Skim Milk Flocculation Concentrates White Spot Syndrome Virus in Seawater for Detection Using A Monoclonal Antibody Based Flow-Through Assay

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Abstract

Since its emergence, white spot syndrome virus has caused huge loss to shrimp aquaculture. However, these viruses are present in very low copy number in water and hence, concentrating them prior to detection is very much essential. In the present study, skim milk (0.01%) was employed for one step concentration of white spot syndrome virus from seawater under acidic (pH 3.5) and neutral (pH 7.4) conditions. The concentrated virus was detected using a monoclonal antibody based flow-through assay, RapiDot and compared its performance with Polymerase Chain Reaction (PCR). In an *invitro* experiment, the recombinant VP28 protein of white spot syndrome virus was flocculated successfully by skim milk in 50 mL measuring cylinders and detectable in both sub-surface and floc samples by RapiDot. Further, these results were validated by experimental infection studies using virus infected water for skim milk flocculation and detected the virus in floc samples 6 h earlier by RapiDot, compared to 1-step PCR. Overall, a simple method to concentrate white spot syndrome virus in water by skim milk flocculation for detection by RapiDot has been developed and may be adaptable to field conditions.

Keywords: Detection; Flocculation; RapiDot; Recombinant VP28 protein; Skim milk; White spot syndrome virus;

Introduction

The increase in demand of shrimp due to improved incomes among the ever increasing population has led to the expansion and intensification of shrimp aquaculture globally [1-3]. With the quest to satisfy this demand, many shrimp farmers fail to uphold best management practices in aquaculture resulting to deterioration of water quality and subsequently disease outbreaks. Viral diseases have posed severe threats to shrimp aquaculture [4]. Till date, of the several viral diseases, white spot disease caused by White Spot Syndrome Virus (WSSV) has been notorious and devastative, incurring more than 15 billion USD loss to the shrimp industry [5,6]. VP28 protein is one of the major coat proteins of WSSV that implicates in virulence [7,8]. WSSV is an envelope, bacilliform, double-stranded DNA virus [9,10]; transmit through vertical and horizontal route and also through carrier organisms [11,12]. At present, the rate at which WSSV outbreaks have reached places that were deemed as WSSV free zones is alarming and of great concern [13,14]. However, transmission of WSSV through water is probably the major route of entry into the culture system hence, regular monitoring by checking the water before pumping into ponds for managing the WSSV entry is very much essential [15-17]. Passive diagnosis of viruses in sick animals using molecular tools cannot predict future outbreaks hence; the study of the epidemiology of viruses in the environmental water becomes imperative. However, the bottleneck to virus detection in environmental water is that their copy numbers are extremely low and can hardly be detected by conventional PCR, thus concentrating virus particles should become obligatory prior to their detection [18-21].

Virus concentration and detection methods in water are many [18,22,23] however protocols that have been developed for WSSV include tangential flow ultra filtration [24] followed by nested PCR (Polymerase Chain Reaction) and qPCR detection, combined ferric colloid adsorption and foam separation [25]

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followed by PCR detection, centrifugation [26] and membrane filtration [27] followed by PCR detection. Recently, [28] used inorganic flocculent, alum at 15 and 30 ppm to concentrate WSSV from water and detected successfully by monoclonal antibody based flow-through assay, RapiDot and PCR. However, most of these methods employed to concentrate WSSV are costly, may result in loss of virus particles and also not farmer friendly. Moreover, inorganic flocculents result in secondary pollution which is harmful to the environment. Alternatively, the eco-friendly organic flocculents are found to be more advantageous as they are biodegradable and lead to faster floc formation because of their high settling velocity [29,30]. In this study, skim milk has been employed as an organic flocculent to concentrate WSSV in seawater and its detection using a monoclonal antibody based RapiDot test, compared with PCR. Overall, the results obtained from *invitro* experiments and experimental infection studies, indicate that WSSV in seawater could be easily flocculated by skim milk for detection by monoclonal antibody based RapiDot.

Materials and Methods

Source of white spot syndrome virus (WSSV)

Shrimp (*Penaeus vanammei*) with white spots were collected from a farm in Kannur District, Kerala, and transported on ice to the Aquatic Animal Health Management Laboratory, College of Fisheries, Mangaluru. WSSV infection in shrimp was confirmed by PCR [31,32]. Similarly, wild shrimp were collected from fish landing centre, Mangaluru for the preparation of negative control. A pair of pleopods and gills of each shrimp were fixed in absolute methanol and assayed for WSSV by nested PCR (nPCR).

