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Application of PCR screened fry for increasing production of *Penaeid* shrimp in growout ponds of West Bengal, India

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Abstract

In the present study the effectiveness of polymerase chain reaction (PCR) screened fry for controlling white spot disease in semi-intensive culture of penaeid shrimp, *Penaeus monodon* was evaluated. PCR tested negative post-larvae and without PCR screened post-larvae were stocked for culture for 135 days in treatment ponds and control ponds respectively at a stocking density of 15 m⁻² under similar management protocols. PCR surveillance resulted negative in initial, intermediate and pre-harvest culture phases in treatment ponds. However, emergency harvest occurred prematurely in 66.6% of control ponds with symptoms of white spot disease. Harvest outcome showed significantly higher ($p < 0.05$) average body weight and yield resulting in higher net profit, benefit cost ratio and return on investment in treatment ponds than the control. Thus, the study reveals that stocking of PCR negative fry become an effective biosecurity tool and increased production of *penaeid* shrimp in growout ponds of West Bengal, India.

Keywords: PCR Screened, *Penaeid* Shrimp, Biosecurity Tool, White Spot Disease

1. Introduction

White spot disease (WSD) caused by white spot syndrome virus (WSSV) in shrimp occurs worldwide and causes high mortality and considerable economic damage, thus poses the greatest threat to the sustainability in shrimp farming industry^[1-3]. A conservative total loss in shrimp farming industry due to disease over the past 15 years was estimated 15 billion US\$^[4], of which WSD caused a loss of around 4-6 billion US\$^[3]. WSSV belongs to the family *Nimaviridae*, and characterized by the presence of a large circular double-stranded DNA^[5]. This virus was reported to be widely present in shrimp brood stock and its larvae^[6,7] and produced symptoms like white spots in the exoskeleton of infected shrimp in culture ponds^[2]. Transmission of WSSV was reported to occur vertically from infected brood stock to larvae or horizontally through water and/or infected animals (mainly crabs and wild shrimp)^[8,9]. The main causes of WSD in shrimp includes stocking of infected post larvae^[10], addition of infected water in the pond^[11], presence of WSSV carriers in the pond or in flow water^[12], occurrence of stress factors^[13], and the oral ingestion of infected shrimp or fresh feed^[11]. Since no therapeutic treatments are available for WSD, thus the best management strategy is to prevent WSSV from entering a shrimp farming facility by means of biosecurity practices^[4,14]. In the recent years advanced biosecurity measures was followed for semi-intensive shrimp culture^[4,15], which was adopted in the present experiment (see material and methods section).

Now-a-days shrimp brood stock collection still depends on wild catch and the chance of infection in post larvae (PL) is more by vertical transmission. Thus it is very much necessary to stock negative WSSV PL tested by nested-PCR (improves the sensitivity for detection of the target DNA sequence) along with surveillance to local crustaceans^[16]. Lightner pointed out that no significant resistance to this disease had been reported so far for any species of shrimp^[8]. Walker and Mohan reported that the disease is still remained uncontrollable and the situation of the negative impact has yet been changed^[17]. Many aquaculture practitioners and farmers are still unaware of the usefulness of PCR technique and proper biosecurity implementation in shrimp farming. Thus, the ability of the biosecured growout culture of *P. monodon* and other penaeid shrimp to prevent outbreak of WSD need to be explored by screening PCR negative PL before stocking and practicing general biosecurity norms during culture period. Therefore, the objectives of the present study were to assess the use of biosecurity and PCR test PL for controlling WSD in the light of: (i) preventing WSSV to avert WSD in shrimp culture; (ii) achieving cost effective sustainable shrimp culture.

2. Materials and Methods

2.1 Pond characteristics

In the present study (conducted during March to August, 2011) the black tiger shrimp, *Penaeus monodon* Fabricius, 1798 were cultured in the earthen ponds in the shrimp farm situated at Keshabpur, East Medinipur, India (Lat 21°55' N, Long 88°46' E). Experimental ponds were uniform in size (0.5 hectare and rectangular) having both inlet and outlet facilities. Water exchange (0-10%) was performed once weekly, although water was added to maintain losses due to seepage and evaporation. Chlorine treated water was used for maintaining water level and exchange during whole culture period. Aeration was maintained for 8-10 hr per day.

