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Association of risk factors: WSSV proliferation in the shrimp (*Penaeus monodon*) farms of south-west coastal region of Bangladesh

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ABSTRACT

In the shrimp *(P. monodon)* culture, various diseases, white spot syndrome virus (WSSV) in particular, has become a serious constraint in Bangladesh. Under the study, 72 culture sites from four different locations of Bagerhat (Kochua, Rampal, and Fakirhat) and Khulna (Paikgacha) district were investigated from January to June 2011. Throughout the study period, 20 factors regarding farm management and water quality were keenly considered inferring the association of WSSV outbreak and the farms either claimed infected or not were confirmed by PCR test. Study revealed significant correlation with some factors like accessibility of cattle into the farms (r=0.630, p≤0.01), and farms linked up with surrounding water bodies/farms (r=0.754, p≤0.01) within a cluster (Spearman's rho correlation coefficient test). Pearson Correlation coefficient for salinity found to have significant correlation with the risk of WSSV infection (r= -0.727, p≤0.01), followed by temperature (0.624, p≤0.01) and the average depth (-0.618, p≤0.01). However, feeding kept 30.6% farms away from the outbreak followed by sludge removal (26.39% farms). Uses of river water directly into the farms showed 38.9% risk of being attacked which was nil and 1.4% for the underground and rainwater respectively. March to June found to be the disease prone months and out of the four locations, Fakirhat found to be less infected due to better management. Therefore, to cope with the risk of WSSV infection, proper farm management practice, virus free PL (post larvae), awareness buildup at the farmer level and switch into community based farm management can be broadly brought into practice.

Keywords: WSSV; Risk factor; Shrimp *(P. monodon);* Farm management

INTRODUCTION

A major portion of the world shrimp culture production comes from South East Asia. However, in recent years, the production of cultured shrimp has markedly decreased because of serious viral disease outbreaks; especially the increased severity of widespread White Spot Syndrome Virus (WSSV) infection became the most serious threat to stable aquaculture production. Features of this WSSV were that the diseased prawns were often showing obviously White spots on their carapace and that the high mortality was occurred from 80% to 100% in only few days after infection (Nakano et al., 1994; Chou et al., 1995). A recent major outbreak of WSSV infection in China, Japan, Taiwan, Bangladesh, Thailand, and India (Otta et al., 2003; Zhan and Wang, 1998; Pongmaneerat et al., 2001; ASCC, 1996), led to higher farm losses, has raised significant concerns in aquaculture around the world (Islam et al., 2007). In Bangladesh, farmed shrimp production reduced down to 87,972 metric Ton in the year of 2009-10 from 1,02,854 metric Ton in the year of 2008-09; and till 2012, the production rate, however; raised slightly, due to horizontal spreading and substitution by giant freshwater prawn, *Macrobrachium rosenbergii*. (DoF, 2011).

Treatment of such viral diseases found to be less efficient than the prevention to avoid outbreaks (Iqbal et al., 2011; Menasveta, 2002). However, several studies have been carried out to investigate the effect of disinfectants on WSSV infection (Chang et al., 1998; Maeda et al., 1998; Balasubramanian et al., 2006) along with the use of vaccines and immunostimulant to control WSSV outbreak (Citarasu et al., 2006; Satoh et al., 2008; Sajeevan et al., 2009). However, in the context to the culture practice of Bangladesh, vaccines and immunostimulants can hardly withstand the disease outbreak as most of the farms are being managed almost traditionally. Therefore, bio-security measures have been suggested by some scientists to exclude the pathogen or reduce the risk (Lotz, 1997; Mohan et al., 2005). Most of the research carried along the line, dealt on the carrier organism(Lo et al., 1996; Suppamataya et al.,

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1998; Otta et al., 1999; Corsin et al., 2001; Hossain et al., 2001; Yan et al., 2004; Liu et al., 2006), transmission (Suppamataya et al., 1998; Corsin et al., 2001; Peng et al., 1998), effect of water physicochemical parameters (Vidal et al., 2001; Granja et al., 2003; Guan et al., 2003; Rahman et al., 2006; Reyes et al., 2007), and genetics (Wongteerasupaya et al., 2003; Dieu et al., 2004; Musthaq et al., 2006).

Only few studies reported the association of risk factors related to pond culture. In some research, WSSV infection has been found positively correlated with proximity of the pond to the sea and negatively to ponds closely located within a given cluster (Mohan et al., 2008). Sludge removal, ploughing, liming, complete system dry-out between culture cycles, water filtration through 300 μm mesh screen and phosphorus application through fertilization were reported to reduce the risk of WSSV infection (Corsin et al*.,* 2001; Velasco et al., 2002; Mohan *et al.,* 2008) in Philippines. Corsin et al. (2001) found no association between stocking density and WSSV infection.