Confirmation of WSSV infection in shrimp by polymerase chain reaction (PCR)

DNA extraction: Gills and pleopods from shrimp were used for DNA extraction according to DNA express kit (Himedia). Briefly, 50 mg of pleopods and gills were homogenised in 1 mL DNA express reagent using micro pestle in a 1.5 mL micro-centrifuge tube. 1 mL chloroform was added and the homogenate was centrifuged at 10,000 x g for 10 min at Room Temperature (RT). 1 mL ethanol was added and centrifuged at 5,000 x g for 4 min at RT. The supernatant was decanted and the pellet washed in ethanol and further centrifuged at 12,000 x g for 2 min. Finally, the pellet was solubilised in 50 μl Tris-EDTA (TE) buffer.

First-step and nested PCR: In the first-step PCR, one μl of DNA extract was added to PCR tube containing 29 μl of PCR reaction mixture from Genei, Bangalore (75 mMTris-HCl pH 9.0, 20 mM $(NH_4)_2 SO_4$, 0.01% Tween 20, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1.5 units of Taq DNA polymerase) and (100 pM of each primers (146F1/146R1) BioServe, Hydrabad. The primer sequence of 146F1 is 5'-ACTACTAACTTCAGCCTATCTAG-3' and 146R1 is 5'-TAATGCGGGTGTAATGTTCTTACGA-3'. The First round DNA amplification was performed using C1000 Touch Thermo cycler (Biorad) by subjecting the reaction mixture to initial denaturation of 94°C for 5 min, followed by 30 cycles of amplification (denaturation, 94°C/90 sec; annealing, 55°C/180sec; extension, 72°C/90sec and a final extension at 72°C for 5 min).

In the nested PCR, two μl first-step PCR product was added to PCR tube containing 18 μl of PCR reaction mixture (200 μM of each dNTP, 100 pM of 146F2, 100 pM of 146R2, 1.5 units of Taq polymerase, Taq buffer with 1.5 mM $MgCl_2$) and subjected to similar amplification conditions as above. The primer sequence of 146F2 is 5'-GTAACTGCCCCTTCCATCTCCA-3' and 146R2 is 5'-TACGGCAGCTGCTGCACCTTGT-3'. The positive control (previously tested 1-step PCR positive WSSV infected shrimp pleopods and gills) and negative control (previously tested nested PCR negative normal shrimp pleopods and gills) were treated similarly along with the samples. The PCR products were electrophoresed onto 1.5% agarose gel under Tris Acetate-EDTA (TAE) buffer (Trizma base, acetic acid and EDTA, pH 8.5) at 120 V for 60 min. The PCR amplicons were visualised under U.V. trans-illuminator (Biorad).

Recombinant VP28 protein (rVP28) of WSSV preparation and purification

The recombinant His-tagged VP28 protein containing plasmid construct, pET28a in DH5α *E. coli* cells was received from Prof. Just M Vlak, Wageningen University, The Netherlands as courtesy and stored at -80°C and prepared according to [33] and purified using Ni-NTA (Nickel-nitrilotriacetic acid) column following manufacturer's instructions(Qiagen, USA). In brief, cells harvested by centrifugation (11000 x g for 5 min) were lysed in 700 μl 7M Urea pH 8.0 by keeping at RT for 15 min under agitation.This cell lysate was centrifuged at 12000 x g for 15 min at RT, the clarified cell lysate (about 600 μl) containing His-tagged protein was loaded onto a Ni-NTA spin column and centrifuged at 270 x g for 5 min. After thorough washing of spin column with 8M Urea pH 6.3, the spin column was centrifuged at 890 x g for 2 min to elute purified proteins and the elution was done twice in 200 μl 8M urea pH 4.5. Dialysed eluted proteins for 7 h at 4°C were concentrated and stored at 4°C. The protein content of this purified VP28 was quantified using the A280 method by NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) following the suppliers instructions.

