ICAR-Central Institute of Brackishwater Aquaculture (CIBA), Chennai
Coastal Aquaculture Authority (CAA) &
Marine Products Development Authority (MPEDA)
Rajiv Gandhi Centre for Aquaculture (RGCA)

3rd PCR Ring test to evaluate the skill levels and capacity of polymerase chain reaction (PCR) diagnostic laboratories, in India. 1st phase completed with white spot syndrome virus (WSSV) as a model

About the PCR ring test organised and conducted by ICAR-CIBA, Chennai

Emergence of lethal viral pathogens such as white spot syndrome virus (WSSV) in the shrimp farming sector in India and overseas, has caused catastrophic mortalities and crop losses since the nineties, which lead to the use of new generation diagnostics such as polymerase chain reaction (PCR) for the detection and avoidance of the virus from the shrimp seed. The specific, sensitive and fast PCR technique which can detect even a few viral copies and non-lethal method of screening has opened up a new health management regime in shrimp farming. This has resulted in the establishment of PCR labs in the private sector, using both the Indian and imported PCR kits for diagnosis of WSSV. Later, the farmers found the quality of the results coming out of these labs being compromised, and at large farmers started losing the confidence in the private labs. At this juncture, the only way out to maintain the quality of the PCR labs and to bring these diagnostic labs under some kind of quality evaluation by national intuitions of repute for assuring confidence among shrimp farmers on the diagnostic labs. The first ring test in India was jointly organised by ICAR-CIBA-MPEDA and NACA in 2005. 46 labs with PCR diagnostic capabilities were evaluated under this ring test, and 21 came out with accreditation standard specified. The rest of the labs were given corrective training. The second voluntary PCR ring testing exercise was conducted in the year 2009 by ICAR-CIBA along with MPEDA, in which 34 laboratories participated and 31 participants provided their results. 21 laboratories successfully completed the 2nd PCR ring testing exercise in the year 2009.

What is PCR ring test?

Uniformity of testing results and reporting of the PCR results by the laboratories servicing the aquaculture sector has been often questionable, and this factor has underpins

greater attention to standardization and harmonization. The scope of harmonization includes aspects of laboratory testing, use of terminology and units, report formats, decision limits, as well as test profiles and criteria for the interpretation of results. The ring testing is also aimed to provide an overview of the current quality of WSSV PCR testing in participating laboratories in India. The process of ring test includes distribution of a panel of coded tissue samples by the referral laboratory, and the participating laboratories are expected to return results in a standard report format within reasonable period of time. Participation in the ring test was voluntary and the laboratories would be free to use the protocol of their choice. In effect, this exercise not only provides a step towards accreditation and also gives participants an opportunity to assess their own performance and offer the opportunity to compare their results with other laboratories. The PCR ring test helps in maintaining the skill levels and capacity of the PCR diagnostic laboratories in the country.

Post SPF vannamei introduction scenario

Indian shrimp farming sector witnessed the introduction of exotic SPF vannamei to tide over productivity related issues associated with tiger shrimp in addition to crop losses due to WSSV infections. Farming of vannamei triggered a spectacular growth in the Indian shrimp farming sector and during 2010 to 2015, the production increased to four fold. But during this period, screening of seed to ensure their quality decreased with a conviction that SPF seeds need not be tested, especially for the endemic vial pathogens such as WSSV. The result was that, most of the PCR laboratories and in-house PCR labs in hatcheries stopped functioning and the whole process of screening of shrimp seeds for potential viral pathogens almost stopped. CIBA initiated an active surveillance programme in Andhra Pradesh and Tamil Nadu under the National Surveillance Project for Aquatic Animal Diseases (NSPAAD) in 2013 with support from NFDB-DAHD&F, and the results for the two years since then have revealed serious issues including increased prevalence of WSSV emergence of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in the shrimp farming in India. The prevalence of WSSV in AP and TN was about 37 % (with about 6.5% outbreak cases) and IHHNV generally linked to slow growth was prevalent in about 5 % of farms investigated. Further, prevalence of emerging pathogens such as *Enterocytozooan hepatopenaei* (EHP) has also come to the fore. We also need to keep a close watch on the early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) pathogen, so far not detected in India. In this backdrop, CIBA along with CAA-DAD&F and MPEDA took up the initiative to conduct a PCR ring test to bring the focus of shrimp seed

testing again to keep the quality of seeds and to protect the shrimp farming sector, sliding into the era of disease problems and crop failures, mainly due to the lethal viral pathogens such as WSSV and emerging pathogens such as IHHNV and EHP.

