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Rapid immunochromatographic test strip to detect swimming crab *Portunus trituberculatus* reovirus

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ABSTRACT: Swimming crab reovirus (SCRV) is the causative agent of a serious disease with high mortality in cultured *Portunus trituberculatus.* A rapid immunochromatographic assay (ICA) was developed in a competitive assay format and optimized for the detection of SCRV. The gold probe-based ICA test comprised SCRV antigen and goat anti-chicken egg yolk antibody (IgY) sprayed onto a nitrocellulose membrane as the test line and control line, respectively. IgY-gold complexes were deposited onto the conjugate pad as detector reagents. The method showed high specificity with no cross-reactivity with other related aquatic pathogens. The detection limit of the ICA strip was 50 µg ml^{-1}. To evaluate the performance of the ICA test, the strip and an enzyme-linked immunosorbent assay (ELISA) were applied to the same samples (n = 90 crabs). The strip successfully detected SCRV in all of the artificially infected samples. Furthermore, the ICA strip and ELISA tests had high consistency (98.28%). The strip assay requires no instruments and has a detection time of less than 10 min. It is portable and easy to perform in the field. These results indicated that the developed strip could be a promising on-site tool for screening pooled crabs to confirm SCRV infection or disease outbreaks.

KEY WORDS: *Portunus trituberculatus* · Reovirus · Rapid detection · Immunochromatographic strip · Gold nanoparticle label · Competitive assay · Egg yolk antibody (IgY)

INTRODUCTION

The swimming crab *Portunus trituberculatus* (Crustacea: Decapoda: Brachyura), also known as the Japanese blue crab, gazami crab or horse crab, is the most widely fished species of crab in the world (Ren et al. 2013). It is a commercially important species in East Asian countries, particularly in China, Korea and Japan (Wu et al. 2010). Due to its high nutritional value and the increasing market demands for this species, pond-culture of swimming crabs has been developed and spread quickly in the east coast regions of China since the 1990s (Wang et al. 2014). However, swimming crab aquaculture has suffered substantial economic losses in China due to an explosive epidemic with high mortality in recent years. Conventional molecular epidemiological investigations and artificial infection tests have shown that the pathogenic agent of the disease is swimming crab reovirus (SCRV) (Li 2012, Li et al. 2012). The complete SCRV particle is approximately 30 ± 10 nm in diameter, icosahedral and nonenveloped. It cannot infect mud crabs. SCRV can provoke severe hemorrhaging in swimming crabs and causes a mortality rate up to 100% within 10 days in autumn. This disease is a major epidemic that is endangering the swimming crab industry (Li 2012, Li et al. 2012, Fang et al. 2015). Early detection of SCRV levels is crucial for successful monitoring and control of the disease in crab farms.

To date, various methods for the detection of pathogens have been proposed, including receptor-ligand

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binding assays (de Jong et al. 2005), loop-mediated isothermal amplification (LAMP) (Nagamine et al. 2002), polymerase chain reaction (PCR) (Telenti et al. 1993), amplified rDNA restriction analysis-PCR (ARDRA) (Mendoza-Espinoza et al. 2008), enzyme-linked immunosorbent assay (ELISA) (Gan & Patel 2013) and high-performance liquid chromatography-electrospray-tandem mass spectrometry assay (HPLC–MS/MS) (Taylor 2005). However, these approaches require advanced instruments and skilled operators, and the sample preparation is labor intensive and time consuming (Lu et al. 2012). Thus, none of the aforementioned methods are suitable for field detection of SCRV in aquaculture, and it is necessary to develop a rapid and economical method for the detection of such pathogens in the field. The use of the gold immunochromatographic assay for diagnosis is much more advanced and user-friendly in terms of operator simplicity, short assay time, visual evaluation of results, low cost of production and long-term storage, and is suitable for large sample detection (Byzova et al. 2011, Wang et al. 2013).

The immunochromatographic assay (ICA) has been a popular tool for diagnosis since its introduction in the late 1980s. Human pregnancy and urine glucose test strips were the first tests to be commercialized (Bazin et al. 2010). Currently, ICA has gained considerable interest in various areas, including agriculture, environmental science, medicine, veterinary medicine and the food industry (Molinelli et al. 2008, Zhu et al. 2008, Zhou et al. 2009). However, few colloidal gold-based ICA methods have been reported for the detection of aquatic animal pathogens.

