The effect of different salinities on mortality and histopathological changes of SPF imported *Litopenaeus vannamei*, experimentally exposed to White Spot Virus and a new defferential hemocyte staining method

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Abstract

Shrimp farmers have been suffering from loss of production due to White Spot Disease (WSD) in the last decades. A few studies focused on the environmental factors predisposed WSD. Our study aimed to investigate the effects of the different salinities on occurrence of WSD. The treatments were 30 ppt, 40 ppt and 50 ppt in triplicate and corresponding untreated control groups. White Spot Virus (WSV) was intramuscularly injected to the shrimp in whole treatments. PCR and histopathological findings within the measurement of some hemolymph parameters such as Differentiated Hemocyte Count (DHC), Total Hemocyte Count (THC) and Total Plasma Protein (TPP) were carried out. A positive result was observed for the occurrence of the antigen of the WSV obtained from the samples of each treatment of salinity due to the challenging treatments with the WSV. Mortality at the salinity of 30 ppt began at 72 h post inoculation as well as the salinity of 40 ppt, but at 50 ppt it started after 50 h. The Minimum and maximum count of mortality at the salinity of 30, 40 and 50 ppt were 3.5, 8.5; 0.5, 4.5 and 1.5, 7.5, respectively. No mortality was observed in the untreated control groups of 30, 40 and 50 ppt during the experiment. It is concluded that the higher and the lower salinity, lesser or greater than the normal condition in exposed to WSV could lead to severe mortality of WSD.

Keywords: WSD, Salinity, *Litopenaeus vannamei*, THC, TPP, DHC, Mortality

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Introduction

Inattention to the effects of climate changes on the fluctuations in physico-chemical parameters of the water ponds during the shrimp culture particularly in stocking phase and on the time of harvesting, the yield resulted in a few epizootic of White Spot Diseases (WSD) in Iran in the last decade (Soltani et al., 2000; Kakoolaki, 2004). Shrimp farmers have been suffering from loss of production due to the WSD (Soltani et al., 2009). Appropriate shrimp health management applied in Choybdeh shrimp site in south west Iran in 2010 resulted in a relative control of the WSD (Kakoolaki et al., 2010c). WSD causes severe mortality and kills the cultured shrimps during 3-10 days (Lightner, 1996). The outbreak of WSD was first reported from *Marsupenaeus japonicus* shrimp ponds in Japan in 1992-1993 (Lightner, 2004). Causative agents of WSD were consequently dispersed to elsewhere in Asia and other continents where pandemics of the disease occurred (Huang et al., 1995). Marine penaeid shrimps are susceptible to be infected by the White Spot Virus (WSV) (Rodriguez et al., 2003) whereas other marine and fresh water crustaceans though to be susceptible as well (Hameed et al., 2003). The salinity of sea water in the Persian Gulf is 40ppt-42ppt and that of the shrimp ponds reach up to 50ppt to 52ppt in hot days (Kakoolaki, 2004). The effect of salinity on Total Hemocyte Count (THC) and Total Plasma Protein (TPP) was studied in shrimp (Vargas-Albores et al., 1998; Cheng and Chen, 2000). Salinity is one of the most important environmental factors that affects the occurrence of WSD (Liu et al., 2006; Kakoolaki et al., 2010b). Some studies were taken to evaluate the effect of salinity on the occurrence of viral diseases of the shrimp (Yu, Z. M. et al., 2003; Afsharnasab et al., 2006; Liu et al., 2006; Ramezani Fard, 2006; Ramirez-Rodriguez et al., 2006; Annies and Rosamma, 2007; Kakoolaki et al., 2010b; Kumlu et al., 2010; Yu, X. et al., 2010). Ramezani Fard (2006) indicated that isosmotic media of *P. vannamei* is obtained while shrimp are reared at the salinity of 35-40 ppt in which no more energy waste for osmoregulation. The main objective of the study was to determine differences in mortality rates, Total Plasma Protein (TPP) and Total Hemocyte Count (THC) between the samples of variant salinities.