In Bangladesh, every year WSSV grounds for catastrophic loss to the shrimp *(P. monodon)* industry by mass mortality of shrimp *(P. monodon)* since poor information available on the etiology of the disease in this region. Therefore, epidemiological approach thought to be the right one for understanding this contagious disease due to different factors of culture area and management practices affecting WSSV infection. Hence, this study hypothesized that factors related to farm site and farm management affect the occurrence of WSSV. Therefore, by developing a well-structured questionnaire these risk factors could be identified, using the epidemiological approach.

MATERIALS AND METHODS

Study area

A total number of 72 shrimp *(P. monodon)* farm/farms (both affected and unaffected) have been investigated randomly from four spots *viz.* Paikgacha, Kochua, Rampal and Fakirhat of Bagerhat district. Sampling was carried out from January to June, 2011. This period of time considered the most crucial period in WSSV infection and proliferation in this region for decades. Investigation of the farm/farms carried out according to the following design (Table 1).

Data collection

Each sampling consists of the data regarding water quality parameters, general aqua-ecology and management practices, etc. attained by a pre-tested well-structured questionnaire comprised of several groups of variables viz., pond preparation, water management, culture practices and bio-security measures, etc. Along with the interviews, 25 no's of shrimp *(P. monodon)* samples were collected randomly from each of the farms, for investigation of WSSV pathogen by PCR until the characteristic white spherical spots appear on the carapace and telson ensuring WSSV infection visually in the farmed shrimp *(P. monodon)* drawing a firm conclusion regarding WSSV association. Water samples collected and tested on the spot using a water test kit (HACH FF-2 with digital tritator) between 8.00 to 10.00 A.M. consists of temperature, salinity, pH, dissolved oxygen, total alkalinity and total ammonia.

WSSV identification using PCR

Collected shrimp *(P. monodon)* samples from the sites were then subjected to PCR test in the shrimp *(P. monodon)* health management laboratory of Bangladesh Fisheries Research Institute using Genie's kit. DNA extraction and amplification were done by the supplied protocol of the respected kit.

Statistical analysis

All date were analyzed using SPSS (Windows Evaluation Ver. 14.0, released September 5, 2005). For the categorical data, nonparametric correlation test, spearman's rho has been performed inferring the degree of WSSV association whereas, for the parametric data, Pearson correlation coefficient has been considered. Values for WSSV infection taken 1 and 2 for WSSV positive and negative, for PL (post larvae) source 1, 2 and 3 stands for wild, hatchery and mix sources of PL, simultaneously for water source, 1, 2, 3 and 4 stands for the river, canal, underground and rain-fedwater. A positive correlation coefficient indicates that an increase in the corresponding variable will increase the risk of WSSV occurrence. On the contrary, a negative correlation coefficient implies that an increase in the level of the respective variable will reduce the risk.

RESULTS

The presence of WSSV in the shrimp *(P. monodon)* was confirmed by Polymerase Chain Reaction (PCR test). The test was performed until the symptoms of the disease (Opaque white spherical spot in cephalothorax, abdomen and telson mainly) could be reckoned visibly through appearance of spherical opaque spots in cephalothorax, abdomen and telson. PCR test obtained no presence of WSSV in the month of January and February (Fig 1. A and B) but in early March 2 *farms* (in case of 2 and 3 no. samples) in Rampal Upazila showed WSSV positive result at 300 bp for nested PCR (Fig 1. C).In the same month, among the rest of the farms 5, 6, 9, 10 and 12 showed WSSV positive both at 600 and 300 bp for the first step and nested PCR respectively, along with identical opaque white spherical spots as external sign (Fig 1. C and D).
Only the WSSV data for January and February differ significantly compared to other months (Table 3) alike the data of Fakirhat in comparison with other areas (Table 4). Regarding the parametric and non-parametric data analysis, six water quality parameters of the eight parametric data, collected throughout the crucial period of disease occurrence from the four sampling sites were analyzed by Pearson correlation test using SPSS (Table 5). Salinity and Temperature found to have significant correlation ($r= -.727$ and $.624$, $p\leq0.01$) with WSSV infection followed by the average depth ($r= -.618$, $p\leq0.01$). Study depicted, increase of average temperature from 21° C at January to 33° C at June, number of infectedfarm/farm reached from nil to 10 (Figure 2) .
On the other hand, for the categorical data, Spearman's rho test was performed to infer the level of significance (Table 6). Sharing same water in several ponds/farms (Linked up with other farms) showed significant influences ($r=.754$, $p\leq0.01$) in WSSV infection followed by infection due to the accessibility of cattle or other animals into the farms ($r= .630$, $p\leq0.01$).
Among the farms investigated, 43.1% found to be infected by WSSV where the accessibility of cattle was frequently compared to the farms free from cattle grazing (4.2%) (Fig. 2).
Removal of sludge (black soil) was also found to have influences on WSSV infection. Sludge removed farms were found to be infected at 15.28% whereas farms that did not remove sludge were found to be infected at 31.94% (Fig. 3)
Liming prior to the beginning of culture found to have influence reducing the risk of WSSV infection up to 36.11%, whereas the risk rose up to 29.17% in the farms that did not apply lime during as well as at the beginning of culture (Fig. 4)
Feeding, however, is an important issue in any cultural practice, farmers in the study area found to be reluctant in feeding showed 38.9% risk of being infected by WSSV, which can be reduced to 8.3% by providing supplementary feed (Fig. 5)
Another important factor found to be the source of PL. Risk of WSSV infection to the hatchery PL was up to 6.94% which could be up to 16.67% and 23.61% of the PL of wild source and a mix of wild and hatchery source respectively (Fig. 6)
The most interesting thing found in the study was the source of water. WSSV risk was found up to 38.9% and 6.9% for the farms having water directly from the rivers and canals, which were just nil, and 1.4% **of** the underground and rain fed water respectively (Fig. 7)

