รายงานสรุปผลการวิจัยฉบับสมบูรณ์

การพัฒนาชุดทดสอบไวรัสตัวแดงดวงขาวอย่างง่าย

Development of the assay for White Spot Syndrome Virus (WSSV)

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รายการภาพประกอบ

รายการภาพประกอบ

5. กิตติกรรมประกาศ

โครงการวิจัยนี้ได้รับทุนสนับสนุนจากงบประมาณแผ่นดิน มหาวิทยาลัยสงขลานครินทร์ ประจำปีงบประมาณ 2558-2559 รหัสโครงการ SCI580310S

6. บทคัดย่อภาษาไทยและภาษาอังกฤษ

บทคัดย่อ

อุตสาหกรรมการเพาะเลี้ยงกุ้งและการส่งออกกุ้งในประเทศไทยสร้างรายได้ให้ ประเทศปีละหลายหมื่นล้านบาท แต่เกษตรกรผู้เพาะเลี้ยงกุ้งยังคงพบปัญหาในการเพาะเลี้ยงกุ้ง ปัญหาหนึ่งที่พบบ่อยคือการติดเชื้อไวรัสอย่างรวดเร็วและรุนแรง โดยเฉพาะอย่างยิ่งไวรัสตัวแดงดวงขาว ดังนั้นการพัฒนาวิธีการตรวจวัดไวรัสตัวแดงดวงขาวที่สามารถตรวจวัดได้ง่าย รวดเร็ว มี ความจำเพาะ และสามารถนำไปใช้ภาคสนามได้ จึงเป็นประโยชน์อย่างมากกับเกษตรกรผู้เพาะเลี้ยง กุ้งสำหรับใช้ตรวจวัดไวรัสตัวแดงดวงขาวในน้ำที่เพาะเลี้ยงกุ้ง จากการศึกษาก่อนหน้านี้มีการรายงาน ว่า โปรตีน White Spot Syndrome Binding Protein (WBP) ที่มีน้ำหนักโมเลกุล 3 กิโลดาลตัน สามารถจับได้อย่างจำเพาะกับโปรตีน VP26 ซึ่งเป็นโปรตีนของไวรัสตัวแดงดวงขาวและได้มีการนำโปรตีน VP26 ไปทำการฉีดในกระต่ายเพื่อผลิตโพลิโคลนอลแอนติบอดี้ต่อโปรตีน VP26 (anti-VP26) ดังนั้นในการศึกษาครั้งนี้ผู้วิจัยจึงสนใจที่จะนำทั้งโปรตีน WBP และ anti-VP26 มาใช้เป็นวัสดุชีวภาพ สำหรับการพัฒนาวิธีการตรวจวัดไวรัสตัวแดงดวงขาว โดยใช้โปรตีน WBP ซึ่งผลิตโดย E.coli สายพันธุ์ HB2151 ที่มียีน pCANTAB-WBP และ anti-VP26 ซึ่งได้จากการศึกษาก่อนหน้านี้ นำมาติดฉลากด้วยอนุภาคนาโนทอง (AuNPs) โดยจะเรียกว่า Colloidal gold probe เพื่อนำไปตรวจวัดไวรัสตัวแดงดวงขาวด้วยวิธี Dot-blot Immunogold Assay (DBIA) ที่ได้ท าการพัฒนาขึ้นในงานวิจัยนี้ และใช้แผ่นไนโตรเซลลูโลสเมมเบรนเป็น Detection pad หากตรวจวัดพบไวรัสตัวแดงดวงขาวจะปรากฏเป็นจุดสีแดงขึ้นบนแผ่นไนโตรเซลลูโลสเมมเบรนภายในเวลา 2-5 นาที ซึ่งสามารถมองเห็นได้ด้วยตาเปล่า ทำการตรวจวัดไวรัสตัวแดงดวงขาวที่ทราบค่าความเข้มที่ 10^3-10^7 copies μl^{-1} และ ทดสอบความจำเพาะของ Colloidal gold

probe โดยทำการตรวจวัดกับไวรัสหัวเหลืองและไวรัสทอร่า พบว่าสามารถตรวจวัดไวรัสตัวแดงดวงขาวได้ที่ความเข้มข้นต่ำสุด 10^5 copies μl^{-1} และไม่ปรากฏจุดสีแดงขึ้นบนแผ่นไนโตรเซลลูโลสในการตรวจวัดไวรัสหัวเหลืองและไวรัสทอร่าแสดงให้เห็นว่า Colloidal gold probe ที่ผลิตขึ้นมีความจำเพาะในการตรวจวัดไวรัสตัวแดงดวงขาวเพียงอย่างเดียว และ Colloidal gold probe สามารถนำใช้ในการตรวจวัดซ้ำได้โดยให้ผลชัดเจน มีประสิทธิภาพที่ดีในช่วง 2-3 วันแรก โดยทำการเก็บ Colloidal gold probe ที่ 4 องศาเซลเซียส จากนั้นนำวิธี DBIA ไปตรวจวัดไวรัสตัวแดงดวงขาวในตัวอย่างน้ำที่ใช้ในการเพาะเลี้ยงกุ้ง โดยไม่ต้องมีการเตรียมตัวอย่างน้ำ เก็บตัวอย่างน้ำ 3 ตัวอย่างจากบ่อเพาะเลี้ยงกุ้ง ตัวอย่างที่ 1 จากบ่อเพาะเลี้ยงกุ้งที่ติดเชื้อ ไวรัสตัวแดงดวงขาว และตัวอย่างที่ 2 และ 3 จากบ่อเพาะเลี้ยงกุ้งที่สุขภาพดี ผลการตรวจวัดพบว่ามีเพียงตัวอย่างที่ 1 ที่ตรวจพบไวรัสตัวแดงดวงขาวได้ ในขณะที่ตัวอย่างที่ 2 และ 3 ไม่พบไวรัสตัวแดงดวงขาว จากนั้นนำตัวอย่างที่ 2 และ 3 ทำการ Spike ไวรัสตัวแดงดวงขาวที่ทราบความเข้มข้นที่ 10^3-10^7 copies μl^{-1} ตรวจวัดด้วยวิธี DBIA เป็นผลให้ปรากฏเป็นจุดสีแดงขึ้นบนแผ่นไนโตรเซลลูโลสเมมเบรน สามารถตรวจวัดไวรัสตัวแดงดวงขาวได้ที่ความเข้มข้นต่ำสุด 10^4 copies μl^{-1} แสดงให้เห็น ว่าวิธี DBIA ที่พัฒนาขึ้นเป็นวิธีการที่เชื่อถือได้ในการตรวจวัดไวรัสตัวแดงดวงขาวในตัวอย่างน้ำที่ใช้เพาะเลี้ยงกุ้ง จากนั้นทำการยืนยันผลการตรวจวัดไวรัสตัวแดงดวงขาวด้วยเทคนิค PCR พบว่าผลการ ทดสอบด้วยวิธี PCR และ วิธี DBIA มีความสอดคล้องกัน ซึ่งยืนยันได้ว่าประสิทธิภาพของวิธี DBIA สามารถนำไปใช้ในการตรวจวัดไวรัสตัวแดงดวงขาวในตัวอย่างน้ำได้อย่างถูกต้อง วิธี DBIA ที่พัฒนาขึ้นสามารถตรวจวัดไวรัสตัวแดงดวงขาวได้ง่าย รวดเร็ว มีความจำเพาะในการตรวจวัดไวรัสตัวแดงดวง ขาวและไม่ต้องมีการเตรียมตัวอย่างน้ำก่อนการตรวจวัด โดยที่สามารถใช้เป็นเครื่องมือตรวจสอบเบื้องต้นที่เกษตรกรผู้เพาะเลี้ยงกุ้งสามารถนำมาใช้ในภาคสนามเพื่อป้องกันและควบคุมการ แพร่กระจายของไวรัสตัวแดงดวงขาวเพื่อลดความเสียหายให้กับเกษตรกรผู้เพาะเลี้ยงกุ้งได้

ABSTRACT

Shrimp farming and export has become a multi-billion dollar business in Thailand. However, shrimp farmers continue to experience problems from severe virus infections that spread rapidly, especially the White Spot Syndrome Virus (WSSV). Therefore, we developed a simple, rapid and specific method of detecting the WSSV that can be used in the field. It is very useful for shrimp farmers to be able to detect the WSSV in the culture water. Previous studies have reported that the White Spot Syndrome Binding Protein (WBP) (Molecular Weight 3 kDa) bound specifically to the VP26 protein of the WSSV and recombinant VP26 protein (rVP26) has been used to produce a polyclonal antibody against the VP26 (anti-VP26). Thus, both WBP and anti-VP26 were interesting biological materials to use in the development of the method of WSSV detection we propose in this study. The WBP was produced from E.coli HB2151 containing pCANTAB-WBP and the polyclonal antibody against VP26 protein (anti-VP26) was obtained from our previous work. They were labeled with gold nanoparticles (AuNPs) to create a colloidal gold probe and a nitrocellulose membrane was used as a detection pad. Then, we developed a dot-blot immunogold assay (DBIA) to detect WSSV in shrimp culture water. The results showed that, when the target WSSV bound to the colloidal gold probe, a reddish dot, visible to the naked eye, appeared on the surface of the membrane within 2 to 5 minutes. The sensitivity, specificity and reproducibility of the DBIA were tested

with 3 viruses (White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV) and Taura Syndrome Virus (TSV)) in concentrations ranging from 10^3 to 10^7 copies μl^{-1}. The dot-blot immunogold assay (DBIA) had a detection limit as low as 105 copies µl-1 of the WSSV. The specificity of DBIA was tested with YHV and TSV, and no reddish dot appeared on the surface of the membrane. Therefore the results showed the good specificity of the developed colloidal gold probe for WSSV detection. Stored at 4 °C, the colloidal gold probe could be reused for up to 2 to 3 days, when reddish dots were still appearing clearly on the surface of the membrane. In real sample analysis, the dot-blot immunogold assay (DBIA) was applied to water samples used for shrimp cultivation without any sample preparation. Three water samples were tested: sample 1 was collected from a culture pond with WSSV-infected shrimp and samples 2 and 3 were collected from culture ponds with healthy shrimp. The WSSV was only detected in sample 1 and not in the other two samples. Then, sample 2 and 3 were spiked with WSSV in concentrations from 10^3 to 10^7 copies μl^{-1} and again tested by DBIA. Reddish dots appeared on the surface of the membrane with a detection limit as low as 104 copies µl-1 of WSSV. These results suggested that the developed DBIA is a reliable method of detecting WSSV in water samples. The detection of WSSV by PCR replicated the results of the developed DBIA, which supported the application of the DBIA for WSSV detection in real water samples. The DBIA is simple, rapid, specific and no sample preparation is needed for real sample analysis. This developed DBIA can be used as a screening tool and applied in the field to prevent and control the spread of WSSV in cultivated shrimp populations.

7. บทสรุปผู้บริหาร (Executive Summary) ประกอบด้วย

7.1 บทนำ

Shrimp farming and export has become a multi-billion dollar business in Thailand (Patmasiriwat et al. 1998) but shrimp farmers are constantly confronted with various problems. One frequently encountered problem is hostile virus infection, especially with the white spot syndrome virus (WSSV), which can cause up to 100% mortality within 3 to 10 days after shrimp are infected. It can infect all varieties of economically important shrimp resulting in large losses to the shrimp farming industry (Lightner 1996). To date, various methods of WSSV detection have been developed. These methods consist polymerase chain reaction (PCR) (Lo et al. 1996, Kim et al. 1998, Tapay et al. 1999, Yoganandhan et al. 2003), histopathological procedures (Yoganandhan et al. 2003), in situ hybridization (Mccoll et al. 2004), and loop-mediated isothermal amplification (Kono et al. 2004). Each method has different advantages and disadvantages in terms of specificity, sensitivity, cost and convenience. However, all these methods are very expensive and complex, requiring trained skilled technicians. Thus, it would be very useful to have an accurate, sensitive, specific, rapid and simple diagnostic method for the detection of this virus.

The protein components of the WSSV virion (viral protein or VP) have been well established by proteomic methods and almost 40 WSSV structural proteins have been identified (Tsai et al. 2004), of which 22 are envelope proteins. Four major proteins consisting of VP28, VP26, VP24, and VP19 have been isolated from infected shrimps. VP24 and VP26 were found to be associated with nucleocapsids and VP19 and VP28 were associated with the envelope (Van Hulten et al. 2000). Further research revealed that VP28 and VP26 are the two most abundant structural proteins observed in the envelope (Tang et al. 2007).

In previous studies, immuno-dot blot assays for the detection of WSSV in shrimp have been developed from the monoclonal and polyclonal antibodies specific to the viral proteins of WSSV. The dot-blot nitrocellulose enzyme immunoassay (DB-NC-EIA) can detect WSSV and YHV in infected shrimp. Rabbit antiWSSV IgG-HRP and rabbit anti-YHV IgG-HRP were tested on WSSV-antigen and YHVantigen spotted onto a nitrocellulose membrane. The assay required at least 3 h to conclude (Nadala Jr et al. 2000). In further studies, immuno-dot blot assays showed false positives when labeled with an enzyme (Zhan et al., 2003, 2004). A subsequent development was the dot-immunogold filtration assay (DIGFA) for the detection of WSSV in shrimp, which used nitrocellulose membrane (NCM) as a support for WSSV and anti-WSSV monoclonal antibodies (MAbs) 6A4 and 1D5 conjugated gold nanoparticles. The test procedure was achieved within 3 min without incubation or any device but false positive results presented visibly. Thereafter, an immunochromatographic test (ICT) was developed for detecting WSSV in shrimp. The test used gold nanoparticles conjugated to anti-WSSV monoclonal antibodies (MAbs) 2E6 and 2A3 as a detection buffer, and anti-WSSV MAb 1D5 as a capture antibody immobilized on a nitrocellulose membrane (NCM). The ICTs were achieved in less than 10 min and the detection limit was 1 μg ml-1 of WSSV without false positive results. However, this technique also has a complicated procedure and uses many antibodies (Wang et al. 2006). The sensitivity of immuno-dot blot assays for WSSV detection in shrimp was improved by using gold nanoparticles coupled with alkaline phosphatase conjugated secondary antibody, which in turn recognized primary antiserum raised against WSSV. This immuno-dot blot assay can detect as little as 1 ng mL-1 of WSSV (Thiruppathiraja et al. 2011). However, the test is a sandwich assay consisting of many steps, which makes it unsuitable for use in the field.

