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# Tangential flow ultrafiltration for detection of white spot syndrome virus (WSSV) in shrimp pond water

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A B S T R A C T

Water represents the most important component in the white spot syndrome virus (WSSV) transmission pathway in aquaculture, yet there is very little information. Detection of viruses in water is a challenge, since their counts will often be too low to be detected by available methods such as polymerase chain reaction (PCR). In order to overcome this difficulty, viruses in water have to be concentrated from large volumes of water prior to detection. In this study, a total of 19 water samples from aquaculture ecosystem comprising 3 creeks, 10 shrimp culture ponds, 3 shrimp broodstock tanks and 2 larval rearing tanks of shrimp hatcheries and a sample from a hatchery effluent treatment tank were subjected to concentration of viruses by ultrafiltration (UF) using tangential flow filtration (TFF). Twenty to 100 l of water from these sources was concentrated to a final volume of 100 mL (200–1000 fold). The efficiency of recovery of WSSV by TFF ranged from 7.5 to 89.61%. WSSV could be successfully detected by PCR in the viral concentrates obtained from water samples of three shrimp culture ponds, one each of the shrimp broodstock tank, larval rearing tank, and the shrimp hatchery effluent treatment tank with WSSV copy numbers ranging from 6 to 157 $mL^{-1}$ by quantitative real time PCR. The ultrafiltration virus concentration technique enables efficient detection of shrimp viral pathogens in water from aquaculture facilities. It could be used as an important tool to understand the efficacy of biosecurity protocols adopted in the aquaculture facility and to carry out epidemiological investigations of aquatic viral pathogens.

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## 1. Introduction

White spot syndrome virus (WSSV) is an enveloped double stranded DNA virus and belongs to the family *Nimaviridae*. It has emerged as a devastating viral pathogen in shrimp farming with an estimated loss to the tune of over US$6 billion from the year 1992 (Lightner et al., 2012). The virus has a wide host range of more than 98 species, including salt and brackish water penaeids, crabs, lobsters, freshwater prawns and crayfish (Escobedo-Bonilla et al., 2008). The prevalence of WSSV has been reported widely among wild and cultured shrimp. It has been reported that the WSSV prevalence in wild *Penaeus monodon* was high (56.2%) in Chennai, Tamil Nadu followed by Digha, West Bengal (10.9%), and Visakhapatnam, Andhra Pradesh (0.6%) in India (Dutta et al., 2013). In another study, the prevalence of WSSV was reported to be only about 3.6% in wild invertebrates such as crabs, blue, white and brown shrimps and vertebrates in the environment surrounding shrimp farms along the Pacific coast of Mexico (Macías-Rodríguez et al., 2014). Transmission of WSSV is known to occur vertically from infected broodstock to larvae (Lo et al., 1997) or horizontally through cannibalism of moribund shrimp or through carriers such as polychaete worms, bait shrimp, rotifers and possibly even birds (Lotz, 1997; Vanpatten et al., 2004; Yan et al., 2004; Vijayan et al., 2005; Esparza-Leal et al., 2009). Routine water exchange and wastewater released during white spot disease (WSD) emergency harvests can often preserve WSSV in carrier organisms in the shrimp farming environment, resulting in its spread through water to neighbouring ponds. WSSV particles enter the water column after the infected animals die and start decomposing. Viral loads can reach significantly high levels in the affected ponds due to the release of virions from infected shrimp suffering rapid mortalities, and get diluted upon discharge into natural water bodies.