DISCUSSION

As an invertebrate, shrimp *(P. monodon)* lacks the key components of the vertebrate adaptive immune response (e.g. immunoglobulins, major-histocompatibility-complex (MHC) antigens, T-cell receptors) that provide a versatile mechanism for natural protection and allow for conventional vaccination against viruses (Arala-Chaves and Sequeira, 2000). Therefore, strategies for health management in shrimp *(P. monodon)* aquaculture are primarily based on the principles of pathogen exclusion and the avoidance of environmental conditions that induce stress, stimulate viral replication or facilitate disease transmission (Walker and Mohan, 2009). Studies revealed that rapid changes in the temperature due to heavy rain increase viral proliferation.

Temperature fluctuation and low temperature are identified as risk factors for WSSV infection, while an increase in temperature can be a risk factor for an outbreak in pond-cultured *P.monodon* (Tendencia et al., 2010). As the temperature raises high to 33-35^{o} C in the month of April, May, and June, the number of infected farms increased also. This may be attributed to sudden rain in those months that reduce the temperature rapidly, as most of the farm found to have too low water depth to resist abrupt change in water temperature. According to Tendencia et al. (2010a) climate is one of the vital WSSV risk factors. Iqbal et al. (2011) reported that the prevalence of WSSV is higher in the months of May to September in Bangladesh. Low atmospheric temperature, consequently affects the water temperature that is considered an important WSSV risk factor. Exposure to low water temperature could lead to WSSV infection in pond cultured *P. monodon* (Tendencia et al., 2010b), owing to the increase in viral replication and the decrease in the immune response of shrimp *(P. monodon)* at low temperature (Vidal et al., 2001; Reyes et al., 2007).

Salinity had been another factor having significant association with WSSV outbreak. Several studies in captivity reported that fluctuation in salinity and temperature could weaken the shrimp's immune system and affect viral proliferation. Yu et al., (2003) reported weaker immune response in Marsupenaeus japonicus as the deviation from the original salinity becomes greater. However, the current study revealed that most of the farms could not withstand against salinity fluctuation due to the lower water depth at sudden rain. Therefore, after successive raining in the aforementioned months, number of WSSV infected farms increased as the harboring salinity falls suddenly.