A number of studies have replaced IgG with IgY in immunodiagnostics. Chicken IgY is the functional equivalent of mammalian IgG. However, IgY offers many advantages compared to IgG. The IgY antibody is unable to interact with human rheumatoid factors, Fc receptors, and the mammalian complement system and cannot bind protein A or G. Therefore, the application of IgY can eliminate the interference caused by IgG (Dias da Silva & Tambourgi 2010). Due to their evolutionary distance, avians can mount a sufficient immune response against highly conserved mammalian proteins. Moreover, the use of IgY decreases cross reactivity and false positive results (Kovacs-Nolan & Mine 2004). IgY is a more hydrophobic molecule than IgG, which allows IgY to achieve stable adsorption to latex particles (Dávalos-Pantoja et al. 2001). Therefore, IgY is very suitable for colloidal gold immunochromatographic technology. Chicken antibodies also represent an attractive alternative to currently used mammalian IgGs for diagnostic applications due to ethical and economic concerns.

An indirect ELISA method was developed to detect SCRV (X. L. Xiao, D. F. Li, L. P. Zhang unpubl. data); however, this assay is time consuming due to its requirement for stationary washing steps and requires a microplate reader, and is not convenient and portable enough for rapid on-site detection. Following the preparation of a specific IgY antibody against SCRV, the present study focused on developing a rapid one-step immunochromatographic strip to detect SCRV.

MATERIALS AND METHODS

Sources of crab samples and pathogens

All crab samples were collected from the markets and farms of Ningbo, China. The positive and negative controls were confirmed by RT-PCR. SCRV, white spot syndrome virus (WSSV), soft-shelled turtle systemic septicemia spherical virus (STSSSV), and the *Aeromonas hydrophila*, *Edwardsiella tarda* and *Citrobacter freundii* strains were stored in our laboratory. The *Pseudomonas putida* and *Pseudomonas aeruginosa* strains were provided by Professor Chen Jiong's laboratory at Ningbo University. The grass carp reovirus (GCRV), spring viremia of carp (SVC) and large yellow croaker iridovirus (LYCIV) strains were provided by Professor Qian Dong's laboratory at Ningbo University.

Purification of SCRV

SCRV was purified from the tissues of artificially infected swimming crabs as described previously (Xie et al. 2005, Li 2012). Briefly, the tissues of infected crabs were collected and homogenized in ice-chilled TNE_2 buffer (50 mM Tris-HCl, 400 mM NaCl, and 5 mM EDTA, pH 8.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and then centrifuged at $8000 \times g$ for 10 min at 4°C. The supernatant was collected and centrifuged at $38\,000 \times g$ for 1.5 h at 4°C. Then, the upper loose pellet was rinsed out carefully, and the lower white pellet was suspended overnight in 30 ml of TM_2 buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, and 15‰ NaCl, pH 7.5) containing 1 mM PMSF. After centrifugation at $3500 \times g$ for 5 min, the virus particles were precipitated by centrifuging at $38\,000 \times g$ for 1.5 h at 4°C. Again, the upper loose pel-

let was rinsed out carefully, and the lower white pellet was suspended in 30 ml of TM_2 buffer. These differential centrifugations and rinse steps were repeated 3 times to remove impurities. The final white pellet was suspended in PBS (0.01 M, pH 7.2) and stored at −80°C prior to use. In this experiment, the tissues of uninfected crabs were used as the control, but no white pellet could be obtained. The purity of the isolated virus was verified by negative-staining transmission electron microscopy (TEM) (Hitachi H-7650). The concentration of the virions was evaluated using the Nanodrop 2000c (Thermo).

Preparation of anti-SCRV chicken IgY

Specific pathogen free (SPF) hens (25 wk old) were immunized with purified SCRV antigen mixed with Freund's adjuvant in a 1:1 ratio on days 0, 14, 21, 28 and 45. Each single dose of the immunogen was 500 µg per hen. Freund's complete adjuvant (FCA) (Sigma) was used for the first immunization and Freund's incomplete adjuvant (FIA) (Sigma) was used for the subsequent booster injections. After the immunizations, the eggs were collected daily, marked and stored at 4°C for the next use.