RapiDot Analysis of rVP28 proteinof WSSV

RapiDot was carried out according to [34] briefly, an aliquot (~0.2μl) each of VP28 protein (0.5μg/μl), estimated using the A280 method as positive and PBS buffer as negative control were dotted onto the upper part of the nitrocellulose membrane in the cassette while the sub-surface and floc samples were dotted onto the lower part. The membrane was air dried for 5 min and thereafter treated stepwise in sequential order with 200 μl each reagent including blocking buffer (2% BSA in PBS), one week old cell culture supernatant of MAb (C-05), wash buffer (6% Tween 20 in PBS), rabbit anti mouse IgG-HRP (1:500 in 2% BSA in PBS), wash buffer and 3, 3', 5, 5'-Tetramethylbenzidine (TMB)/H_2O_2 (Sigma, USA) as a precipitating chromogen-substrate. Finally, the reaction was terminated by adding 400 μl of wash buffer. The development of varied intensities of purple blue dots for different samples were recorded and compared with positive and negative control.

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Experimental set up for concentrating rVP28 protein of WSSV in seawater by skim milk flocculation

To concentrate recombinant VP28 by skim milk flocculation, column purified rVP28 protein was serially diluted to 10^{-10} in 0.2 µm nitrocellulose membrane filtered seawater (pH 7.4, salinity 36 ppt, temperature 25.7°C). One microliter from each dilution was analysed by 1-step PCR [35] and RapiDot [34]. The sequences of primers used to amplify the recombinant VP28 transcript of WSSV are F; 5'CGCGGATCCGATGGATCTTTCTTTCACTCTTTC-3'and R; 5'-CCGGAATTCTTACTCGGTCTCAGTGCCAG-3'. The lowest dilution at which RapiDot detected the rVP28 was tested for concentrating the rVP28 protein by skim milk flocculation.0.5 mL of purified rVP28 protein from the stock was added to a 100 mL beaker containing 100 mL filtered seawater, mixed with 0.01% skim milk. After thorough mixing for at least 10 min, the seawater samples were uniformly distributed into four 50 mL measuring cylinders, and kept undisturbed to allow flocculation of rVP28 protein by gravity. The experiments were conducted under acidic (pH 3.5) and neutral (pH 7.4) conditions in duplicate. At every 2 h interval for the initial 12 h period and 6 h interval till 36 h period, samples from sub-surface (1 cm below from the surface) and floc that is deposited at the bottom in each experimental conditions were collected using sterile sucker pipettes. The floc samples were centrifuged at 10,000 x g for 15 min at 4°C and the resultant pellet was re-suspended in 100 µl PBS, labeled and stored at 4°C for further analysis. All the sub-surface and floc samples were analysed by RapiDot [34] and directly (without performing DNA extraction) by PCR as described above [35].

Experimental infection of WSSV

Healthy *Penaeus monodon* were collected from a farm near Kumta, Karnataka, India and acclimatised to the laboratory conditions for a week. Three shrimps (~15 g each) based on the stocking density practiced in Andrapradesh, India by shrimp farmers, were stocked in aquarium tanks containing 25 L seawater (salinity 16 ppt, specific gravity 1.011, pH 7.4, temperature 28°C) with continuous aeration. Shrimps were injected with 100 µg of semi-purified WSSV at the lateral position of third abdominal segment using 1 mL insulin syringe fitted with 30G x 5/16 (0.30 x 8 mm) needle. The injected shrimps were fed daily with commercial diet at 5% body weight and monitored daily for the development of clinical white spots on carapace and mortality. Semi-purified WSSV (2.93 µg/µl) was prepared from the dead shrimps according to [36] and added back into the aquaria from which water samples were collected in different laboratory glassware (2 and 1 L beakers, and 50 mL measuring cylinders) in duplicate for concentrating WSSV by skim milk flocculation as per the protocol previously explained. The WSSV in the sub-surface and bottom floc samples was analysed by RapiDot and directly (without performing DNA extraction) by1-step and nested PCR as previously described.