2.2 Experimental design

Experiments were conducted in semi-intensive methods in earthen ponds where three ponds each (similar in size and keeping same distance between them) were randomly selected from the said farm and used as- i) control: used PL without PCR test, and ii) treatment: used PCR tested PL. Different farm level biosecurity measures like use of chlorine treated water to avoid entry of carriers of WSSV, crab barrier to avoid entry of crab inside ponds, filtration of water by 250 μ mesh to avoid entry of eggs or larvae of carriers, *i.e.*, wild shrimp, crab; and general disinfection procedures were followed in all experimental ponds to control horizontal transmission of WSSV in the culture ponds [4, 13, 15, 18].

All experimental ponds were stocked with 17 days old post larvae (PL₁₇) with a stocking density of 15 PL/m². The PL was procured from a commercial hatchery (Vaisakhi hatchery, Vishakapatnam, Andhra Pradesh, India) after testing PCR negative whereas wild PL were collected locally and released in the experimental ponds after proper acclimatization. Cultured shrimp in all experimental ponds were tested PCR in three different phases of surveillance, *viz.*, initial (40 days), intermediate (80 days) and pre-harvest (120 days).

2.3 Sampling and estimation procedure

For WSSV PCR sampling, 2% prevalence sampled 150 individuals either PL₁₅ (2 days before lifting in hatchery) or shrimp in grow-out ponds were collected for PCR assay as per the methods described by Lightner [8]. Moribund shrimp in infected ponds were collected for PCR test for confirmation of WSD. Prior to DNA isolation, the gill of shrimp/crab, and whole body of PL was preserved in 70% ethanol and subjected to PCR analysis in laboratory following the method of Corriveau *et al.* [19]. WSSV infection was confirmed when shrimp tested found PCR positive. An outbreak was reported when mass mortalities were observed in WSSV positive shrimp.

2.4 DNA isolation and PCR assay in Shrimp PL

Total genomic DNA was isolated from the gills of shrimp by DTAB-CTAB DNA extraction procedure with PCR kits (IQ2000, Taiwan) and subsequently precipitated with ethanol [20]. The isolated DNA pellet was dissolved in DEPC ddH₂O and quantified using NANODROP spectrophotometer [20].

The nested PCR was carried out in a 0.2 ml thin wall PCR tube with 50 ng of genomic DNA, 10 pM of each primers, 50 μ M of dNTPs, 1X PCR buffer, 1.5 mM MgCl₂, and 1.0 U Ampli Taq Gold (Perkin Elmer, USA). Amplification conditions were followed 39 cycles at 94 °C for 4 min, 55 °C for 1 min, 72 °C for 3 min and final extension at 72 °C for 5 min in a PCR thermocycler. Positive and negative controls were included in all tests. PCR products were subjected to electrophoresis in 2% agarose gel and quantified as per the method of Hoa *et al.* [20].

2.5 Pond management

All the experimental ponds were prepared and managed following the method described by Hasan *et al.* [21]. Pond bottom was dried and ploughed well and pond water was chlorinated (60 ppm with 60% active ingredients) to kill all potential carriers of WSSV including rotifers and crab eggs. Shrimp were fed with balanced commercial diet (protein 38%, fat 5%, fiber 4%; procured from CP Aquaculture, Chennai, India) and the feed ration was divided into 2-4 times a day and quantity was reduced or increased as per regular check tray observations [22]. Shrimp were harvested after a grow-out period of 135 days or depending upon the disease incidence and/or mortality.

2.6 Water quality parameters analyses

Different water quality parameters like temperature, dissolved oxygen (DO), pH were monitored twice a day (6-00 and 16-00 hrs); salinity and transparency once a week (11-00 hours) *in situ* by multi-parameter device (YSI, MP556 mode) and Secchi disc. Other nutrient parameters (NH₄-N, NO₂-N, NO₃-N, PO₄-P) and total alkalinity were monitored in monthly basis during whole production period [23].

2.7 Shrimp health and production

Cast net was used to haul shrimp routinely for checking shrimp growth and health status. Average body weight (ABW) and survival (%) of the shrimp was recorded fortnightly in all the ponds throughout culture period. Soil and water conditions were recorded during sampling. Healthiness and abnormality were recorded during each fortnightly sampling. ABW, survival rate, feed conversion and biomass of shrimp during culture were estimated following the methods of Hasan *et al.* [21].

2.8 Economic and statistical analysis

Production costs, gross revenue, net profit, benefit cost ratio (BCR) and return on investment (ROI) were calculated following standard methods [24]. Results were analyzed by a one-way analysis of variance (ANOVA) with the help of MS Excel and computer software SPSS (version 7.5) and the sample means compared. To test the level of significance, the student's *t*-test was conducted between the results of treatment and control series.