Effective pathogen exclusion practices require attention to all points in the shrimp *(P. monodon)* production cycle, from spawning to harvest, at which viruses may be encountered or recycled into the environment (Fegan & Clifford 2001). According to the study, there is a significant correlation between accessibility of cattle into the shrimp *(P. monodon)* farms and WSSV proliferation. Accessibility of cattle into the farms poses increased risk of viral infection up to 43.1%, which is quite alarming. Moreover, sharing of the same water and common flow-through system is found to be another threat in the study area, having significant impact on WSSV outbreak. Mohan et al. (2008) reported that WSSV infection is negatively correlated to ponds closely located within a given cluster. May be that the water that is allowed to enter into the pond is mixed with discharge of the farm itself or other farms, that would contaminate the water. This argument also holds for having the same receiving water source as a risk factor. Contamination of water can also come from the sludge removed from the pond bottom. However, according to Tendencia et al. (2011), biosecurity measures did not prevent WSSV occurrence. Biosecurity measures aim to exclude pathogens and carrier organisms from the cultural environment; e.g., farmers installed birds scare and crab fence to prevent the entry of birds and crabs. But, these bio-security measures often do not reach their purpose. Birds still fly over the strings that are supposed to scare them, might defecate above the pond and their feces contaminated with WSSV particles could infect the cultured shrimp *(P. monodon)*. Crab fence, usually made of nets, is installed on the pond dike to prevent the entry of crabs; however, crabs could still enter the pond by creeping through the net or by making holes through the dike. Farmers should optimize their biosecurity measures. Limited access to farm, footbaths, tire baths, and hand disinfection were strictly implemented by farm management. These measures concern humans as carriers. However, the question is whether human can actually carry WSSV particles that could infect the cultured shrimp *(P. monodon)*. This question is not just anecdotic; no scientific evidence proves that humans can transmit WSSV or particles carrying WSSV, although Corsin et al. (2005) found that sharing of personnel between ponds is associated with WSSV. Stocking of WSSV negative fry could prevent the entry of WSSV into the culture system, but shrimp *(P. monodon)* once inside the pond can be infected in several ways.

In agreement with Tendencia et al. (2011), sludge removal also found to be a risk factor of WSSV infection in the shrimp *(P. monodon)* farms. Sludge is made up of accumulated organic matter such as excess feed and feces. Organic matter harbors microorganisms that could be pathogenic to shrimp *(P. monodon)*. In most instances, sludge removed from the pond bottom was placed on the dike. This might allow harmful microorganisms present in the sludge to be washed back into the pond. The amount of sludge that accumulates at the pond bottom was affected by stocking density, which also was another risk factor. The higher the stocking density the more organic matter due to feces and uneaten feed may accumulate on the pond bottom.

Feeding live molluscs to shrimp *(P. monodon)* poses a risk for WSSV infection, which might be transmitted in two ways to the system. Molluscs either being filter feeders can serve as WSSV carriers or molluscs could ingest WSSV particles from the soil and the water column, which could be transferred to the shrimp *(P. monodon)* by feeding. Feeding commercial pellets as a risk factor in polyculture farms might be a consequence of sludge accumulation at the pond bottom due to improper pond preparation in the farms (Tendencia et al., 2011). In the current study, feeding showed a negative correlation with WSSV infection. Farmers provide feed to the farms are likely to have 30% less chance of being infected by WSSV.

Source of post-larvae (PL) also found to have an impact on viral infection in the shrimp (P. *monodon)* farming area of the country because WSSV is highly infective not only for marine penaeid shrimp *(P. monodon)* (Rodriguez *et al.* 2003), but also infect many other crustaceans and marine crabs (Hameed *et al.,* 2003). There are reports that freshwater species such as the crayfish*Pacifastacusleniusculus* (Jiravanichpaisal et al., 2001) and *Macrobrachium rosenbergii* (Chakraborty et al., 2002) are also susceptible to infection.WSSV genomic DNA can be vertically transmitted to *Artemia* cysts, but it is lost during hatching (Li et al., 2003).

Study revealed that farmers using hatchery produced PL was capable of reducing the risk of infection by nearly about 10% than farmers using wild PL in farms. Because as mentioned previuosly, water from different sources could be contaminated with virus particles, and posing a risk of WSSV infection into culture system. Direct rain fed ponds and farmers using underground water, found to be less susceptible to viral infection. Evaporation process for rainwater and different filtration stages of underground water might have an impact reducing the association of WSSV in the farming system. Further study is required to confirm the risk factors responsible for a massive infection of WSSV in the shrimp *(P. monodon)* industry.

Therefore, water or pond bottom quality and immune response of shrimp *(P. monodon)* could be considered as the risk factors for WSSV infection. Rapid changes in salinity and temperature can be minimized by ensuring proper depth in the culture pond/farm. Intensive study is required to confirm the optimum water depth, minimizing the abrupt changes in salinity and temperature due to sudden rainfall. Some WSSV risk factors such as feeding live molluscs should be avoided. Proper liming and feeding can be maintained to ensure good health condition of the shrimp *(P. monodon)*. Sludge removed from the pond should be disposed of in a place where washing back into the system is prevented. The effect of other risk factors can be mitigated by the implementation of the identified protective factors.

