Introduction

The intensifying penaeid shrimp aquaculture is dramatically affected by several existing and emerging viral diseases. Disease surveillance plays a major role to predict, designate and manage disease outbreak in aquaculture sector. Active surveillance for the potential pathogens forms the core of mitigation measures in case of viral disease outbreaks. Surveillance systems are established in order to substantiate the disease status of the area or even a country (OIE, 2010).

A well-structured and planned strategy for all round surveillance is necessary for the pilot-scale and long-term study for identifying and reporting pathogens. For this, the cultured animals have to be tested at various levels at every stage of farming in order to identify probable threats. A regular or periodic study in farms also provides light on those pathogens that can survive long in the pond sediments and thereby provide ample time for planning remedial and management strategies. The study is particularly substantial under Indian context since India has been involved in shrimp production for more than 20 years. The present study may be helpful for the shrimp aquaculture industry to be aware of the importance of disease surveillance and to develop preventive strategies against viral diseases.

Infectious myonecrosis (IMN) is an OIE listed penaeid shrimp disease caused by infectious myonecrosis virus (IMNV) (Flegel, 2006; Lightner, 2011; OIE, 2015). The virus belongs to the family Totiviridae; measures 40 nm in size with icosahedral shape, possessing linear double-stranded RNA (Lightner et al., 2004a; Lightner, 2011). Its natural hosts are Penaeus vannamei (Pacific whiteleg shrimp) (da Silva et al., 2015) and Farfantapenaeus subtilis (southern brown shrimp) (Lightner et al., 2004a, b; Tang et al., 2005; Poulos et al., 2006; Coelho et al., 2009; Dantas et al., 2015; Prasad et al., 2017). The clinical signs exhibited by the infected shrimp are the presence of white patches on abdominal segments especially in the tail region with major histological changes being extensive coagulative necrosis of muscle tissues (Uhrik et al., 1989; Lightner et al., 2004a, b; Nunes et al., 2004; Poulos et al., 2006), also characterised by formation of lymphoid organ spheroids, oedema, haemocyte infiltration in muscle bundles and liquefactive necrosis (Uhrik et al., 1989; Lightner et al., 2004a, b; Poulos et al., 2006). The virus shows both horizontal as well as vertical mode of transmission in P. vannamei (Lightner et al., 2004a; da Silva et al., 2016).

First report of IMNV outbreak was from Brazil in South America (Lightner et al., 2004a) followed by Indonesia in Asia (Senapin et al., 2007). So far, the reports about the disease from other places have been...
found negative but as the seeds are procured from these countries, there is always a potential chance that the virus may find its way into neighbouring Asian countries (Senapin et al., 2011). In this context, the present study was conducted to determine the status of IMNV in Indian shrimp aquaculture by adopting risk-based two stage random sampling survey with imperfect tests. The risk-based two stage random sampling survey was adopted hypothesising that the disease is present at a level equal to or greater than the minimum expected prevalence (Hadorn et al., 2002).

Materials and methods

Sampling design and sampling frame

The necessary sample size for this survey was calculated for a random sampling protocol in a risk-based approach as suggested by Hadorn et al. (2002). Since it is a large -area survey and national wide sampling, we adopted the two-stage random sampling method as recommended by Cameron and Baldock (1998). The World Organisation for Animal Health (Office International des Epizooties, OIE) follows the same standardised statistical sampling procedure in order to declare freedom from an OIE listed disease (Cameron, 2002).

The first stage of sampling involved a random selection of three farms in different geographical locations along the coastal states of Gujarat, Maharashtra, Goa, Kerala, Tamil Nadu, Andhra Pradesh, Odisha and West Bengal in India, where intensive shrimp farming prevailed (Cameron and Baldock, 1998; Cameron, 2002; Hadorn et al., 2002). A list of 60 active shrimp farming areas was first generated and from that, a total of 21 farms/hatcheries were randomly selected using the Epi Tool Free Calc software. The parameters were adjusted based on the economic reasons, convenience as well as the importation risk of the animal to the country. The inputs for the software are given in Table 1. The second stage involved testing of predetermined number of individual animal samples per farm. Cast net, which is a non-selective gear was used to harvest the animals from three different points of the same pond. Sample size was calculated using the software FreeCalc Calculator for freedom testing with imperfect test (Cameron and Baldock, 1998). The input parameters are described Table 1.