Results

Confirmation of WSSV in infected shrimp

The homogenate prepared from *Penaeus vannamei* with white spots (Figure 1a) were found positive for WSSV by 1-step and nested PCR (Figure 1b). The appearance of 1447 and 941 bp amplicons in 1-step and nested PCR products, respectively, when electro phoresed on 1.5% agarose gel electrophoresis re-affirmed the presence of WSSV in shrimp. The preparations from normal shrimp showed no amplification of WSSV specific amplicons by nested PCR.
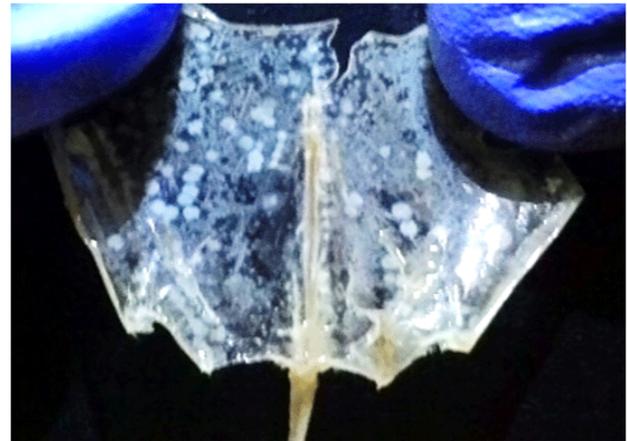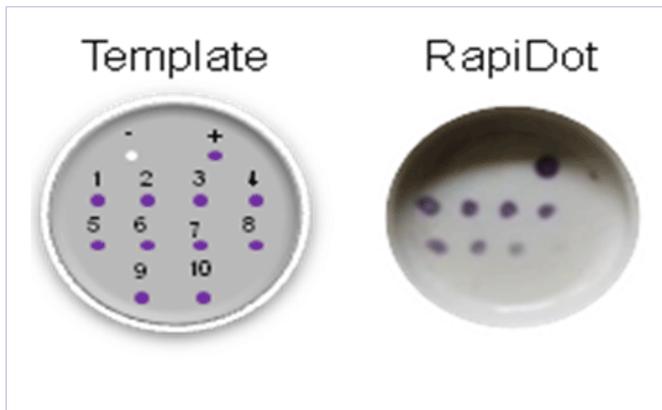

Figure 1a: Presence of white spots (arrow heads) on the carapace of suspected shrimp samples.
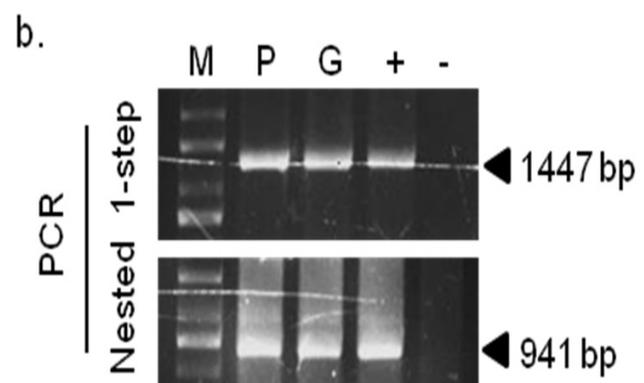
Figure 1b: Confirmation of WSSV in shrimp by PCR. Amplification of WSSV-specific gene of 1447 bp by 1-step and 941 bp by nested PCR in the suspected shrimp samples. Lanes: M, Molecular marker P, Pleopods; G, Gills; +, Positive control; and -, Negative control.

Skim milk concentrates rVP28 protein of WSSV to floc in seawater

Each log dilution of the purified WSSV rVP28 protein stock was analysed by RapiDot and found that the WSSV was detectable up to 10^{-7} dilution (Figure 1c). The estimated concentration of rVP28 protein in the (10^{-7}) dilution was used to find the volume taken from the rVP28 stock using the dilution formula, for further use in the skim milk (0.01%) flocculation experiment. The rVP28 protein was detectable by RapiDot (Figure 2a) and the rVP28 transcript by PCR at 615 bp (Figure 2b) in sub-surface samples, in

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Figure 1c: RapiDot analysis of log diluted samples of the recombinant VP28 protein stock. Appearance of purple blue dots showing the presence of the recombinant VP28 protein in log diluted samples by RapiDot. The order is -, Negative control, +, Positive control and log dilution from 10-1 to 10-10 of the recombinant VP28 protein from stock; depicted as numbers in the template.

