Validation of White Spot Syndrome Virus Infection in *penaeid* Shrimp of Different Geographical Regions using Electrochemical Immunosensor

Natarajan Anusha, Annamalai Senthil Kumar, Raja Sudhakaran

Abstract: ***WSSV being a major threat causing disease in the aquaculture industry is a well-known pathogen, yet there is no proper treatment found to eradicate the virus causing huge economic loss to the farmers. Being lethal and quickly replicative, the morality occurs within 3-10 days post infection. To support the farmers on field level through detection technique, we had developed an electrochemical immunosensor for WSSV using modified GCE (glassy carbon electrode) immobilised methylene blue. In this study, we had collected shrimp samples from different geographical regions of east coast of India to validate the developed immunosensor method in comparison with other molecular methods and commercially available detection kit. The immunosensor developed is observed to be sensitive and has no controversy with other molecular methods; having the potential to be analysed on site at shrimp culture ponds. Validation of the method is done with different samples and further research is required to make it as a gadget.***

Keywords: WSSV; Immunosensor; Diagnosis; Validation

Abbreviations: WSSV-White Spot Syndrome Virus**,** GCE-Glassy Carbon Electrode, DNA- Deoxyribonucleic acid, ELISA- Enzyme-Linked Immunosorbent Assay, PCR-polymerase chain reaction, Ab^2-HRP- Horse radish peroxidase linked Secondary antibody, Ab^1- Primary antibody, WSSV-vp28- White spot syndrome virus-viral protein 28, Ag- Antigen, MB-methylene blue, EDC/NHS-3-dimethyl aminopropylcarbodiimide/N-hydroxysuccinimide, BSA-bovine serum albumin, ABTS-2,2′ -Azinobis (3-Ethylbenzthiazoline-6-Sulfonate) (ABTS), SDS-sodium dodecyl sulphate, Ag/AgCl – Silver/Silver chloride, TMB-3, 3′, 5, 5′ -tetramethylbenzidine, KCl- Potassium chloride, PBS- Phosphate Buffer Solution, CV- Cyclic voltammetry, H_2O_2- Hydrogen peroxidase, NTE buffer- NaCl-Tris-EDTA buffer.

I. INTRODUCTION

The commercial shrimp cultivation has been increased in recent era. In parallel, the ailment including White Spot Syndrome Virus (WSSV) also takes the limelight for researchers in aquaculture field. WSSV is an extremely lethal and contagious shrimp pathogen emerged globally. The outbreaks of WSSV lead to cumulative mortalities of 100% within 3–10 days after the on fall of clinical signs [1, 2].

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The first case of WSSV was reported in northern Taiwan in the year of 1992 [2]. Since its spread, shrimp farms and other aquaculture industries were lead to threats and economic loss [3].

Till date, multiple ways of transmission and multiple hosts' carriers (more than 90 species of arthropods and some carriers belonging to other phyla that may constitute a potential source of infection) has been reported for WSSV [4]. Office of International Epizootic (OIE) Aquatic Animal Health Code has declared WSSV as notifiable disease since 1997 reported by Lightner [5]

Researchers are trying to find a therapeutic treatment to prevent the spread or decrease the antagonistic impact of WSSV. But, no appropriate medicines have been surrounded up until this point. In order to avoid the ease transmission of the virus, regular and intensive surveillance records needs to be maintained in brood-stock as well in larvae [6, 7]. There are several methods to detect the WSSV includes from basic histopathology to complicated conventional detection techniques like DNA based detection (PCR, DNA microarray); Protein based detection such as dot blot, western blot ELISA and antibody based microarray [8]. Based on sandwich immunoassay method many test kits have been developed as well [9, 10]. Due to the limitations in the conventional techniques, we took electrochemical sensor as an alternative and developed a prototype specific and sensitive to WSSV [11].

II. MATERIALS AND METHODS

A. Collection of Animal

The shrimp samples were transported on dry ice and stored at −20°C. Shrimp were collected from south east coastal regions includes Chennai-Tamil Nadu (12°52'02.4"N 80°14'38.4"E),
80°01'09.8"E) and Marakannam, Tamil Nadu (12°11'24.9"N 79°55'28.1"E).

B. Work flow

Challenge of this study was to evaluate WSSV infected shrimp, which were collected from shrimp ponds having an outbreak of white spot syndrome during the study period using the developed prototype. In addition, the prototype results were also compared to conventional PCR (polymerase Chain Reaction), western blot and commercially available strip based WSSV kit called Q-Test (Marine Leader Co., Ltd. Thailand).

