Development of lateral flow immunoassay strip for rapid detection of acute hepatopancreatic necrosis disease-causing PirAB toxins in shrimp

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Abstract

Acute hepatopancreatic necrosis disease (AHPND) is currently the most affected disease of shrimp farms worlwide As rapid and specific detection methods are essential to improve disease management, a lateral flow immunoassay (LFIA) was designed for the detection of $PirA^{vp}$ and $PirB^{vp}$ toxins in shrimp. In this study, polyclonal antibody based lateral flow immunoassay (LFIA) strip has been developed. The recombinant $PirA^{vp}$ and $PirB^{vp}$ toxin-like proteins of *Vibrio parahaemolyticus* (VP_{AHPND}) was used to immunize rabbits with the highest antibody titre after the third booster. This polyclonal rabbit antiserum was purified and used to develop a LFIA strip for the detection of AHPND pathogen. This test strip could detect $PirA^{vp}$ and $PirB^{vp}$ toxins up to 125 ng/mL by naked eye within 15 min. No cross-reactivity was observed with $VP_{non\text{-}AHPND}$. Furthermore, the sensitivity and specificity of LFIA were 94.0% and 98.0%, respectively. This LFIA strip could be used as a handy tool by the farmers and technicians *in situ*.

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Keywords

AHPND, polyclonal antibody, PirAvp and PirBvp toxins, lateral flow immunoassay strip

1 Introduction

In recent years, shrimp production has declined due to the emergence of a bacterial disease known as acute hepatopancreatic necrosis disease (AHPND), which causes high mortality in two commercially cultured shrimp species, including *Penaeus monodon* and *P. vannamei* in many countries. The economic losses due to this disease were estimated over USD 7 billion annually [1]. This disease is mainly caused by *Vibrio parahaemolyticus* that carry a 70 kbp-virulent plasmid encoding for binary toxins similar to the Photorhabdus insect-related (Pir) toxin. In addition, *Vibrio harveyi*, *V. campbelli*, and *V. owensii* which carry one or more extrachomosomal pVA1 plasmids containing Pir toxin genes, has recently caused AHPND in shrimp [2,3]. Therefore, controlling the disease in shrimp farming is to target the plasmid or plasmid-released proteins like toxins for early detection. Most of the commercially available AHPND diagnostic kits are PCR-based [4-7], or real-time PCR techniques [8], yet these kits are time-consuming, require expensive instruments, and not suitable for most shrimp farmers. Therefore, the need for developing reliable on-site diagnostic kits that facilitate rapid and timely diagnosis, and potentially allow farmers to control the disease using appropriate treatments. The aim of this study is to raise the

polyclonal antibodies against PirAvp and PirBvp toxins, thereby developing LFIA strip for the detection and monitoring of AHPND pathogen shrimp status during cultivation.

2 Materials and methods

2.1 Bacterial strains, rabbits and reagents:

AHPND strain of *Vibrio parahaemolyticus* XN89 and non-AHPND strain XN8 were kindly provided by Dr. Saengchan Senapin National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. *V. cholerae*, *V. vulnificus*, *V. alginolyticus*, and White Spot Syndrome virus were kindly provided by Dr. Tuan V. Vo from University of Agriculture and Forestry, Ho Chi Minh City, Vietnam.

Female, about 2.5 kg New Zealand white rabbits were provided by Central Animal House of Pasteur Institute in Ho Chi Minh City, Vietnam.

MyTaqTM Red Mix was purchased of Bioline, USA. Anti-His-Tag antibody (SC-8036) were purchased from Santa Cruz. Affinity column (Ni-sepharose, Hitrap protein G HP column), membrane and pads (sample pad-81132250, conjugate pad-8133-2250, nitrocellulose membrane (NCM)- 10547004, and absorbent pad-8116-2250) were purchased from Cytiva Life Sciences, Sweden. Colloidal gold solution averages 20 nm in size (CG-020) was purchased from DCNovations, USA. Goat anti-rabbit IgG antibody (R5506) was purchased from Sigma, USA. Culture media were purchased from BD. Other chemicals were of analytical grade.