which virus protein was flocculated by 0.01% skim milk. RapiDot detected the rVP28 protein with low intense dots from 0 h to18 h in acidic and 0 h to 36 h in neutral conditions. PCR detected the rVP28 transcript with very faint bands in acidic conditions at 4 h, while in neutral conditions at 2 h and also 12 h to 30 h respectively. However, the dot intensity in RapiDot was better in sub-surface samples from neutral seawater conditions, compared to acidic conditions.
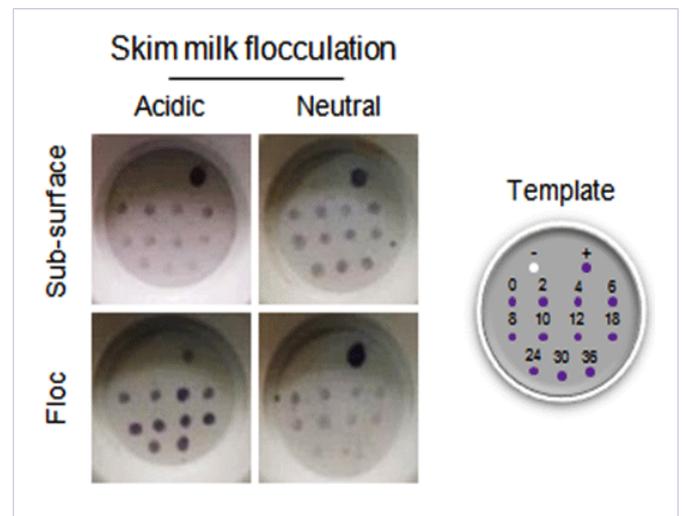
Figure 2a: RapiDot analysis of the recombinant VP28 concentrated by skim milk flocculation in sub-surface and floc samples. Appearance of purple blue dots showing the presence of the recombinant VP28 protein in sub-surface and floc samples under acidic and neutral conditions by RapiDot. The order is -, Negative control, +, Positive control and 0-36, sampling time points in h; as depicted in the template.