C. PCR

A sum of 53 samples was collected (Table1). The animal gills tissues of 20 grams were collected and homogenised with NTE buffer (20 mM Tris–HCl -pH 7.4, 200 mM NaCl, 0.2 mM EDTA). The homogenised tissues were centrifuged and stored at -20ºC and used for the studies. The experiment was started with confirmation of WSSV by performing PCR. Guanidine hydrochloride method was adopted for DNA extraction from dissected tissues [12]. The amplification of DNA cycling conditions are initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30seconds, annealing at 50°C for 30 seconds and extension at 72°C for 30 seconds with a final extension at 72°C for 10 min [11]. The PCR product was checked in 1% agarose gel the results were documented (Figure 1a).

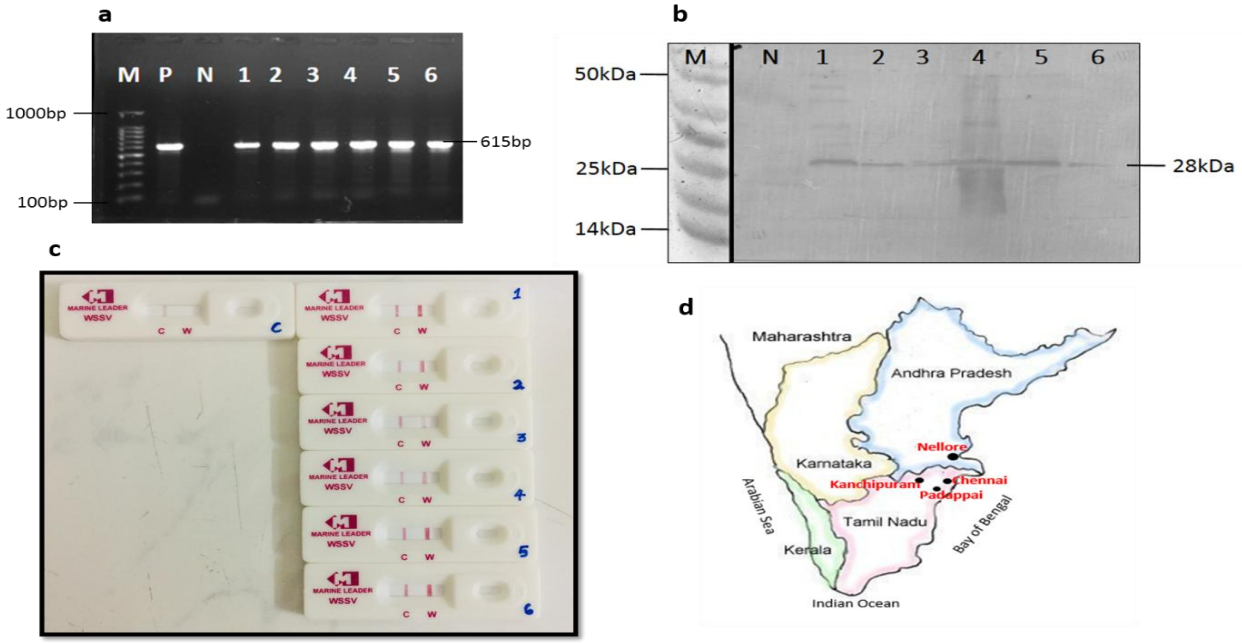


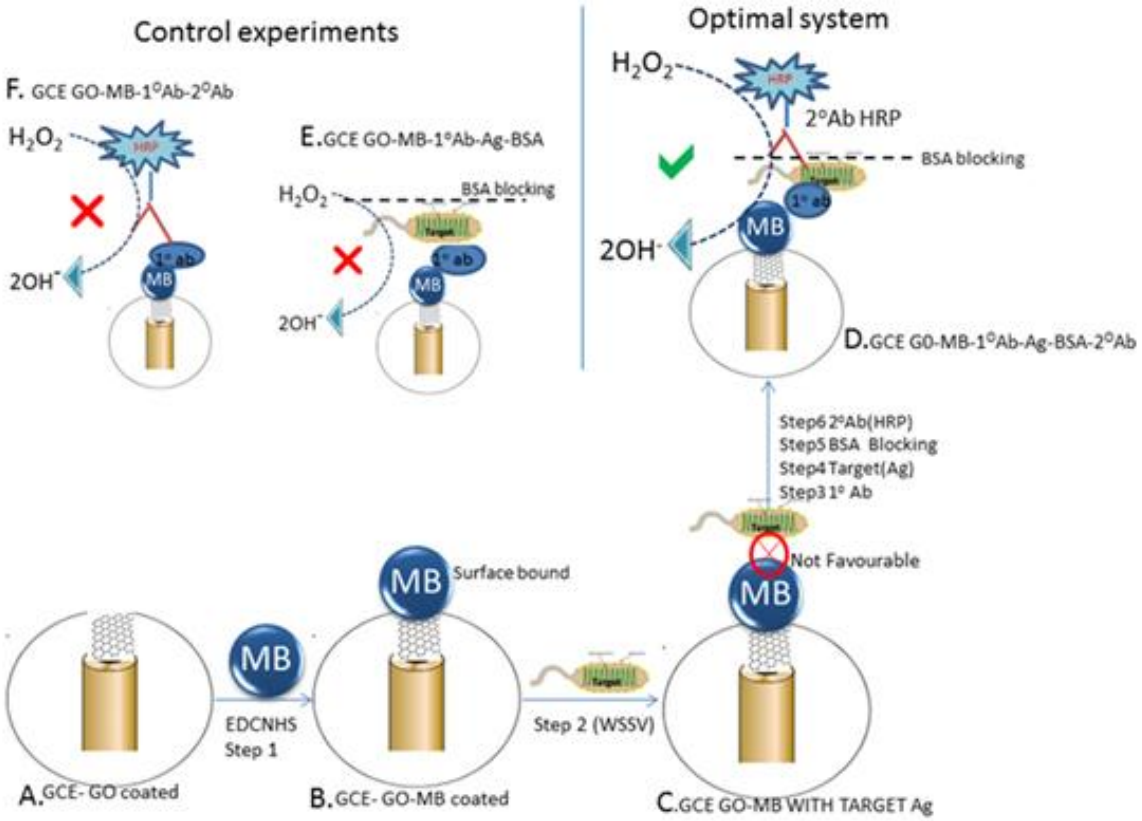
Figure 1. In all the three experiments lane 1-6 were followed same region samples. 1- Chennai-1, 2- Padappai-2, 3- Marakannam-1, 4-Marakannam-2, 5- Nellore-1, 6-Nellore-2. **a)** Amplification of WSSV-vp28 gene was observed in PCR for different field samples. Different field DNA samples were studied for WSSV-vp28. M-100bp marker, P-positive control, N- Negative control. All region samples were found to be positive for WSSV. **b)** Protein expression of WSSV-vp28 from the sample collected was observed in Western Blot. Western Blot was performed and protein expression of different samples was seen. M-Marker, N-Negative control. **c)** Q-Test's (Diagnosis strip test WSSV) commercial kit results. C- Control shows negative for WSSV and WSSV infection which is indicated by a red line in W. **d)** Location of sample collection along South Indian coastal regions. The red colour indicates the regions we collected the samples.

D. Western Blot