IgY was isolated from the egg yolk via the water dilution method (Zhou et al. 2012). The egg yolk was diluted 10-fold with acetic acid–sodium acetate (0.05 M, pH 5.0) and mixed thoroughly. The suspension was incubated overnight at 4°C and centrifuged at $10\,000 \times g$ for 20 min. Subsequently, saturated ammonium sulfate was added to the supernatant to a final concentration of 40%. The mixture was maintained under stirring for 6 h prior to centrifugation at $12\,000 \times g$ for 20 min at 4°C. The precipitate was dissolved in double-distilled water at half of the initial volume, followed by the addition of saturated ammonium sulfate. The mixture was stirred for 4 h and then centrifuged again ($12\,000 \times g$, 20 min, 4°C). The obtained IgY precipitate was suspended in 5 ml of PBS (0.01 M, pH 7.2) and dialyzed against this same solution overnight. The purity of IgY was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the titer was evaluated by ELISA. The concentration of IgY was determined using the Nanodrop 2000c.

Preparation of gold nanoparticles

Colloidal gold nanoparticles with a 20 nm average diameter were produced following the procedure of Frens (1973) with a slight modification. In this study, several different diameters of gold nanoparticles were prepared by adding different volumes of trisodium citrate. Briefly, 100 ml of 0.01% gold chloride trihydrate solution was heated to reflux, and then 2, 1.8, 1.5, and 1.2 ml of 1% trisodium citrate solution was added rapidly with constant stirring. After the color changed to wine red, the solution was boiled for another 10 min. The preparation was cooled and restored to its original volume. The solution was stored at 4°C in a dark bottle with 0.02% sodium azide. The average diameters of the obtained gold particles were estimated with TEM. The maximum absorption (λ_{max}) wavelengths of the colloidal gold were scanned using a spectrophotometer (Biochrom) in the visible spectrum.

Preparation of colloidal gold-IgY conjugates

The pH of the colloidal gold was adjusted to 5.4 with 0.1 M K_2CO_3. A series of 6 dilutions (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg ml^{-1}) of anti-SCRV IgY antibodies were prepared. A total of 10 µl of each concentration was added to 1.0 ml of colloidal gold solution. The mixtures were incubated for 15 min at room temperature. Subsequently, 100 µl of 10% NaCl was added to each tube and incubated for 15 min. The lowest antibody concentration which prevented a color change was chosen as the minimum concentration for conjugation.

For conjugation, the IgY solution was used at 20% excess of the minimal concentration. With gentle stirring, purified IgY antibodies (using their selected concentration above) were added drop by drop to 100 ml of pH-adjusted colloidal gold solution. The mixtures were gently mixed for 30 min and blocked with 10% BSA solution (final concentration 1%) for 30 min. The free gold pellets were removed by centrifugation at $1000 \times g$ for 15 min at 4°C. Subsequently, the supernatant was centrifuged at $18\,000 \times g$ for 1 h at 4°C to remove unbound antibodies. The sediments were then re-suspended with 25 ml of PBS (0.01 M, pH 7.2) containing 1% BSA by another centrifugation. Finally, the gold pellets were suspended in PBS (0.01 M, pH 7.2) containing 5% sucrose, 1% trehalose and 1% BSA. A final concentration of 0.02% sodium azide was added for long-term storage, and the probes were stored at 4°C for use. The conjugation was evaluated by UV visible spectroscopy at wavelengths from 400 to 700 nm to ensure that the antibody and colloidal gold connected successfully.

Preparation of the immunochromatographic strip

A schematic diagram and the composition of the immunochromatographic strip are shown in Fig. 1. The colloidal gold probe solution (30 µl) was deposited onto conjugate pads and dried in a vacuum freezing dryer (Labconco) for 2 h. SCRV was diluted with PBS (0.01 M, pH 7.2) containing 5% trehalose and 1 mM PMSF to protect the antigen. The test and control regions comprised the SCRV antigen and goat anti-chicken IgY, respectively. The distance between the test and control line was approx. 5 mm. The sample pads and conjugate pads were treated with PBS (0.01 M, pH 7.2) containing 1% BSA, 1% trehalose, 2.5% sucrose, 1% Tween-20, and 0.02% sodium azide for 30 min and dried at 37°C prior to use. The sample pad, conjugate pad, nitrocellulose membrane and absorbent pad were overlapped and pasted onto a PVC plate in sequence. Then, the well-assembled plate was cut into 4 mm-wide strips. All strips were sealed in a plastic bag and stored at 4°C under dry conditions.