A few studies have attempted detection of WSSV in water. Hossain et al. (2004) tested sediments obtained upon centrifugation of water samples by polymerase chain reaction (PCR) and reported that 5 of the 12 water samples from shrimp ponds and ghers were

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positive for WSSV. Quang et al. (2009) reported that WSSV could be detected in coastal environments such as diseased shrimp ponds and surrounding canals using membrane filtration followed by PCR. These studies used centrifugation and membrane filtration, and no virus concentration methods were employed. These methods capture only viruses adhering to particulate matter in seawater on a filter membrane (Quang et al., 2009) and centrifugation (Hossain et al., 2004). The non-adherent viral particles would pass through the filters and remain in suspension upon low speed centrifugation. Hence, these protocols are likely to underestimate the occurrence of WSSV in water. Free WSSV is reported to be viable for a period of 12 days in seawater of 27 ppt salinity, pH of 7.5 at 29–33 °C in the absence of any hosts (Satheesh Kumar et al., 2013), and continue to persist depending on the availability of hosts for their multiplication and the physicochemical conditions of the aquatic ecosystem. It has been also confirmed that the particulate fractions act as vehicles for WSSV dispersion via the viruses associated with microalgae or zooplankton (Esparza-Leal et al., 2009). Using a differential filtration technique, it was reported that WSD could be induced in healthy shrimp by particulate fraction and liquid fractions (Esparza-Leal et al., 2009). However, they reported that they could not consistently detect WSSV in all fractions/replicates. Recently, Samanman et al. (2011) described the use of capacitive biosensor for quantitative detection of WSSV in water. However, this tool is not feasible in most laboratories. A combined ferric colloid adsorption and foam separation-based method for concentration of WSSV and its detection in sea water has been recently described (Suzuki et al., 2011). However, this method requires steps to dissociate the WSSV particles from the colloidal foam prior to DNA extraction for its detection.

An assay for detection, abundance and distribution of WSSV in water in the aquaculture environment will help to analyse risks of its transmission through water. It would be advantageous for surveillance of possible reservoirs and to monitor the persistence of WSSV in the environment. The objective of this study was to examine the utility and efficiency of ultrafiltration (UF) using tangential flow filtration (TFF) for concentration of viruses in water samples from brackishwater aquaculture system for specific detection of WSSV and other shrimp viral pathogens using PCR.

## 2. Materials and methods

### 2.1. Samples

A total of 19 water samples (20–100 L) from shrimp aquaculture facilities, comprising three creeks, ten shrimp culture ponds, five shrimp hatcheries (three broodstock tanks and two larval rearing tanks), and a shrimp hatchery effluent treatment tank (Table 1) were collected in clean disinfected carboys and transported to the laboratory and processed for concentration of viruses in water.

### 2.2. Concentration of viruses from water

Viruses in the water samples were concentrated by microfiltration (MF) followed by UF (Thurber et al., 2009). Water samples were pre-filtered using 5 μm cartridge filter to remove particulate matter and then subjected to MF using TFF system (Quixstand™ Benchtop system, GE Health Care Bio Sciences Corp., USA) fitted with 0.2 μm pore size TFF cartridge (CFP-2-E-4MA with a surface area of 420 $cm^2$ or CFP-2-E-9A with a surface area of 8400 $cm^2$) to remove bacteria and particulate matter. The permeate was then subjected to UF for concentration of viruses with 100 kDa MWCO TFF cartridge using either a UFP-100-C-4MA with a surface area of 650 $cm^2$ or UFP-100-C-9A with a surface area of 1.15 $m^2$ (Fig. 1). Finally, 20–100 L of water samples was thus concentrated to 200–1000 fold. After every use, the filter system was sanitized with 200 ppm of free chlorine and 0.5 M sodium hydroxide solution.

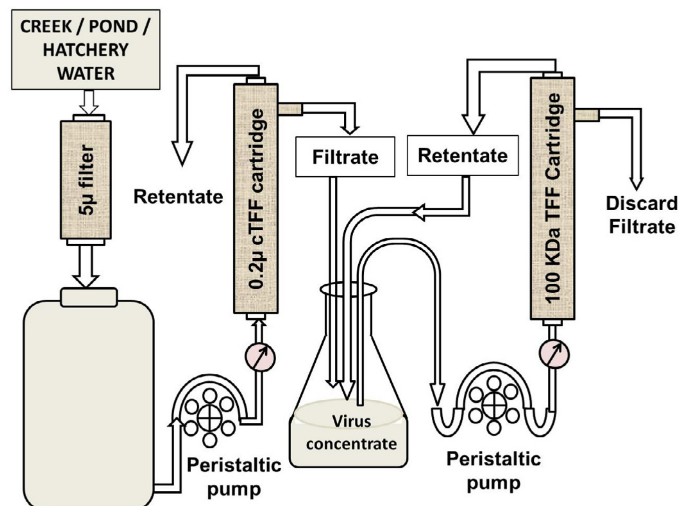


**Fig. 1.** Schematic diagram showing concentration of viruses from water samples from shrimp hatcheries, aquaculture ponds and creeks using tangential flow filtration.