Materials and methods

Shrimps and experiment protocol

Five hundred and forty two juveniles of *P. vannamei* (7.988±0.543) were collected from a research shrimp farm located in Heleh site in north of Bushehr and transferred to the Iran Shrimp Center for experimental and laboratory examinations. According to the PCR result (Fig.1), normal distribution of the juvenile shrimp (n=542) was allowed the study to be began. Shrimp were then acclimated (Kakoolaki, 2004; Kakoolaki et al., 2010c) to the allocated conditions (T1=30ppt, T2=40ppt and T3=50ppt salinity as treatments 1,2 and 3) of the study, distributed to each 18 glass aquariums (50×50×60cm) with well clean aerated sea water (100 litre per each aquarium). It was prepared in 50ppt under the sunshine condition and diluted to 40ppt and 30ppt by adding non-chlorinated tap water. The shrimp were reared in the current condition for 40 days. They were being fed with a commercial dry diet twice a day and residual feed were removed daily by siphon. Inter-moult stage of the shrimp selected to evaluate the pathogenecity caused by variant salinity during the experiment. Three treatments in triplicate were designed in exposure to WSV along with the same three triplicate control groups without exposing to WSV.

Preparation of WSV stock solution

The infected hemolymph of *Astacus leptodactylus* with the code no. of wssv/irn/1/2011 which has already been prepared by Motamedi lab in Iran (Motamedi Sedeh et al., 2011) in which they study to produce a vaccine of WSD. Virus with the titre of $LD_{50}= 1\times10^{5.4}$ were offered to our research and used as virus in challenging with the treatments but control groups were left unchallenged with WSV. The mixture was stored at -80^{O}c until use.

WSV inoculation

After 40 days passed for acclimation, shrimp in the all treatments were intramuscularly inoculated with 50 µl containing the median infectious titre of $10^{5.4}$ virulent WSSV, which caused a serious mortality in Bushehr in 2006.

PCR and Histopathological examinations

After the mortality observation, 3 moribund shrimp from each treatment in which the mortality occurred, were prepared to transfer to histopathology and PCR labs. The appendages and gill tissues were cut, placed in Eppendorf micro tubes containing ethanol 70% for PCR technique using IQ2000 WSV commercial kit (Afsharnasab et al., 2009). The remaining tissues were placed in tubes containing Davidson's fixative. They were then transferred to the lab. The tubes containing Davidson's fixative were discarded and replaced with ehanol 70% after 48-72 h in the lab and stained by H & E method(Lightner, 1996).

Hemolymph analysis

Collecting the hemolymph

After the mortality observation, 0.2ml of Hemolymph of 3 moribund shrimp from each triplicate in treatments and controls were withdrawn from the second leg's basement of ventral segments using 1ml syringe along with 26 gauge needle. Each syringe was pre-filled with 0.8 ml Alsever solution as anticoagulant (Kondo, 2003; Kakoolaki et al., 2010a).

THC, Total Hemocyte Count

THC was carried out using the Neubauer type's Hemocytometer. A drop of hemolymph-anticuagulant mixture was then placed on the Hemocytometer and the cells were counted under a light microscope ×10 as follows:

THC (Cells/ml)=Average count of 5 cells$\times5\times10^{4}\times1$/dilution (Kakoolaki et al., 2010a)

DHC, Differentiated Hemocyte Count

0.2ml of Hemolymph were withdrawn from the second leg's basement of ventral segments leg using 1ml syringe along with 26 gauge needle. Each syringe was pre-filled with 0.1 ml fixative. Differential Hemocyte Count (DHC) was carried out using a slide, a drop of mixture solution was then placed on it and staining with May-Grundwald Giemsa (MGG) method begun after that.