Principle of strip and test determination

A competitive immunoassay format was used in the present study. The analyte in the sample and the immobilized analyte (test line) compete for the limited binding sites of the gold-labeled antibody. When the test solutions (100 µl) are dripped onto the sample pad, the liquid migrates along the nitrocellulose membrane driven by capillary action into the absorbent pad. The antibody-gold complexes can be dissolved and flow forward with the sample liquid. The results are observed after 10 min by the naked eye. If the target analyte (SCRV) exists in the sample, the gold-labeled conjugate reacts with the SCRV in the sample first, leaving few conjugates to bind with the antigen coated on the test line. Therefore, a deeper color intensity of the test line indicates a lower antigen concentration in the test sample. The visual limit of detection (LOD) was defined as the minimum concentration that resulted in the complete invisibility of the test line and the detection of only one red line (the control line) for the positive samples. If there was no SCRV in the sample solution, the gold-labeled IgY complexes were captured by the antigen (SCRV) coated on the test line and the goat anti-chicken IgY on the control line. Thus, the negative result was judged by the appearance of 2 red bands in the test and control zones. The test strip was considered to be invalid if only the test line or no line was present.

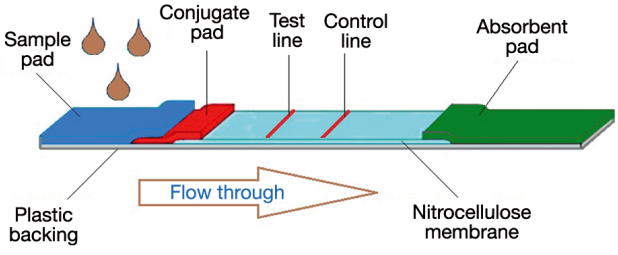

Fig. 1. Schematic diagram of the immunochromatographic assay device

Detection limit of the test strip

SCRV standard solutions at concentrations of 0, 10, 20, 40, 50, 75 and 100 µg ml^{-1} were prepared by diluting purified SCRV with PBS buffer to determine the sensitivity of the strip. Five preparations for each concentration were tested. Each dilution was applied to the test strip, and then the detection limit was determined.

Cross-reactivity of the test strip

The specificity of the strips was tested in a cross-reaction study with several different viruses (GCRV, SVC, LYCIV, WSSV, STSSSV), and bacteria (*P. putida*, *P. aeruginosa*, *Vibrio anguillarum*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and *C. freundi*). Each virus was prepared at a concentration of 100 µg ml^{-1} and each bacterium was prepared at a concentration of 10^7 CFU ml^{-1} in PBS buffer. SCRV-infected crabs were used as positive controls, while PBS buffer and healthy swimming crabs were used as negative controls. The assay was performed with the developed strips and ELISA to evaluate the cross-reactivity.

Detection of SCRV in swimming crabs using ELISA

The methods of ELISA described by Couzens et al. (2014) were followed. First, high-binding 96-well plates were coated with 200 µl of the sample supernatants and incubated overnight at 4°C. Each supernatant was added in duplicate to 2 different wells. Carbonate buffer (CBS) (0.05 M, pH 9.6) was used as a blank control. After 3 washes with PBS containing 0.05% Tween 20 (PBS-T), 100 µl of 1% BSA was added and incubated for 1 h at 37°C. After 3 washes with PBS-T, 200 µl of a 1:2000 dilution of purified

anti-SCRV IgY in diluting buffer (PBS-T containing 1% BSA, PBS-T/B) was added to alternate columns of wells, with common IgY used as the control. The plates were incubated for 2 h at 37°C and washed 3 times with PBS-T. A total of 100 µl of horseradish peroxidase-conjugated goat anti-chicken IgY diluted 1:10 000 in PBS-T/B was added to the wells and incubated for 1 h at 37°C. After 5 washes with PBS-T, the color was developed using *o*-phenylenediamine/H_2O_2 and stopped after 15 min by the addition of 50 µl of 2 M H_2SO_4. The absorbance in each well was read at 492 nm. The net optical density (OD) for each sample tested was obtained by subtracting the corresponding OD values obtained in the wells with CBS buffer. The final result for each sample was evaluated using the formula: p = OD experiment ÷ OD control. The sample was considered to be positive when p > 2.1, and negative when p < 1.5. The sample was considered to be weakly positive between p = 1.5–2.1.

Analysis of consistency between ICA and ELISA

The specificity of the ELISA was tested in a cross-reaction study with several of the different viruses and bacteria described above. ELISA exhibited good specificity (no cross-reaction with other related aquatic pathogens) and was consistent with the strip. The sensitivity of the ELISA was 0.35 µg ml^{-1}. To evaluate the performance of the ICA strip, the strip and ELISA were applied to detect the same samples.