The tissue homogenate was analysed for total protein using Bradford's protein estimation procedure by Bradford [13] and Western blot was performed. Tissue was from purified for WSSV-vp28 protein, served as a positive control, while healthy the tissue was used as an uninfected negative control. The anti-mouse vp28 antibodies raised in rabbit were used as primary antibody (Ab^1) with the dilution of 1: 10,000 and primary antibody linked with HRP at a dilution of 1: 10,000 were used as secondary antibody (Ab^2-HRP) for detection of vp28 (goat raised anti-rabbit). Nitro-cellulose membrane were used observe the results developed using the TMB (3, 3′, 5, 5′ -tetramethylbenzidine) blotting substrate solution. These were also used in the prototype (figure 1b).

E. Electrochemical sensor

For this study, we chose a cost effective electrode as a working electrode- glassy carbon electrode (GCE) along with reference and counter electrode. Graphene oxide (GO) was used as the carbon material. Methylene blue (MB) serves as a redox mediator. Fabrication of GCE/GO@MB modified electrochemical immunosensor. EDC-NHS (Ethyl (Dimethylaminopropyl) Carbodiimide - N-Hydroxysuccinimide) was used as a linker molecule. It immobilizes the antigen on electrode surface without any hindrance of toxicities and prevents the leaches. GCE was coated with 5µl of Go solution and immobilised in 5mg of methylene blue (MB) in 500µl of ethanol. After every preparation step the cyclicvolatmmetric (CV) was run at 10 mV s^{-1}. The procedure were followed with coating primary antibody (Ab^1), BSA was used for blocking, EDC-NHS as linker, followed by coating the antigen of WSSV (Ag). Secondary antibody (Ab^2-HRP) linked with horse radish peroxidase was coated. The system was taken as GCE/GO@MB-Ab^1-Ag-BSA-Ab^2-HRP was subjected to CV study with 500 µ M H_2O_2 dissolved pH 7 PBS at v = 10 mV s^{-1} (Figure 2). From the developed prototype, we were able to detect the presence or absence of WSSV within 35±5 min time [11]. We have designed a working scheme for the electrochemical immunosensor for better understanding. (Scheme 1)