In our previous studies, the WSSV-binding protein (WBP), which had been reported to bind specifically to the VP26 protein of WSSV (Youtong et al. 2011), and the recombinant VP26 protein (rVP26) were used to produce the polyclonal antibody against the VP26 (anti-VP26) WSSV nucleocapsid protein in order to develop a label-free impedimetric biosensor, which is a quantitative and sensitive method of WSSV detection (Loyprasert-Thananimit et al. 2012). Subsequently, WBP and antiVP26 were further developed into an immuno-based colorimetric assay for detecting the VP26 protein on the envelope of WSSV in shrimp pond water

(LoyprasertThananimit 2014). In other work, WBP and anti-VP26 have been used together to develop a label-free affinity immunosensor for the quantitative detection of WSSV, also in shrimp pond water (Waiyapoka 2015). However, these developed methods still require equipment and trained technicians so the tests are not portable to the field.

In this study, we tried to develop a method of on-site testing that could be used immediately by untrained personnel using naked eye observation. Therefore, the dot-blot immunogold assay (DBIA) technique was adopted for the detection of WSSV in water from shrimp cultivation ponds, using WBP and anti-VP26 as biological elements immobilized on gold nanoparticles (AuNPs). When WBP and anti-VP26 bind to the target VP26 protein of WSSV, the color developed on the membrane can easily be seen with the naked eye. The developed DBIA was simple, rapid and applicable as a screening tool in the field to prevent and control the spread of WSSV and reduce losses to the shrimp cultivation industry.

7.2 วัตถุประสงค์

7.2.1 ผลิตโปรตีน WBP และตรวจสอบการจับกันของคู่แอฟฟินิตีเบื้องต้นโดยวิธี Western blot ระหว่างการจับกันของโปรตีน WBP กับ WSSV

7.2.2 พัฒนาชุดทดสอบอย่างง่าย (test kit) ในการหาปริมาณไวรัสตัวแดงดวงขาว

7.2.3 ประยุกต์ใช้ชุดทดสอบอย่างง่ายที่พัฒนาขึ้นในการตรวจหาปริมาณไวรัสในน้ำจากบ่อเลี้ยงกุ้ง

OBJECTIVES

To study and develop a simple test kit for detection WSSV. Two subproject were studies as follows.

Subproject I: To study the immobilization method using protein WBP and AntiVP26 with gold nanoparticles.

Subproject II: To develop the assay for detection of WSSV and applied in shrimp pond water.

7.3 สรุป (สรุปผลการทดลองทั้งหมดของงานวิจัยทั้งชุดโครงการ ทั้งที่ตีพิมพ์แล้ว และยังไม่ตีพิมพ์)

Subproject I: To study the immobilization method using protein WBP and Anti-VP26 with gold nanoparticles.

Previous studies have reported VP26 to be one of the protein components of the WSSV virion isolated from infected shrimp (Van Hulten et al. 2000). One WSSV binding protein (WBP) gene was found to consist of 171 bp that had no matches in the NCBI database. The white spot syndrome binding protein (WBP) produced from the WBP gene was shown to bind

specifically to the VP26 protein of the WSSV (Youtong et al. 2011) and, in a previous study, we produced the polyclonal antibody against VP26 (anti-VP26) from recombinant protein VP26 (LoyprasertThananimit et al. 2012). Thus, both WBP and anti-VP26 are interesting biological elements for immobilization with AuNPs in the development of an assay for detecting the VP26 protein on the envelope of WSSV.

White spot syndrome binding protein (WBP) was produced by E. coli HB2151 containing pCANTAB-WBP and purified using a Sephadex G-25 column. WBP appeared as a 3 kDa on 15% SDS-PAGE as shown in Figure 1 (Paper I). WBP was immobilized with AuNPs. The AuNPs were synthesized by the citrate reduction method (Loyprasert et al. 2010) and characterized by TEM. The average particle diameter of the AuNPs was 20±4 nm (Figure 2 (1)). The AuNPs were characterized by UV-Vis spectroscopy as shown in Figure 2 (2) and the characteristic UV-Vis absorption spectrum shows a maximum absorbance at 532 nm (Paper I). The WBP was immobilized with AuNPs (colloidal gold probe) prepared according to X. Wang et al., 2006. This colloidal gold probe was used to detect WSSV at 1.6 × 106 copies µL-1. The gold nanoparticles appeared as a reddish dot which indicated that binding between the WSSV and the colloidal gold probe had occurred. The results show that colloidal gold probes can be used to detect WSSV. However, the WBP couldn't be reproduced in later study.

Subproject II: To develop the application of the assay for detection of WSSV in shrimp pond water.

In further study, the size of the AuNPs was improved. The AuNPs were synthesized by the citrate reduction method (Brown et al. 2000). The AuNPs solution was intensely red in color. TEM images (Figure 1A) (Paper II) show a monodispersion of gold nanoparticles with an average particle diameter of 5±0.3 nm. The UV-Vis absorption spectrum shows a maximum absorbance at 515 nm (red curve) (Figure 1C (Paper II)), down from 532 nm (Paper I) as the size of the AuNPs decreased from 20 nm to 5 nm. According to previous studies, as the size of AuNPs increased, the maximum absorbance increased and the maximum peak wavelength red-shifted as the relative particle size got bigger (Hong et al. 2013). If the size of the AuNPs is reduced, the higher surface area should increase the efficiency of immobilization. Polyclonal antibody against the VP26 (anti-VP26), obtained from our previous work (Loyprasert-Thananimit et al. 2012), was immobilized with AuNPs (Ab-AuNPs). In this study, a gold aggregation test (GAT) was used to determine the optimal condition of immobilization using NaCl which caused the aggregation of the AuNPs and shifted the maximum absorbance peak from 515 nm to 580 nm. The optimum concentration of anti-VP26 for avoiding AuNPs aggregation was determined by observing when the solution did not change color from red to blue after the addition of 10% NaCl (Figure 2A, 2B) (Paper II) and by the difference in absorbance (UAbs change) from 515 nm (dispersed AuNPs) and at 580 nm (aggregated AuNPs), as shown in Figure 2C. The results indicated that the minimum

concentration required for full coverage of the surface of all the AuNPs was 50 µg mL-1. In this study, the selected concentration of anti-VP26 was 200 µg mL-1 in order to prevent salt-induced aggregation. After immobilizing the antibody on the AuNPs, the resulting Ab-AuNPs had an average diameter of 8±0.3 nm (Figure 1B). When the antibodies were immobilized on the AuNPs the absorption band changed from 515 nm (red curve) to 518 nm (blue curve).

The optimal pH was determined by adjusting AuNPs solutions with 0.1 M Na2CO3 to pH values of: 5.5, 7, 8, 9 and 10. A color change occurred after adding NaCl at pH 5.5, indicating the aggregation of AuNPs. However, there was no color change at pH values from 7 to 10, as shown in Figure 2. These results indicated that the optimum pH of the AuNPs for immobilizing anti-VP26 was between 7.0 and 10. In this study, a pH of 9 was selected for immobilization because a low pH value causes the AuNPs to agglomerate, while a high pH value tends to generate unstable AbAuNPs (Paper II). The optimal concentrations of BSA for nonspecific site blocking were established by a GAT. Based on the results shown in Figure 3 (Paper II), the optimum condition was a BSA concentration of 1 mg mL-1. The assay called "Dot Blot Immunogold Assay (DBIA)" was developed for detection of WSSV following the method of Wang et al 2006. The WSSV, YHV and TSV were tested using the DBIA with concentrations in the range of 103 to 107 copies µl-1. In these experiments, the viruses were tested with 3 replicates from the same batch of Ab-AuNPs to determine the specificity, sensitivity, and reproducibility of the assay. Figure 4 (Paper II) shows images of the DBIA after testing with a standard virus. The increased intensity in the color is clear to see: a result of the increased concentration of WSSV in the sample. YHV and TSV were used to test the specificity of the DBIA. Figure 4 shows that the presence of these two viruses was not indicated by the appearance of the reddish dot on the surface of the membrane which indicates the presence of WSSV within 2 to 5 minutes. Therefore, the developed DBIA had good specificity for WSSV detection. In terms of sensitivity, the assay had a detection limit as low as 105 copies µl-1 of the WSSV, which means the assay is sufficiently sensitive because the number of WSSV in water must exceed 1.6 x 104 copies µl-1 to be infective to shrimp (Loyprasert-Thananimit et al. 2012). The reproducibility of the DBIA was evaluated every day over a period of 7 days under storage at 4 °C. The reddish dots that indicate a positive result on the surface of the membrane were clearly produced for 2 to 3 days. However, the performance of the test deteriorated in the following days. Therefore, the developed test should be used within 2 to 3 days and stored at 4 °C (Figure 5).

Seawater samples were collected from ponds used for shrimp cultivation. All the seawater samples were tested using the DBIA .Sample 1 produced a positive result, while the absence of a dot in samples 2 and 3 indicated negative results (Figure 6). Thereafter, samples 2 and 3 were spiked with concentrations of WSSV ranging from 103 to 107 copies µl-1 and tested by DBIA . Reddish dots appeared on the surface of the membranes within 2

to 5 minutes .The detection limit was 104 copies µl-1 of WSSV (Figure 7). These results suggest that the developed DBIA is a reliable method for the detection of WSSV in seawater (Paper I). To confirm the performance of the developed DBIA, we carried out a detection of WSSV by PCR. Seawater samples from a pond used for shrimp cultivation were spiked with various concentrations of WSSV ranging from 2 × 102 to 107 copies µl-1 and the seawater samples collected from a shrimp pond (Sample 1-3) tested by PCR .The results of the tests on the spiked seawater samples showed that the PCR products of the WSSV (about 400 base pairs) serially decreased corresponding to the number of copies of WSSV in the sample (Figure 8) Lanes 4-9. The PCR product of WSSV was found in Lane 10 but none was found in Lanes 11-12 .These results are the same as those from the DBIA and confirmed that the DBIA could be used for the quantification of WSSV.

7. ภาคผนวก

7.1 ผลการวิจัยส่วนที่ยังไม่ได้ตีพิมพ์หรือตีพิมพ์ไม่ได้ แต่อยู่ในวัตถุประสงค์ของโครงการวิจัย

Methods

7.1.1 Production of White Spot Syndrome Binding Protein (WBP)

The procedure followed was that of Youtong et al., 2011. Briefly, White Spot Syndrome Binding Protein (WB) was produced by E.coli HB2151 containing the plasmid pCANTAB-WBP. One colony was cultured in LBG (LB+ 2%glucose) at 30 °C for 16-18 h. Then 1 mL was transferred into 10 mL of LBG (LB + 2% glucose + 80 µg/mL of ampicillin) and incubated at 30 °C for 1 h. The culture was centrifuged at 1500 rpm at room temperature for 20 min and the pellet was resuspended in 10 mL of LB containing 1 mM IPTG and 80 µg/mL of ampicillin and incubated at 30 °C for 24 h and again centrifuged at 1500 rpm for 20 min. The protein in the supernatant was further purified by elution from a Sephadex™ G-25 column (GE Healthcare Bio-Sciences AB, Sweden) connected to an AKTA prime plus (GE Healthcare Bio-Sciences AB, Sweden). The obtained protein obtained from the eluted fractions were checked by PAGE on 15% SDS and kept at -80 °C for immobilization with colloidal gold nanoparticles (AuNPs).

7.1.2 Preparation of gold nanoparticles

The procedure followed was that of Loyprasert-Thananimit et al., 2010. All glassware used in the preparation was rigorously cleaned by soaking in freshly prepared HNO3 (10% in MilliQ processed water) overnight and thoroughly rinsed. Five hundred milliliters of aqueous solution containing 2.5mM HAuCl4 and 3.75mM sodium citrate (C6H5O7Na3) was prepared (Brown et al., 2000). Five milliliters of 1.25M NaBH4 (Sigma-Aldrich, USA)solution was rapidly added into the gold solution while being vigorously stirred. The mixture was continuously stirred overnight and the prepared gold colloidal solution were then stored in a dark glass

bottle at 4 °C for further use. The gold nanoparticles were inspected by UV-visible spectroscopy. The sizes of the gold nanoparticles were verified using a transmission electron microscope (TEM).

7.1.3 Immobilization of WBP with gold nanoparticles

The procedure followed was that of X. Wang et al., 2006. To prepare colloidal gold probes, 150 µL of 0.5 g/L WBP was mixed with 10 mL colloidal gold solution. The reaction was allowed to proceed for 10 min with slow stirring and then overnight at 4 °C without stirring. The reaction mixture was centrifuged at 18,000×g 110 min 4 °C and the precipitate was suspended in 1 mL of 0.01 M PBS (pH 7.4) containing 1% bovine serum albumin (BSA) and 0.5% Tween-20. The prepared colloidal gold probes were then stored at 4 o C until used.

7.1.4 Detection of WSSV

2 µL of WSSV concentrations of 1.6 × 106 copies/µL was dripped onto a nitrocellulose membrane (NCM) then dried at room temperature. Then, 100 µL of blocking agent (0.01M PBS containing 1% BSA and 0.05% Tween-20, pH 7.4) was added onto the nitrocellulose membrane, followed by the addition of 100 µL of the colloidal gold probe while soaked in. Finally, a 100 µL of the solution (0.01M PBS containing 0.05% Tween-20, pH 7.4) was added to soak into the membrane. The appearance of a purple dot indicated a positive reaction.

7.1.5 Synthesis of gold nanoparticles

All glassware and the magnetic stirring bar used in the AuNPs synthesis were rigorously cleaned by soaking in freshly prepared 10% HNO_3 overnight and thoroughly rinsed with Milli-Q water, then oven-dried prior to use.

The AuNPs, which were 5 nm in diameter, were prepared by the citrate reduction of $HAuCl_4 \bullet 3H_2O$ following the procedure described by Brown and Natan (Brown et al. 2000). Briefly, 10 ml of 1% $HAuCl_4 \bullet 3H_2O$ was added to 900 ml of Milli-Q water at room temperature. After 1 minute of stirring, 20.0 ml of 38.8 mM Tri-sodium citrate (TSC) was added. Then 1 minute later, 10 ml of fresh 0.075% $NaBH_4$ was added. The AuNPs were stirred for an additional 5 minutes and stored in a dark bottle at 4 °C for further use. They were characterized using ultraviolet/visible spectroscopy (UV-Vis) (NanoDrop 2000c spectrophotometer) in a wavelength range between 400 and 800 nm. The sizes of the AuNPs were verified by transmission electron microscopy (TEM) using a JEOL 2000FX microscope (JEOL Ltd, Japan) fitted with a Gatan (Pleasanton, CA) CCD camera.