CONCLUSION

Shrimp *(P. monodon)* export is the second largest source of earning foreign currency in Bangladesh. However, in recent years, the production of cultured shrimp *(P. monodon)* has markedly decreased because of serious viral disease outbreaks. Especially, the increased severity of widespread White Spot Syndrome Virus (WSSV) infection, the most serious threat to stable aquaculture production. Several studies to control the disease have been done. However, tank experiments identified WSSV risk factors related to the physicochemical properties of the water, a few studies reported pond level WSSV risk factors. In this study, initiative taken to find out the risk factor associated with WSSV outbreak in the shrimp *(P. monodon)* farms, which will be helpful in reducing the risk of massive disease outbreak. Study revealed that proper pond/farm preparation, stocking of SPF (Specific Pathogen Free) PL and maintaining optimum water depth (not less than 3 feet) can withstand against the invasion of the viral pathogen into the culture area. Flow-through system within the farms along with cattle grazing in the culture area also found to have fatal impact on viral spreading from which some management an approaches can help to overcome the problem with ease. In a word, it can be said that proper farm management could have adequate impact on preventing mass mortality due to WSSV infection, as there is no sustainable treatment has been developed yet. Therefore, proper initiatives to be taken to grow awareness among the farmers that can play vital roles in minimizing the loss through WSSV outbreak of shrimp sector.

Table 1. Study area and sampling design

Sl. No.	Sampling spot	Type of water body	No. of sampling/month
01	Paikgacha	Local Shrimp Farms	3
02	Kochua		
03	Rampal		
04	Fakirhat		
Total			12 farm/month×6 months=72

Table 2. List of different types of data considered

Sl. No.	Categorical Data	Parametric Data
1	Month	Tem
2	Region	DO
3	Pond Drying	Salinity
4	Ploughing	Ph
5	Sludge Removal	Ammonia
6	Water Source	Alkalinity
7	Water Sharing	Stocking Density
8	Linked With Farms	Avg. Depth
9	Cattle Accessibility	
10	Pl Source	
11	Liming	
12	Feeding	

Table 3. Descriptive Statistics of month wise WSSV infection

Month		Mean	Std. Deviation	Mean Difference	Sig.
January	February	2.00	.000	.000	1.000
	March	1.42	.515	.583*	.005
	April	1.33	.492	.667*	.001
	May	1.25	.452	.750*	.000
	June	1.17	.389	.833*	.000
February	January	2.00	.000	.000	1.000
	March	1.42	.515	.583*	.005
	April	1.33	.492	.667*	.001
	May	1.25	.452	.750*	.000
	June	1.17	.389	.833*	.000
March	January	2.00	.000	-.583*	.005
	February	2.00	.000	-.583*	.005
	April	1.33	.492	.083	.994
	May	1.25	.452	.167	.889
	June	1.17	.389	.250	.592
April	January	2.00	.000	-.667*	.001
	February	2.00	.000	-.667*	.001
	March	1.42	.515	-.083	.994
	May	1.25	.452	.083	.994
	June	1.17	.389	.167	.889
May	January	2.00	.000	-.750*	.000
	February	2.00	.000	-.750*	.000
	March	1.42	.515	-.167	.889
	April	1.33	.492	-.083	.994
	June	1.17	.389	.083	.994
June	January	2.00	.000	-.833*	.000
	February	2.00	.000	-.833*	.000
	March	1.42	.515	-.250	.592
	April	1.33	.492	-.167	.889
	May	1.25	.452	-.083	.994

*. The mean difference is significant at the 0.01 level. Dependent Variable: WSSV Infection

Table 4. Descriptive Statistics of area wise WSSV infection

Upazilla		Mean	Std. Deviation	Mean Difference	Sig.
Rampal	Kochua	1.389	0.502	0.000	1.000
	Fakirhat	1.944	0.236	-.556*	0.002
	Paikgacha	1.389	0.502	0.000	1.000
Kochua	Rampal	1.389	0.502	0.000	1.000
	Fakirhat	1.944	0.236	-.556*	0.002
	Paikgacha	1.389	0.502	0.000	1.000
Fakirhat	Rampal	1.389	0.502	.556*	0.002
	Kochua	1.389	0.502	.556*	0.002
	Paikgacha	1.389	0.502	.556*	0.002
Paikgacha	Rampal	1.389	0.502	0.000	1.000
	Kochua	1.389	0.502	0.000	1.000
	Fakirhat	1.944	0.236	-.556*	0.002

*. The mean difference is significant at the 0.01 level.
Dependent Variable: WSSV Infection

Table 5. Pearson correlation coefficient for parametric data

		Tem	DO	Salinity	pH	Ammonia	Alkalinity	Avg. Depth	Stocking Density
Pearson Correlation	WSSV Infection	.624(**)	.070	-.727(**)	-.261(*)	.175	-.256(*)	-.618(**)	.043
	Sig. (1-tailed)	.000	.280	.000	.013	.071	.015	.000	.359
	N	72	72	72	72	72	72	72	72

** Correlation is significant at the 0.01 level (1-tailed).
* Correlation is significant at the 0.05 level (1-tailed).

Table 6. Spearman's rho correlation coefficient for non-parametric data.