2.2 Polyclonal antibody production against PirAvp and PirBvp toxins

The gene coding for PirAvp and PirBvp toxins was obtained from *V. parahaemolyticus* AHPND strain XN89 by PCR method with specific primers. Next, PirAvp gene and pET22b plasmid were digested with two restriction enzymes, i.e. NdeI and XhoI and ligating using T4 ligase. The ligated product was transformed into *E. coli* DH5α and cultured on Luria broth (LB) agar containing 100 µg/mL ampicillin and incubated overnight at 37 °C. After incubated, the colonies on LB agar were screened by PCR. After that, recombinant vector pET22b- PirAvp was transformed into *E. coli* BL21(DE3) for PirAvp protein expression. IPTG was added into the culture medium with a final concentration of 1 mM and cultured at 37 °C, for 4 hours. The bacterial pellet was collected by centrifugation, followed by SDS-PAGE with Coomassie Blue staining and Western blot probed to confirm the protein expression. The recombinant PirAvp protein was purified by Histrap column HP and dialysed against PBS. The purified PirAvp protein was quantified by Bradford method and verified by SDS-PAGE with silver staining, and analyzed by Gel Analyzer software. The procedure was similar for PirBvp protein.

Polyclonal antibodies were obtained from immunizing rabbits, testing blood, and evaluating titer results by ELISA. Six rabbits were divided two groups (3 rabbits/group) for each individual toxin. The rabbits were immunized by 4 subcutaneously injections (28 days interval each time) with the same dose (300 µg/rabbit) but different adjuvant. An initial injection was given using complete Freund's adjuvant and booster injections were performed using incomplete Freund's adjuvant. Blood was collected before the first injection and two weeks after the third injection. Rabbits sera were determined their titers by ELISA. The serum with high antibody titer was precipitated by 50% ammonium sulphate, dialyzed against PBS, and passed through Hitrap protein G column to purify antibodies. The purified antibodies were determined amount by Bradford assay and analyzed by SDS-PAGE.

2.3 Preparation of pAb-gold Conjugate

pAb-Gold conjugate was performed by the antibodies passive adsorption onto colloidal golds surface. One mL of colloidal gold solution was adjusted pH 9 using 0.2M K_2CO_3. Aliquots of 75 µL of antibody solution at the concentration of 137 µg/mL were added to adjust the pH of gold solution, as instructed by manfacturer. The mixtures were maintained under orbital shaking for 30 minutes at room temperature. Then, 10% BSA solution was added to the suspension and incubated for 1 hour at room temperature. After that, the conjugate solution was centrifuged at 12,000 rpm for 20 minutes. The pellet was resuspended in 5 mM Borate, 1% BSA, pH 9.0, and stored at 4 °C until use. The formation of colloidal gold-polyclonal antibody conjugate was recorded with UV/Vis spectroscopy at the wavelength ranging from 400 to 700 nm (UV1800, Shimadzu).

2.4 Preparation of lateral flow immunoassay strip (LFIA)

LFIA strip was composed of a sample pad, a conjugate pad, a nitrocellulose membrane (NC), an absorbent pad, and an adhesive plastic backing plate. A test line of anti-PirAvp, and anti-PirBvp antibody were dispensed with a lateral flow reagent dispenser (ClaremontBio, USA) on a NC. In order to validate the correct performance of lateral flow tests, a control line of goat anti-rabbit IgG antibody was also dispensed onto the same NC, 5 mm apart from the lines of antibodies. The membrane was dried at 37 °C for 2 hours. The pAb-gold conjugate solution (10 µg/mL) was diluted in 2 mM borate buffer with 5% sucrose and dropped 5 µL on conjugate pad, and the pad was dried at 37 °C for 2 hours. After drying, they were fixed on plastic backing cards coated with a pressure-sensitive adhesive along with sample pad and absorbent pad. Finally, strips were cut to a width of 5 mm using Programmable strip cutter (Shanghai Kinbio Tech Co., Ltd, China). The test strips were stored in a desiccator chamber at 4 °C until use.

2.5 Procedure for LFIA

Sample was prepared in 5 mM phosphate buffer, pH 7.2, 1% Triton X-100 buffer. A volume of 100 µL was dropped on the sample pad of the test strip. LFIA strip were examined visually after 10 to 15 min. The appearance of red bands at the test line and the control line indicated a positive result. Only one colored control line indicated a negative result, as shown in Figure 1.