### 2.3. Detection of shrimp pathogenic DNA viruses in viral concentrates from water

The viral DNA was extracted from the 5 mL virus concentrates (UF retentates) obtained from water samples (Xie et al., 2005). Detection of WSSV was carried out as per OIE protocols (2012) using the first step PCR primers 146F1 (5′-ACT-ACT-AAC-TTC-AGC-CTA-TCTAG-3) and 146R1 (5′-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A-3′) amplifying 1447 bp product and 146F2 (5′-GTA-ACT-GCCCCT-TCC-ATC-TCC-A-3′) and 146R2 (5′-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3′) for the nested PCR reaction that produces an amplicon of 941 bp size. OIE protocol (2012) was used for detection of infectious hypodermal and hematopoietic necrosis virus (IHHNV) and hepatopancreatic parvo virus (HPV) detection was carried out according to the protocol of Manjanaik et al. (2005) using primers H441F: 5′-GCA-TTA-CAA-GAG-CCA-AGC-AG-3′ and H441R: 5′-ACA-CTC-AGC-CTC-TAC-CTT-GT-3′ producing a 441 bp PCR amplicon. Monodon baculovirus (MBV) was detected using protocol of Stalin Raj (2007) using primers MBVF: CGA TTC CAT ATC GGC CGA ATA and MBVR: TTG GCA TGC ACT CCC TGA GAT, which amplifies an amplicon of 596 bp. The DNA was also extracted from MF retentates and similarly tested to enable detection of these viruses adherent to particulate matter of >0.2 μm size.

### 2.4. WSSV stock and quantification

The WSSV stock was prepared and quantified as described by Satheesh Kumar et al. (2013). Briefly, WSSV infected tiger shrimp of 16–26 g size were obtained in dry ice from a tiger shrimp farm in Nellore, Andhra Pradesh, India. About 10 g of WSSV infected shrimp tissue (gills and pleopods) was pooled in 100 mL TN buffer (20 mM Tris–HCl, 400 mM NaCl, pH 7.4), homogenized and then centrifuged at 5000 g for 10 min at 4 °C. The pellet was again suspended in TN buffer, homogenized and centrifuged. The resultant supernatant was pooled and filtered through 400 μm pore size nylon net and then subsequently through 0.45 μm filter and used as viral preparation (Xie et al., 2005). The viral DNA was extracted and the WSSV copy number was estimated using WSSV detection and quantitative real time PCR kit (LabIndia Life Sciences, Gurgaon, India) as per manufacturer's instructions using Applied Biosystems Step One™

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**Table 1**
Detection of WSSV in water samples from shrimp hatcheries, shrimp culture ponds and creeks.