7.1.6 Optimization of antibody-immobilized on gold nanoparticles

A gold aggregation test (GAT) was used to determine the optimal condition of immobilization using NaCl which caused the aggregation of the AuNPs and shifted the maximum absorbance peak from 515 nm to 580 nm, resulting in a difference in absorbance ($UAbs_{change}$) that was calculated from equation (1) (Ambrosi et al. 2007).

$$UAbs_{Change} = [UAbs_{515} - UAbs_{580}]_{Before\ NaCl\ addition} - [UAbs_{515} - UAbs_{580}]_{After\ NaCl\ addition} \quad (1)$$

The optimal concentrations of antibody immobilized on the gold nanoparticles were determined by performing a GAT according to the method described in Chamero-Garcia et al., with some modifications. Briefly, 1 ml of AuNPs was placed in each well of a multi-well plate. The pH of the AuNPs was adjusted with 0.1 M Na_2CO_3 to pH values of: 5.5, 7, 8, 9 and 10. Then 100 µl of antibody at different concentrations (0, 10, 50, 100, 200 µg ml^{-1}) were added to each well. The plates were incubated under gentle mixing at room temperature. The optimal concentration of antibody was determined by adding 100 µl of 10% NaCl solution to each well and incubating it under gentle mixing for 10 minutes at room temperature. NaCl causes the aggregation of AuNPs and changes the color from red to blue. If the color does not change the antibody- immobilized on the AuNPs (Ab- AuNPs) contains the optimal concentration of Ab for fully covering the surface of all the AuNPs (Omidfar et al. 2010).

Following the GAT, optimized concentrations of bovine serum albumin (BSA) for nonspecific site blocking were determined. Briefly, 1 ml of AuNPs was placed in each well of a multi-well plate. The pH of the AuNPs was adjusted with 0.1 M Na_2CO_3 to pH values of: 5.5, 7, 8, 9 and 10. Then 100 µl of BSA solution at different concentrations (0, 0.1, 0.5, 1, 2 mg mL^{-1}) were added to each well. The plates were incubated under gentle mixing at room temperature. Then 100 µl of 10% NaCl solution were added to each well and incubated under gentle mixing for 10 minutes at room temperature. The optimum concentration of BSA for blocking any remaining active surface of the Ab-AuNPs was the concentration of Ab-AuNPs that did not change color from red to blue after the addition of 10% NaCl.

7.1.7 Preparation of antibody-immobilized on gold nanoparticles

The AuNPs modified with anti-VP26 were prepared according to Chamero-Garcia et al. using the optimal condition optimized by the GAT. Briefly, the pH of the gold nanoparticles was adjusted to 9 using 0.1 M Na_2CO_3. Next 100 µl of 200 µg ml^{-1} anti-VP26 was incubated with 1 ml of gold nanoparticles for 30 minutes under gentle mixing at room temperature. Then the blocking step was performed by adding 100 µl of 1 mg ml^{-1} BSA incubated for 30 minutes at room temperature. Finally, the Ab- AuNPs were separated from the excess of reagents by centrifugation at 15000 x g and 4 ºC for 45 minutes and the pellet of conjugates was resuspended in 200 µl of phosphate buffered saline (PBS), pH 7.4 and kept at 4 ºC for further use.

7.1.8 Characterization of the gold nanoparticles and the gold nanoparticles- antibody conjugates

Ultraviolet/visible (UV-Vis) measurements were carried out using a NanoDrop 2000c spectrophotometer using a standard 10 mm path length quartz cuvette. Spectra were obtained from 400 to 800 nm. Milli-Q water was used as the blank for the AuNPs and as the buffer used for the Ab-AuNPs.High resolution TEM images were recorded with a JEOL 2000FX microscope (JEOL Ltd, Japan) fitted with a Gatan (Pleasanton, CA) CCD camera. Samples were

prepared by placing 2 µL of AuNPs and Ab-AuNPs onto a copper grid. The grid was then air-dried.

7.1.9 Dot-blot Immunogold assay

The DBIA for the detection of WSSV followed the method of Wang et al. The detection procedure was as follows: 2 µl of concentrations in the range of 10^3-10^7 copies of WSSV serially diluted in PBS (0.01 M, pH 7.4) were spotted onto a nitrocellulose membrane (0.45 µm pore size). The spots were dried and the membrane was blocked with a blocking agent (0.01 M PBS containing 1% BSA and 0.05% Tween-20, pH 7.4) for 15 minutes at room temperature. Then the membrane was washed three times for 10 minutes with PBS buffer. Subsequently, the membrane was incubated with Ab-AuNPs for 30 minutes at room temperature. Finally, the membrane was washed with lotion (0.01 M PBS containing 0.05% Tween-20, pH 7.4). The appearance of a reddish dot on the surface of the membrane indicated a positive result, while the absence of such a dot was indicative of a negative result.

7.1.10 Performance of the DBIA

The sensitivity, specificity and reproducibility of the DBIA was tested with 3 viruses, WSSV, YHV, TSV with concentrations in the range of 10^3-10^7 copies μl^{-1} diluted in PBS (0.01 M, pH 7.4) and phosphate-buffered saline (PBS), pH 7.4 as a control. All the viruses were tested using the DBIA. The appearance of a reddish dot on the surface of the membrane indicated a positive result, while the absence of such a dot was indicative of a negative result.

7.1.11 Detection of WSSV from a sea water sample by DBIA.

Sea water samples were collected from a pond used for culturing shrimp in Songkhla province, Thailand. The sample 1 was collected from cultured pond with WSSV-infected shrimp and Sample 2, 3 were collected from cultured pond with healthy shrimp. The sea water samples were spiked with various concentrations of WSSV in the range of 10^3-10^7 copies μl^{-1} and were used for the detection of WSSV by DBIA.

7.1.12 Comparison of the sensitivity of the DBIA and Polymerase Chain Reaction

White spot syndrome virus, WSSV solutions concentrations between $2x10^3$ to $2x10^7$ copies μl^{-1}, was tested by using DBIA. The same samples were also tested with a PCR to compare the performance of the DBIA developed. The PCR was performed in a 25 µL reaction volume containing 400 ng of DNA extracted from seawater, 1 µM of each primer, 4 mM of deoxynucleotide (dNTP), 2.5 mM of $MgCl_2$, 1X PCR buffer and 2.5 U tag DNA polymerase. The primer used was from the shrimp WSSV isolate Japan 98 VP19 gene, GenBank accession number AY249447.1. The PCR was performed according to the method described in Youtong et al. Briefly, it was conducted with an initial denaturation step at 94 °C for 5 minutes, followed by 39 cycles of 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 2 minutes. The PCR product was analyzed by electrophoresis on a 1.5% agarose gel.

Results and Discussions

7.2.1 Production of WBP from pCANTAB-WBP E. coli (HB2151)

WBP was produced by E.coli HB2151 containing pCANTAB-WBP and purified using a Sephadex G-25 column. WBP appeared as a 3 kDa on 15% SDS-PAGE as shown in Figure 1.

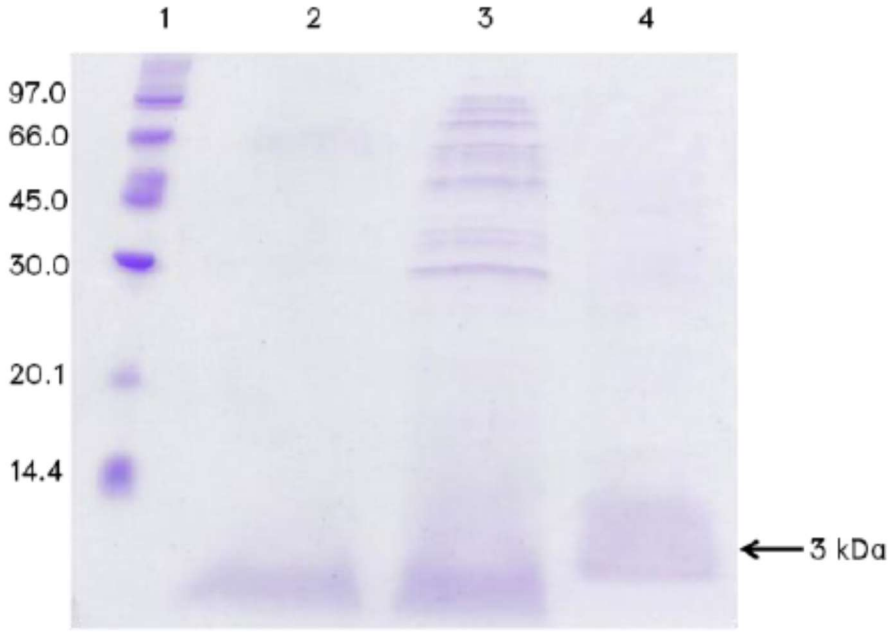

Figure 1 15% SDS-PAGE (1) low molecular weight standard markers, (2) induced protein from bacteria containing pCANTAB-WBP plasmid, (3) non-induced protein from bacteria containing pCANTAB-WBP plasmid and (4) WBP purified protein (3 kDa).

7.2.2 Preparation of AuNPs

Structural characterics of the gold nanoparticles were verified by transmission electron microscope (TEM). The TEM image showed an average size of the particles of 20±4 nm. A UV-vis wave scan (400-800 nm) of the nanoparticle suspension showed a clear absorption maximum 532 nm.

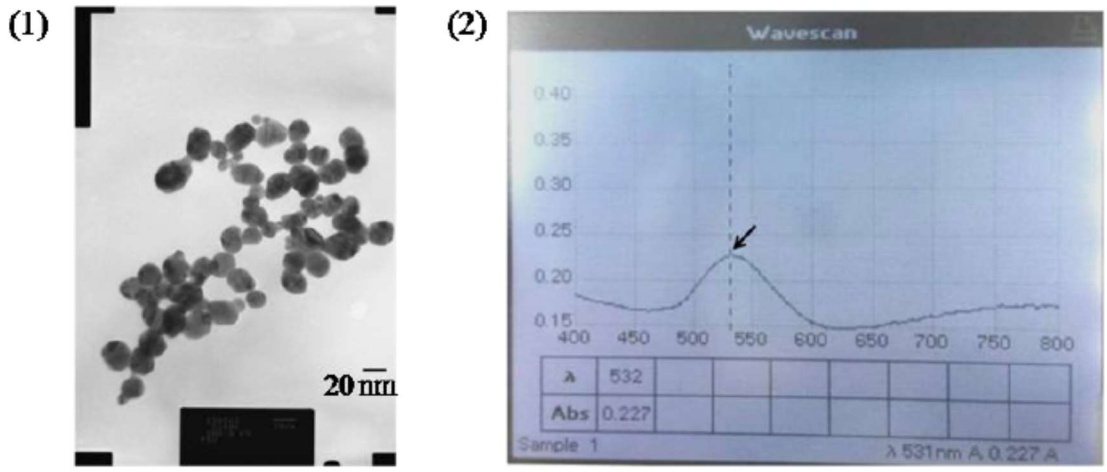

Figure 2 The TEM images (scale bars: 20 nm) (1) and the optical absorption spectra of AuNPs solution (2).

7.2.3 Detection of WSSV

A WSSV solutions concentration of 1.6 x 106 copies/μL was tested. The results showed the positive purple dot with WSSV sample while no purple dot was appear on the membrane by using the control (PBS) and yellow head virus (YHV) used as a negative control.

Figure 3 The results of colloidal gold probe to detect the WSSV virus.

7.2.4 Characterization of gold nanoparticles and antibody-immobilized on gold nanoparticles

The AuNPs were synthesized by the citrate reduction method. The AuNPs solution is intensely red in color. The synthesized AuNPs were characterized by TEM images and Figure 4A shows a monodispersion of gold nanoparticles with an average particle diameter of 5±0.3 nm. After immobilizing the antibody on the Ab-AuNPs, the resulting Ab-AuNPs had an average diameter of 8±0.3 nm (Figure 4B). The AuNPs were characterized by UV-Vis spectroscopy. As shown in Figure 4C, the characteristic UV-Vis absorption spectrum shows a maximum absorbance at 515 nm (red curve), which evidences the formation of a well-dispersed colloidal suspension.

When the antibodies were immobilized on the AuNPs the absorption band changed from 515 nm (red curve) to 518 nm (blue curve). This result indicated that the Ab-AuNPs had good colloidal stability without aggregation.

