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Surveillance for infectious myonecrosis virus in Indian shrimp aquaculture

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ABSTRACT

Disease surveillance programmes provide information on diseases that mitigate aquaculture production. Infectious myonecrosis virus (IMNV) has emerged recently as one of the OIE (Office International des Epizooties, the World Organisation for Animal Health) listed viral diseases in penaeid shrimps, caused by dsRNA virus. The present study was conducted to investigate the prevalence of IMNV along the Indian coastal states. Samples of the Pacific whiteleg shrimp *Penaeus vannamei* and the green tiger shrimp *Penaeus monodon* were collected from 21 randomly selected active shrimp farms located along the coast using a risk-based two stage random sampling survey. Screening of the sampled shrimps based on nested RT-PCR diagnostic tool, did not detect IMNV in any of the samples examined indicating absence of IMN or IMNV incidence in shrimp aquaculture along Indian coastal states.

Keywords: Infectious myonecrosis, *Penaeus monodon*, *Penaeus vannamei,* Risk-based two stage random sampling, RT-PCR, Shrimp aquaculture

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 (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 targeted 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

Table 1. Input parameters and output values for the sample size estimation

Input parameter	First stage (Farm level)	Second stage (Animal level)	Output (Result)	First stage (Farm level)	Second stage (Animal level)
	Input value			Output value	
Population size	60	100000	Required sample size	20	59
Test sensitivity	0.95	0.95	Cut-point number of reactors	1	2
Test specificity	0.99	0.99	Type-I error	0.0424	0.0474
Designed prevalence	0.2	0.1	Type-II error	0.0169	0.0215
Diseased elements	12	10000	Herd-level sensitivity	0.9576	0.9526
Analysis method	Modified hypergeometric exact	Simple binomial (large population)	Herd-level specificity	0.9831	0.9785
Type-I error	0.05	0.05	Interpretation	Probability that the population is diseased at a prevalence of 0.2 is 0.0424.	Probability that the population is diseased at a prevalence of 0.1 is 0.0474.
Type-II error	0.05	0.05			
Population threshold for infinite probability formula (default)	1000	1000			
Maximum sample size	20	60	Method	Modified hypergeometric exact	Simple binomial (large population)

Table 2. RT-PCR screening report of shrimp samples collected from coastal state for IMNV

Name of State	Site no. (Date of collection)	Name of species	Life stage collected	Tissue collected	Sample size	Nested RT-PCR reactions	IMNV status
Gujarat	1. Surat (17/07/2015)	*Penaeus vannamei*	Post-larvae	Whole PL	120	1	Negative
	2. Dhandi (10/03/2016)	*P. vannamei*	Subadult	Gill, Muscle, Hemolymph	60	3	Negative
Maharashtra	3. Panvel (09/11/2015)	*P. vannamei*	Subadult	Gill, Muscle, Hemolymph, LO	60	4	Negative
	4. Dahanu (19/11/2015)	*Penaeus monodon*	Subadult	Gill, Muscle, Hemolymph, LO	60	4	Negative
	5. Palghar (28/12/2015)	*P. vannamei*	Subadult	Gill, Muscle, Hemolymph, LO	60	4	Negative
Goa	6. Mapusa (28/01/2016)	*P. vannamei*	Subadult	Gill, Muscle, Hemolymph	60	3	Negative
	7. Bicholim (28/01/2016)	*P. vannamei*	Subadult	Gill, Muscle, Hemolymph	60	3	Negative
	8. Bicholim (29/01/2016)	*P. vannamei*	Juvenile	Gill, Muscle	60	2	Negative
Kerala	9. Kodungalloor (28/10/2015)	*P. monodon*	Subadult	Gill, Muscle, Hemolymph, LO	60	4	Negative
	10. Aluva (29/10/2015)	*P. monodon*	Subadult	Gill, Muscle, Hemolymph	60	3	Negative
	11. Kozhikkode (29/10/2015)	*P. monodon*	Post-larvae	Whole PL	120	1	Negative
Tamil Nadu	12. Chennai (20/02/2016)	*P. vannamei*	Post-larvae	Whole PL	120	1	Negative
	13. Pattukottai (22/02/2016)	*P. vannamei*	Juvenile	Gill, Muscle,	60	2	Negative
	14. Pattukottai (22/02/2016)	*P. vannamei*	Post-larvae	Whole PL	120	1	Negative
Andhra Pradesh	15. Kakkinada (15/09/2015)	*P. monodon*	Post-larvae	Whole PL	120	1	Negative
	16. Sriramapuram (01/03/2016)	*P. vannamei*	Post-larvae	Whole PL	120	1	Negative
	17. Konapapapetta (01/03/2016)	*P. vannamei*	Post-larvae	Whole PL	120	1	Negative
Odisha	18. Gopalapur (02/03/2016)	*P. vannamei*	Post-larvae	Whole PL	120	1	Negative
	19. Berhampur (02/03/2016)	*P. vannamei*	Post-larvae	Whole PL	120	1	Negative
West Bengal	20. Contai (03/06/2016)	*P. vannamei*	Juvenile	Gill, Muscle	60	2	Negative
	21. Digha (03/06/2016)	*P. vannamei*	Juvenile	Gill, Muscle	60	2	Negative

from the distant sampling points were preserved in *RNA later* 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 Insittute 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, 1992; 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.