A total of 40 swimming crab samples were collected from local markets and farms in Ningbo (Zhejiang Province, China). To increase the number of infected samples, 50 healthy crabs were artificially infected with SCRV via injection and tissue-sampled post-mortem. A total of 3 g of tissue (gill, hepatopancreas or muscle) from each crab was homogenized in 5 ml of PBS buffer and centrifuged at 3000 × *g* for 5 min at 4°C. The supernatants were collected as the test samples. All of the samples were analyzed 5 times using the test strips and ELISA, respectively. The consistency rate was expressed as a percentage of the ratio of the number of positive results between the strip and ELISA.

RESULTS

Preparation of SCRV and anti-SCRV chicken IgY

TEM observations revealed that the purified virions were pure and intact (Fig. 2) (Fang et al. 2015).

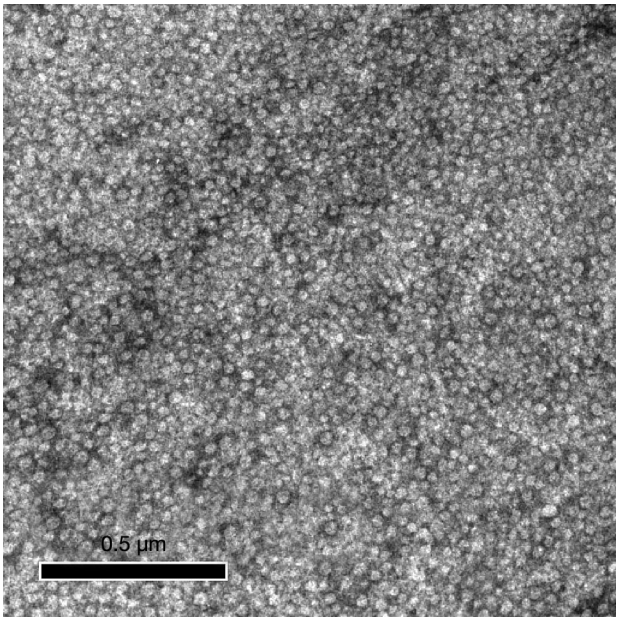

Fig. 2. TEM image of purified, intact swimming crab reovirus virions

The concentration of the virions was 5.8 mg ml^{-1}. Five weeks after immunization, the anti-SCRV IgY titer in the egg yolk determined by ELISA was 1:128 000.

Optimization of assay conditions

Because the whole immunoassay was based on the interaction of the colloidal gold and antibody through hydrophobic and ionic bonds, the amount of antibody coating the colloidal gold (which was one of the most important components for the successful performance of the strip) needed to be optimized (Chen et al. 2007). Preliminary experiments were performed to search for the optimal concentration of IgY antibody based on the color of the colloidal gold solution. The optimum concentration of anti-SCRV chicken IgY coated onto the colloidal gold was found to be 10 µg ml^{-1}, which provided the best stabilization of the conjugates. Different diameters of colloidal gold nanoparticles were used to conjugate IgY antibodies. As a result, the 20 nm colloidal gold nanoparticles were found to be sensitive and possessed the best stability compared to the other diameters. Furthermore, various concentrations of coating antigens (SCRV) and goat anti-chicken IgY were optimized to balance the color intensity of the test line and the control line. The concentrations of SCRV and goat anti-chicken IgY that resulted in test and control

lines with the same color intensity were found to be 2.5 mg ml^{-1} and 0.4 mg ml^{-1}, respectively.

Characterization of the colloidal gold and the colloidal gold probe

Colloidal gold solution was prepared by the reduction of chloroauric acid with trisodium citrate. To examine the size and distribution of the gold particles, the colloidal gold solution was scanned under a TEM. The TEM image revealed that the average diameter of the colloidal gold particles was approx. 20 nm. The gold particles were almost all the same diameter and were well-dispersed, which provided an ideal condition for antibody conjugation. The conjugation was confirmed by UV visible spectroscopy. The results revealed that the naked colloidal gold only had one maximum absorbance at 522 nm, which was the characteristic absorbance peak of colloidal gold. There was a red shift in the maximum absorption wavelength of gold-labeled IgY (528 nm) compared with the naked colloidal gold nanoparticles (522 nm) (Fig. 3), which demonstrated that anti-SCRV IgY was successfully labeled.