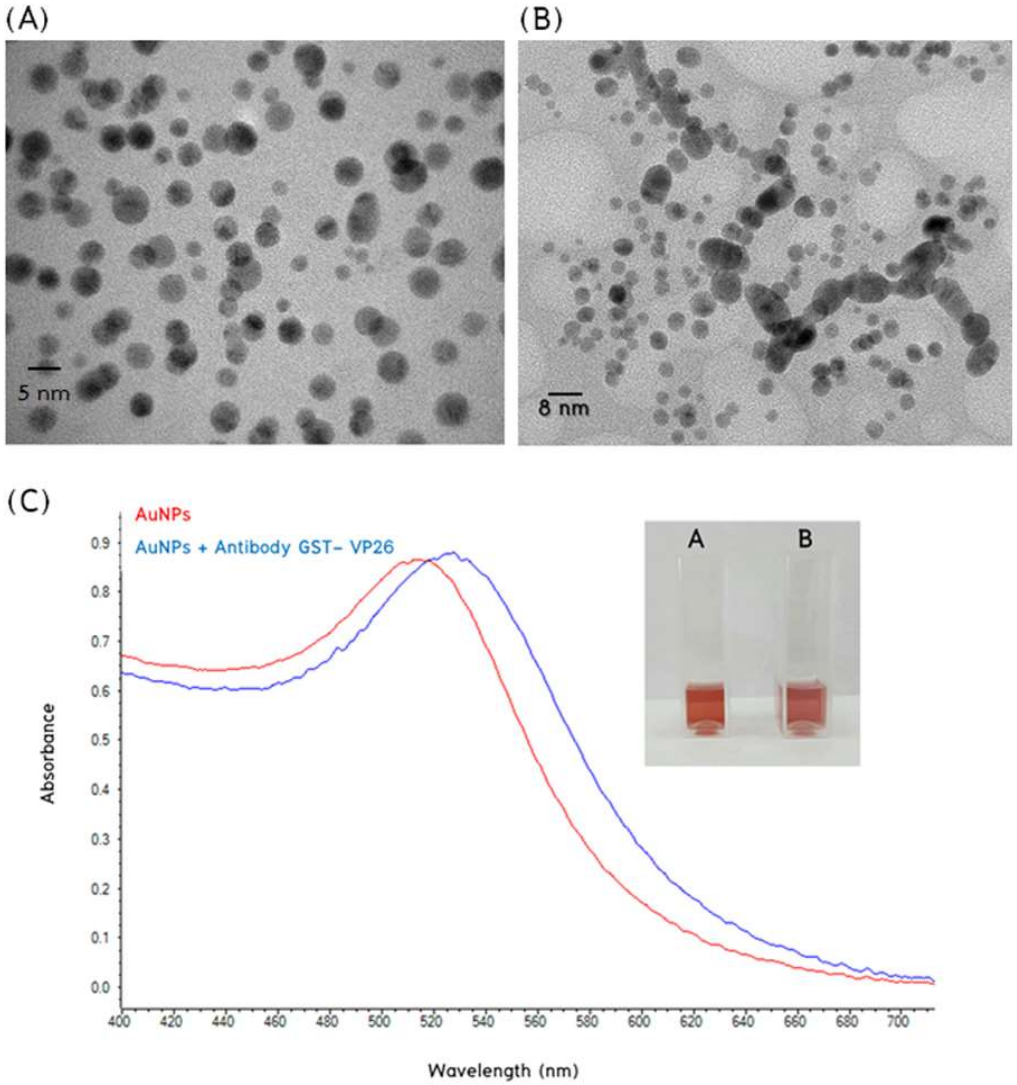

Figure 4 TEM image of the AuNPs; scale bar indicates 5 nm (A), TEM image of the Ab-AuNPs; scale bar indicates 8 nm (B) and UV-Vis spectra of the AuNPs at 515 nm (red curve) and Ab-AuNPs at 518 nm (blue curve) (C).

7.2.5 Optimization of antibody-immobilized on gold nanoparticles

A GAT was preliminarily carried out to determine the optimum conditions for immobilization and to estimate the minimum amount of antibodies required to fully cover the surface of all the AuNPs. NaCl dislocates surface charges on the AuNPs causing their aggregation (color change from red to blue) while Ab-AuNPs do not aggregate since NaCl is not able to break the Au–S bond (Chamorro-Garcia et al. 2016). The optimum concentration of anti-VP26 for avoiding AuNP aggregation was determined based on observing when the solution did not change color from red to blue after the addition of 10% NaCl (Figure 5A, 5B)

as well as the result of the difference in absorbance (UAbs change) from 515 nm (dispersed AuNPs) and at 580 nm (aggregated AuNPs) as shown in Figure 5C. The results of the UAbs change were tested for significance in a two-way analysis of variance (ANOVA) using SPSS version 16.0 (p-value ≥ 0.05). The results indicated that the minimum concentration required for full coverage of the surface of all the AuNPs was 50 μg ml^{-1}. In this study, however, the concentration of anti-VP26 selected to be used was 200 μg ml^{-1} in order to prevent salt-induce aggregation.

In terms of pH, the results show a color change after adding NaCl at pH 5.5, indicating the aggregation of the AuNPs. However there was no color change for pH values from 7 to 10 as shown in Figure 5. These results indicated that the optimum pH of the AuNPs for immobilizing anti-VP26 was between 7.0 and 10.0. In this study, a pH of 9 was selected for immobilization because a low pH value causes the AuNPs to agglomerate, while a high pH value tends to generate unstable Ab-AuNPs. The optimal concentrations of BSA for nonspecific site blocking were established by a GAT. Based on the results shown in Figure 6, the optimum conditions were a BSA concentration of 1 mg ml^{-1}.

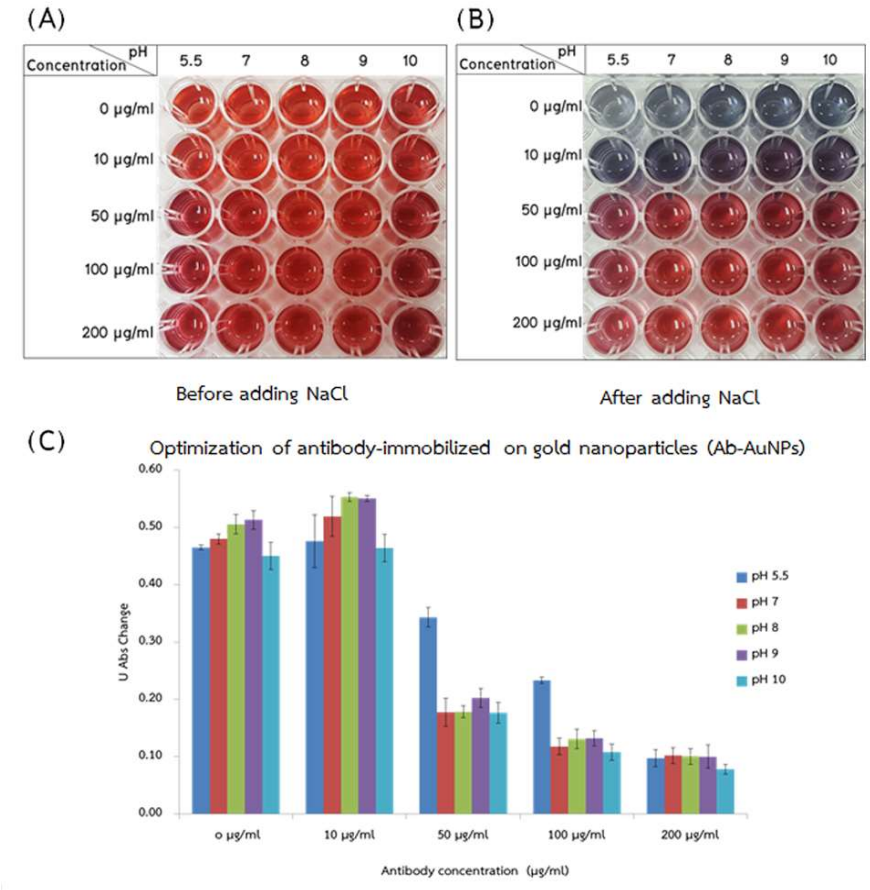

Figure 5 Images of the solutions with various concentrations of anti-VP26 and pH values (A) and Difference in absorbance (Abs 515 - Abs 580) plotted against concentration of anti-VP26 in the GAT for different pH values (B).

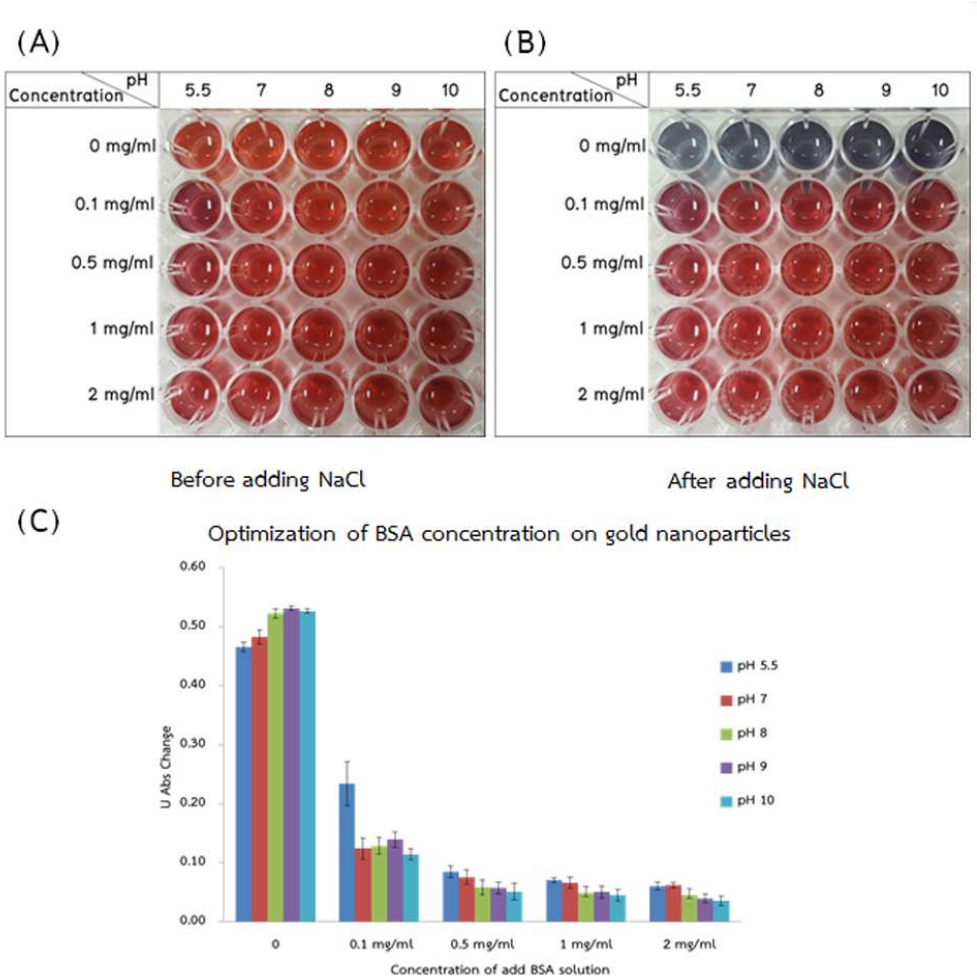

Figure 6 Images of the solutions with different concentrations of BSA and pH values (A) and Difference in absorbance (Abs 515 - Abs 580) plotted against concentration of BSA in the GAT for different pH values (B).

7.2.6 Performance of the DBIA

The WSSV, YHV and TSV were tested using the DBIA with concentrations in the range of 10^3-10^7 copies μl^{-1}. In these experiments, the viruses were tested with 3 replicates from the same batch of Ab-AuNPs to determine the specificity, sensitivity, and reproducibility of the DBIA. Figure 7 shows images of the DBIA after testing with a standard virus clearly showing an increase in the observed color intensity as a result of the increase in WSSV concentration. YHV and TSV were used to test the specificity of the DBIA and the results, showed that no reddish dot appeared on the surface of the membrane within 2 to 5 minutes as occurred with the WSSV, as can be seen in Figure 7. Therefore the results showed that the DBIA developed had good specificity for WSSV detection.

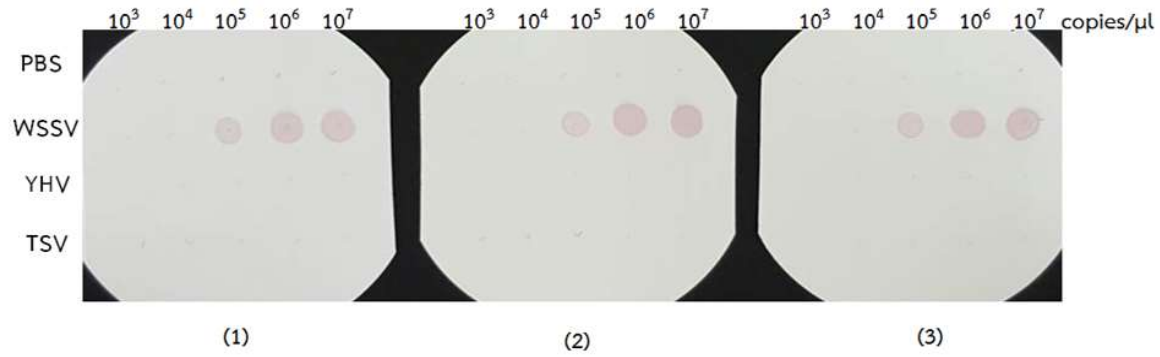

Figure 7 The sensitivity and specificity for detection of WSSV by the DBIA; Detection as low as 10^5 copies μl^{-1} of WSSV. PBS was used as the negative control.

In terms of sensitivity, the DBIA has a detection limit as low as 10^5 copies μl^{-1} of the WSSV. The DBIA is sensitive enough to detect WSSV in water because the amount of WSSV in water that is infective and lethal in shrimp needs to be above 1.6×10^4 copies μl^{-1} (Loyprasert-Thananimit et al. 2014). The reproducibility of the DBIA was evaluated every day over a period of 7 days under storage at 4 °C. The results showed that reddish dots on the surface of the membrane were clearly observed within 2 to 3 days although its performance reduced in the following days. Therefore the test developed should be used within 2 to 3 days and stored at 4 °C (Figure 8).