A new method for fixation and staining of the hemolymph (modified after Kondo, 2003) was carried out as follows:

Fixative solution: 4% formalin in 0.45 M NaCl

MGG solution:

-1.5 ml of MGG working solution was added on the slide to cover it (0.25 g of MGG were mixed in to the 100ml of Methanol) for 5 minutes.

-1.15M of Phosphate buffer were added to MGG (Mixture of 6 volumes of 1.15 M KH_2PO_4 and 4 volumes Of 1.15 M Na_2HPO_4, pH 6.6) for 5 minutes.

-The previous solution were discarded and Giemsa one added (4ml of Phosphate buffer mixed with 1ml of stocking solution of Giemsa adding by 18 ml of 2-times distilled water).

-The slides were slightly rinsed with distilled water.

TPP, Total Protein Plasma

TPP was examined using centrifuge, (3000 rpm for 10 min. at 4^{o}c) the mixture of distinct anticoagulant and collected hemolymph (*n*=3) of each triplicate of the treatments. The supernatants were removed and transferred after 24 h to the lab in freezing conditions to examine TPP of the treatments using a biochemical autoanalyzer (Technicon, RA 1000).

Statistical analysis

The effects of variant salinities on mortality rate were analyzed using the nonparametric Kruskal-wallis test. The Mann-Whitney test was used in cases where the Kruskal-Wallis test showed significant differences to evaluate the ranks between the treatments two by two. One-way ANOVA & Benfrroni multi-comparison test were used to compare Epigastric hematopoietic spheroid values between treatments and also to compare THC, TPP and various DHC between treatments after challenging the virus against the treatments.

Results

The results of PCR for the WSV of the pooled samples (*n*=5) randomly collected from the Heleh site were negative (Fig.1). Figure 2 shows the positive result for the occurrence of the antigen of the WSV within the samples of each treatment of salinity due to the challenging treatments with the WSV (based on the IQ2000, WSV commercial kit).

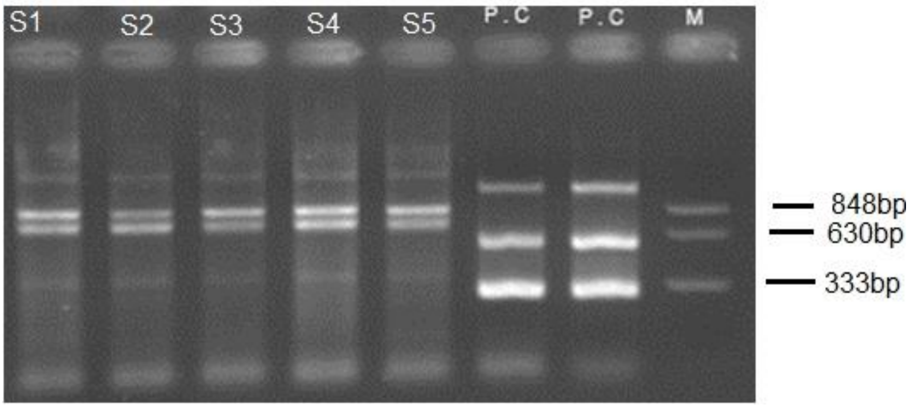

Figure 1: The negative result of PCR for the WSV of the 5- pooled samples (S_1-S_5) collected from the shrimp stock. P.C.=Positive control, M.=Marker (IQ2000, WSV commercial kit)