3. Results

3.1 PCR results

During pre-stocking phase, PL₁₇ was screened for PCR negative and cultured in treatment ponds (Fig. 1a). After stocking in treatment ponds, cultured shrimp were resulted WSSV negative on 40 days of culture (DOC; initial phase) in all three ponds, the same was found negative during other culture phases (Fig. 1b). Moribund and weak shrimp in two infected ponds of control (DOC 89 and 105) resulted positive for WSSV (Fig. 2b).

3.2 WSD incidence in shrimp ponds

In the present experiment, first WSD infected shrimp were found on 88 DOC by abnormal behavior of shrimp, red discoloration and stopped feeding in one of the ponds of control; and outbreak of mortality started from DOC 89. Second time the same incidence occurred on DOC 105 in another control pond; and outbreak occurred on the same day. All the WSD infected ponds were made for emergency harvest on the day of outbreak and subsequently confirmatory test by PCR resulted positive for WSSV. All the three treatment ponds showed no abnormality and any symptoms of WSD which were harvested after 135 DOC (Fig. 2a).

3.3 Water quality parameters

The results of different physico-chemical parameters in all the experimental ponds are presented in table -1. All parameters were found suitable and optimum for shrimp culture in all culture ponds. Average morning temperature in control and treatment ponds was 28.2 ± 1.6 , and 27.9 ± 1.7 °C respectively, while pH (7.91 ± 0.09 and 7.94 ± 0.1) was alkaline throughout the culture period in all the ponds. Mean DO was 4.2 ± 0.9 and 3.9 ± 0.7 mg l^{-1} in the control and treatment ponds respectively during the morning. Average transparency and salinity of water in the treatment ponds was 50.3 ± 6.3 , 47.8 ± 6.9 cm; and 11.5 ± 2.8 , 10.7 ± 2.5 ppt respectively. The total alkalinity was within optimum levels in both control and treatment ponds (117.5 ± 14.9 , 112.2 ± 11.8 mg l^{-1} respectively). On the other hand, the mean values of NH_4-N were higher in control (0.13 ± 0.05) than treatment (0.09 ± 0.04). The amount of nitrate-N in control and treatment ponds was 0.56 ± 0.28 and 0.62 ± 0.17 and orthophosphate was same (0.08 ± 0.03) in both the treatment and control series. It was found that all the parameters showed insignificant result ($p > 0.05$) while *t-test* was done between treatment and control series (table-1).

3.4 Production outcome

Final average body weight (ABW), survival (%), feed conversion ratio (FCR) and yield of cultured shrimp have been presented in table-2. At harvest, ABW was higher in treatment (29.0 ± 0.36) than control ponds (24.2 ± 5.1). Before the disease outbreak, lesser ABW (19.1 and 24.3 g) was achieved in two ponds of control ponds; and 27.1 g was resulted in one pond of treatment series (table -2 and Fig. 2a). Higher survival rate (79.2 ± 5.7) of cultured shrimp was recorded in treatment ponds than control (60.5 ± 9.4) at harvest (Fig. 2b). Survival was reduced for mortality during outbreak in two ponds (52.1 and 57.4% respectively) of control series but the difference was statistically insignificant ($p > 0.05$). On the other hand, FCR increased as days of culture progresses for all the series but it was lower and best result was achieved in treatment ponds (1.72 ± 0.04) than control ponds (mean 1.95 ± 0.2) where higher FCR observed in two ponds of infection (1.96 and 2.14 respectively) and the difference was also statistically insignificant ($p > 0.05$) (Fig. 2c). Finally, an increase in average production of cultured shrimp was obtained in treatment ponds (4599 ± 366.4) than control ponds (2968.4 ± 280.8), where final yield (biomass) was lesser in two ponds of control ponds (1492.7 kg and 2092.2 kg respectively) and the differences was statistically significant ($p < 0.05$) (Fig. 2d). Moribund shrimp or shrimp having clinical signs of WSD (white spot on carapace) was observed only in two of control ponds (out of three), thus confirmed WSD prevalence (infection and outbreak) as 66.6% in control ponds, whereas it was absent in case of treatment ponds.