2.6 Specificity and sensitivity assays

The specificity and sensitivity of the LFIA was determined using VP_{AHPND}-derived PirAvp and PirBvp proteins spiked in shrimp samples. VP_{AHPND}-free shrimps were spiked with bacteria at 5×10^3 CFU/ml and incubated at 30 °C in 30 mL of TSB medium. For negative samples and positive samples, $VP_{non\text{-}AHPND}$ strain XN8 and VP_{AHPND} strain XN89 were added, respectively. After 18 h, shrimp heads were crushed in 5 mL of the running buffer and 100 µL of the supernatant was applied to the test strip. The result was read as instructed above.

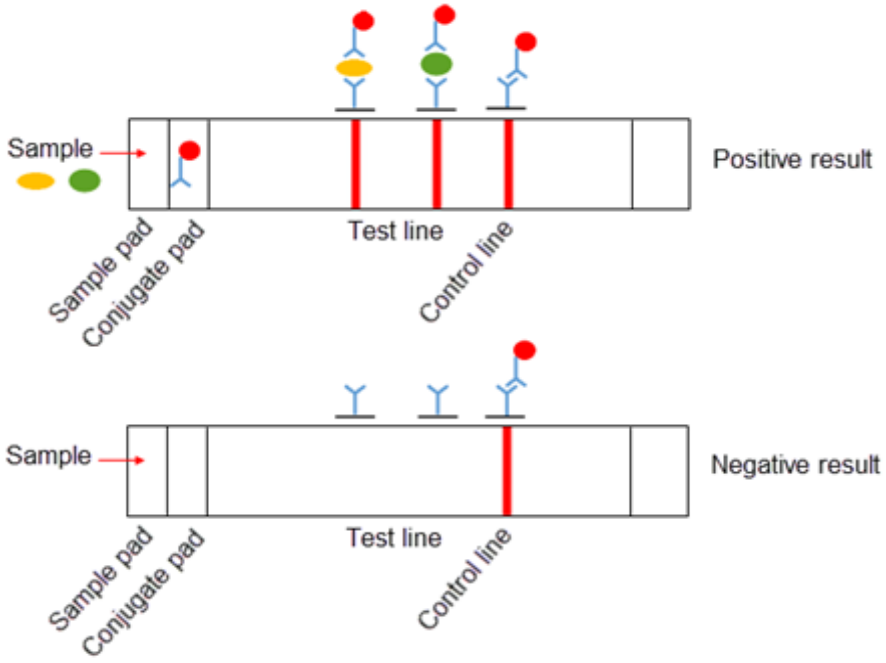

Fig. 1 Schematic of lateral flow immunoassay strip.

3 Results and discussion

3.1 Polyclonal antibody production against $PirA^{vp}$ and $PirB^{vp}$ toxins

Initially, PirAvp and $PirB^{vp}$ toxins were prepared and confirmed the expression of recombinant $PirA^{vp}$ and $PirB^{vp}$ proteins by SDS-PAGE. PirAvp and $PirB^{vp}$ proteins were over-expressed as a recombinant protein about 14 kDa and 50 kDa (Figure 2 A andB), and confirmed indirectly using Western blot probed with Anti-His-Tag antibody at final concentration of 0.08

µg/mL (Figure 2 A*-B*). After that, PirA^{vp} and PirB^{vp} proteins were purified by Histrap column HP with obtained concentration at 0.76 and 2.90 mg/mL. These purified fractions were analyzed using SDS-PAGE with silver staining and Gel Analyzer software (Figure 2C). Evaluation by Gel Analyzer showed that PirA^{vp} and PirB^{vp} proteins in elution fraction had a purity of 94.49% and 97.85%, respectively.