Visual limit of detection of the test strip

The detection capacity of the assay was characterized for samples containing different concentrations of SCRV. The results were determined to be positive or negative on the basis of the absence or presence of the red colored test line. The test line disappeared when the concentration of SCRV was 50 µg ml^{-1} or more (Fig. 4), indicating that the visual limit of detection (LOD) of the ICA was 50 µg ml^{-1}.

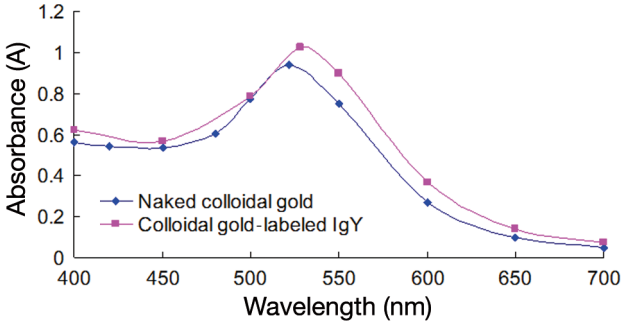

Fig. 3. Visible spectroscopy of naked colloidal gold and colloidal gold-labeled IgY. The red shift in the maximum absorption peak illustrated that the anti-SCRV IgY was successfully labeled

Fig. 4. Detection limit of the immunochromatographic strip. SCRV standard solutions were tested at concentrations of 0, 10, 20, 40, 50, 75 and 100 µg ml^{-1} (from left to right). The test line disappeared (i.e. showing a positive result) at concentrations above 50 µg ml^{-1}

Cross-reactivity of the test strip

The selectivity of the immunochromatographic strip was evaluated in comparison with other aquatic pathogens. The symptoms of SCRV can be associated with distinct viruses such as GCRV, SVC, LYCIV, STSSSV or WSSV or bacterial pathogens such as *P. putida*, *P. aeruginosa*, *V. anguillarum*, *V. parahaemolyticus*, *V. alginolyticus* or *C. freundii*. All of the above pathogens were tested by the ICA strips and ELISA, but yielded negative results; in contrast, SCRV yielded positive results. Thus, the test strip had no cross-reactivity with these examined pathogens. Furthermore, this result demonstrated the specificity of the strip for SCRV detection.

Stability of the test strip

The stability of the assay was determined by running the immunochromatographic strips from the same batch after storage for 1 wk and 1, 2, 3 and 6 mo at 4°C with a zero concentration of SCRV. The intensity of the red color on the test line and control line after 3 mo was exactly the same as the assays performed after 1 wk. However, the density of the red color in the test region and control region after 6 mo seemed to be slightly weaker compared to the tests

performed after 1 wk, although the loss of the density of the red lines was not obvious (Fig. 5). There was no doubt that the immunochromatographic strips were still usable after 6 mo of storage at 4°C.

Analysis of consistency between ICA and ELISA

To evaluate the accuracy rate and practicability of the SCRV test strip in real samples, 50 artificially infected crabs and 40 different swimming crab samples from local markets and farms in Ningbo were collected and analyzed using the ICA test strips and ELISA (see Table 1). A total of 7 samples from endemic areas and 50 artificially infected samples showed positive results in the ICA tests. The other samples gave negative results, indicated by 2 red lines on the ICA strip. The ELISA and ICA agreed in 32 out of 32 negative cases and 57 out of 58 positive cases (Table 1). Therefore, among the 90 samples, the ICA and ELISA results agreed for 89 crabs, with 1 discrepancy; and the ICA strip had a positive accuracy rate of 98.28% (57/58). Although ICA exhibited lower sensitivity than ELISA, it was a more cost and time effective procedure and did not require special equipment. Therefore, ICA can be applied on-site as a preliminary screening method for rapid SCRV detection in crab samples.

Fig. 5. Stability test of the immunochromatographic strips after storage at 4°C for (from left to right) 1 wk and 1, 2, 3 and 6 mo

Table 1. Comparison of immunochromatographic assay (ICA) test strips and enzyme-linked immunosorbent assay (ELISA) methods for the detection of SCRV in crab samples

Sample source	Total number of samples	ELISA positive/ negative	ICA positive/ negative
Local supermarket	8	0/8	0/8
Vegetable market	8	0/8	0/8
Healthy farm 1	8	0/8	0/8
Healthy farm 2	8	0/8	0/8
Endemic farm	8	8/0	7/1
Artificially infected samples	50	50/0	50/0

DISCUSSION

In the present study, a rapid gold-based immunochromatographic assay (ICA) for SCRV detection in swimming crab samples was successfully developed and optimized based on the preparation of a special IgY antibody against SCRV. This report represented the first time that the ICA was applied for the detection of SCRV.