Estimated sample size of farms or hatcheries in the first stage was 18 and within each farm and 59 individual animals from each farm. We surveyed three different farms from each state and the number of samples collected varied with the life stages of the shrimp being 60 for adult, sub-adult and juvenile stages and 120 for post-larval stages, from the identified sampling areas along the west and east coasts of India (Table 2). The harvested shrimps were pooled and required number of samples were collected randomly. The whole sampling frame and process is depicted as a flow chart (Fig. 1).

Collection of tissue samples

The live animals sampled were transferred to the lab with aeration and the tissues or the post-larvae collected...
from the distant sampling points were preserved in RNA后来 solution. Cold-chain of 4°C was maintained during the entire transportation. Further processing and the screening procedures were conducted in the molecular diagnostics laboratory, Department of Aquatic Animal Health Management, ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Mumbai, India. Live animals were acclimatised for one day to reduce transportation stress and kept in 200 l capacity tubs with aeration.

The collected live adult animals were processed straight away and the downstream procedures were carried out at 4°C, maintaining cold-chain. The samples were pooled into batches of 12, containing 5 animals each. A total of 252 pools of the sampled animals were used to screen for the virus. Haemolymph was collected using sterile 24 G needle and syringe, in sterile anticoagulant containing 450 mm NaCl, 10 mm KCl, 10 mm EDTA, Na\(_2\) and 10 mm HEPES, pH 7.3 (Vargas-Albores et al., 1993) and animals were aseptically dissected after anesthetising on ice. Tissues, mainly gills, 6th abdominal muscle and lymphoid organs (LO) were aseptically collected separately and immediately processed for RNA extraction. Similar tissues collected from each individual in the same batch were pooled into one. For larger animals, cross-contamination during tissue collection was eliminated by sterilising the dissection instruments on flame after an alcohol wipe. Post-larvae (PL) were processed as such, and 10 nos. of whole PL were pooled into one.

### Total RNA extraction

The total RNA was extracted from the collected shrimp tissues using TRIzol reagent (Invitrogen) according to the manufacture’s protocol. The purity of extracted total RNA was confirmed by a run on 2% agarose gel followed by visualisation using ethidium bromide staining. The resulting RNA was stored at -80°C, for further use.
Selection of coastal state

Selection of geographic locations based on intensive shrimp farming

Random selection of three farms in the chosen locations as first stage of sampling

The second stage involved the testing of predetermined number of individual animal samples per farm

21 Locations which includes 14 shrimp farms and 7 hatcheries

Cast-netting at the three different points

60 no. of animals juveniles/sub-adults

Animals were pooled into 12 batches having 5 animals each

Animals were pooled into 12 batches having 10 post-larvae each

From each pool, the whole post-larvae were screened using RT-PCR

From each pool, tissues were dissected out and separately screened using RT-PCR

G

M

LO

H

12 nested RT-PCR reactions

12 nested RT-PCR reactions

4 nested RT-PCR reactions

8 nested RT-PCR reactions

9 nested RT-PCR reactions

Totally 45 nested RT-PCR reactions were carried out to screen the collected animal samples

cDNA synthesis

cDNA was synthesised using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) as per the manufacturer’s protocol and the resulting cDNA was stored at -20°C, for further use.

Polymerase chain reaction (PCR)

Nested PCR reaction was performed with two sets of specific OIE Universal Primers (Poulos and Lightner, 2006). In the second step nested-PCR, 2.0 µl of the primary PCR product was used as the template. Universal IMNV specific primers (IMNV-4587F, IMNV-4914R, IMNV-4725 NF, IMNV-4863 NR) of amplicon size of 328 bp in the first step and 139 bp in nested step were used for the detection (Poulos and Lightner, 2006). The non-template control tube added with nuclease free water served as negative control in the first step and first step non-template control reaction product formed the negative control in the nested reaction. Cloned fragment of first step PCR product was used as the positive control. The given reaction conditions were used for PCR amplification and PCR was carried out in a thermal cycler (Takara, Japan). PCR products were visualised on 2% agarose gel with a standard 100 kbp plus DNA ladder (Thermo Scientific).
Results and discussion

To our knowledge, this study forms the first targeted surveillance survey to document the IMNV infection status in Indian shrimp aquaculture. A total of 45 nested RT-PCR reactions were carried out to screen the targeted tissues from the pooled samples. In our study, none of the samples were found positive with the nested RT-PCR diagnostic method. The results also fall in a sensitivity limitation of the diagnostic tool used. The probability of the presence of infection at lower levels less than a viral copy number of 10 cannot be eliminated (Poulos and Lightner, 2006; OIE, 2012). The present study confirmed the absence of IMNV and statistically provided a valid, reliable justification for the freedom of the same in Indian shrimp aquaculture via a risk-based two stage random sampling survey. The test reports are consistent with the evidence of negative finding recorded from the nested RT-PCR results showing absence of IMNV positive product.