Relevance of PCR ring test:

There is no therapeutic option available for the control of virus and hence biosecurity is the most important means of disease management strategies in shrimp aquaculture. Early pathogen detection is crucial in preventing disease spread in shrimp aquaculture. The elimination of infected seed prior to stocking is one of the most important measures in reducing the disease risks in shrimp farming. Accurate, specific and sensitive diagnosis plays an important role in achieving biosecurity in shrimp hatcheries and farms. This can be achieved by the proficient DNA based PCR testing of broodstock and seed. The PCR technique is widely used as a diagnostic tool for shrimp pathogens such as WSSV since almost two decades. Several commercial kits are in market with varying degrees of sensitivities, while the OIE protocol is regarded as gold standard. However, there are various issues related to the application and the reliability of PCR diagnostic results due to the inconsistencies in the testing methods and the results, mainly on account of different levels of technical competency. Often, technicians themselves are unable to prove beyond doubt the veracity of the diagnostic tests and the procedures adopted. This variance in the quality of testing and accuracy of results has contributed to an erosion of the confidence among shrimp farmers on PCR testing. Hence harmonization or an inter-calibration of the PCR testing capabilities of different service providing laboratories has become essential.

PCR ring test with WSSV Pathogen as a model

WSSV is an extremely virulent viral pathogen and the causative agent of devastating white spot disease (WSD) of shrimp. Since its emergence in 1994 in shrimp farming regions of Asia and the Americas, it has caused huge economic loss as high as US$ 8-15 billion to global aquaculture sector. It has been estimated about Rs.300 crores is lost annually by the Indian shrimp farming sector due to WSD. Considering this devastating nature, achieving biosecurity in shrimp farming would be immensely helpful in minimising its losses to the aquaculture sector in the country. Hence, WSSV has been taken here as a model for the PCR ring test with the objective to enhance capacity of the PCR laboratories in correctly diagnosing and providing quality service to the shrimp farmers in the country.

3rd PCR ring test organised by ICAR-CIBA, CAA-DAHD&F and MPEDA: The process

CIBA initiated the process of ring test involving the CAA under DAHD&F, the coastal aquaculture regulatory body and RGCA-MPEDA the brackishwater aquaculture promotional body to address the issue to train the technical personnel involved in PCR diagnostic laboratories through an intensive capacity building drive. Expression of interest (EOI) for participation in the ring test was invited by CAA and widely publicised through advertisements in newspapers and on websites of CAA, ICAR-CIBA and RGCA during March-April 2016. The exercise comprised three stages. In the first stage, PCR infrastructure available with laboratories was ascertained through inspection by team from CIBA and RGCA-MPEDA. This was followed by hands-on training programme during 3rd to 5th October 2016 for laboratory technicians at CIBA, Chennai on PCR for a period of three days, covering theoretical and practical classes on nucleic acids, principles and practice of PCR for WSSV, EHP and an RNA virus, infectious myonecrosis virus (IMNV). A training manual was provided to the trainees having comprehensive information on important shrimp diseases, basics of nucleic acids and PCR, protocols for shrimp sampling, nucleic acid extraction, performing PCR, electrophoresis to reporting the PCR results in addition to a section on trouble shooting.

The final phase of "Ring test" included sample distribution, submission of results by the participating laboratories and evaluation of the ring test. Each laboratory was provided with a panel of four coded tissue samples comprising positive and negative samples for WSSV fixed in 95% ethanol for testing during the first week of January 2017. Participation in the ring testing was voluntary and the laboratories were free to use the protocol of their choice. A standard report format was provided, and the laboratories were given a maximum of five working days to analyse the samples and submit results. For the reports to be considered complete, the primer sets or commercial kits, extraction methods, PCR conditions and gel photographs were expected to be included. Laboratories which employed real-time PCR were expected to include the chromatograms in the results section of the report. Results are provided here (Table 1) to for the participants through the specific codes which are known to only individual laboratories and the nodal institute, ICAR-CIBA and RGCA. ***Since the purpose of the ring test is not only to determine proficiency, but to help improve performance, feedback would be provided to those laboratories experiencing problems with the analysis, so that they get an opportunity to provide a quality service to the farmer.***

A total of 25 laboratories comprising 8 labs from Tamil Nadu, 11 from Andhra Pradesh, 3 labs from Karnataka and 3 labs from Gujarat participated in the ring test 2016-17.