3.5 Economic analysis

Economic analysis of shrimp production and income generation in Indian Rupees (INR.) has been summarized and presented in table -3. The application of PCR tested PL had a positive effect on the gross revenue of the harvested shrimp from treatment ponds (INR. 1176357) than control ponds (INR. 620706). Net profit and BCR among the experimental ponds changed as gross revenue was higher BCR (0.47) in treatment ponds as compared to control ponds (-0.10) amply speaks for effective net profit (INR. 374828) and return on investment (ROI) of 47% after harvest. Variable cost was found to be higher in treatment than control (INR. 543284) due to additional cost incurred for PCR assay before stocking. However, net profit and ROI was found

higher and attractive in treatment ponds than control.

4. Discussion

In recent days shrimp farming, development of sensitive diagnostic tools using molecular approaches may be useful not only for the identification and comparative studies of the viruses but also for the screening of carriers in shrimp larvae and parental brood stocks [8]. In the present study both farm level biosecurity and PCR tested PL were applied in the culture ponds as an diagnostic tools for controlling WSD of *P. monodon* in a semi-intensive culture system. The result showed that in WSSV infected ponds (two ponds of control series) premature harvest took place as viral loads might be severe in shrimp due to WSD infection [13]. It was reported that in natural situation, shrimp become infected through oral and water-borne routes and the gills were thought to be a major point of viral entry [25, 26]. Thus it can be said that horizontal transmission might have been took place in these ponds by means of untreated water or crustacean carriers or through contaminations [11, 12].

The present result showed that the confirmatory diagnosis (PCR) of WSSV occurrence was found when mortality occurred in two-third of control ponds where PL was not PCR screened before stocking (Fig. 1b), which may be occurred due to the vertical transmission of WSSV-positive spawners to their offsprings [16]. One-third control pond was not affected by WSD, as there was chance of PL free of WSSV used during stocking unknowingly or viral loads were in the lower range. The present finding depicts similar opinion that possible source of this virus may be either brooders or PL (vertical transmission) as no PCR screening was performed in control ponds before stocking but only biosecurity measure was followed. The treatment ponds were not infected with WSSV as hypothesized that in these ponds PCR screened PL was stocked and farm level biosecurity practices were followed which was in agreement with previous study [27]. During routine sampling for health monitoring, the shrimp found apparently healthy and no microscopic white spots were visible before the infection in all ponds in the experiment. Results of environmental surveillance (wild shrimp/crab) showed positive for PCR and hence horizontal transmission was barred by implementing biosecurity measures in both control and treatment ponds (Fig. 1b). It was reported that adoption of better farm management practices by using biosecurity practices, PCR screening in particular, is of utmost importance for success of present day aquaculture [27]. However, no stress for temperature and salinity or other physico-chemical parameters were observed in the culture ponds (table-1) which could be a risk factor for appearance of WSD as reported by earlier workers [11, 28].

It was also found that body weight increases as DOC increases in all the experimental ponds but it was found that mean ABW was lesser (24.3 g) in control ponds which might be stopped due to feeding of shrimp in two ponds on DOC 88 and 105 respectively (Fig. 2a). Finally shrimp started dying in the control ponds and symptoms of WSD observed. It was assumed that WSD during shrimp culture resulted due to stress for higher viral loads [27]. Survival of cultured shrimp was reduced as days of culture progresses in all the experimental ponds (Fig. 2b). In the present study, maximum survival (85.5%) was achieved during harvest in one of the treatment ponds and minimum survival (52.1%) was found in one of the control ponds which may happen due to mortality caused by WSD. FCR in control ponds resulted higher as shrimp losses were observed in two ponds of control during outbreak of WSD (Fig. 2c). FCR was higher in the two infected ponds of control (1.96 and 2.14) as

biomass losses (mortality with decay) were found during the day of outbreak and harvest (Fig. 2c). However, no infection and mortality was observed in treatment ponds as PCR diagnosis was followed during stocking (table-2) was in agreement with the previous study [4]. Lower FCR and higher yield with bigger size of shrimp in treatment ponds fetched better ROI amply proved economically cost efficient even though there have been an additional expenses made for PCR tests before stocking (Fig. 2c, 2d); but stability in production yield was achieved unlike control ponds. The study revealed that treatment ponds was the best and success to adopt for sustainable and stable production as an improved aquaculture technique by using both PCR test and adoption of biosecurity measures during culture. Of late, shrimp farming is regarded as the best economic and high pay-off activity in terms of returns to investment and found good net profit in different shrimp farms of India [24] also in agreement of the present result (see table-3). Huge mortality incurred in two ponds of control caused economic losses was also corroborated with previous studies [2, 3, 8]. However, the success with good harvest in treatment ponds seems to be an effect of better management practices (PCR and biosecurity practices) which reduced the risk of crop losses from WSD and improve productivity and profitability of small-scale shrimp farms [27]. The realistic economic analysis performed in the on-farm trial showed combination of both PCR screening and biosecurity practices substantially increased net profit, BCR and ROI when compared to ponds cultured without PCR diagnosis before stocking.