The 95% level of confidence, the minimum expected prevalence of the disease within a farm and 20% amongst farms were assumed to be 10% based on the knowledge of the biology of the disease and the nature of the screening test (Cameron and Baldock, 1998; Hadorn et al., 2002). The nature of the surveillance method took into consideration that at present no evidence of the disease and if the disease is present, likely to be in the small proportion of the farms (Cameron and Baldock, 1998; Hadorn et al., 2002).

All collected samples were screened by OIE recommended nested RT-PCR diagnostic tool using IMNV specific universal primer. The results obtained were all negative. The first IMNV disease outbreak occurred in 2002 in Brazil, followed by an outbreak in Indonesia in 2006 (Lightner et al., 2004a, b; Senapin et al., 2007; Taukhid and Nur’aini, 2009). Rumors on IMNV disease outbreaks in other Asian countries including India have been reported since 2007 (Taukhid and Nur’aini, 2009). Results of the present study are in compliances with the findings of Senapin et al. (2011) who did not record any form of IMNV infection in whiteleg shrimp in Asia. Our results are also on par with the suggestion that false reports may be a result of contamination occurring during the screening procedures.

It is not possible to prove that a population is free from a disease and the minimum disease prevalence cannot be 0 for a surveillance survey (Cameron, 2002). The two-stage random sampling method was recommended by the OIE to detect a disease or to demonstrate freedom from a disease (OIE, 2012). Cameron and Baldock (1998) statistically proved that the two-stage random sampling design can be used to designate the freedom from a disease and advocated that Free Calc software can be used to calculate the required sample size for the survey (Cameron and Baldock, 1998). The same sampling strategy was adopted by East et al. (2005) to verify freedom from WSSV and YHV in Australia and Pinheiro et al. (2007) to find out the epidemiological status of TSV and IMNV in Brazil. So, the two-stage random sampling adopted in the present study is in agreement with the international surveillance sampling protocol. According to Manual of Diagnostic Tests for Aquatic Animals (OIE, 2012), nested RT-PCR is the recommended method for targeted surveillance for reasons of diagnostic specificity and sensitivity, utility and availability (OIE, 2012). So the study utilised the same diagnostic tool to screen the shrimp samples for the presence or absence of IMNV. The first step of RT-PCR can generate results in an infection with a minimum of 10 IMNV copies and the nested RT-PCR can detect up to 10 IMNV copies (OIE, 2012). Baumgartner et al. (2009) used the nested RT-PCR technique for the primary diagnosis and surveillance of WSSV in wild and farmed crawfish (Procambarus clarkii and Procambarus clarkii) in Louisiana, USA along with the shrimp bioassays and real-time PCR. They followed the same protocol as suggested by OIE (Baumgartner et al., 2009). The same RT-PCR method has evidently proven its sensitivity in the case of longitudinal surveillance survey of human picornavirus infection in children with weekly sampling protocol (Winther et al., 2006). Griffiths and Melville (2000), also utilised RT-PCR to visualise the presence of infectious salmon anaemia virus (ISAV) in gill mucus of Atlantic salmon parr according to OIE guidelines (Griffiths and Melville, 2000). Altogether, the materials and methods followed throughout the present study is valid and meets recommendations of the international standards (OIE, 2006; 2010; 2012).

Presently, among Asian countries, IMNV is prevalent only in Indonesia even though the shrimp farming is actively practiced in the nearby countries (Senapin et al., 2007; Taukhid and Nur’aini, 2009). Indonesian shrimp farming practices almost coincide with Indian context, except for the stocking of seeds which is at a higher rate in the range of 70 to 300 pieces per m² (Gesteira, 2006). High density stocking leads to increased chances of contact between the individual animals and also escalates the probability of ingestion of IMNV contaminated tissues (Graf et al., 2004). The onset of the disease is related to stress factors ranging from changes in salinity and temperature to rough handling during partial harvest or sampling by cast net. Temperature also plays a crucial role in the occurrence of disease. Exposure to an elevated temperature for more time can lead to excessive feeding which subsequently leads to elevated ammonia concentrations in the culture.
system (OIE, 2012). Indian climatic conditions like high atmospheric temperature can lead to the occurrence of stress conditions in the shrimps thereby rendering them susceptible to IMNV infection (unpublished information).