The performance of the participating laboratories was rated as pass or fail based on the results returned by them. Twenty-flour out the 25 laboratories performed the tests and submitted the results. 71% (17/24) of the labs scored 4 and passed the ring test by correctly diagnosing all four samples (Fig 1). 17% (4/24) of the labs scored 3 and failed in the ring test by incorrectly diagnosing one of the four samples. 8% of the labs scored 2 and failed in the ring test by incorrectly diagnosing two of the four samples. 4 % (1/24) of the labs scored 1 and failed in the ring test by incorrectly diagnosing three of the four samples. Eleven of the 13 laboratories employing commercial diagnostic kits such as IQ Plus™ WSSV Kit with POCKIT System, IQ 2000 qRT PCR, and other real time PCR kits came up with correct diagnosis. However, success rate of correct diagnosis was lower (6 out of 11 or about 54%) using conventional nested PCR protocols and kits. Some of these labs reported positive reactions in negative samples indicating problems with test cross contamination. Some labs failed to detect positive samples indicating a problem of their test sensitivity. This is a cause for concern and needs to be addressed on a case by case basis through assistance with resourcing, technical advice and training.

Conclusion and the way forward

In the current PCR ring test, only 24 laboratories participated and about 71% of the laboratories had the capacity to provide correct diagnosis of WSSV using PCR. However, reporting pattern of some of these laboratories requires improvement. The laboratories which failed to achieve expected results require further technical training. During the pre-vannamei period, a number of laboratories were provided subsidy by the MPEDA. Further, as per the CAA guidelines, the approved SPF shrimp hatcheries need to have in house PCR facilities to provide tested disease free quality shrimp seed to the shrimp farmers. About 276 shrimp hatcheries are currently registered under CAA for import of SPF *P. vannamei* broodstock for seed production. This capacity building and harmonisation of PCR diagnostics has to include all these service providing laboratories to improve their skills and provide disease-free quality shrimp seed to the shrimp farming sector in the country. This would be one of the important steps towards enhancing shrimp aquaculture production in the country.

Despite repeated emphasis on the importance and need for the participation of the stake holders in the PCR ring test through personal communications and advertisements in newspapers and websites etc., the response has been poor, and final participation was only average. This shows the complacency from the sector, in that the common attitude ‘When things are going OK, let’s relax and when problems strike, panic and running from pillar to

post attitude'. Considering the common wisdom 'the effective method of disease control in aquaculture is Prevention rather than attempting treatment, the sector needs to be proactive and participation needs to be higher in the activities such as PCR Ring tests. CIBA along with CAA-DAHD&F and MPEDA will take this forward, with the active participation of stake holders, so that the sustainability, profitability and social relevance of the brackishwater aquaculture will have continuous growth trajectory.

Fig 1. Score obtained by PCR laboratories for diagnosis of WSSV during Ring test 2017 by ICAR-CIBA Chennai (17 labs scored 4 and passed the ring test by correctly diagnosing all four samples; labs scoring 3, 2 and 1 failed in the ring test by incorrectly diagnosing one, two and three of the four samples)