In floc samples, the skim milk concentrated recombinant VP28 protein was detectable by RapiDot (Figure 2a) and the rVP28 transcript by PCR (Figure 2b) under both acidic and neutral conditions. RapiDot detected the rVP28 protein from 2 h to 36 h in acidic and 2 h to 24 h in neutral conditions. PCR detected the rVP28 transcript from 2 h to 36 h in acidic conditions whereas in neutral conditions it was not discernible throughout the flocculation period.
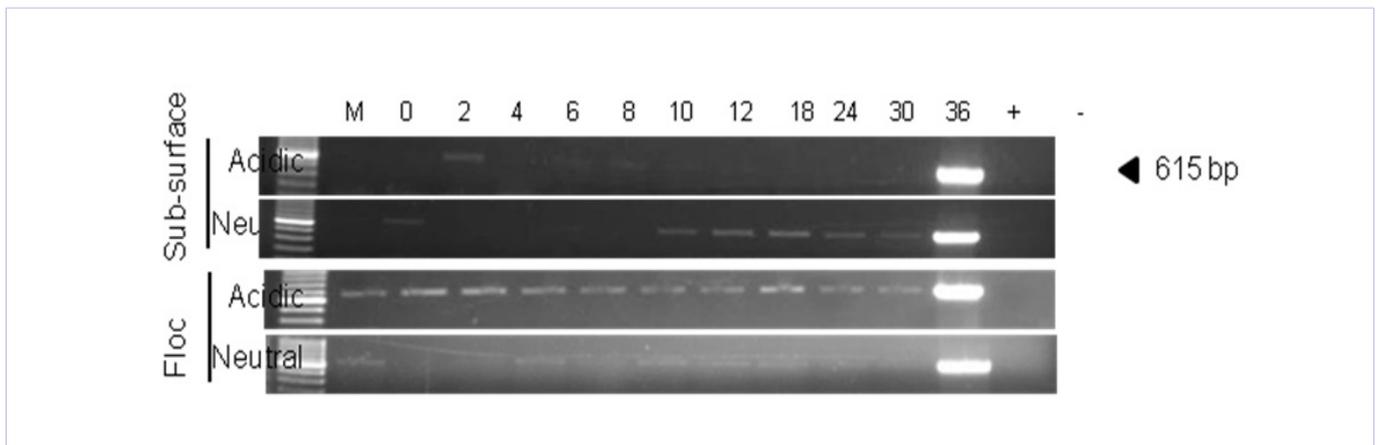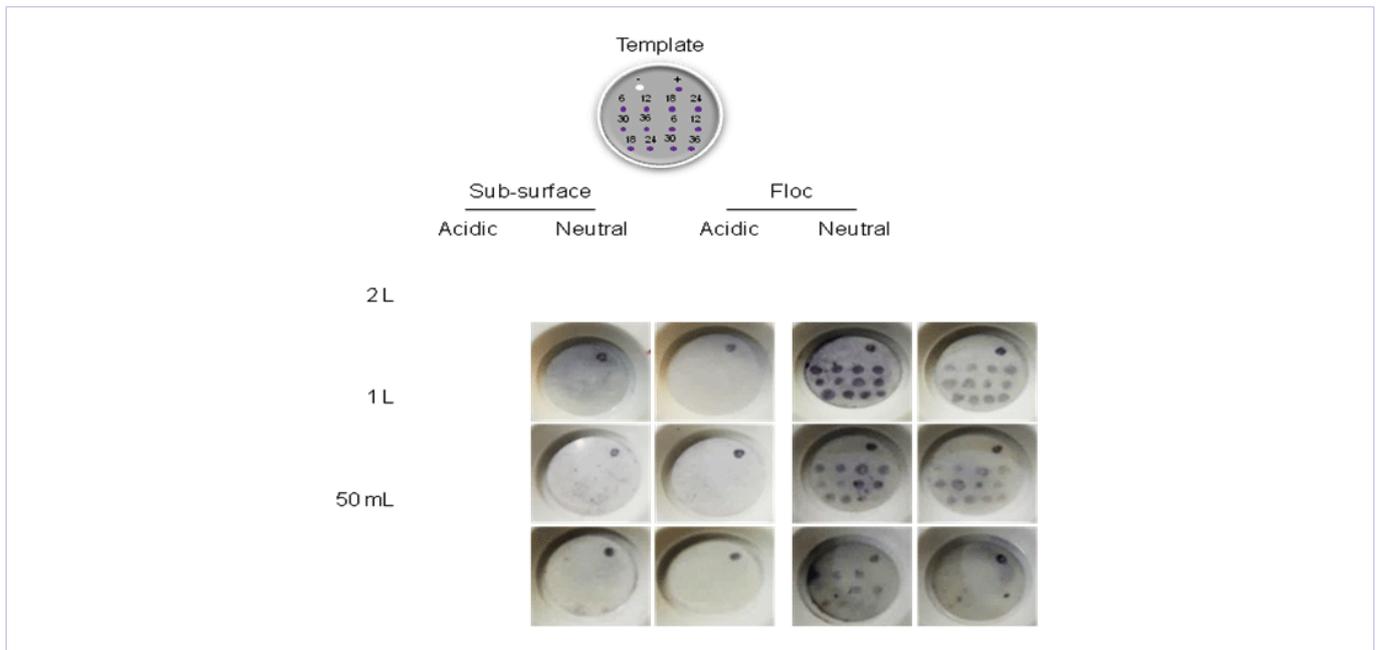

Figure 2b: PCR analysis of the recombinant VP28 concentrated by skim milk flocculation in sub-surface and floc samples. Amplification of the rVP28 transcript amplicons at 615 bp in sub-surface and floc samples by 1-step PCR following flocculation by skim milk under acidic and neutral conditions. Lanes: M, Molecular marker; 0-36, Sampling time points in h; +, Positive control and -, Negative control.

WSSV from experimentally infected shrimp in seawater concentrates to floc by skim milk

WSSV from experimentally infected shrimp water in 2 and 1 L beakers, and 50 mL measuring cylinders was successfully concentrated by flocculation using 0.01% skim milk. The WSSV was not detectable by RapiDot (Figure 3) and 1-step

PCR (Figure 4) from 6 h to 36 h in sub-surface samples from all of the different laboratory glassware under acidic and neutral conditions. In contrast, nested PCR detected the virus in sub-surface samples under acidic and neutral conditions with low gel band intensity at different time intervals in 2 and 1 L beakers, and 50 mL measuring cylinders (Figure 4). In floc samples, the WSSV was detectable by RapiDot (Figure 3), 1-step and nested

