a Pab was used in the F1 strip kit for the assays, a distinct specificity can still be attained from the strips.

Detection of *Y. pestis* in various matrices

Simulated samples (*Y. pestis* diluted in human serum, milk powder, and flea slurry) were assayed using the *Y. pestis* test strips to evaluate the effect of these emulated, naturally occurring conditions. The results showed that the detection sensitivity was unchanged in serum, homogenized flea, and milk powder (Fig. 6). Besides this, none of the blank simulated samples yielded positive results. Furthermore, the detection limit (10^5 CFU/ml) obtained for all three simulated samples was equivalent to that of *Y. pestis* in PBS, suggesting that the strips were suitable for use in these simulated sample conditions.

Discussion

Although *Y. pestis* no longer causes pandemic disease, currently the bacterium remains a serious public health problem

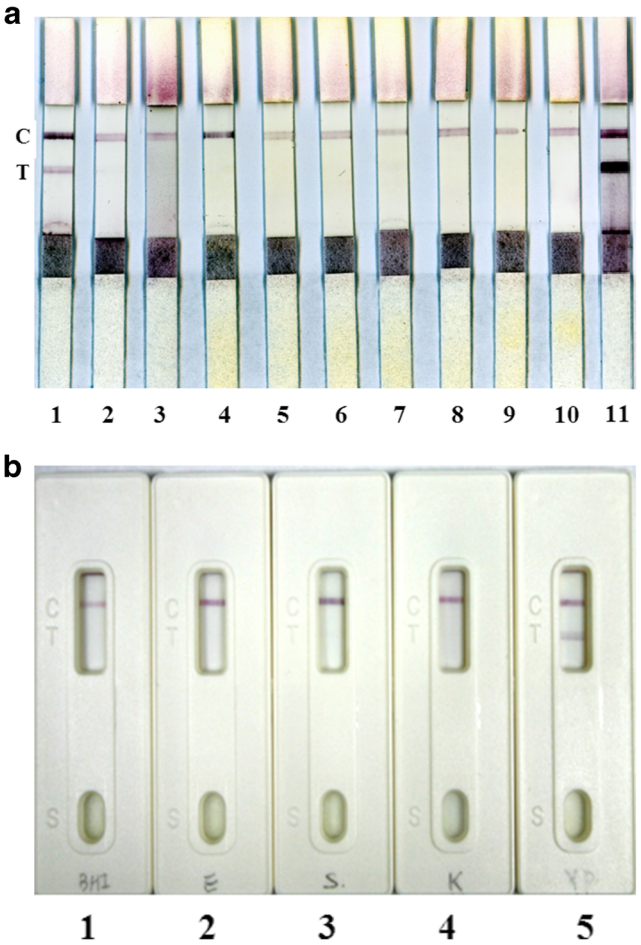

Fig. 5 Cross-reactivity of *Y. pestis* F1strips. **a** Various *Yersinia* spp. (more than 10^8 CFU/ml) were analyzed by *Y. pestis* F1 strips. *Lane 1*, 1 μg/ml F1; *lane 2*, brain heart infusion broth; *lanes 3–10*, the tested *Yersinia* spp., including *Y. enterocolitica* (ATCC 27729), *Y. frederiksenii* (ATCC 29912), *Y. intermedia* (ATCC29909), *Y. mollaretii* (ATCC43969), *Y. pseudotuberculosis* (ATCC29910), *Y. rohdei* (ATCC43380), *Y. aldovae* (ATCC35236), and *Y. rucheri* (ATCC29473), respectively; *lane 11*, *Y. pestisyreka* strain IPM00722. **b** Various capsule-producing *Enteobacteriaceae* strains were applied onto *Y. pestis* F1 strips. *1*, Brain heart infusion broth; *2*, *E. coli* ATCC12710; *3*, *Salmonella typhi* ATCC167; *4*, *Klebsiella pneumonia* ATCC8044; *5*, *Y. pestisyreka* strain IPM00722

in the Far East and Asian subcontinent. During World War II, plague was initially used as a biological weapon in warfare, but over time, with the increasing risk of bioterrorism, the effective transmission and the deadly potential of plague made governments and civilians around the world reconsider the potential dangers of *Y. pestis*, especially as this highly dangerous bacterium can be easily obtained from infected patients. Once been infected, the amount of *Y. pestis* in blood ranges from 10^6 to 10^8 CFU/ml (Norkina et al. 1994). The traditional diagnosis for *Y. pestis* confirmation is by cultivation of the bacteria, which requires at least 24 h in a BSL-3 facilities. Therefore, to develop a rapid, highly sensitive and specific tool to monitor the bacterium is greatly desirable. Unfortunately, current plague detection techniques do not