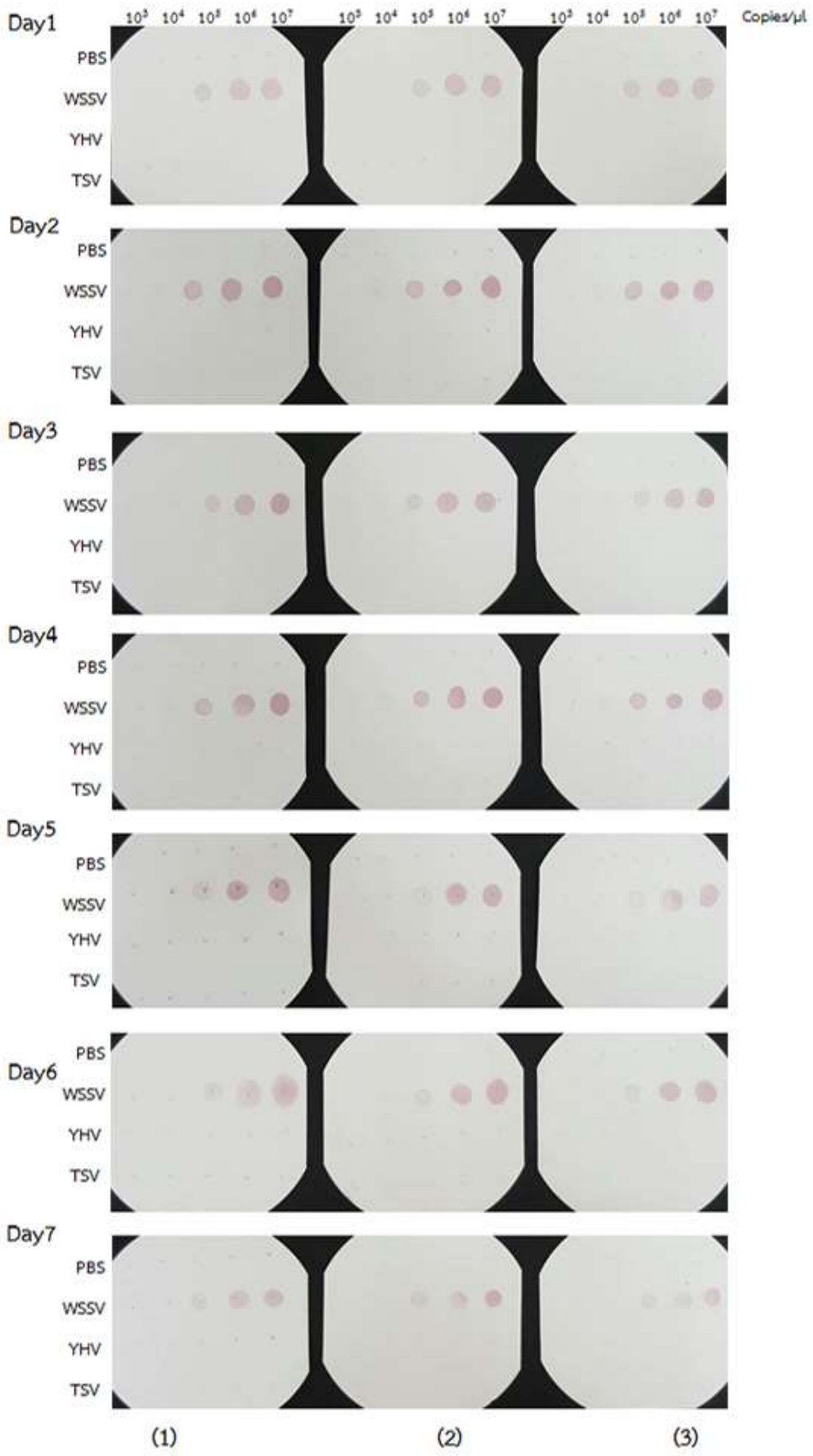

Figure 8 The reproducibility of the DBIA was tested by use every day on 7 consecutive days.

This developed DBIA can be used as a screening tool for shrimp farmer and applied in the field to prevent and control the spreading of WSSV in shrimp cultivation, ensures that DBIA has many advantages. First, the DBIA could be applied for WSSV detection in real water samples without the sample preparation while other mainly used shrimp gill tissues and the sample preparation is needed (Nadala and Loh. 2000, Thiruppathiraja et al. 2011, Wang and Zhan. 2006). Second, the study of Dot-blot nitrocellulose enzyme immunoassays (DB-NC-EIA) (Zhan et al. 2003, Zhan et al. 2004) and Dot-immunogold filtration assay (DIGFA) (Wang et al. 2006) showed false positives. Third, DIBA results showed that when the target WSSV bound to the colloidal gold probe a reddish dot appeared on the surface of the membrane within 2 to 5 minutes could be seen with the naked eye without incubation or any equipment. The analysis time of our work is faster compared to immunochromatographic test (ICT) (Wang and Zhan. 2006) while those process was completed within 10 min. Finally, the sensitivity of DIBA detection limit is $2x10^5$ copies μl^{-1} of WSSV (0.087 ng ml^{-1}). The DBIA has greater sensitivity compared to immunochromatographic test (ICT); 1µg ml^{-1} (Wang and Zhan. 2006) and immuno-dot blot assay; 1 ng ml^{-1} (Thiruppathiraja et al. 2011). The following table is the comparison of the Dot-blot Immunogold assay (DBIA) with other methods for the detection of WSSV

7.2.7 Detection of WSSV in seawater sample by DBIA

Three seawater samples were collected from ponds used for shrimp cultivation. All the seawater samples were tested using the DBIA. All the experiment in this study were performed in three replicates. The results were that, sample 1 indicated a positive result because a reddish dot appeared on the surface of the membrane within 2 to 5 minutes, while the absence of a dot in samples 2 and 3 was indicative of a negative result (Figure 9).

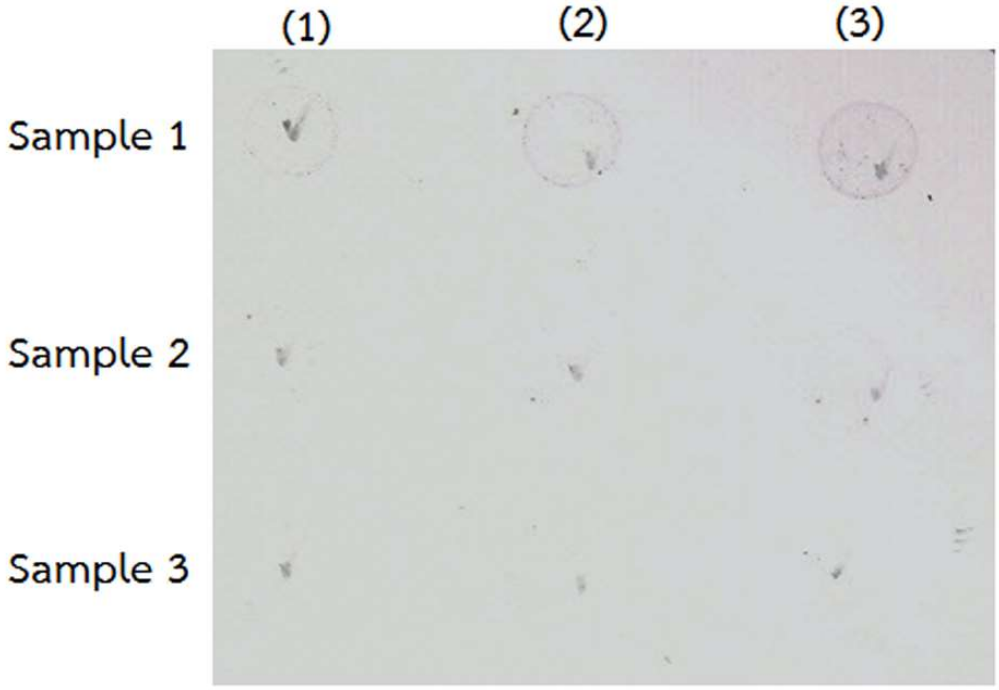

Figure 9 The three seawater samples (Samples 1, 2 and 3) used for the detection of WSSV by DBIA.

Thereafter, samples 2 and 3 were spiked with WSSV from 10^3-10^7 copies μl^{-1} and tested by DBIA. As a result, reddish dots appeared on the surface of the membranes within 2 to 5 minutes. The detection limit was $2x10^4$ copies μl^{-1} of WSSV (Figure 10) without the sample preparation. These results suggest that the DBIA developed is a reliable method for the detection of WSSV in seawater samples.

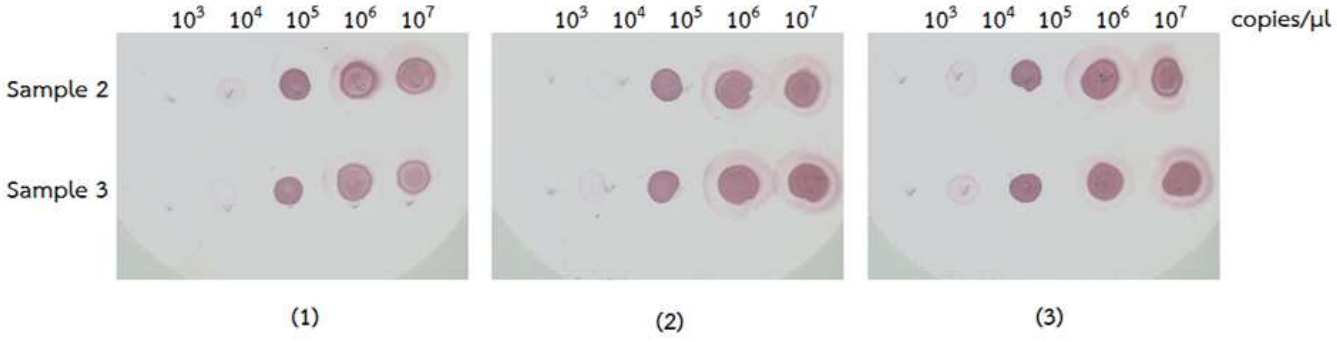

Figure 10 Two of the three seawater samples (Samples 2, 3) were spiked with WSSV at various concentrations of WSSV in the range of 10^3-10^7 copies μl^{-1} for the detection of WSSV by DBIA.

7.2.8 Determination of WSSV by PCR

The detection of WSSV by PCR was carried out to confirm the performance of the DBIA developed. Seawater samples from a pond used for culturing shrimp were spiked with various concentration of WSSV in the range of 2×10^2 to 10^7 copies μl^{-1} and the seawater samples collected from a shrimp pond (Samples 1-3) were tested by PCR. The results of the tests on

the spiked seawater samples showed that the PCR products of the WSSV (about 400 base pairs) serially decreased corresponding to the number of copies of the WSSV (Figure 11) Lane 4-9. The PCR product of the WSSV was found in lane 10 but none was found in Lanes 11-12. These results are the same as those found by DBIA and confirmed that the performance of the DBIA could be used for the quantification of WSSV.

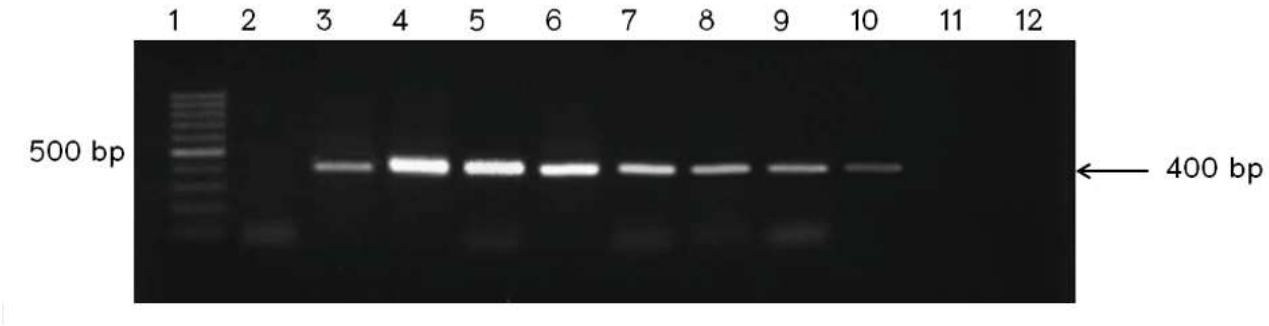

Figure 11 The PCR products of different numbers of copies of WSSV using the 5'FB and 3'RX primer and sea water samples from a pond used for culturing the shrimp analyzed by electrophoresis on a 1.5% agarose gel, staining with ethidium bromide. Lane 1, 100 base pair DNA marker; 2, negative control; 3, positive control; 4–9, PCR product of WSSV at 2×10^7 to 10^2 copies; respectively and 10-12, seawater samples from a pond used for culturing the shrimp.

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7.2 ข้อคิดเห็นและข้อเสนอแนะสำหรับการวิจัยต่อไป

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7.3 บทความวิจัยที่นำเสนอที่ประชุมวิชาการ (Proceeding) และบทความที่ตีพิมพ์แล้ว

ดังแนบ

T3-PB44

Development of a simple test for detecting the White Spot Syndrome Virus (WSSV)

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Abstract. In this manuscript we have described an assay for White Spot Syndrome Virus)WSSV(using White Spot Syndrome Binding Protein)WBP(that has a MW of 3 kDa and binds WSSV. The WBP was produced by *E.coli* HB2151 containing pCANTAB-WBP and purified using a Sephadex G-25 column. WBP was then immobilized with colloidal gold nanoparticles. This colloidal gold probe was used to detect WSSV by dripping onto a nitrocellulose membrane containing WSSV. The color of the gold nanoparticles appears as a purple dot indicating that the colloidal gold probe bound to the WSSV. In the test, the colloidal gold probes detect WSSV at concentrations as low as 1.6×10^6 copies/µL.

Keywords: gold nanoparticles; Nitrocellulose membrane; White Spot Syndrome Virus (WSSV)

1. Introduction

Culturing and exporting shrimp has contributed to Thailands income from aquaculture by around 60,000 – 80,000 million baht a year. Nowadays the industry has to continuously meet with growing problems. One of the most frequent problems is to deal with rapid unexpected and severe virus infections, especially from White Spot Syndrome Virus, WSSV. All shrimp infected with white spot disease in a pool dye within 3 – 10 days (Lighter, 1996). Hence a rapid and sensitive method for the detection of shrimp infections will be useful and will help to decrease the damage of shrimp cultivation. Recently, molecular diagnostic techniques, including real-time polymerase chain reaction (real-time PCR) was the techniques for analysis of WSSV (Tang and Lightner, 2000 and Lo et al., 1996), however, this methods are quite expensive and require many steps as well as an expert operator.

In this work, we have developed an assay for WSSV using the White Spot Syndrome Binding Protein (WBP) that has a MW of 3 kDa and binds with White Spot Syndrome Virus (WSSV). WBP was immobilized with colloidal gold nanoparticles and this colloidal gold probe was employed to detect the WSSV virus.

2. Materials and Methods

2.1 Materials

WSSV stock solutions contained 1.6×10^{10} copies/µL. Chloroauric acid ($HAuCl_4$) and sodium citrate ($C_6H_5O_7Na_3$) were from Sigma-Aldrich (USA). Helper phage M13K07 and Nitrocellulose membranes (pore size, 0.45µm) were obtained from Life Technologies (USA) and Sartorius Stedim Biotech GmbH (Germany). All other chemicals used were of analytical grade.

2.2 Production of White Spot Syndrome Binding Protein (WBP)

The procedure followed was that of Youtong et al., 2011. Briefly, White Spot Syndrome Binding Protein (WB) was produced by *E.coli HB2151* containing the plasmid pCANTAB-WBP. One colony was cultured in LBG (LB+ 2%glucose) at 30 °C for 16-18 h. Then 1 mL was transferred into 10 mL of LBG (LB + 2% glucose + 80 µg/mL of ampicillin) and incubated at 30 °C for 1 h. The culture was centrifuged at 1500 rpm at room temperature for 20 min and the pellet was resuspended in 10 mL of LB containing 1 mM IPTG and 80 µg/mL of ampicillin and incubated at 30 °C for 24 h and again centrifuged at 1500 rpm for 20 min. The protein in the supernatant was further purified by elution from a Sephadex™ G-25 column (GE Healthcare Bio-Sciences AB, Sweden) connected to an AKTA prime plus (GE Healthcare Bio-Sciences AB, Sweden). The obtained protein obtained from the eluted fractions were checked by PAGE on 15% SDS and kept at -80 °C for immobilization with colloidal gold nanoparticles (AuNPs).