The present study demonstrated that Indian shrimp farms located along the coastal areas are free from IMNV or IMN and provides preliminary statistical information on the current status of the virus in shrimp aquaculture. This is the first report on a risk-based targeted disease surveillance of the shrimp RNA virus, IMNV from India. Results affirm the validity of risk-based two stage random sampling design and its reliability based on these findings.

Acknowledgements

The authors thank Dr. Rajeev Kumar Jha, CP Prima Indonesia, a stakeholder who had shared the test material and the Director, ICAR-CIFE, Mumbai for providing all necessary facilities for the research work. We also thank National Surveillance Programme for Aquatic Animal Disease (NSPAAD) Project, ICAR-CIFE, India for the support in conducting this research work.

References

Baumgartner, W. A., Hawke, J. P., Bowles, K., Varner, P. W. and Hasson, K. W. 2009. Primary diagnosis and surveillance of white spot syndrome virus in wild and farmed crawfish (Procambarus clarkii, P. zonangulus) in Louisiana, USA. Dis. Aquat. Org., 85(1): 15-22.

Cameron, A. R. and Baldock, F. C. 1998. A new probability formula for surveys to substantiate freedom from disease. Prev. Vet. Med., 34(1): 1-17.

Cameron, A. 2002. Survey toolbox for aquatic animals: a practical manual and software package. ACIAR Monograph, 94, 375 pp.

Coelho, M. G. L., Silva, A. C. G., Nova, C. M. V. V., Neto, J. M. O., Lima, A. C. N., Feijo, R. G., Apolinar, D. F., Maggioni, R. and Gesteira T. C. V. 2009. Susceptibility of the wild southern brown shrimp (Farfantepenaeus subtilis) to infectious hypodermal and hematopoietic necrosis (IHHN) and infectious myonecrosis (IMN). Aquaculture, 294: 1-4.

da Silva, S. M. B. C., da Silva, A. D. R., Lavander, H. D., Chaves, T. C. B., Peixoto, S., Gavez, A. O. and Coimbra, M. R. M. 2015. Analysis of new isolates reveals new genome organisation and a hypervariable region in infectious myonecrosis virus (IMNV). Virus Res., 203: 66-71.

East, I. J., Black, P. F., Findlay, V. L. and Bernoth, E. M. 2005. A national survey to verify freedom from white spot syndrome virus and yellow head virus in Australian crustaceans. Dis. Aquat. Org., 15-26.

Flegel, T. W. 2006. Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture, 258: 1-33.

Gesteira, T. C. V. 2006. Enfermidadesinfecciosasregistradas nas carcais de camarão (P. vannamei) em diferentes regiões do Brasil. ABRAPA, Maringa, p. 137-158.

Graf, C., Gervais, N., Fernandes, M. P. C. and Ayala, J. C. 2004. Transmissão da doença do necrose epidemica muscular (NIM) em Litopenaeus vannamei. Aqualider (www.aqualider.com.br).

Griffiths, S. and Melville, K. 2000. Non-lethal detection of ISAV in Atlantic salmon by RT-PCR using serum and mucus samples. B. Eur. Assoc. Fish Path., 20(4): 157-162.

Hadorn, D. C., Rufenacht, J., Hauser, R. and Stark, K. D. 2002. Risk-based design of repeated surveys for the documentation of freedom from non-highly contagious diseases. Prev. Vet. Med., 56(3): 179-192.

Lightner, D. V. 2011. Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): A review. J. Invert. Pathol., 106: 110-130.

Lightner, D. V., Pantoja, C. R., Poulos, B. T., Tang, K. F. J., Redman, R. M., Andreas, T. and Bonami, J. R. 2004a. Infectious myonecrosis (IMN): a new virus disease of Litopenaeus vannamei. In: Aquaculture 2004, Book of Abstracts, World Aquaculture Society, Baton Rouge, LA, USA, 353 pp.