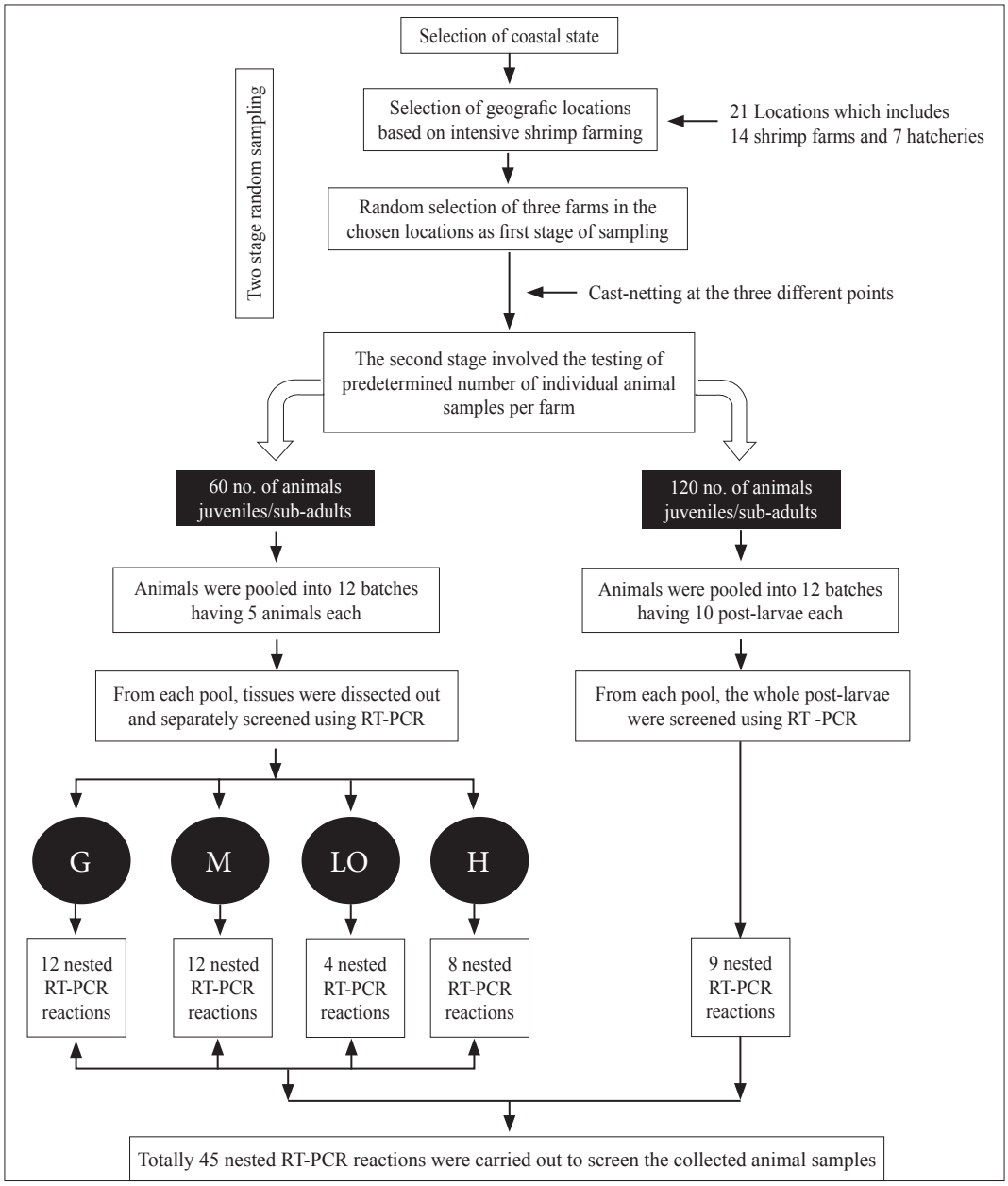

Fig. 1. Flow diagram showing the sampling frame and screening process. G: Gill, M: Muscle, LO: Lymphoid organ, H: Hemolymph

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 100 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 zonangulus*) 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 m^2 (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.

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