2.3 Preparation of gold nanoparticles

The procedure followed was that of Loyprasert-Thananimit et al., 2010. All glassware used in the preparation was rigorously cleaned by soaking in freshly prepared HNO_3 (10% in MilliQ processed water) overnight and thoroughly rinsed. Five hundred milliliters of aqueous solution containing 2.5mM $HAuCl_4$ and 3.75mM sodium citrate ($C_6H_5O_7Na_3$) was prepared (Brown et al., 2000). Five milliliters of 1.25M $NaBH_4$)Sigma-Aldrich, USA (solution was rapidly added into the gold solution while being vigorously stirred. The mixture was continuously stirred overnight and the prepared gold colloidal solution were then stored in a dark glass bottle at 4 °C for further use. The gold nanoparticles were inspected by UV-visible spectroscopy. The sizes of the gold nanoparticles were verified using a transmission electron microscope (TEM).

2.4 Immobilization of WBP with gold nanoparticles

The procedure followed was that of X. Wang et al., 2006. To prepare colloidal gold probes, 150 µL of 0.5 g/L WBP was mixed with 10 mL colloidal gold solution. The reaction was allowed to proceed for 10 min with slow stirring and then overnight at 4 °C without stirring. The reaction mixture was centrifuged at 18,000×*g* 110 min 4 °C and the precipitate was suspended in 1 mL of 0.01 M PBS (pH 7.4) containing 1% bovine serum albumin (BSA) and 0.5% Tween-20. The prepared colloidal gold probes were then stored at 4 °C until used.

2.5 Detection of WSSV

2 µL of WSSV concentrations of 1.6×10^6 copies/µL was dripped onto a nitrocellulose membrane (NCM) then dried at room temperature. Then, 100 µL of blocking agent (0.01M PBS containing 1% BSA and 0.05% Tween-20, pH 7.4) was added onto the nitrocellulose membrane, followed by the addition of 100 µL of the colloidal gold probe while soaked in. Finally, a 100 µL of the solution (0.01M PBS containing 0.05% Tween-20, pH 7.4) was added to soak into the membrane. The appearance of a purple dot indicated a positive reaction.

3. Results and Discussion

3.1 Production of WBP from pCANTAB-WBP *E. coli* (HB2151)

WBP was produced by *E.coli* HB2151 containing pCANTAB-WBP and purified using a Sephadex G-25 column. WBP appeared as a 3 kDa on 15% SDS-PAGE as shown in Figure 1.

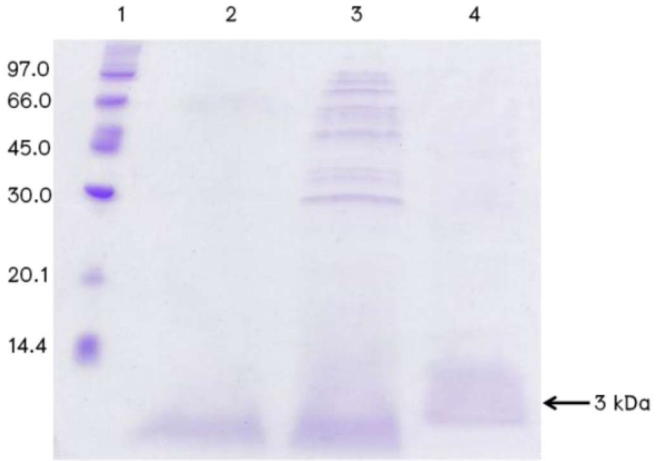

Fig. 1. 15% SDS-PAGE (1) low molecular weight standard markers, (2) induced protein from bacteria containing pCANTAB-WBP plasmid, (3) non-induced protein from bacteria containing pCANTAB-WBP plasmid and (4) WBP purified protein (3 kDa).

3.2 Preparation of AuNPs

Structural characterics of the gold nanoparticles were verified by transmission electron microscope (TEM). The TEM image showed an average size of the particles of 20±4 nm. A UV-vis wave scan (400-800 nm) of the nanoparticle suspension showed a clear absorption maximum 532 nm.

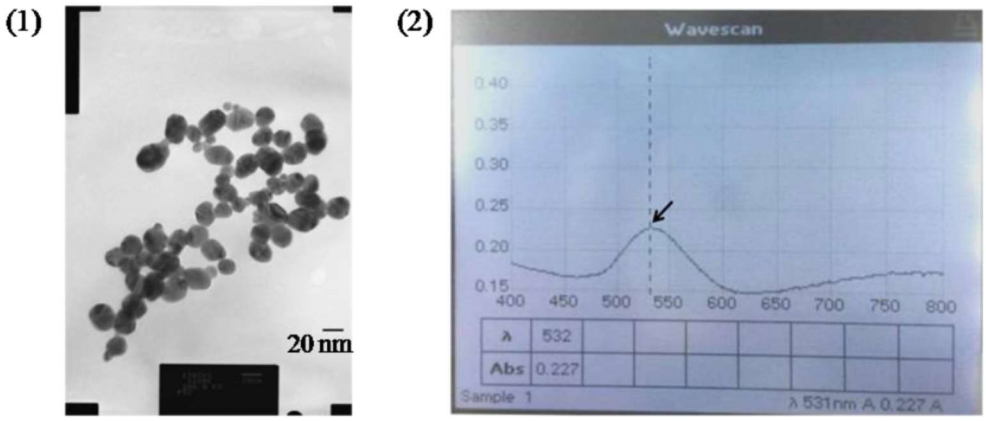

Fig. 2. The TEM images (scale bars: 20 nm) (1) and the optical absorption spectra of AuNPs solution (2).

3.3 Detection of WSSV

A WSSV solutions concentration of 1.6 x 10^6 copies/µL was tested. The results showed the positive purple dot with WSSV sample while no purple dot was appear on the membrane by using the control (PBS) and yellow head virus (YHV) used as a negative control.

Fig. 3. The results of colloidal gold probe to detect the WSSV virus.

4. Conclusions

The WBP produced by *E.coli* HB2151 containing pCANTAB-WBP and purified using a Sephadex G-25 column was immobilized with colloidal gold Nanoparticles. This colloidal gold probe was used to detect WSSV by dripping on a nitrocellulose membrane. The color of the gold nanoparticles appeared as a reddish dot and indicated that binding between the WSSV and the colloidal gold probe had occurred . In the preliminary study, WSSV solutions was detected at concentrations 1.6 x 10^6 copies/µL.

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Semiquantitative dot-blot immunogold assay for specific detection of white spot syndrome virus

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Abstract

A dot-blot immunogold assay (DBIA) was developed to detect white spot syndrome virus (WSSV) using the polyclonal antibody VP26 (anti-VP26). The anti-VP26 was immobilized on gold nanoparticles (Ab-AuNPs), and a nitrocellulose membrane was used as a detection pad. When the target WSSV bound to the Ab-AuNPs a reddish dot appeared on the surface of the membrane used within 2–5 Min, which could be seen with the naked eye. The test was able to detect WSSV at concentrations as low as 10^5 copies μL^{-1} of WSSV. The DBIA developed had good specificity, and the colloidal gold probe can be applied within 2–3 days when stored at 4 °C. For real sample analysis, the DBIA was applied to samples of seawater used for shrimp cultivation without sample preparation. The results indicate that sample 1 showed a positive result, whereas samples 2 and 3 produced negative results. Then, samples 2 and 3 were spiked with WSSV for method validation. To confirm the performance of the DBIA developed, polymerase chain reaction (PCR) was conducted and the PCR results were the same as those found by the DBIA. Therefore, the DBIA developed could be applied for WSSV detection in real water samples. Volume 00, Number 0, Pages 1–8, 2018

Keywords: *dot-blot immunogold assay, nitrocellulose membrane, polyclonal antibody, shrimp farming, white spot syndrome virus*

1. Introduction

The shrimp farming industry has become an important source of exports in Thailand and a multibillion dollar industry [1]. However, nowadays shrimp farmers are continuously facing many problems. One frequently encountered problem is rapid and severe virus infections, especially with the white spot syndrome virus (WSSV), which can cause up to 100% mortality within 3–10 days in commercial shrimp farms. It can infect all important varieties of economically important shrimps, resulting in large financial losses to the shrimp fishery industry [2]. At present, there are various laboratory-based methods by which WSSV detection can be carried out. These include polymerase chain reaction (PCR) [3–6], *in situ* hybridization [7], histopathological [6] and immunological methods [8], and loop-mediated isothermal amplification [9]. Each of these methods has different disadvantages and advantages in terms of cost, convenience, specificity, and sensitivity. However, all these methods require many steps and trained technicians as well as being quite expensive.

The protein components of the WSSV virion (viral protein or VP) have been well established by proteomic methods, and almost 40 WSSV structural proteins have been identified [10] of which 22 are envelope proteins. Four major proteins consisting of VP28, VP26, VP24, and VP19 have been isolated from infected shrimps. VP26 and VP24 were found to be associated with nucleocapsids, and VP19 and VP28 were associated with the envelope [11]. In a further study, it was found that VP28 and VP26 are the two most abundant structural proteins observed in the envelope [12].

In previous studies, the monoclonal and polyclonal antibodies specific to the VPs of WSSV have been developed into

Abbreviations: *Anti-VP26, A Polyclonal Antibody against the Viral Protein26; AuNPs, Gold Nanoparticles; DBIA, Dot-Blot Immunogold Assay; GAT, A gold aggregation Test; PCR, Polymerase Chain Reaction; WSSV, White Spot Syndrome Virus.*

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an immuno-dot blot assay for the detection of WSSV in shrimp [13–16]. Dot-blot nitrocellulose enzyme immunoassay (DB-NC-EIA) can detect WSSV and yellow head virus (YHV) in infected shrimp. Rabbit anti-WSSV IgG-HRP and rabbit anti-YHVIgG-HPR were tested on WSSV-antigen and YHV-antigen spotted onto nitrocellulose membrane. The analysis time using DB-NC-EIA is at least 3 H [8]. Furthermore, the study of immunodot blot assays has showed false positives when labeled with an enzyme [25, 26].

Therefore, a method for the detection of WSSV in shrimp by dot-immunogold filtration assay using a nitrocellulose membrane (NCM) as a support and anti-WSSV monoclonal antibodies (MAbs) 6A4 and 1D5 conjugated gold nanoparticles (AuNPs) was developed. The analysis can be completed within 3 Min without any equipment or incubation. However, false positive results were again visibly present [13]. In addition, an immunochromatographic test (ICT) was developed as a method for the detection of WSSV in shrimp using anti-WSSV MAb 1D5 as a capture antibody immobilized on an NCM and anti-WSSV the MAbs, 2E6 and 2A3, conjugated AuNPs as a detection reagent. The ICT required less than 10 Min to complete, and the detection limit was 1 μg mL^{-1} of WSSV without any false positive results. However, this technique also entails a complicated process and the development of the ICT used many antibodies [27].

Hence, the sensitivity the of the immuno-dot blot assay for WSSV detection in shrimp was enhanced by using AuNPs coupled with a secondary antibody conjugated alkaline phosphatase allowing WSSV to be detected at concentrations as low as 1 ng mL^{-1} [16]. However, the test is a sandwich assay, which still uses many steps.

In our previous studies, the WSSV-binding protein (WBP) was found to bind specifically to the VP26 protein of WSSV [17], and the recombinant VP26 protein (rVP26) was used to produce a polyclonal antibody against the VP26 (anti-VP26) WSSV nucleocapsid protein to develop a label-free impedimetric biosensor, which is a sensitive and quantitative method for WSSV detection [18]. Furthermore, WBP and anti-VP26 have been developed into an immuno-based colorimetric assay for detecting the VP26 protein on the envelope of WSSV in shrimp pond water [19]. Moreover, WBP and anti-VP26 have been used together to develop a method for detecting WSSV in shrimp culture water using a label-free affinity immunosensor [20]. However, these methods are not portable and cannot be used in the field since they require equipment and trained skilled technicians to conduct testing.

In this study, a dot-blot immunogold assay (DBIA) was developed for the detection of WSSV in water from shrimp cultivation ponds, using anti-VP26 immobilized on AuNPs. When the target VP26 bound to the anti-VP26, the color that developed on the membrane could be seen with the naked eye. The DBIA developed is simple and rapid and is an accurate tool ,which can be applied in the field to prevent and control the spread of WSSV to reduce damage to shrimp cultivation.

2. Materials and Methods

2.1. Materials

The WSSV, YHV, and Taura syndrome virus (TSV) stock solutions used in this study contained 2×10^8 , 9×10^9 , and 3×10^7 copies μL^{-1}, respectively. The stock solutions were provided by the Center for Genomics and Bioinformatics Research, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The anti-VP26 was retained for use from Loyprasert-Thananimit et al. [18]. The primer was designed from the shrimp WSSV isolate Japan 98 VP19 gene (GenBank accession number AY249447) forward primers VP19-FB: 5'CGGGATCCATGGCCACCACGACTAA 3′, and reverse primers VP19-RX: 5′GCCTCGAGCCTGATGTTGTGTTTTCTATA 3′. Hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4 \cdot 3H_2O$), trisodium citrate ($C_6H_5O_7Na_3$), and sodium borohydride ($NaBH_4$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cellulose nitrate membranes (pore size 0.45 μm) were purchased from Sartorius Stedim Biotech (Germany). All other chemicals were of analytical grade. All solutions were prepared with ultrapure water from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA)

2.2. Synthesis of AuNPs

All glassware and the magnetic stirring bar used in the AuNPs synthesis were rigorously cleaned by soaking in freshly prepared 10% HNO_3 overnight and thoroughly rinsed with Milli-Q water, then oven-dried prior to use.

The AuNPs, which were 5 nm in diameter, were prepared by the citrate reduction of $HAuCl_4 \cdot 3H_2O$ following the procedure described by Brown and Natan [21]. Briefly, 10 mL of 1% $HAuCl_4 \cdot 3H_2O$ was added to 900 mL of Milli-Q water at room temperature. After 1 Min of stirring, 20.0 mL of 38.8 mM tri-sodium citrate was added. Then 1 Min later, 10 mL of fresh 0.075% $NaBH_4$ was added. The AuNPs were stirred for an additional 5 Min and stored in a dark bottle at 4 °C for further use. They were characterized using ultraviolet/visible spectroscopy (UV–vis) (NanoDrop 2000c spectrophotometer) in a wavelength range between 400 and 800 nm. The sizes of the AuNPs were verified by a transmission electron microscope (TEM) using a JEOL 2000FX microscope (JEOL, Tokyo, Japan) fitted with a Gatan (Pleasanton, CA, USA) CCD camera.