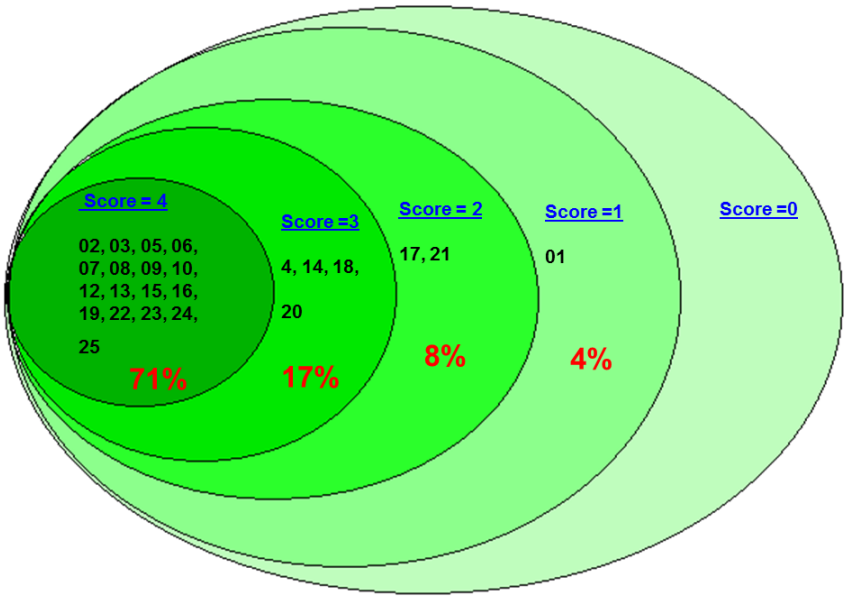

Table 1. WSSV PCR ring test results 2016-17

Sl. No	Group code	Sample code	Expected result	Submitted result	Score	Result
1	A	A1	Positive High	Negative	1	FAIL
		A2	Negative	Positive High		
		A3	Positive Low	Negative		
		A4	Negative	Negative		
2	B	B1	Negative	Negative	4	PASS
		B2	Positive High	Positive High		
		B3	Negative	Negative		
		B4	Positive Low	Positive Low		
3	C	C1	Positive Low	Positive Low	4	PASS
		C2	Negative	Negative		
		C3	Positive High	Positive High		
		C4	Negative	Negative		
4	D	D1	Negative	Negative	3	FAIL
		D2	Positive Low	Positive Low		
		D3	Negative	Negative		
		D4	Positive High	Negative		
5	E	E1	Positive Low	Positive Low	4	PASS
		E2	Negative	Negative		
		E3	Positive High	Positive High		
		E4	Negative	Negative		
6	F	F1	Negative	Negative	4	PASS
		F2	Positive High	Positive High		
		F3	Negative	Negative		
		F4	Positive Low	Positive Low		
7	G	G1	Positive High	Positive High	4	PASS
		G2	Negative	Negative		
		G3	Positive Low	Positive Low		
		G4	Negative	Negative		
8	H	H1	Negative	Negative	4	PASS
		H2	Positive High	Positive High		
		H3	Negative	Negative		
		H4	Positive Low	Positive Low		
9	I	I1	Positive Low	Positive Low	4	PASS
		I2	Negative	Negative		
		I3	Positive High	Positive High		
		I4	Negative	Negative		

10	J	J1	Negative	Negative	4	PASS
		J2	Positive Low	Positive Low		
		J3	Negative	Negative		
		J4	Positive High	Positive High		
11	K	K1	Positive Low			
		K2	Negative			
		K3	Positive High			
		K4	Negative			
12	L	L1	Negative	Negative		
		L2	Positive High	Positive High		
		L3	Negative	Negative		
		L4	Positive Low	Positive Low		
13	M	M1	Positive High	Positive High	4	PASS
		M2	Negative	Negative		
		M3	Positive Low	Positive Low		
		M4	Negative	Negative		
14	N	N1	Negative	Negative	3	**FAIL**
		N2	Positive High	Positive		
		N3	Negative	Positive		
		N4	Positive Low	Positive		
15	O	O1	Positive Low	Positive Low	4	PASS
		O2	Negative	Negative		
		O3	Positive High	Positive High		
		O4	Negative	Negative		
16	P	P1	Negative	Negative	4	PASS
		P2	Positive Low	Positive Low		
		P3	Negative	Negative		
		P4	Positive High	Positive High		
17	Q	Q1	Positive Low	Positive High	2	**FAIL**
		Q2	Negative	Positive Low		
		Q3	Positive High	Positive High		
		Q4	Negative	Positive Low		
18	R	R1	Negative	Negative	3	**FAIL**
		R2	Positive High	Positive High		
		R3	Negative	Positive Low		
		R4	Positive Low	Positive Low		
19	S	S1	Positive High	Positive High	4	PASS
		S2	Negative	Negative		
		S3	Positive Low	Positive Low		
		S4	Negative	Negative		

20	T	T1	Negative	Negative	3	**FAIL**
		T2	Positive High	Positive High		
		T3	Negative	Positive		
		T4	Positive Low	Positive Low		
21	U	U1	Positive High	Positive High	2	**FAIL**
		U2	Negative	Positive High		
		U3	Positive Low	Positive High		
		U4	Negative	Positive High		
22	V	V1	Negative	Negative	4	PASS
		V2	Positive High	Positive High		
		V3	Negative	Negative		
		V4	Positive Low	Positive Low		
23	W	W1	Positive Low	Positive Low	4	PASS
		W2	Negative	Negative		
		W3	Positive High	Positive High		
		W4	Negative	Negative		
24	Y	Y1	Positive Low	Positive Low	4	PASS
		Y2	Negative	Negative		
		Y3	Positive High	Positive High		
		Y4	Negative	Negative		
25	Z	Z1	Negative	Negative	4	PASS
		Z2	Positive High	Positive High		
		Z3	Negative	Negative		
		Z4	Positive Low	Positive Low		


TARSONS