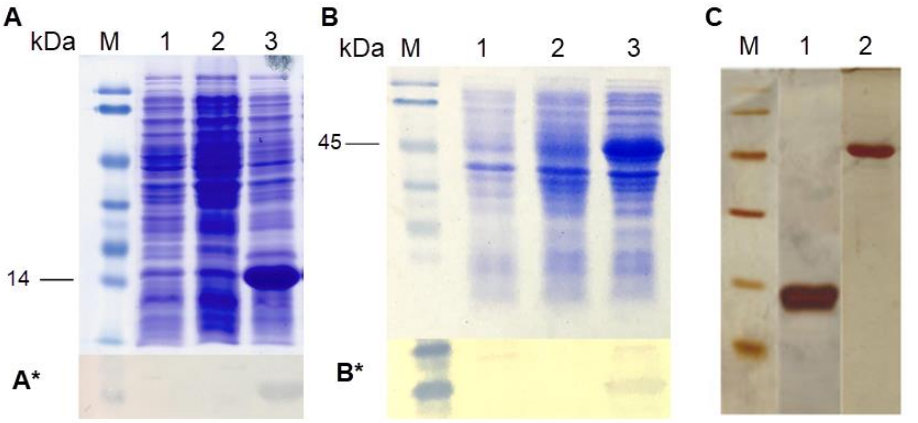

Fig. 2 SDS-PAGE showing the expression of recombinant PirA^{vp} and PirB^{vp} proteins with Coomassie Blue staining (A-B), Western blot (A*-B*), and silver staining (C). In (A-A*): Lane M: protein ladder; Lane 1: *E. coli* BL21(DE3) (+IPTG); Lane 2: *E. coli* BL21(DE3)/pET22b (-IPTG); Lane 3: *E. coli* BL21(DE3)/pET22b-PirA^{vp} (+IPTG). In (B-B*): Lane M: protein ladder; Lane 1: *E. coli* BL21(DE3) (+IPTG); Lane 2: *E. coli* BL21(DE3)/pET22b (-IPTG); Lane 3: *E. coli* BL21(DE3)/pET22b-PirB^{vp} (+IPTG). In (C): Lane M: protein ladder; Lane 1: purified PirA^{vp} protein; Lane 2: purified PirB^{vp} protein.

Rabbits were injected subcutaneously at four booster doses on a monthly basis. Evaluation of immune response to the injections was performed by ELISA. The result showed that the level of antibody started to increas steadily from the 1st injection until the end of the 4th injection (Figure 3A). This result was similar to the published study [9]. The immunogenic competence of recombinant PirA^{vp} and PirB^{vp} proteins were shown in its ability to stimulate humoral response against native PirA^{vp} and PirB^{vp} proteins with the titer of antibody reached up to 1:2,560,000 and 1:5,120,000, respectively (Figure 3B). This titer was higher than the published study [10].

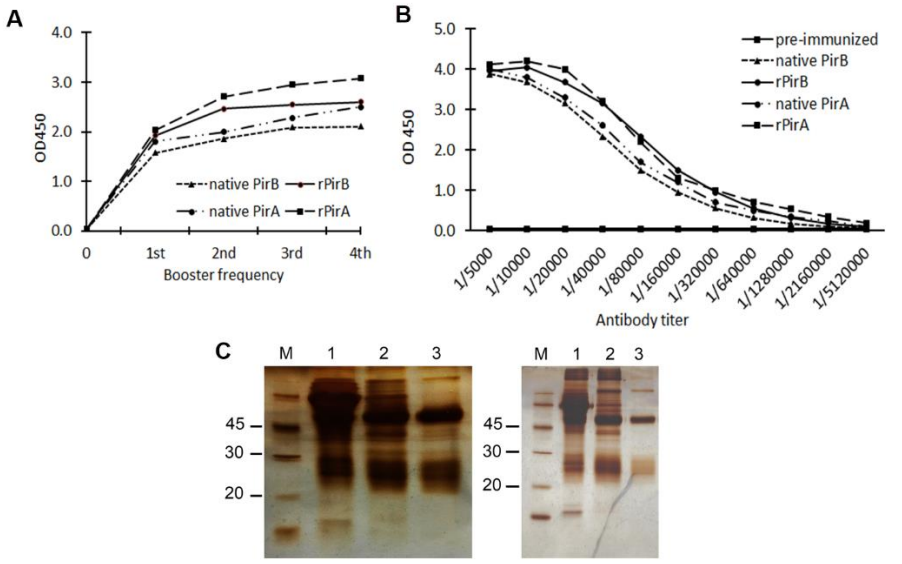

Fig. 3 ELISA for antibody level per booster (A) and for antibody titer (B), and SDS-PAGE of purified antibodies (C). In (C): polyclonal antibodies against PirA^{vp} (left) and polyclonal antibodies against PirB^{vp} (right): Lane M: protein ladder; Lane 1: immunized antiserum; Lane 2: purified IgG with ammonium sulfate; Lane 3: purified IgG with Hitrap protein G.