2.3. Optimization of antibody-immobilized on AuNPs

A gold aggregation test (GAT) was used to determine the optimal condition of immobilization using NaCl, which caused the aggregation of the AuNPs and shifted the maximum absorbance peak from 515 nm to 580 nm, resulting in a difference in absorbance ($UAbs_{change}$) that was calculated from Eq. (1) [22]:

$$UAbs_{Change} = [UAbs_{515} - UAbs_{580}]_{Before\ NaCl\ addition} - [UAbs_{515} - UAbs_{580}]_{After\ NaCl\ addition} \quad (1)$$

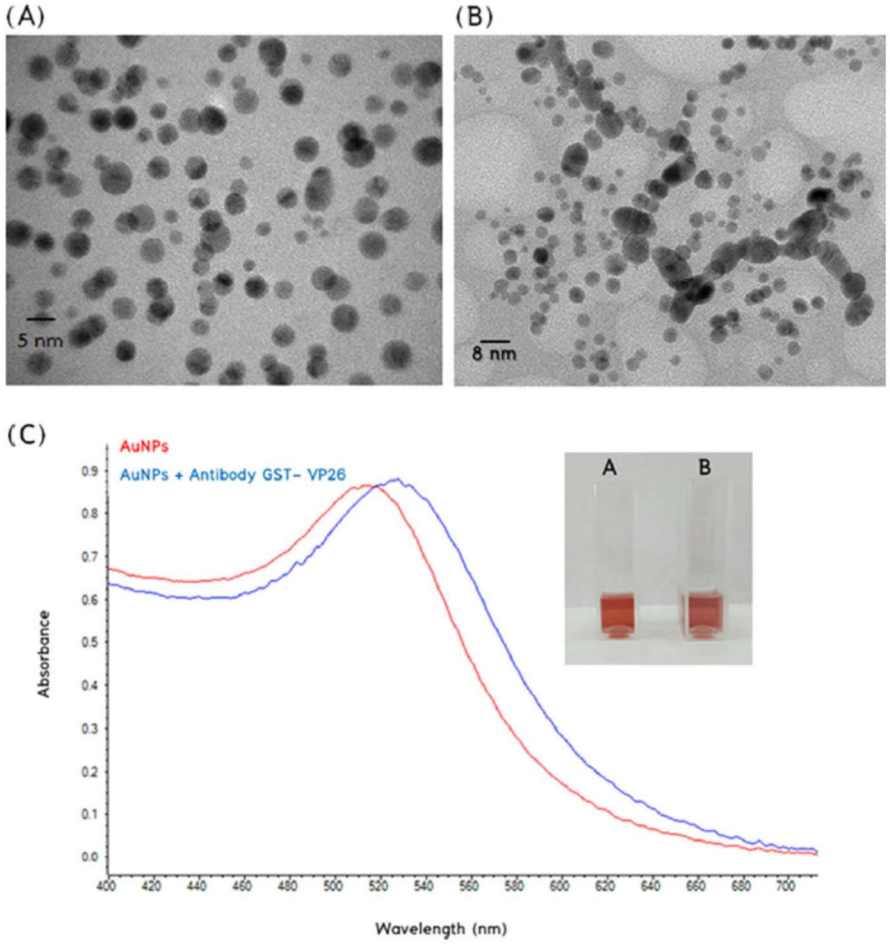

FIG. 1 *TEM image of the AuNPs; scale bar indicates 5 nm (A), TEM image of the Ab-AuNPs; scale bar indicates 8 nm (B), and UV–Vis spectra of the AuNPs at 515 nm (red curve) and Ab-AuNPs at 518 nm (blue curve) (C).*

The optimal concentrations of antibody immobilized on the AuNPs were determined by performing a GAT according to the method described in Chamorro-Garcia et al. [23], with some modifications. Briefly, 1 mL of AuNPs was placed in each well of a multiwell plate. The pH of the AuNPs was adjusted with 0.1 M Na_2CO_3 to pH values of 5.5, 7, 8, 9, and 10. Then 100 μL of antibody at various concentrations (0, 10, 50, 100, 200 μg mL^{-1}) was added to each well. The plates were incubated under gentle mixing at room temperature. The optimal concentration of antibody was determined by adding 100 μL of 10% NaCl solution to each well and incubating it under gentle mixing for 10 Min at room temperature. NaCl causes the aggregation of AuNPs and changes the color from red to blue. If the color does not change, the antibody immobilized on the AuNPs (Ab-AuNPs) contains the optimal concentration of Ab for fully covering the surface of all thex AuNPs [24].

Following the GAT, optimized concentrations of bovine serum albumin (BSA) for nonspecific site blocking were determined. Briefly, 1 mL of AuNPs was placed in each well of a multiwell plate. The pH of the AuNPs was adjusted with 0.1 M Na_2CO_3 to pH values of 5.5, 7, 8, 9, and 10. Then 100 μL of BSA solution at different concentrations (0, 0.1, 0.5, 1, 2 mg mL^{-1}) was added to each well. The plates were incubated under gentle mixing at room temperature. Then 100 μL of 10% NaCl solution was added to each well and incubated under gentle mixing for 10 Min at room temperature. The optimum concentration of BSA for blocking any remaining active surface of the Ab-AuNPs was the concentration of Ab-AuNPs that did not change color from red to blue after the addition of 10% NaCl.

2.4. Preparation of antibody-immobilized on AuNPs

The AuNPs modified with anti-VP26 were prepared according to Chamorro-Garcia et al. [23]. Using the condition optimized by the GAT, the pH of the AuNPs was adjusted to 9 using 0.1 M Na_2CO_3. Briefly, the pH of the AuNPs was adjusted to 9 using 0.1 M Na_2CO_3. Next, 100 μL of 200 μg mL^{-1} anti-VP26 was incubated with 1 mL of AuNPs for 30 Min under gentle mixing

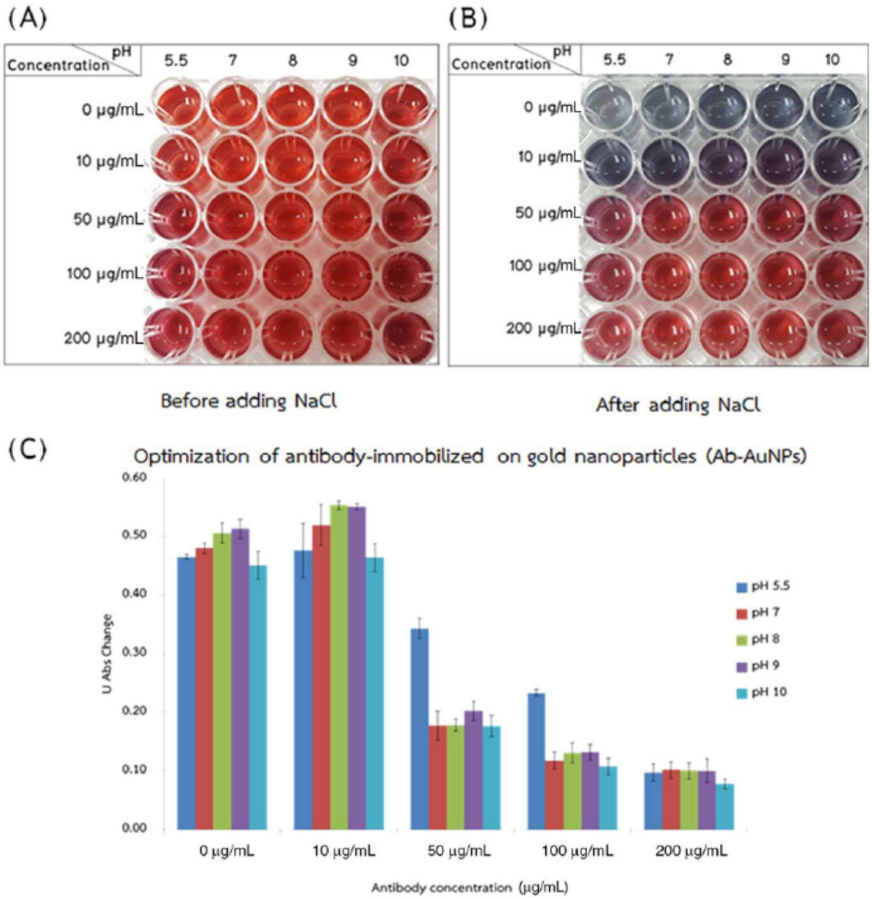

FIG. 2 *Images of the solutions with various concentrations of anti-VP26 and pH values (A) and difference in absorbance (Abs 515 – Abs 580) plotted against concentration of anti-VP26 in the GAT for different pH values (B).*

at room temperature. Then the blocking step was performed by adding 100 μL of 1 mg mL^{-1} BSA incubated for 30 Min at room temperature. Finally, the Ab-AuNPs were separated from the excess of reagents by centrifugation at 15,000*g* and 4 °C for 45 Min and the pellet of conjugates was resuspended in 200 μL of phosphate buffered saline (PBS), pH 7.4 and kept at 4 °C for further use.

2.5. Characterization of the AuNPs and the AuNPs–antibody conjugates

UV–vis measurements were performed using a NanoDrop 2000c spectrophotometer using a standard 10 mm path length quartz cuvette. Spectra were obtained from 400–800 nm. Milli-Q water was used as the blank for the AuNPs and as the buffer used for the Ab-AuNPs. High-resolution TEM images were recorded with a JEOL 2000FX microscope (JEOL, Japan) fitted with a Gatan (Pleasanton, CA, USA) CCD camera. Samples were prepared by placing 2 μL of AuNPs and Ab-AuNPs onto a copper grid. The grid was then air-dried.

2.6. Dot-blot immunogold assay

The detection procedure was as follows: 2 μL of concentrations in the range of 10^3–10^7 copies of WSSV serially diluted in PBS (0.01 M, pH 7.4) was spotted onto a nitrocellulose membrane (0.45 μm pore size). The spots were dried, and the membrane was blocked with a blocking agent (0.01 M PBS containing 1% BSA and 0.05% Tween-20, pH 7.4) for 15 Min at room temperature. Then the membrane was washed three times for 10 Min with PBS buffer. Subsequently, the membrane was incubated with Ab-AuNPs for 30 Min at room temperature. Finally, the membrane was washed with lotion (0.01 M PBS containing 0.05% Tween-20, pH 7.4). The appearance of a reddish dot on the surface of the membrane indicated a positive result, whereas the absence of such a dot was indicative of a negative result.

2.7. Performance of the DBIA

The sensitivity, specificity, and reproducibility of the DBIA was tested with three viruses, WSSV, YHV, TSV with concentrations in the range of 10^3–10^7 copies μL^{-1} diluted in PBS (0.01 M,

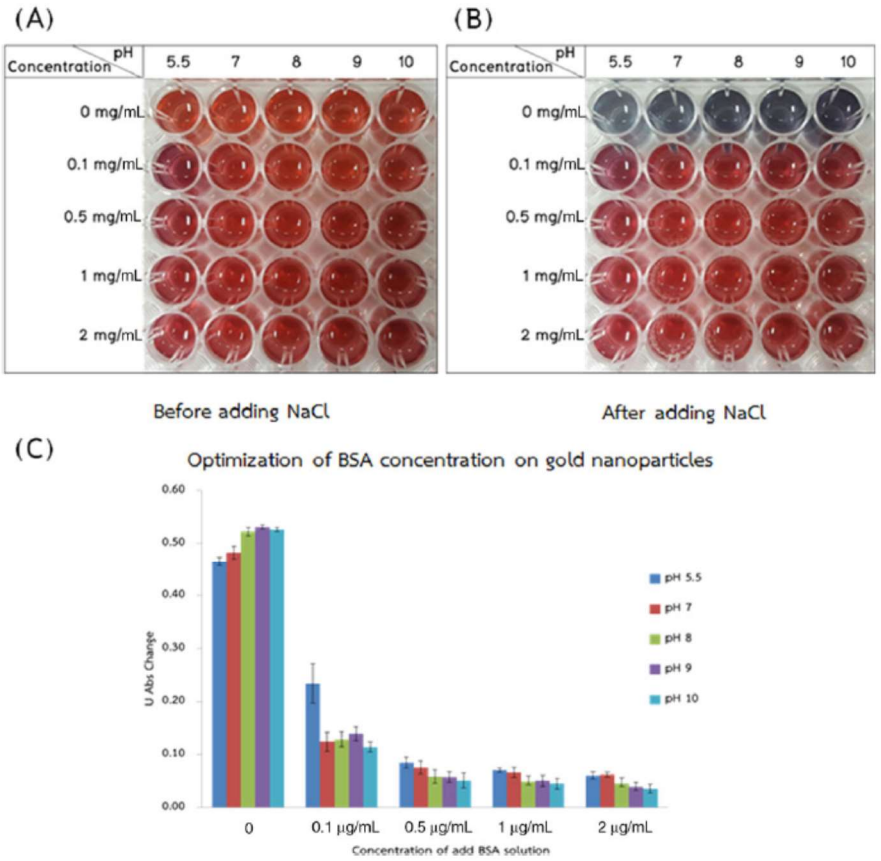

FIG. 3 *Images of the solutions with different concentrations of BSA and pH values (A) and difference in absorbance (Abs 515 – Abs 580) plotted against concentration of BSA in the GAT for different pH values (B).*

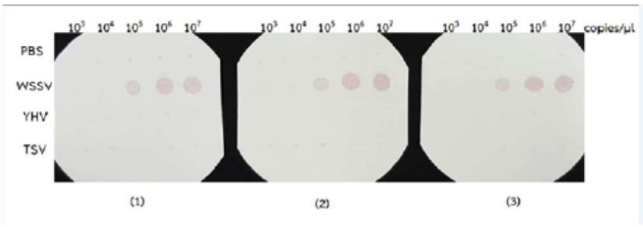

FIG. 4 *The sensitivity and specificity for detection of WSSV by the DBIA; Detection as low as 10^5 copies μL^{-1} of WSSV. PBS was used as the negative control.*

pH 7.4) and PBS, pH 7.4 as a control. All the viruses were tested using the DBIA. The appearance of a reddish dot on the surface of the membrane indicated a positive result, whereas the absence of such a dot was indicative of a negative result.