Table 1: Water quality characteristics of shrimp growout ponds (experimental ponds)

Variables	Control	Treatment	Acceptable limit
Temperature (°C)	28.2 ± 1.6	27.9 ± 1.7	27-33 *
Salinity (mg l ⁻¹)	11.5 ± 2.8	10.7 ± 2.5	10-30 *
Transparency (cm)	50.3±6.3	47.8 ± 6.9	25-50 **
pH	7.91 ± 0.09	7.94± 0.10	7.5-9.0 ***
Dissolved Oxygen (mg l ⁻¹)	4.2 ± 0.9	3.9 ± 0.7	4-8.5 *
Nitrate-N (mg l ⁻¹)	0.56±0.28	0.62±0.17	<1*
Ammonium-N (mg l ⁻¹)	0.13±0.05	0.09±0.04	<0.1***
Ortho-phosphate (mg l ⁻¹)	0.08±0.03	0.08±0.03	0.04-0.06 ****
Total Alkalinity (mg l ⁻¹)	117.5±14.9	112.2±11.8	40-160 *

Sources: *Fast and Lester, 1992; **Soundarapandian and Gunalan, 2008; ***Tsai, 1989; ****Das et al., 1996

Table 2: Production outcome after harvest including emergency harvest in experimental ponds (Mean± SD). (Ranges in parenthesis)

Variables	Control	Treatment
ABW (g)	24.2± 1.1 (19.1-29.3)	29.0±0.36 ^a (28.6-29.3)
Survival (%)	60.5±9.4 (52.1-70.4)	79.2±5.7 (74.2-85.5)
FCR	1.95 ± 0.2 (1.74-2.14)	1.72±0.04 (1.69-1.76)
Yield (kg ha ⁻¹)	2968.4 ± 280.8 (1990.2-4125.4)	4599±336.4 ^a (4244.2-4976.1)
Days of culture (d)	109.7 ± 23.4 (89-135)	135

^a in the same row with different superscripts are significantly different. *p* values: ^a <0.05. WSD and mortality occurred in two control ponds in 83 and 105 DOC.

Table 3: Cost and economic analysis of *P. monodon* in experimental

Particulars	Control	Treatment
(Variable costs)		
Pond preparation	12500	12500
Fertilizers	2400	2400
Probiotics	109513	132700
Post larvae (fry)	111000	123000
Feed	259854	340610
PCR	0	10500
Biosecurity	12800	12800
Miscellaneous	35217	48650
Total variable cost	543284	672660
Interest for 4.5 months@12%	24448	29694
Fixed cost (depreciation + interest)	98600	98600
Production		
Total shrimp yield (kg ha ⁻¹)	2968.4	4599
Price of shrimp (INR. kg ⁻¹)	256.3	310
Economic analysis		
Total production cost (INR.)	666332	801529
Gross revenue (INR.)	620706	1176357
Net profit (INR.)	-45625	374828
Benefit cost ratio (BCR)	-0.10	0.47
Return of investment (ROI) %	-10.6	47

*US\$ 1 = ₹ 54.2 Indian Rupees (INR.)

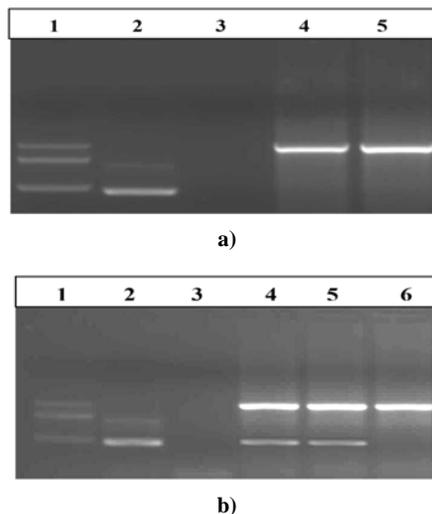
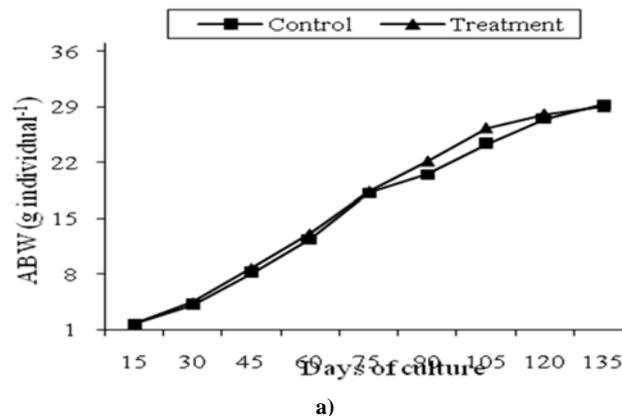