| Serial number | Hatchery/farm/creek | Volume of seawater processed (in L) | Volume of viral concentrate in mL from seawater | WSSV Nested PCR | | WSSV copies estimated by RT-PCR in water ($mL^{-1}$) |
|---|---|---|---|---|---|---|
| | | | | 0.2 μ Retentate | 100 kDa Retentate | |
| 1 | Effluent treatment tank, MES shrimp hatchery | 60 | 100 (600) | +ve[a] | +ve | 25 |
| 2 | Larval rearing tank, MES shrimp hatchery | 40 | 100 (400) | −ve | −ve | NA |
| 3 | Larval rearing tank, Marine hatchery, Marakanam | 100 | 100 (1000) | −ve | +ve | 11 |
| 4 | Brooder tank, shrimp hatchery, Kalpakkam | 90 | 100 (900) | −ve | +ve | 61 |
| 5 | Brooder tank, Marine hatchery, Kanathur | 100 | 100 (1000) | −ve | −ve | −ve |
| 6 | Brooder tank, shrimp hatchery, Marakanam | 40 | 100 (400) | −ve | −ve | NA |
| 7 | Shrimp culture pond, Bhimavaram (F-1) | 60 | 100 (600) | +ve[a] | +ve | 157 |
| 8 | Shrimp culture pond, Ongole | 60 | 100 (600) | +ve[a] | +ve[a] | 18 |
| 9 | Shrimp culture pond-1, Kalpakkam | 80 | 100 (800) | −ve | −ve | NA |
| 10 | Shrimp culture pond-7, Kalpakkam | 50 | 100 (500) | −ve | −ve | NA |
| 11 | Shrimp culture pond-10, Kalpakkam | 50 | 100 (500) | −ve | −ve | NA |
| 12 | Shrimp culture pond, Bhimavaram (F-2) | 40 | 100 (400) | −ve | −ve | NA |
| 13 | Shrimp culture pond, Nellore | 60 | 100 (600) | −ve | −ve | NA |
| 14 | Shrimp culture pond, Kattur | 60 | 100 (600) | −ve | +ve[a] | 6 |
| 15 | Shrimp culture pond, Bhimavaram (F-3) | 20 | 100 (200) | −ve | −ve | NA |
| 16 | Shrimp culture pond, Marakanam | 80 | 100 (500) | −ve | −ve | NA |
| 17 | Buckingham canal, Kalpakkam | 80 | 100 (800) | −ve | −ve | NA |
| 18 | Creek, Bhimavaram | 50 | 100 (500) | −ve | −ve | NA |
| 19 | Kandaleru creek, Pudiparthy, Nellore | 40 | 100 (400) | −ve | −ve | NA |

NA: not applicable; figures in parenthesis under third column indicate number-fold concentration.
[a] Nested positive.

Real-Time PCR system (California, USA). The WSSV copy number in the original stock was estimated to be 12,070,299 copies $\mu L^{-1}$, which was diluted 60,000 fold in TN buffer to obtain working suspension having 186 WSSV copies $\mu L^{-1}$ as estimated by quantitative real time PCR.

### 2.5. WSSV-free sterile seawater

Sand-filtered, UV treated seawater from a shrimp hatchery was obtained and filtered through 0.2 μm TFF cartridge, the permeate was collected, and again subjected to TFF using a 100 kDa cartridge and then sterilized by autoclaving, to obtain virus-free sterile seawater.

### 2.6. Efficiency of recovery of WSSV by tangential flow filtration (TFF)

The efficiency of recovery of WSSV by TFF was examined as described earlier by Suttle et al. (1991) by spiking 10 L virus free sterile seawater to a final concentration of 186 or 279 WSSV copies $mL^{-1}$. Such WSSV spiked seawater was allowed to mix thoroughly by aeration, and after an hour, subjected to virus concentration by TFF using 100 kDa cartridge (UFP-100-C-9A of filter area of 1.15 $m^2$) to a final volume of 100 mL. This experiment was carried out five times. Before and after every experiment, the TFF system was sanitized by circulating 100 mg $L^{-1}$ sodium hypochlorite solution for 30 min. The TFF system was flushed with sterile seawater until sodium hypochlorite was completely removed. The pH (∼7.0) of the permeate was checked to ensure complete removal of the sodium hypochlorite. DNA was extracted from 5 mL of these viral concentrates (Xie et al., 2005) and the WSSV copy number was determined by real-time PCR using the LABINDIA Life Sciences kit (Satheesh Kumar et al., 2013). The efficiency of this method of recovery of WSSV was calculated in percentage using the following equation.

$$\%\ \text{recovery of WSSV by TFF} = \frac{\text{Total no. of WSSV copy numbers in the retentate} \times 100}{\text{Initial total no. of WSSV copy numbers spiked in 10 L}}$$