		WSSV Infection	Pond Drying	Ploughing	Sludge Removal	Linked with	Water Sharing	Water Source	Accessibility of Cattle	Feeding	Liming
Spearman's rho WSSV Infection	Correlation Coefficient	1.000	-.273(*)	-.276(**)	-.179	**.754(**)**	.308(**)	**.416 ** **	**.630(**)**	-.219(*)	-.302(**)
	Sig. (1-tailed)	.	.010	.009	.067	.000	.004	.000	.000	.032	.005
	N	72	72	72	72	72	72	72	72	72	72

* Correlation is significant at the 0.05 level (1-tailed).
** Correlation is significant at the 0.01 level (1-tailed).

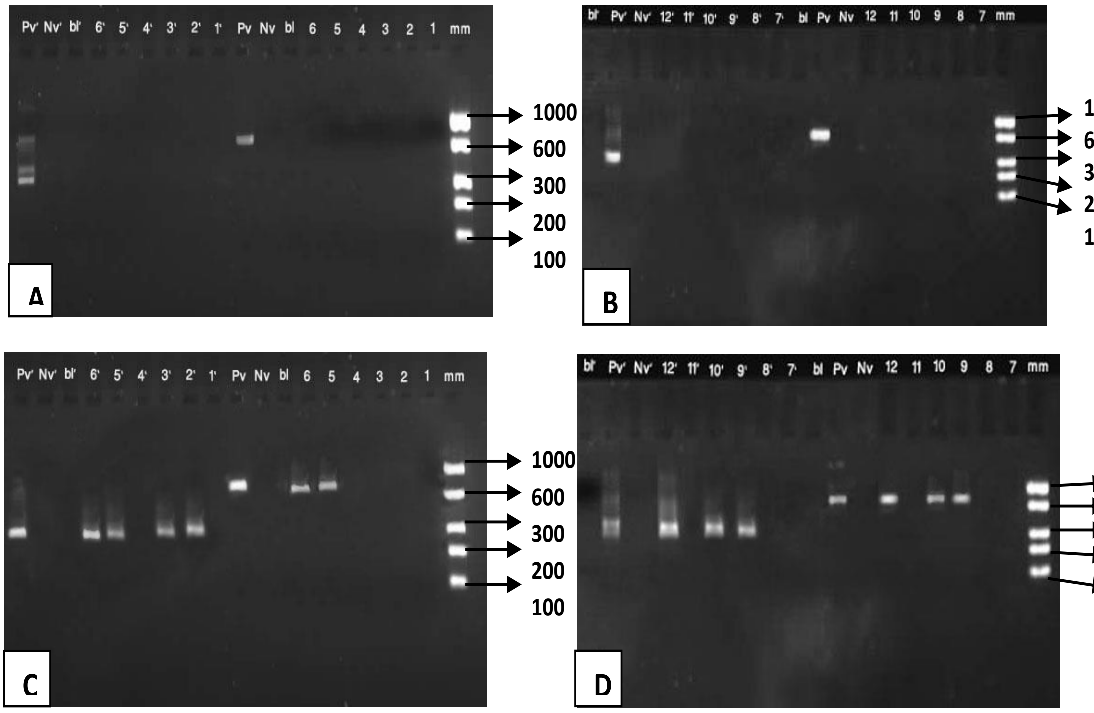

Figure 1. Electrophoretic pattern of PCR product (mm: molecular marker; 1~12: samples for 1st step PCR; bl: reagent control; Nv: negative; Pv: positive; 1'~12': samples for nested (2nd step) PCR; bl': nested reagent control; Nv': nested negative control; Pv': nested positive control).