Further, pAbs were subjected to affinity chromatography purification and found no reactivity to $V_{non\text{-}AHPND}$ (XN89), as well as other *Vibrio* species (*V. alginolyticus*, *V. cholerae*, and *V. vulnificus*), and White Spot Syndrome virus by dot blotting (Figure 4). Therefore, pAbs were further used in developing on-site diagnostic kit. In a recent study, scientists have developed monoclonal antibodies against $PirA^{vp}$ and $PirB^{vp}$ of *V. parahaemolyticus* and characterized them using VP_{AHPND} isolates and other AHPND-free bacterial isolates 012 and developed polyclonal antibodies against $PirA^{vp}$.

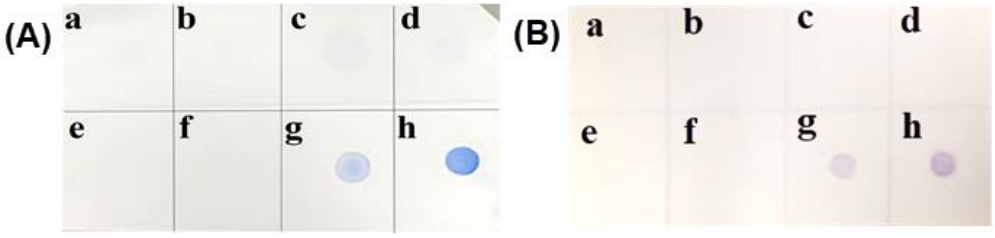

Fig. 4 Dot blot for specific interaction between anti-$PirA^{vp}$ antibody (A), anti-$PirB^{vp}$ antibody (B) and bacterial strains. (a): *V. alginolyticus*; (b): *V. vulnificus*; (c): *V. cholera*; (d): $V_{non\text{-}AHPND}$ (XN8); (e): White Spot Syndrome virus; (f): BSA; (g): *V. parahaemolyticus* XN89; (h): recombinant protein.

3.2 Preparation of pAb-gold conjugate

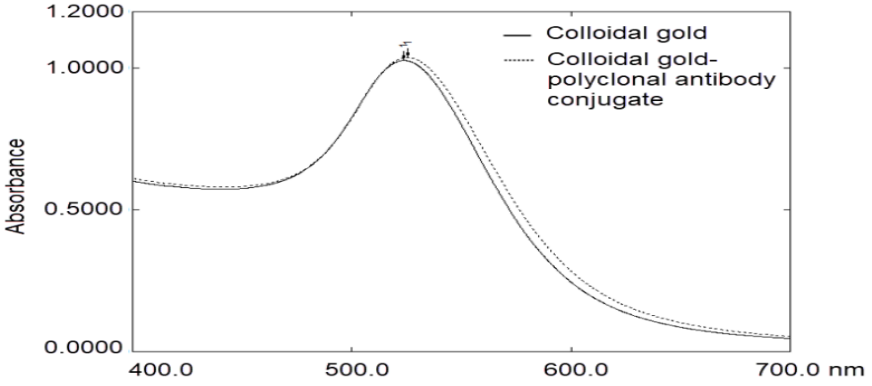

Fig. 5 UV/Vis spectra of colloidal gold and the colloidal gold-polyclonal antibody conjugate.

Preparation of pAb-gold conjugate is one of the most important stages of typical test strip production. Colloidal gold solution is the most widely used label today in commercial LFIA for many reasons. It is relatively easy and inexpensive to prepare in the laboratory. The color is intense, and no development process is needed for visualization. To synthesize pAb-Gold conjugate, physical interaction is the simple method and this conjugate should remain stable with its conjugated parts, as compared to the unbound forms. In the present study, the difference between the colloidal gold particles before and after absorption of antibody was observed by UV-Vis spectroscopy at the wavelength ranging from 400 to 700 nm. The maximum absorption for colloidal gold solution was at the wavelength of 523 nm. After labelling with antibodies, adsorption peak shifted from 523 nm to 526 nm, indicating good protein conjunction (Figure 5).