## 3. Results

### 3.1. Occurrence of shrimp pathogenic DNA viruses in viral concentrates from water

A total of 19 water samples from various shrimp culture ponds, hatcheries, creeks and a shrimp hatchery effluent treatment tank were subjected to virus concentration by TFF. WSSV could be detected in six of these UF viral concentrates by PCR. All the samples were negative for other shrimp pathogenic DNA viruses, viz., MBV, HPV or IHHNV. Five water samples comprising two shrimp culture ponds, a sample each of broodstock rearing tank, a larval rearing tank and effluent treatment tank were first step PCR positive for WSSV, while one *Litopenaeus vannamei* shrimp culture pond with of 75 days duration of culture (DOC) was nested PCR positive (Fig. 2). WSSV count in these water samples ranged from 6 to 157 copies $mL^{-1}$ as estimated by quantitative real time PCR (Table 1).

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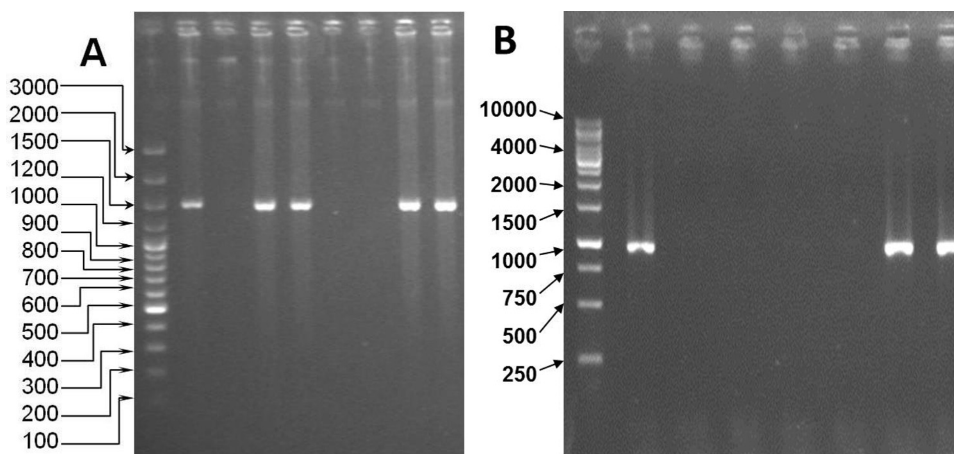


**Fig. 2.** (A) Detection of WSSV in100 kDa TFF viral concentrates from shrimp hatchery/pond/creek water by PCR. M: Molecular weight marker; Lane 1: MES shrimp hatchery effluent treatment tank; Lanes 2 and 3: larval rearing tanks of MES shrimp hatchery and Marine hatchery, Marakanam; Lanes 4–6: brooder tanks of tiger shrimp hatchery, Kalpakkam, Kanathur and Marakanam; Lanes 7 and 8: shrimp culture pond samples from Bhimavaram (F-1) and Ongole; Lane 9: negative control; Lane 10: positive control. (B) Detection of WSSV in 0.2 μ TFF retentates by nested PCR (OIE, 2012). Lane 1: MES shrimp hatchery effluent treatment tank; Lanes 2 and 3: larval rearing tanks of MES shrimp hatchery and Marine hatchery, Marakanam; Lanes 4 to 5: brooder tanks of tiger shrimp hatchery, Kalpakkam, and Kanathur; Lanes 6 and 7: shrimp culture pond samples from Bhimavaram (F-1) and Ongole.

The MF retentates comprise particles and microbes of >0.2 μ size. The viruses adherent to these particles, including some WSSV virions are likely to remain in this fraction. Hence, all MF retentates were also tested for shrimp viruses. The MF retentates of three of the 19 water samples comprising one sample from the hatchery effluent treatment tank and two shrimp culture ponds tested positive for the WSSV by nested PCR (Fig. 2; Table 1). The corresponding UF viral concentrates of these three samples were positive for WSSV by first step PCR. However, all the 19 MF retentates were negative for other shrimp pathogenic DNA viruses, viz., MBV, HPV and IHHNV.