Scheme1 Illustration for the development of WSSV electrochemical immunosensor using GCE/GO@MB chemically modified electrode (Step-1) by sequential immobilization of WSSV target Ag (Step-2)was not favorable, Ab^1(Step3) coated with WSSV target Ag (Step-4), Bovine Serum Albumin blocking(Step-5) and Ab^2 was coated(Step6)(A-D) and its relevant control experiments (E & F).

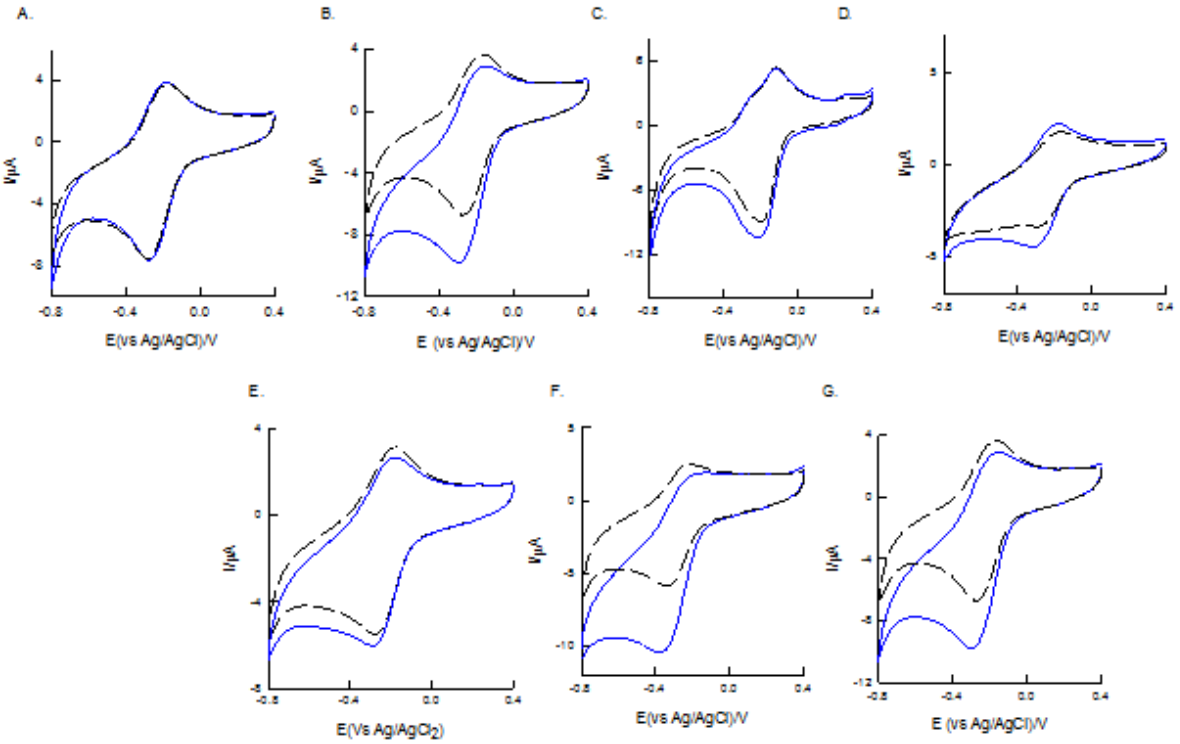


Figure 2 CV responses of GCE/GO@MB-Ab^1-BSA-Ag-Ab^2-HRP with 500µM of H_2O_2 in pH 7 PBS at v = 10 mVs^{-1}. (A) Control (B) Chennai-1 (C) Padappai-2 (D) Marakannam-1 (E) Marakannam-2 (F) Nellore-1 (G) Nellore-2

F. Kit Test

Commercially available kit Q-Test (Diagnosis strip test WSSV) was brought from Thailand. The results obtained were compared to the other experiments performed. The kit was provide with a buffer, pestle and a strip. The procedure was followed as given in the kit description. The tissue sample (1 strip of gill) from the WSSV suspect animal was taken and squashed in 0.3ml buffer provided in the microcentrifuge using pestle. From that 0.15ml-0.2ml was withdrawn applied in the sample well and the results were read after 15-30mins. The reddish purple colour band appearance on C refers the control and W refers to presence of WSSV (Figure 1c). We collected different samples from each region (Figure1d) and they were checked for the WSSV infection using PCR as the first step. The PCR was performed thrice and confirmed. The animals from each region's field was taken and proceeded with different experiments including the immunosensor technique.