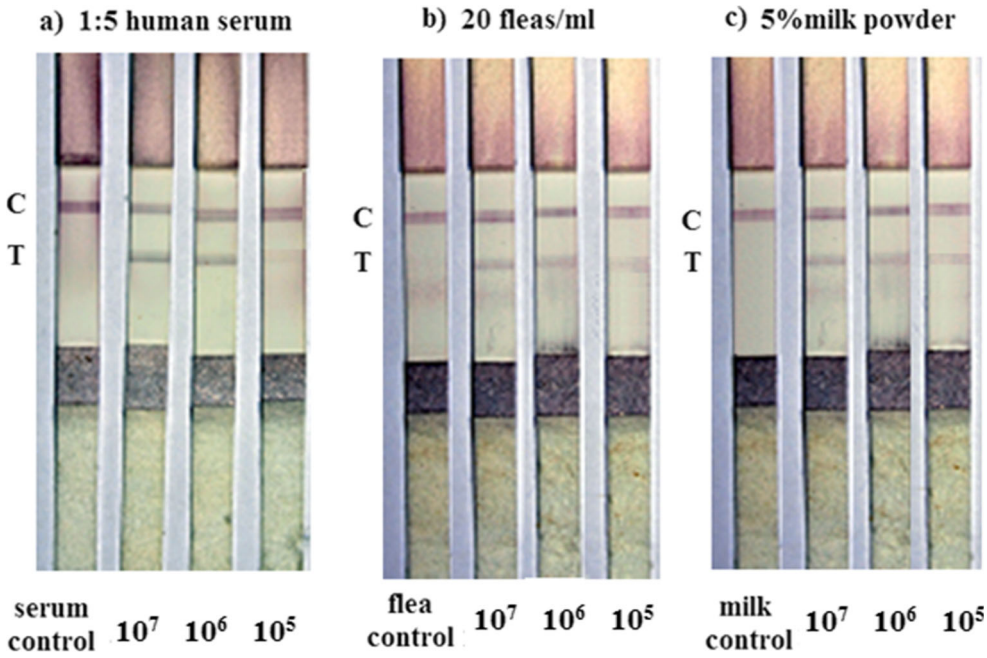

Fig. 6 Detection of *Yersinia pestis* in various matrices. Simulated samples were assayed by *Y. pestis* F1 test strip. All the *Y. pestis* containing samples were diluted in the control reagents that diluted in PBS (**a** serum 1:5 dilution; **b** flea slurry (20 fleas/ml in PBS); **c** milk powder, 5 % *w*/*v*). All the simulated samples have same detection limit (10^5 CFU/ml), which was the same as that of *Y. pestis* in PBS

sufficiently meet these requirements, and besides this fact, most of these techniques require sophisticated equipment and trained personnel. In this paper, a lateral flow assay for *Y. pestis* detection was developed. In 2007, Tomaso et al verified that among several immunographic assays, including LFA, ELISA, flow cytometry and immunofluorescence microscopy, the LFA proved to be the best identification technique. In LFA, when F1-containing samples were applied, the F1 protein initially reacted with anti-F1-Pab coated on colloidal gold particles and then reacted with the same Pab fixed on the membrane. These reactions resulted in the appearance of a red line in the detection zone, with intensity proportional to the F1 concentration. Formation of a red line in the control region but not the test region was designated as a negative result, while no red line in the control region was treated as an invalid result and thus need to be reanalyzed.