### 3.2. Efficiency of recovery of WSSV from water

In the present study, 20–100 L water from hatcheries, grow-out ponds and creeks was concentrated by UF for viruses to a final volume of 100 mL, working out to 200–1000 fold (Table 1) concentration of viruses in water. The efficiency of recovery of WSSV from water by TFF was found to be cent-percent (all the times WSSV could be recovered). However, the number of WSSV copy numbers as estimated by quantitative real time PCR greatly varied, and ranged from 12 to 136 out of 186 WSSV genome copies added to virus-free seawater (i.e., 7.5–89.61%), with an average recovery of 57.84% of WSSV genome copies (Table 2).

### 3.3. Time required for the concentration of viruses

Sixteen water samples were processed using smaller MF and UF cartridges (CEP-2-E-4MA and UFP-100-C-4MA) with filter surface areas of 420 and 650 $cm^2$ respectively and the rest of the samples were processed using larger cartridges (CFP-2-E-9A and UFP-100-C-9A) with filter surface areas of 8400 $cm^2$ and 1.15 $M^2$. The MF using the smaller cartridge, with a flow rate ranging from 239 to 462 $mL\,min^{-1}$, required about 4.10–21.20 h. The time was drastically reduced to 3–4.30 h using the larger cartridge with a flow rate of 1.6–1.8 $L\,min^{-1}$. The UF with a flow rate of 650 $mL\,min^{-1}$, the time required for the concentration of viruses ranged from 3.30 to 17.10 h using the smaller cartridge could be completed in 2.10–2.50 h using the larger cartridge. The time to obtain the viral concentrate through various steps of coarse filtration, MF and UF using the smaller TFF cartridges required about 7.40–38.30 h, and this time was considerably reduced to less than 7.2 h using the TFF cartridges with larger surface areas (Table 3).

## 4. Discussion

### 4.1. Detection of WSSV in water by ultrafiltration

In this study, occurrence of WSSV in water in shrimp aquaculture settings was examined. Water samples from hatcheries, grow-out ponds and creeks were subjected to virus concentration by TFF. WSSV was detected by nested PCR in two of the five hatchery water samples, one hatchery ETP tank and three of the ten shrimp farm water samples tested. The WSSV load in these water samples ranged from 6 to 157 viral copies $mL^{-1}$ as determined by quantitative real-time PCR. In this study, six ultrafiltration retentates were positive for WSSV. Three of these corresponding microfiltration water samples (retentates with particles >0.2 μm) including the one from the hatchery effluent and two from aquaculture ponds were WSSV positive by nested PCR, indicating the necessity for ultrafiltration. Of these three samples, while the reasons for the high viral load in the shrimp pond could be attributed to WSD outbreak and was preparing for emergency harvest, the reasons for the high viral load in the hatchery effluent could not be ascertained. The other three WSSV positive water samples (ultrafiltration retentates) comprised two water samples from shrimp hatchery and one sample from an aquaculture pond. Personnel in the two shrimp hatcheries were unaware of the presence of WSSV in their facilities. Similarly, the pond water that was nested PCR positive with (6–61 WSSV copies $mL^{-1}$) also did not have WSD outbreak at the time of sampling. These three samples required >600–1000 fold concentration to detect WSSV, possibly due to relatively low viral load compared to the other samples. However, within the next 2 days, the farm personnel informed of WSD outbreak in the pond. The three water samples from creeks and canals were WSSV negative. The creek (Kandaleru creek, Nellore district, Andhra Pradesh) runs about 36 km and is used as a source of seawater for shrimp farming and also receives farm waste water. The creek is influenced by tidal amplitudes and gets flushed regularly.