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Figure 3: RapiDot analysis of WSSV concentrated by skim milk flocculation in sub-surface and floc samples from seawater experimentally inoculated with virus. Appearance of purple blue dots showing the presence of WSSV in sub-surface and floc samples under acidic and neutral conditions from 2 L, 1 L and 50 mL laboratory glassware. The order is -, Negative control, +, Positive control and 6-36, sampling time points in h, as depicted in duplicate in the template.
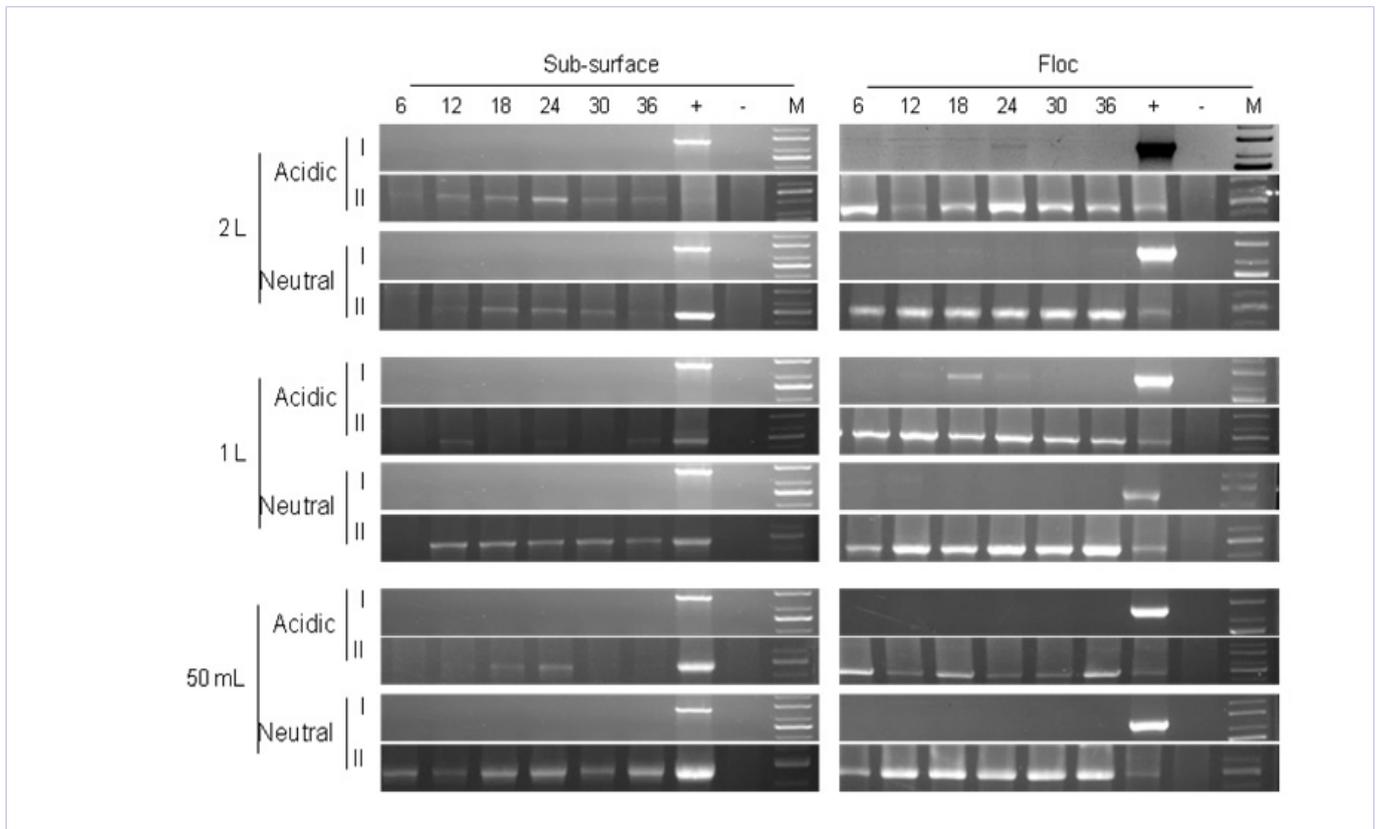
Figure 4: PCR analysis of WSSV concentrated by skim milk flocculation in sub-surface and floc samples from seawater experimentally inoculated with virus. Amplification of WSSV-specific amplicons of 1447 bp by 1-step and 941 bp by nested PCR in sub-surface and floc samples under acidic and neutral conditions from 2 L, 1 L and 50 mL laboratory glassware. Lanes: 6-36, Sampling time points in h; +, Positive control; -, Negative control and M, Molecular marker.