2.8. Detection of WSSV from a seawater sample by DBIA

Seawater samples were collected from a pond used for culturing shrimp in Songkhla province, Thailand. Sample 1 was collected from cultured pond with WSSV-infected shrimp, and samples 2 and 3 were collected from cultured pond with healthy shrimp. The seawater samples were spiked with different concentrations of WSSV in the range of 10^3–10^7 copies μL^{-1} and were used for the detection of WSSV by DBIA.

2.9. Comparison of the sensitivity of the DBIA and PCR

WSSV solutions in concentrations between 2×10^3 and 2×10^7 copies μL^{-1} were tested using the DBIA. The same samples were also investigated with a PCR to compare the performance of the DBIA developed. The PCR was performed in a 25-μL reaction volume containing 400 ng of DNA extracted from seawater, 1 μM of each primer, 4 mM of deoxynucleotide (dNTP), 2.5 mM of $MgCl_2$, 1× PCR buffer, and 2.5 U tag DNA polymerase. The primer used was from the shrimp WSSV isolate Japan 98 VP19 gene (GenBank accession number AY249447.1). The PCR was carried out according to the method described in Youtong et al. [17]. Briefly, it was conducted with an initial denaturation step at 94 °C for 5 Min, followed by 39 cycles of 94 °C for

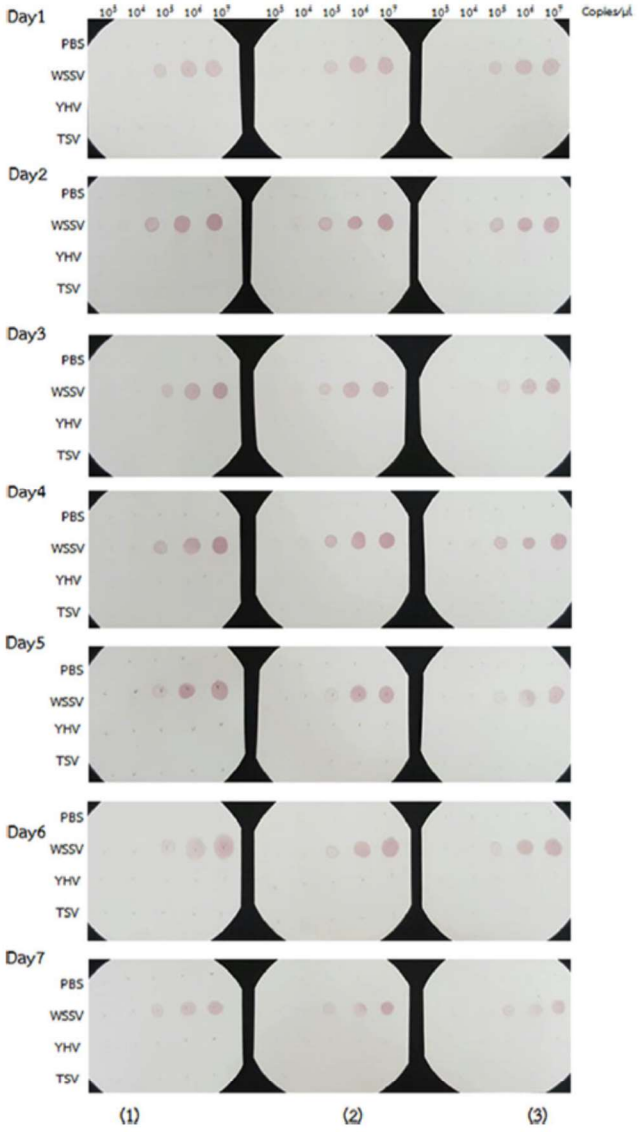

FIG. 5 *The reproducibility of the DBIA was tested by use every day on 7 consecutive days.*

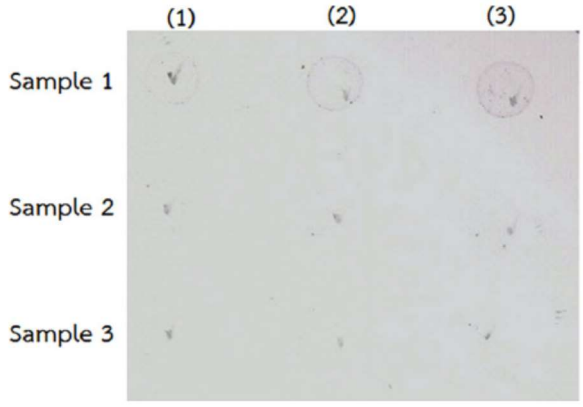

FIG. 6 *The three seawater samples (samples 1, 2, and 3) used for the detection of WSSV by DBIA.*

1 Min, 55 °C for 1 Min, and 72 °C for 2 Min. The PCR product was analyzed by electrophoresis on a 1.5% agarose gel.

3. Results and Discussion

3.1. Characterization of AuNPs and antibody-immobilized on AuNPs

The AuNPs were synthesized by the citrate reduction method. The AuNPs solution is intensely red in color. The synthesized AuNPs were characterized by TEM images, and Fig. 1A shows a monodispersion of AuNPs with an average particle diameter of 5 ± 0.3 nm. After immobilizing the antibody on the Ab-AuNPs, the resulting Ab-AuNPs had an average diameter of 8 ± 0.3 nm (Fig. 1B). The AuNPs were characterized by UV–vis spectroscopy. As shown in Fig. 1C, the characteristic UV–vis absorption spectrum shows a maximum absorbance at 515 nm (red curve), which evidences the formation of a well-dispersed colloidal suspension. When the antibodies were immobilized on the AuNPs, the absorption band changed from 515 nm (red curve) to 518 nm (blue curve). This result indicated that the Ab-AuNPs had good colloidal stability without aggregation.

3.2. Optimization of antibody-immobilized on AuNPs

A GAT was preliminarily carried out to determine the optimum conditions for immobilization and to estimate the minimum amount of antibodies required to fully cover the surface of all the AuNPs. NaCl dislocates surface charges on the AuNPs causing their aggregation (color change from red to blue), whereas Ab-AuNPs do not aggregate since NaCl is not able to break the Au–S bond [23]. The optimum concentration of anti-VP26 for avoiding AuNP aggregation was determined based on observing when the solution did not change color from red to blue after the addition of 10% NaCl (Figs. 2A and 2B) as well as the result of the difference in absorbance (UAbs change) from 515 nm (dispersed AuNPs) and at 580 nm (aggregated AuNPs) as shown in Fig. 2C. The results of the UAbs change were tested for significance in a two-way analysis of variance (ANOVA) using SPSS version 16.0 (P value ≥ 0.05). The results indicated that the minimum concentration required for full coverage of the surface of all the AuNPs was 50 μg mL^{-1}. In this study, however, the concentration of anti-VP26 selected to be used was 200 μg mL^{-1} to prevent salt-induced aggregation.

In terms of pH, the results showed a color change after adding NaCl at pH 5.5, indicating the aggregation of the AuNPs. However, there was no color change for pH values from 7 to 10

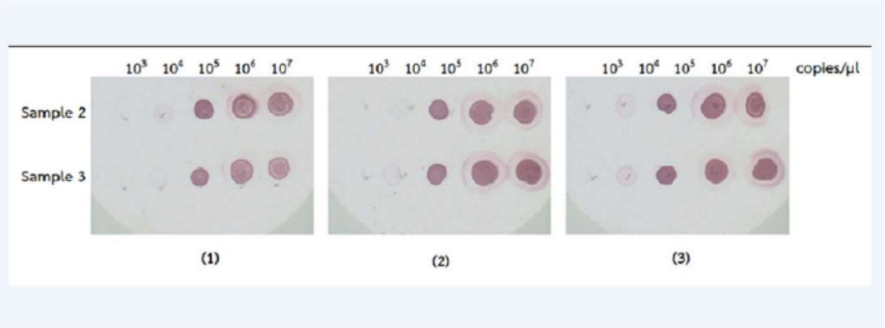

FIG. 7 *Two of the three seawater samples (samples 2 and 3) were spiked with WSSV at various concentrations of WSSV in the range of 10^3–10^7 copies μL^{-1} for the detection of WSSV by DBIA.*

as shown in Fig. 2. These results indicated that the optimum pH of the AuNPs for immobilizing anti-VP26 was between 7.0 and 10.0. In this study, a pH of 9 was selected for immobilization because a low pH value causes the AuNPs to agglomerate, whereas a high pH value tends to generate unstable Ab-AuNPs. The optimal concentrations of BSA for nonspecific site blocking were established by a GAT. Based on the results shown in Fig. 3, the optimum conditions were a BSA concentration of 1 mg mL^{-1}.

3.3. Performance of the DBIA

The WSSV, YHV, and TSV were tested using the DBIA with concentrations in the range of 10^3–10^7 copies μL^{-1}. In these experiments, the viruses were tested with three replicates from the same batch of Ab-AuNPs to determine the specificity, sensitivity, and reproducibility of the DBIA. Figure 4 shows images of the DBIA after testing with a standard virus, clearly showing an increase in the observed color intensity as a result of the increase in the WSSV concentration. YHV and TSV were used to test the specificity of the DBIA, and the results showed that no reddish dot appeared on the surface of the membrane within 2–5 Min as occurred with the WSSV, as can be seen in Fig. 4. Therefore, the results showed that the DBIA developed had good specificity for WSSV detection.

In terms of sensitivity, the DBIA has a detection limit as low as 10^5 copies μL^{-1} of the WSSV. The DBIA is sensitive enough to detect WSSV in water because the amount of WSSV in water that is infective and lethal in shrimp needs to be above 1.6×10^4 copies μL^{-1} [19]. The reproducibility of the DBIA was evaluated every day over a period of 7 days under storage at 4 °C. The results showed that reddish dots on the surface of the membrane were clearly observed within 2–3 days although its performance reduced in the following days. Therefore, the test developed should be used within 2–3 days and stored at 4 °C (Fig. 5).

This DBIA developed can be used as a screening tool for use by shrimp farmers and can be applied in the field to prevent and control the spread of WSSV in shrimp cultivation. The DBIA has many advantages. First, the DBIA can be applied for WSSV detection in real water samples without sample preparation whereas other tests mainly use shrimp gill tissues, and sample preparation is needed [8, 16, 27] Second, the study of DB-NC-EIAs [25, 26] and DIGFAs [13] has shown false positive results. Third, the DBIA results showed that when the target WSSV bound to the colloidal gold probe, a reddish dot appeared on the surface of the membrane within 2–5 Min, which could be seen with the naked eye without incubation or any equipment. The analysis time for our work is faster compared to the ICT [27] with the process being completed within 10 Min. Finally, the sensitivity of DBIA detection limit is 2×10^5 copies μL^{-1} of WSSV (0.087 ng mL^{-1}). The DBIA has greater sensitivity compared to the ICT where the limit of detection is 1 μg mL^{-1} [27] and immuno-dot blot assay where the limit is 1 ng mL^{-1} [16].

3.4. Detection of WSSV in seawater samples by DBIA

Three seawater samples were collected from ponds used for shrimp cultivation. All the seawater samples were tested using the DBIA. All the experiments in this study were performed in three replicates. The results were as follows: sample 1 indicated a positive result because a reddish dot appeared on the surface of the membrane within 2–5 Min, whereas the absence of a dot in samples 2 and 3 was indicative of a negative result (Fig. 6). Thereafter, samples 2 and 3 were spiked with WSSV from 10^3 to 10^7 copies μL^{-1} and tested by DBIA. As a result, reddish dots appeared on the surface of the membranes within 2–5 Min. The detection limit was 2×10^4 copies μL^{-1} of WSSV (Fig. 7) without sample preparation. These results suggest that the DBIA developed is a reliable method for the detection of WSSV in seawater samples.

3.5. Determination of WSSV by PCR

The detection of WSSV by PCR was carried out to confirm the performance of the DBIA developed. Seawater samples from a pond used for culturing shrimp were spiked with various concentrations of WSSV in the range of 2×10^2 to 10^7 copies μL^{-1}, and the seawater samples collected from a shrimp pond (samples 1–3) were tested by PCR. The results of the tests on the spiked seawater samples showed that the PCR products of the WSSV (about 400 base pairs) serially decreased corresponding to the number of copies of the WSSV (Fig. 8, lanes 4–9). The

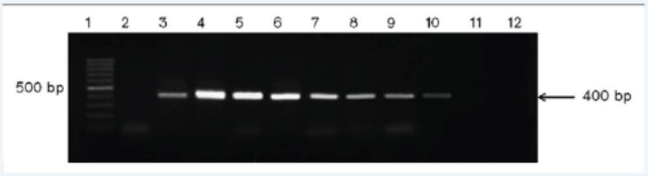

FIG. 8 *The PCR products of different numbers of copies of WSSV using the 5′FB and 3′RX primer and seawater samples from a pond used for culturing the shrimp analyzed by electrophoresis on a 1.5% agarose gel, staining with ethidium bromide. Lane 1, 100 base pair DNA marker; lane 2, negative control; lane 3, positive control; lanes 4–9, PCR product of WSSV at 2 × 10^7 to 10^2 copies; respectively and lanes 10–12, seawater samples from a pond used for culturing the shrimp.*

PCR product of the WSSV was found in lane 10, but none was found in lanes 11 and 12. These results are the same as those found by DBIA and confirmed that the performance of the DBIA could be used for the detection of WSSV.

4. Conclusion

A DBIA was developed for the detection of WSSV using the polyclonal antibody anti-VP26, which specifically binds to the VP26 protein of WSSV. The Anti-VP26 was immobilized as Ab-AuNPs. The procedure used for the detection of WSSV in seawater samples from a pond used for culturing shrimp was as follows: Samples were spotted onto a nitrocellulose membrane, and the membrane was incubated with Ab-AuNPs. The detection of WSSV indicated a positive result because reddish dots appeared on the surface of membranes within 2–5 Min, and the test was able to detect WSSV at a concentration as low as 10^4 copies μL^{-1}, whereas the absence of such a dot was indicative of a negative result.

The DBIA is simple, rapid, specific, and no sample preparation is needed for real sample analysis. This DBIA developed can be used as a screening tool for shrimp farmers and applied in the field to prevent and control the spread of WSSV in shrimp cultivation.

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The authors indicate no potential conflicts of interest.

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