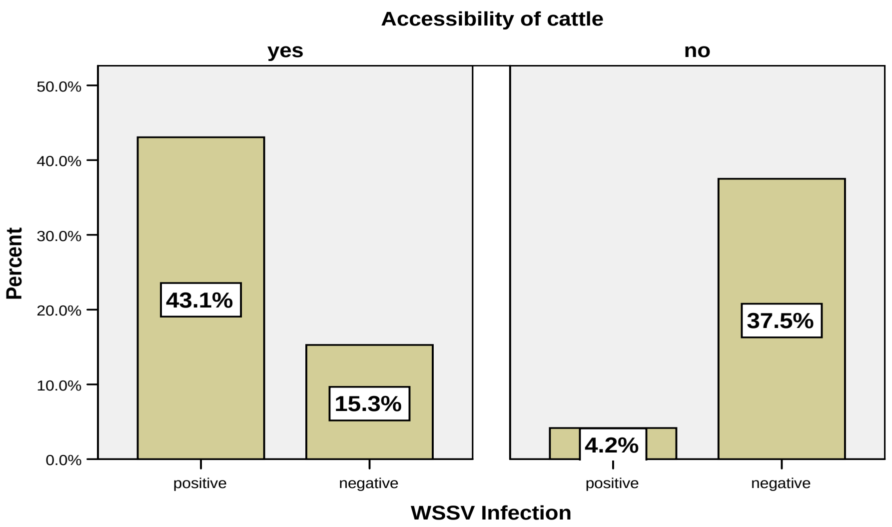

Fig 2. Percentages of WSSV infection and cattle accessibility

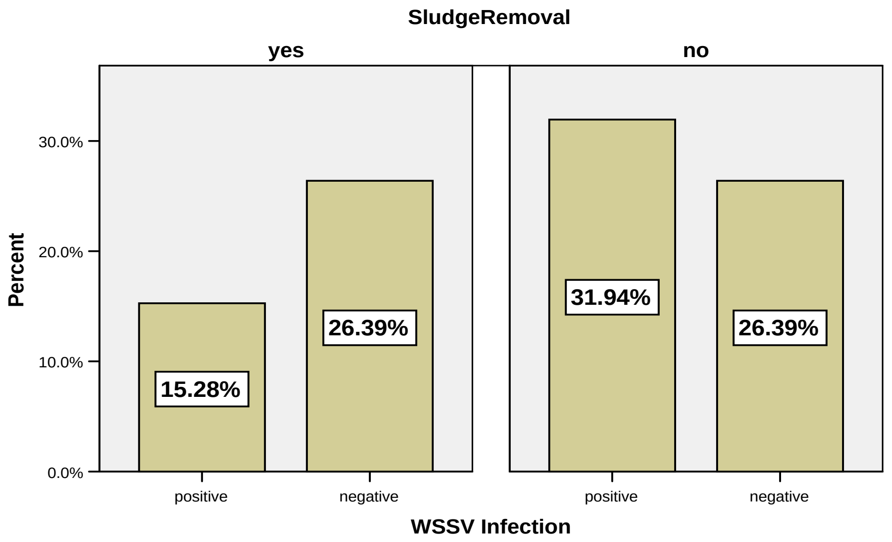

Fig 3.percentages of WSSV infection and sludge removal

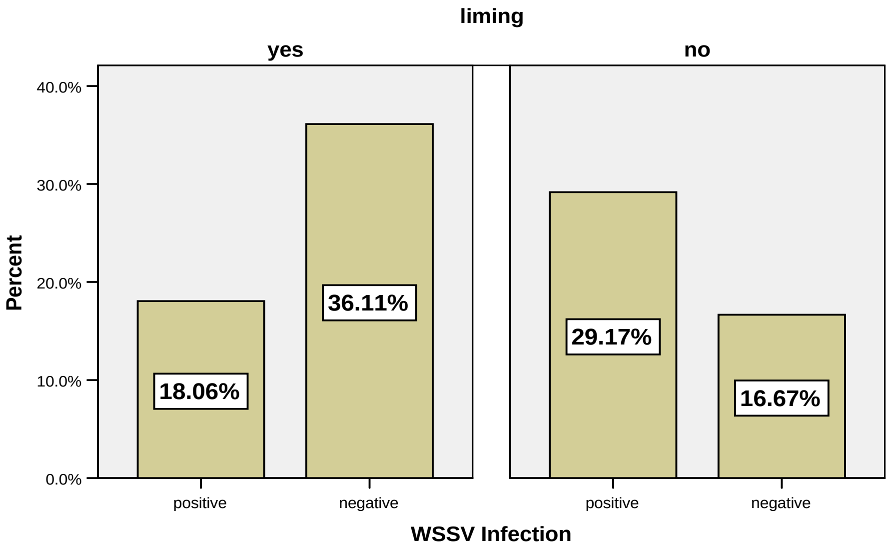

Fig 4. Percentages of WSSV infection and liming

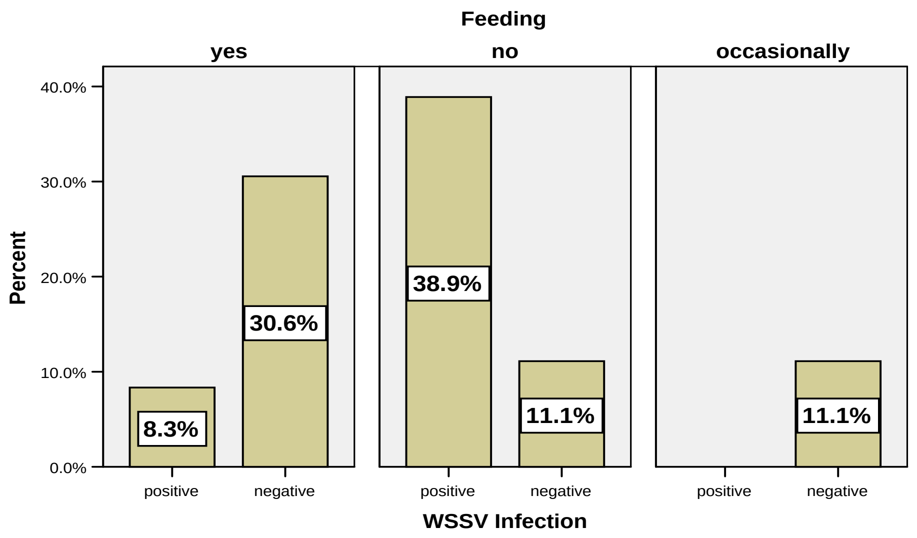