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PCR (Figure 4) in all the different laboratory glassware. The RapiDot dot intensities were more intense for the floc samples under acidic conditions in all the different laboratory glassware, compared to the neutral conditions, but these dot intensities were decreased over the flocculation period. In contrast, 1-step PCR detected WSSV with less intensity (1447 bp) under acidic conditions at 24 h for 2 L and 18 h for 1 L beaker floc samples, but not detected in 50 mL measuring cylinder floc samples from acidic and neutral conditions at any period of flocculation. Furthermore, nested PCR detected WSSV in acidic and neutral conditions for floc samples from 2 and 1 L beakers, and 50 mL measuring cylinders throughout the flocculation duration. In general, the gel band thickness and intensities were increased under neutral conditions while decreased over period of time for acidic conditions.

Discussion

Despite bio security measures being adopted for the management of WSSV, it continues to elicit huge loss to shrimp aquaculture. Hence, early detection of this virus, especially in water is paramount for its management and control [37]. However, numerous interacting biological, physical and chemical factors, including predation by higher organisms, sunlight inactivation, disinfection and proteolytic enzymes in the natural aquatic environment are known to reduce the viral copy numbers [38,39]. This low copy number of WSSV in natural water hampers its detection and can only be solved by concentrating the virus from water. In the present study, attempts have been made to develop a simple method to concentrate the WSSV using skim milk flocculation and its detection by MAb based RapiDot. In preliminary experiments, the lowest dilution, in which RapiDot detected the recombinant VP28 protein, was used to determine the volume taken from therVP28 stock of WSSV for concentrating the rVP28 protein from water by flocculation using skim milk. RapiDot detected the rVP28 protein of WSSV in sub-surface and floc samples under both acidic and neutral conditions. PCR could detect the rVP28 transcript of WSSV in sub-surface samples under both acidic and neutral conditions while in floc samples, PCR could detect only under acidic conditions. VP28 protein is one of the major coat proteins of WSSV that implicates in virulence [7] and has been widely used to detect WSSV from the shrimp homogenate [40,41,34,35].

The concept of concentrating WSSV by skim milk flocculation was further evaluated by simulating the WSD outbreak in the laboratory aquaria through experimental infection studies using *P. monodon.* RapiDot, 1-step and nested PCR detected the WSSV in floc samples from acidic and neutral conditions, but the intensity of dots and gel bands varied with the flocculation duration. Normally, concentrating environmental viruses from water requires large volumes of water i.e. groundwater and tap water need about 100 L while recreational water 10 L for processing. In another study, WSSV was concentrated and detected by PCR after >60 L of water was processed [24]. However, this study has demonstrated that WSSV can be concentrated by skim milk flocculation from less volume of infected water for detection. Additionally, similar results were observed in experimental infection studies and

PCR method. Nevertheless, RapiDot and nPCR can detect WSSV in apparently healthy shrimp and pond soil [34,42-44] with very low viral load which could not be detected in 1-step PCR.

Moreover, it was observed that flocculation of WSSV by skim milk was improved under acidic conditions, as indicated by high intensity dots in RapiDot and good amplification by 1-step and nested PCR. Similarly, observations were made earlier wherein the real time PCR (qPCR) of human and animal viruses showed more viral recovery when concentrated by skim milk under acidified environment [19,20,45]. The better performance of flocculation at pH 3.5 could be attributed to the change in the isoelectric point of virus that made them aggregate and precipitate out of the water [21,46]. Though these results were found better under acidic conditions, but the requirement of acid and special equipment makes it impracticable for the farmers. Nevertheless, the concentration of WSSV under neutral conditions which is more farmer-friendly, simple, cheap and equally performed well can be recommended instead.

In conclusion, a one-step method for concentrating WSSV by skim milk flocculation has been developed in this study and enhanced detection of virus by RapiDot under acidic conditions. Furthermore, both the concentration and detection method that has been developed are rapid, simple, cheap and may be adaptable to field level as an early warning tool to monitor the dynamics of WSSV over-time in the aquatic environment before an outbreak.

Acknowledgements

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