Other than the recent description of concentrating viruses prior to detection in aquaculture and fisheries sector (Suzuki et al., 2011), review of the literature showed that only very few studies have attempted to concentrate viruses prior to detection. While the

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**Table 2**
Efficiency of recovery of WSSV from water by TFF.

| Serial Number | No. of WSSV genome copies added to 10 L virus free water | No. of WSSV genome copies detected in TFF concentrate | Percentage recovery |
|---|---|---|---|
| 1 | 279 | 188 | 67.38 |
| 2 | 279 | 250 | 89.61 |
| 3 | 186 | 96 | 51.6 |
| 4 | 186 | 136 | 73.1 |
| 5 | 186 | 12 | 7.5 |

marine birnavirus was concentrated using a glass fibre filter pre-coated with bovine serum albumin (Kamata and Suzuki, 2003), the infectious salmon anaemia virus (ISAV) (Løvdal and Enger, 2002) and the infectious hematopoietic necrosis virus (IHNV) (Batts and Winton, 1989; Watanabe et al., 1988) were concentrated by TFF. TFF has been the most preferred method used to concentrate viruses from natural waters because it reduces filter clogging and allows concentration of viruses from the hundreds of litres of sample that are often necessary for genomic and metagenomic analyses of aquatic viral populations (Wommack et al., 2010). This technique has several advantages over the adsorption-elution based methods with increased virus recovery efficiencies (Muscillo et al., 1997) and is currently the most efficient means of concentrating viruses from large volumes of water, with virus recoveries varying from 11 to 98% (Colombet et al., 2007).

### 4.2. Virus recovery by ultrafiltration

The question with regard to the detection of viral pathogens in water that arises would be, about the appropriate volume of water required to be concentrated for the efficient recovery of shrimp viruses present in low counts (Bosch et al., 2011). It is logical that virus concentration would be directly proportional to the number fold concentration and the volume of water processed. The chances of detection of shrimp pathogenic viruses would depend on the extent of contamination of pond/hatchery water. Recovery and retention of virus particles by TFF have been reported to be directly or indirectly associated with many variables such as experimental conditions, total cartridge filter area, pump speed, pump pressure, molecular and particle size and shape, concentration, and particle polarity that might induce electrostatic attraction in the seawater electrolyte, etc. (Rodriguez et al., 1998). Further, the pH, conductivity, turbidity, presence of particulate matter, and organic acids, can also affect the efficiency of recovery of viruses from water (Rzeżutka and Carducci, 2013). Groundwater and potable water usually contain fewer viruses and about 100 L or more amount of water has to be processed, while recreational fresh or marine waters may contain relatively more viruses, and hence processing about 10 L samples may be sufficient. In the present study, it appears that >60 L of water was required to be

**Table 3**
Flow rates and time required to concentrate viruses from water samples from aquaculture facilities.

| Serial Number | Hatchery/farm/creek | Micro-filtration flow rate (mL $min^{-1}$) | Time taken (h) for micro-filtration | Ultra-filtration flow rate (mL $min^{-1}$) | Time taken (h) for ultra-filtration | Total time taken (h) for concentration of viruses |
|---|---|---|---|---|---|---|
| 1 | Effluent treatment tank, MES shrimp hatchery | 336 | 12.50 | 650 | 10.40 | 23.30 |
| 2 | Larval rearing tank, MES shrimp hatchery | 378 | 08. 20 | 650 | 07.10 | 15.30 |
| 3 | Larval rearing tank, Marine hatchery, Marakanam | 357 | 20.00 | 650 | 17.00 | 37.00 |
| 4 | Brooder tank, shrimp hatchery, Kalpakkam | 378 | 17.00 | 650 | 15.40 | 32.40 |
| 5 | Brooder tank, Marine hatchery, Kanathur | 336 | 21.20 | 650 | 17.10 | 38.30 |
| 6 | Brooder tank, shrimp hatchery, Marakanam | 399 | 07.00 | 715 | 06.20 | 13.20 |
| 7 | Shrimp culture pond, Bhimavaram (F-1) | 252 | 17.10 | 650 | 10.40 | 27.50 |
| 8 | Shrimp culture pond, Ongole | 239 | 17.50 | 650 | 10.40 | 28.30 |
| 9 | Shrimp culture pond-1, Kalpakkam | 336 | 17.10 | 650 | 13.30 | 30.40 |
| 10 | Shrimp culture pond-7, Kalpakkam | 273 | 13.20 | 715 | 08.30 | 21.50 |
| 11 | Shrimp culture pond-10, Kalpakkam | 252 | 14.30 | 650 | 08.30 | 23.00 |
| 12 | Shrimp culture pond, Bhimavaram (F-2) | 420 | 07.10 | 715 | 06.00 | 13.10 |
| 13 | Shrimp culture pond, Nellore | 378 | 11.10 | 780 | 08.20 | 19.30 |
| 14 | Shrimp culture pond, Kattur | 462 | 10.30 | 650 | 06.20 | 16.50 |
| 15 | Shrimp culture pond, Bhimavaram (F-3) | 378 | 04.10 | 650 | 03.30 | 07.40 |
| 16 | Shrimp culture pond, Kalpakkam | 336 | 17.20 | 650 | 13.30 | 30.50 |
| 17 | Buckingham canal, Kalpakkam | 1600 | 03.30 | 3800 | 02.40 | 6.10 |
| 18 | Creek, Bhimavaram | 1700 | 04.30 | 3800 | 02.50 | 7.20 |
| 19 | Kandaleru creek, Pudiparthy, Nellore | 1800 | 03.00 | 3600 | 02.10 | 5.10 |