Fig 5. Percentages of WSSV infection and feeding

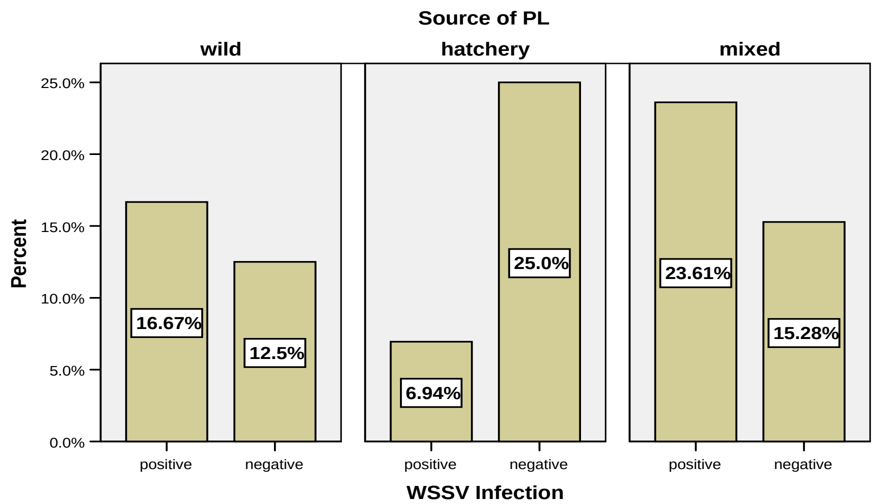

Fig 6.Percentages of WSSV infection and source of PL

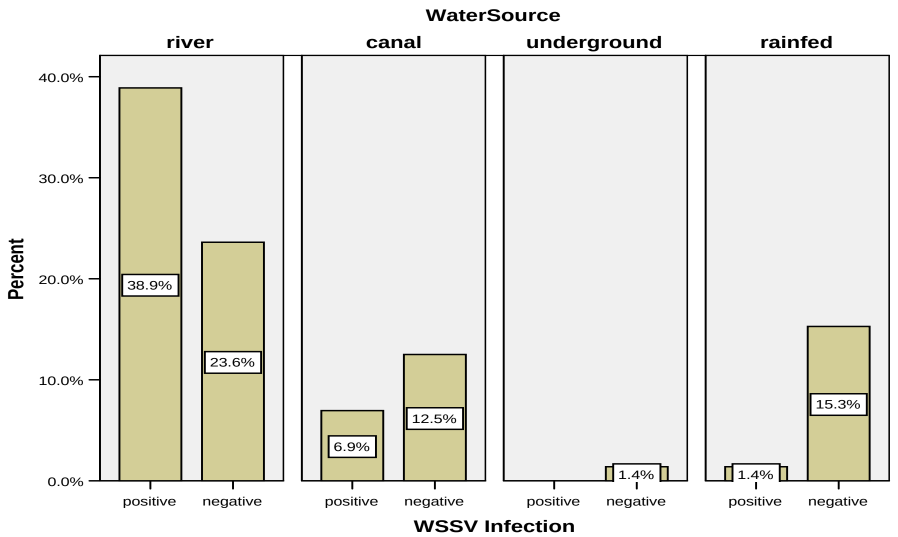

Fig 7. Percentages of WSSV infection and source of water

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