3.3. Specificity and sensitivity assays

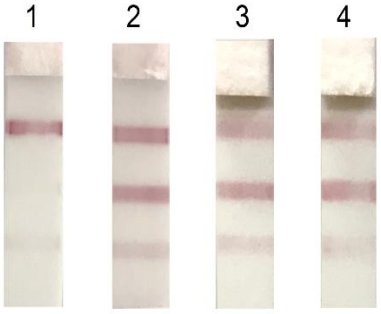

Fig. 6 The performance of the LFIA. 1: negative spiked sample ($VP_{non\text{-}AHPND}$); 2-4: positive spiked samples (VP_{AHPND}). Top band: control band; middle band: $PirB^{vp}$; bottom band: $PirA^{vp}$.

The specificity and sensitivity assays were conducted by adding bacteria to VP_{AHPND}-free shrimps. For negative samples and positive samples, $VP_{non\text{-}AHPND}$ strain XN8 and VP_{AHPND} strain XN89 were added, respectively (Figure 6). A positive LFIA test result was found in 47 out of 50 positive spiked samples and 1 out of 50 negative spiked samples (Table 1). As a result, the sensitivity and specificity of the LFIA were 94.0% and 98.0%, respectively.

Table 1 Sensitivity and specificity of LFIA in spiked shrimp sample.

		LFIA test		**Total**
		Positive	**Negative**	
Spiked sample	Positive	47	3	50
	Negative	1	49	50
Total		48	52	100

4 Conclusion

LFIA based on pAbs-gold conjugate was a more rapid and sensitive assay for AHPND detection as a point-of-care test than other detection methods, as this naked-eye analysis test did not require the skilled personnel and could be used at the farm site. This proposed method was also successfully applied to the detection of $PirA^{vp}$ and $PirB^{vp}$ proteins in spiked normal shrimps within 15 minutes using only one step. However, further studies on bacterial concentration and shelf-life were warranted.

Acknowledgement

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Phát triển que thử phát hiện nhanh độc tố PirAB của vi khuẩn *Vibrio parahaemolyticus* gây bệnh hoại tử gan tụy cấp trên tôm

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Tóm tắt Hiện nay, bệnh hoại tử gan tụy cấp tính (AHPND) gây ảnh hưởng nghiêm trọng nhất đến các trang trại nuôi tôm của một số nước như Trung Quốc, Việt Nam, Malaysia, Thái Lan, Mexico, Philippines, các nước Nam Mĩ, Bangladesh, Hoa Kì, Đài Loan và Hàn Quốc. Để cải thiện việc quản lí dịch bệnh, các phương pháp phát hiện đặc hiệu, nhanh là cần thiết và xét nghiệm miễn dịch dòng chảy bên (LFIA) được thiết kế để phát hiện độc tố $PirA^{vp}$ và $PirB^{vp}$ trong tôm. Nghiên cứu này phát triển LFIA dựa trên kháng thể đa dòng. Các độc tố tái tổ hợp $PirA^{vp}$ và $PirB^{vp}$ từ *Vibrio parahaemolyticus* (VP_{AHPND}) được sử dụng để gây miễn dịch trên thỏ và sau lần tiêm nhắc lại thứ ba, kháng thể thu được có hiệu giá cao nhất. Kháng thể đa dòng này được tinh chế và sử dụng để phát triển que thử LFIA phát hiện bệnh AHPND. Que thử có khả năng phát hiện độc tố $PirA^{vp}$ và $PirB^{vp}$ tại 125 ng/mL trong vòng 15 phút khi quan sát bằng mắt thường. Không có phản ứng chéo với $VP_{non\text{-}AHPND}$. Độ nhạy và độ đặc hiệu của LFIA là 94,0% và 98,0%. Que thử này có thể sử dụng ngay tại ao nuôi tôm bởi những người nông dân và kỹ thuật viên.

Từ khóa AHPND, kháng thể đa dòng, độc tố $PirA^{vp}$ và $PirB^{vp}$, que thử LFIA.