Samples 17, 18 and 19 were processed using microfiltration and ultrafiltration TFF cartridges with surface area of 8400 $cm^2$ and 1.15 $m^2$ respectively.

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concentrated to enable detection of WSSV in aquaculture waters (Table 1).

During the current study, water was pre-filtered with 5 μm pore size cartridge filter prior to MF and UF. This step alleviates the need for pre-blocking ultrafiltration membranes and increased recovery of viruses in water (Garin et al., 1993). Virus recoveries are generally reduced at various stages of processing such as pre-filtration, microfiltration and ultrafiltration. The virus recovery efficiency in the present study ranged from 7.5 to 89.61% with an average of 57.84% with WSSV spiked in 100 kDa filtered sterile seawater. Colombet et al. (2007) reported that viral recovery efficiency of ultrafiltration averaged 52% (range 11–98%), while comparing pegylation to concentrate viruses. Applying a double filtration method (i.e. concentration of 0.1 μm filtrates with 10,000 molecular weight cut-off ultrafilters), Wommack et al. (1995) reported recovery rates that ranged from 23 to 72% for a mixture of two cultured phages, and from 58 to 100% for natural communities of viral like particles (VLPs) in the Chesapeake Bay. Suttle et al. (1991) reported recovery rates from 75.1 to 99.6% for two specific bacteriophages (PWH3-P1 and LMG1-P4) added to ultrafiltered virus-free seawater and counted by plaque assay method.

It should be noted that mere detection of WSSV DNA does not establish viral viability and infectivity. Regardless of the inability of the PCR to distinguish infectious from non-infectious viral particles, the current study is the first to document the presence of WSSV in water from shrimp hatcheries, shrimp culture ponds and creeks using a standard virus concentration tool. Based on the differential filtration experiments, it was reported that filtration of seawater through 0.2 μm pore sized filters effectively excluded WSSV (Esparza-Leal et al., 2009). However, in our study, we could detect WSSV even in microfiltration permeates (ultrafiltration retentates). It is likely that all the WSSV particles do not pass through filters of 0.2 μm (200 nm) pore size, since intact enveloped WSSV particles are 70–167 nm in width and 210–420 nm in length (Sánchez-Paz, 2010).

### 4.3. Implications

So far, the only option available to the shrimp farmers to prevent the WSD in their shrimp farms is to adhere to the biosecurity protocols or in simple terms, exclusion of the pathogens from their aquaculture facilities. The measures of screening out WSSV positive broodstock or post-larvae in hatcheries using PCR are well placed. So far, the tools for the detection of shrimp pathogens in water, which forms the most important component of aquaculture system were not described. Viral concentration by TFF enables efficient detection of viral pathogens in aquaculture waters and thus helps in understanding the biosecurity status and to take up appropriate actions such as disinfection. Further, this tool can be extended to understand the efficacy of disinfection protocols adopted in shrimp farms, hatcheries and reservoirs.

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