Lightner, D. V., Pantoja, C. R., Poulos, B. T., Tang, K. F. J., Redman, R. M., Pasos de Andrade, T. and Bonami, J. R. 2004b. Infectious myonecrosis: new disease in Pacific white shrimp. Glob. Aquac. Advocate, 7: 85.

Nunes, A. J., Martins, P. C. C. and Gesteira, T. C. V. 2004. Carciniculturaameçada. Produtorrestoform com mortalida desdecorrentes do virus da mionecroseinfecciosa (IMNV). Panorama Aquac., 83: 37-51.

OIE. 2010. Terrestrial aquatic animal health code, 19th edn. Office International des Epizooties, World Organisation for Animal Health, Paris, France, 14 pp.

OIE. 2012. Infectious myonecrosis virus. In: Manual of diagnostic tests for aquatic animals. Office International des Epizooties, World Organisation for Animal Health, Paris, France, p. 138-147.

OIE. 2015. OIE-listed diseases 2015. Office International des Epizooties, World Organisation for Animal Health, Paris, France.
Surveillance for IMNV in Indian shrimp farms

OIE 2006. *Manual of diagnostic tests for aquatic animals*, 5th edn. Office International des Epizooties, World Organisation for Animal Health, Paris, France, 69 pp.

Pinheiro, A., Lima, A., Souza, M., Neto, E., Adriaio, M., Goncalves, V. and Coimbra, M. 2007. Epidemiological status of taura syndrome and infectious myonecrosis viruses in *Penaeus vannamei* reared in Pernambuco (Brazil). *Aquaculture*, 262: 17-22.

Poulos, B. T. and Lightner, D. V. 2006. Detection of infectious myonecrosis virus (IMNV) of penaeid shrimp by reverse-transcriptase polymerase chain reaction (RT-PCR). *Dis. Aquat. Org.*, 73: 69-72.

Poulos, B. T., Tang, K. F. J., Pantoja, C. R., Bonami, J. B. and Lightner, D. V. 2006. Purification and characterisation of infectious myonecrosis virus of penaeid shrimp. *J. Gen. Virol.*, 87: 987-996.

Prasad, K. P., Shyam, K. U., Banu, H., Jeena, K. and Krishnan, R. 2017. Infectious myonecrosis virus (IMNV) - An alarming viral pathogen to penaeid shrimps. *Aquaculture*, 477: 99-105.

Senapin, S., Phewsaiya, K., Briggs, M. and Flegel, T. W. 2007. Outbreaks of infectious myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method. *Aquaculture*, 266: 32-38.

Senapin, S., Phewsaiya, K., Gangnonngiw, W. and Flegel, T. W. 2011. False rumours of disease outbreaks caused by infectious myonecrosis virus (IMNV) in the whiteleg shrimp in Asia. *J. Negat. Results BioMed.*, 10: 10 pp.

Tang, K. F. J., Pantoja, C. R., Poulos, B. T., Redman, R. M. and Lightner, D. V. 2005. *In situ* hybridisation demonstrates that *Litopenaeus vannamei*, *L. stylirostris* and *Penaeus monodon* are susceptible to experimental infection with infectious myonecrosis virus (IMNV). *Dis. Aquat. Org.*, 63: 261-265.

Taukhid, Nuraini, Y. L. 2009. Infectious myonecrosis virus (IMNV) in pacific white shrimp (*Litopenaeus vannamei*) in Indonesia. *ISR J. Aquac.* (Bamidgeh), 61(3): 255-262.

Uhrik, B., Rydlova, K. and Zacharova, D. 1989. The roles of haemocytes during degeneration and regeneration of crayfish muscle fibres. *Cell Tissue Res.*, 255(2): 443-449.

Vargas-Albores, F. 1992. *Sistemas de defensa del camarón cafe (Penaeus californiensis)*. Ph. D. Thesis, Universidad Nacional de Mexico.

Vargas-Albores, F., Guzman, M. A. and Ochoa, J. L. 1993. An anticoagulant solution for haemolymph collection and prophenoloxidase studies of penaeid shrimp (*Penaeus californiensis*). *Comp. Biochem. Phys. Part A: Physiol.*, 106(2): 299-303.

Winther, B., Hayden, F. G. and Hendley, J. O. 2006. Picornavirus infections in children diagnosed by RT-PCR during longitudinal surveillance with weekly sampling: Association with symptomatic illness and effect of season. *J. Med. Virol.*, 78(5): 644-650.

Date of Receipt : 15.01.2017
Date of Acceptance : 25.04.2017