Fig 1: Detection of WSSV in shrimp larvae/shrimp by PCR electrophoresis: a) Lane-1: molecular marker; Lane-2: positive control; Lane -3: negative control; Lane -4 and 5: WSSV negative sample of brood stock shrimp, and b) Lane-1: molecular marker; Lane- 2: positive control; Lane- 3: negative control; Lane- 4 and 5: WSSV infected shrimp of control ponds 1 and 3; Lane- 6: WSSV negative shrimp of control pond-2.



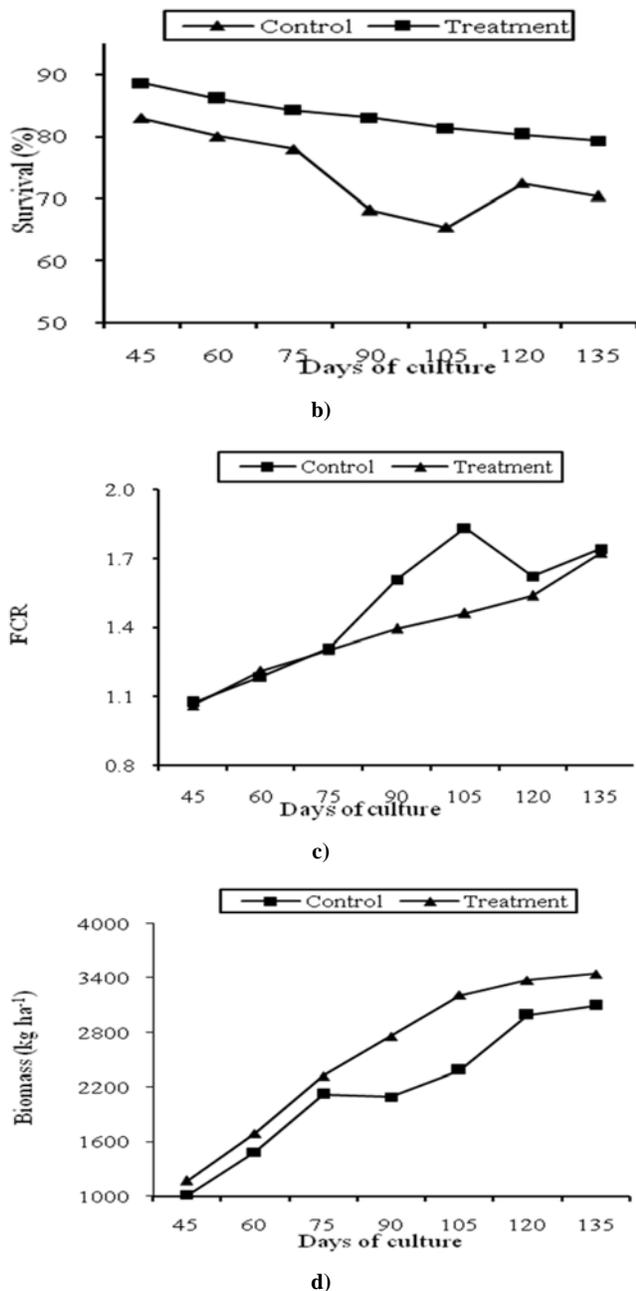


Fig 2: A comparative account of different production parameters in the experimental ponds during different days of culture - (a) average body weight; (b) survival rate; (c) feed conversion ratio; and (d) biomass.

The present study revealed that stocking of PCR screened fry and maintaining biosecurity practices may prevent the WSD and ascertain good harvest for sustainable shrimp culture. Biosecurity practices and PCR diagnosis in particular, may be very much useful to prevent WSSV infection as long as proper implementation and awareness maintained amongst the aquaculture community. PCR screening of WSSV infection for shrimp brood and larval stocks, and other potential viral carriers could be an effective tool in battle against the fatal virus that has almost devastated the shrimp commerce in India and abroad.

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