III. RESULT AND DISCUSSION

From the experiments performed, we were able to observe that the prototype developed to detect WSSV was efficient in detecting the samples which was collected from the farms (Figure 2). We were able to see the prominent H_2O_2 sensing that is the current difference in all the samples. The samples were compared with the control (healthy animal sample). To confirm the infection in the samples, initially DNA was extracted and PCR was performed (Figure1a). Parallely, the protein samples were taken and western blot was performed (Figure 1b). The blot was raised for WSSV confirming the infection in the samples collected. We wanted to cross check our prototype with commercially available immunochromatic product (Figure 1c). The samples responded same the in all the experiments. The prototype developed using methylene blue dye combined with graphene oxide in the modified glassy carbon electrode was found to be much efficient in diagnosing the field samples. This electrochemical immunosensor system is very quick and cost effective. Hydrogen peroxide sensing using HRP linked secondary was sensitive to detect WSSV and no false positive results were observed. In the proposed detection methodology, we have demonstrated testing positive animal samples and negative control target by the innate characteristics of antigen-antibody of vp28 WSSV and have been compared with molecular methods. This detection technique is suitable for preparation of bioassay kits, where the modified electrodes can be prepared in bulk which can be dipped directly into the target antigen sample and in subsequent solutions to read the output. This technique can also be generalized for the detection of other pathogen, provided specific antibodies are available for them. The proposed method can be observed to improve the real-time immunoassay performance and have wide application in the detection of virulent pathogens in aquaculture farming industries.

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Table 1: The shrimp sample collected from different regions are evaluated using molecular techniques, electrochemical immunosensor technique (EIS), and commercial Q-Test kit. The severity of the virus in the animal was notified.

S.No	Geographical Region (District)	Latitude	Sample Nos.	PCR Result	Protein study Result	EIS Result	Q-Test
1	Chennai, TN	Kanathur 12°52'02.4"N 80°14'38.4"E	1	+	+++	+++	+++
			2	+	++	++	+++
			3	+++	++	+	++
			4	+	+++	++	+++
			5	+	+++	+++	+++
			6	+	+	++	++
2	Kanchipuram, TN	Padappai 12°53'15.9"N 80°01'09.8"E	1	++	++	++	++
			2	++	+++	++	++
			3	+++	+++	+++	++
			4	++	+	+	+
			5	+	+	+	+
			6	+++	++	++	++
			7	+++	+++	+++	++
			8	+++	+	++	++
3	Villupuram, TN	Marakannam, Chettinagar 12°05'58.7"N 79°54'17.9"E	1	++	+	+	+
			2	++	+	++	++
			3	+	+	+	-

			4	+	+	+	-
			5	+	+	++	+
			6	+	+	+	+
			7	+	+	+	+
			8	++	+++	+++	+++
			9	+	-	+	-
			10	+	+	+	+
			11	++	++	++	+
			12	+	+	+	+
4	Villupuram, TN	Marakannam 12°11'24.9"N 79°55'28.1"E	1	++	++	+	++
			2	+	+	++	+
			3	+	-	-	-
			4	++	+	++	++
			5	++	++	++	++
			6	+	-	+	+
			7	-	-	-	-
5	Nellore, AP	Nellore 14°26'16.6"N 79°58'59.4"E	1	+++	+++	+++	+++
			2	++	++	+++	++
			3	+++	+++	+++	+++
			4	+++	++	++	++
			5	++	+++	+++	++

			6	+++	++	+++	++
			7	++	+	+	+
			8	+	+	+	-
			9	+	+	++	+
			10	++	++	+	++
			11	+++	+++	+++	+++
6	Nellore, AP	Mypad Village 14°31'09.5"N 80°10'09.1"E	1	++	+	+++	+++
			2	++	++	+++	+++
			3	++	+	+	+
			4	+++	+++	+++	++
			5	++	++	+++	++
			6	++	+	++	++
			7	++	+	+++	+++
			8	+++	++	+++	++
			9	++	+	+++	+++