Immunochromatographic assays using blue latex and colloidal gold have been described previously (Horton et al. 1991; Birnbaum et al. 1992; Sha et al. 2011; Tomaso et al. 2007). In our experience, the use of immunogold detection has three advantages. Firstly, the colloidal gold nanoparticles have better mobility than the blue latex particles when in the porous nitrocellulose membrane. Second, colloidal gold particles are less susceptible to aggregation during the preparation of the test device. Third, the enhancement procedure used with the gold particles may improve the assay sensitivity. The nonenhanced strip assay system and blue latex assays provide similar detection limits; however, the sensitivity of immunogold detection can be increased approximately 1000-fold with the enhancer described above (Fig. 4). In contrast, since the Pab-based *Y. pestis* (F1) test strips had no cross-reaction with other *Yersinia* spp. or other capsule-producing *Enteobacteriaceae* strains, even when 10^7 CFU/ml of bacteria was tested, suggesting that these strips have high specificity for *Y. pestis*.

In this study, several mouse induced anti-F1 MAbs had been applied to develop the LFA before the rabbit anti-F1 Pabs were employed. These anti-F1 MAbs were initially used in an attempt to screen for suitability as a strip assay kit; however, none of the MAb pairs could provide high assay sensitivity (data not shown). Therefore, the anti-F1 Pab was applied instead, to develop the immunochromatographic assay.

The anti-F1 Pab was absorbed by colloidal gold particles as a detection antibody and was also strongly immobilized on the NC membrane as a test antibody. To reduce the nonspecific reaction between antibody and antigen, any remaining protein binding sites on the membrane were blocked with chemicals. Under this format, the detection of *Y. pestis* using this device requires approximately 10 min (we believe that the chances of error increase after 10 min of incubation), a time which is much quicker than that required by an ELISA or radioimmunoassay (Shyu et al. 2002, 2010); besides, the results can be read directly with the naked eye. The user-friendly format, rapid results obtainment, lack of requirement for sophisticated equipment and relatively low manufacturing costs render the assay ideally suited for on-site detection of *Y. pestis*.

Previous studies have mentioned that for plaque diagnosis, hand held kits in detecting F1 were developed (Tomaso et al. 2007; Sha et al. 2011), yet none of the best test is commercially available. Under the format of paired polyclonal and monoclonal antibodies, Tomaso et al. proved that LFA using purified F1 from *Y. pestis* EV76 obtained a detection limit of 3.3 ng/ml in serum. On the other hand, the detection limit of

LFA developed in this study was 50 ng/ml, a much lower sensitivity than that of LFA developed by Tomaso, under the usage of one polyclonal antibody only. These sensitivity variances could be relevant to the purity of the F1 antigen and the strains of *Y. pestis* used, as the amount of theF1 on the bacterial surface is not immutable in variant *Y. pestis* strains (Sha et al. 2011).

Moreover, other studies (Shyu et al. 2010) have shown that in the LFA, some components may interfere with the antigen-antibody reaction in urine, serum, or milk, which may result in a weaker signal. In our study of *Y. pestis*, the strips retained sufficient sensitivity to detect *Y. pestis* in milk powder. We chose serum specimens in our study because blood is the most accessible and frequently assayed bodily fluid, and the most dangerous clinical presentations of plague are primarily from pneumonic and septicemic cases in which no buboes are present (Williams et al. 1972). The detection limit when using silver enhancement was 10^2–10^4 CFU/ml of *Y. pestis*, which was close to (or even better than) the sensitivity of mouse bioassay (10^3 CFU/ml, Quenee et al. 2011). This enhanced strip assay is sufficiently sensitive to support effective in vitro *Y. pestis* distribution analysis and for use in field contamination detection, especially in cases of bioterrorist attacks that use plague as a biological weapon.

In summary, this study has developed a polyclonal antibody-based F1 test strip that can rapidly detect *Y. pestis* without cross-reaction with other *Yersinia* spp. The F1 strip assay described here provides device that requires no further separation steps for the detection of *Y. pestis*. Moreover, the assay is superior to other immunoassays, such as radioimmunoassays and ELISAs, with regard to its overall speed and simplicity. These characteristics suggest that the strip assay is sufficiently sensitive to detect *Y. pestis* contamination in clinical or food samples.

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Conflict of interest The authors declare that they have no competing interests.

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