Reovirus has been identified as a causative agent of mass mortality of cultured swimming crabs (Li 2012, Li et al. 2012). Recently, SCRV has resulted in great economic losses to the crab industry. Therefore, a simple, efficient and fast method to detect SCRV in swimming crabs is greatly desired. The strip developed here met all of these requirements, because the results could be obtained within 5–10 min in the field without any instrumentation.

To evaluate the accuracy rate and the practicability of the strip, a total of 90 different crabs were analyzed using the test strips and ELISA, and showed the consistency rate was 98.28%. Among the 90 samples, only 1 sample tested negative using the strip whilst testing positive using ELISA. The failure to detect SCRV in 1 crab sample by the strip might be because the crab was very lightly infected and the virus concentration was below the detection limit. It is generally recognized that ICA strips are less sensitive than ELISA or PCR (Lui et al. 2002, Chaivisuthangkura et al. 2014). For example, a recent publication on ICA strips for *Pemo*NPV detection demonstrated a sensitivity that was 200-fold lower than the 1-step PCR assay (Wangman et al. 2012). The sensitivity of the ICA strip for YHV detection was 500-fold lower than RT-PCR, with all 55 samples giving positive results by RT-PCR and only 36 samples showing positive results with the test strip (Sithigorngul et al. 2007).

Finally, the WSSV strip test failed to detect samples prior to 12 h post-injection, but RT-PCR provided positive results for all samples collected 1–8 h post-injection (Powell et al. 2006). These results were similar to the results in this report in which one lightly infected sample gave negative results with the SCRV test strip but was positive by ELISA. While ELISA exhibited higher sensitivity (0.35 µg ml^{-1}) than the strip (50 µg ml^{-1}), it entailed various disadvantages, including complicated washing steps, long time of assay (5–8 h) and a requirement for a microplate reader and professional staff. However, the time required to perform the strip assay is significantly reduced (5–10 min) without any requirements for instruments and professional staff. The short time coupled with its simplicity, cost-effectiveness and long-term storage make the strip assay a more attractive method for field tests.

Additionally, 100% of the artificially SCRV-infected samples gave positive results, demonstrating that this developed strip could be used to screen SCRV infections in pooled crab samples. In aquatic disease detection, group detection has gained more attention than individual detection. Once SCRV is detected in some samples, the group of crabs should be processed to avoid further expansion. The accuracy rate of the strip reached 98.28%, and the detection rate could be improved by increasing the amount of samples. Thus, the failure to achieve 100% detection in the present study should not be a limiting factor for the application of the test strip. The ICA strip can be used as a qualitative tool for the rapid screening of SCRV-infected crabs in the field.

Polyclonal antibodies are often associated with cross reactivity. Furthermore, low specificity also hampers the application of polyclonal antibodies in immunodiagnostics. However, the labeling of gold particles with IgY antibodies in this study resulted in considerably high specificity for the detection of different aquatic-associated pathogens.

The successful development of the strip is largely dependent on the antibody used for conjugation with gold nanoparticles and the coating antigen on the test line. IgY is a more hydrophobic molecule than mammalian IgG (Dávalos-Pantoja et al. 2001). The mechanism of coupling of gold with protein is based on the adsorption of the nanoparticle surface through hydrophobic and ionic bonds. It was suggested that colloidal dispersions of IgY-covered latex particles were more stable (Dávalos-Pantoja et al. 2001); therefore, the use of the IgY antibody to prepare the probe is recommended for developing the ICA strip. In the present study, a titration was performed to determine the optimum pH of the colloidal gold solution and the optimum amount of IgY antibody. The purity and concentration of the coating antigen was carefully optimized to ensure that the assay ran satisfactorily, because these factors had a direct influence on the detected coloration of the assay. Under optimum conditions, the strips were found to be stable for at least 6 mo of storage at 4°C without a significant loss of activity.

In summary, the developed ICA was found to be SCRV-specific, sensitive and feasible. With respect to its overall simplicity and speed, this strip can be applied as a preliminary screening tool on site to monitor SCRV in an efficient and quick manner.

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