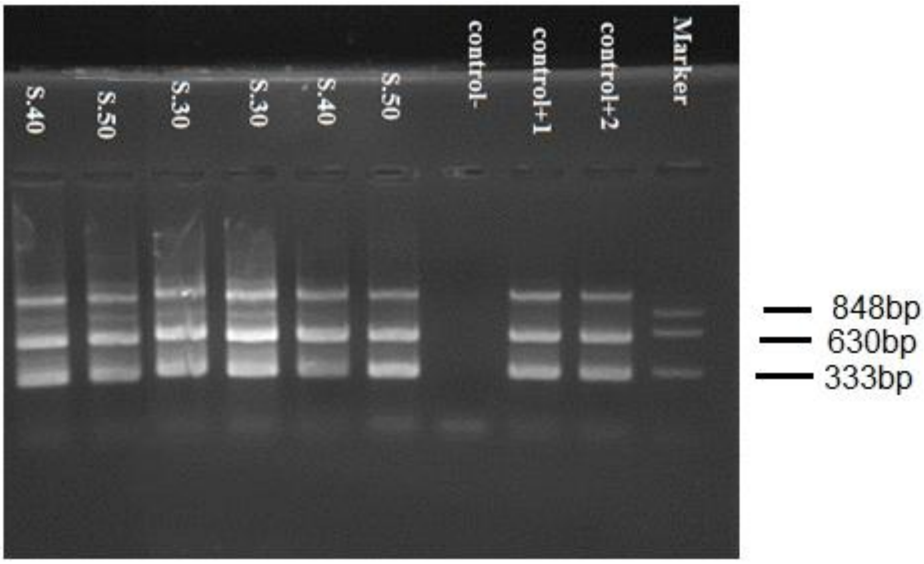

Figure 2: The positive result of PCR for the WSV of the 2- pooled samples (S=Salinity) from each treatment (IQ2000, WSV commercial kit)

According to histopathological findings, the inclusion bodies of the WSV were observed through the tissues such as heart, epithelial cells of gut and epidermis (figs 5, 6, 7). Vacuolation features were observed in hepatopancreas tissue of the shrimp in all treatments (figs 3, 4). The mean value of spheroid formations in epigastric hematopoietic tissue in the shrimps exposed to the salinities of 30 ppt, 40 ppt and 50 ppt were 10.5± 1.14(SEM), 10.00± 1.05, 9.66± 0.61, *n=6*, respectively (Table 1).

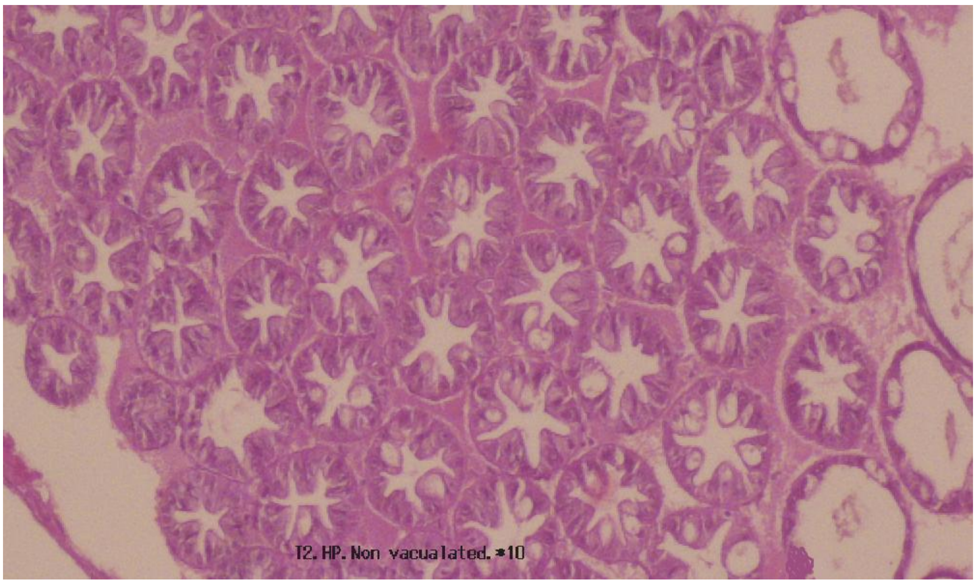

Figure 3: Slight vacuolation forming within the hepatopancreas tissue section at the salinity of 40 ppt, H & E, ×10

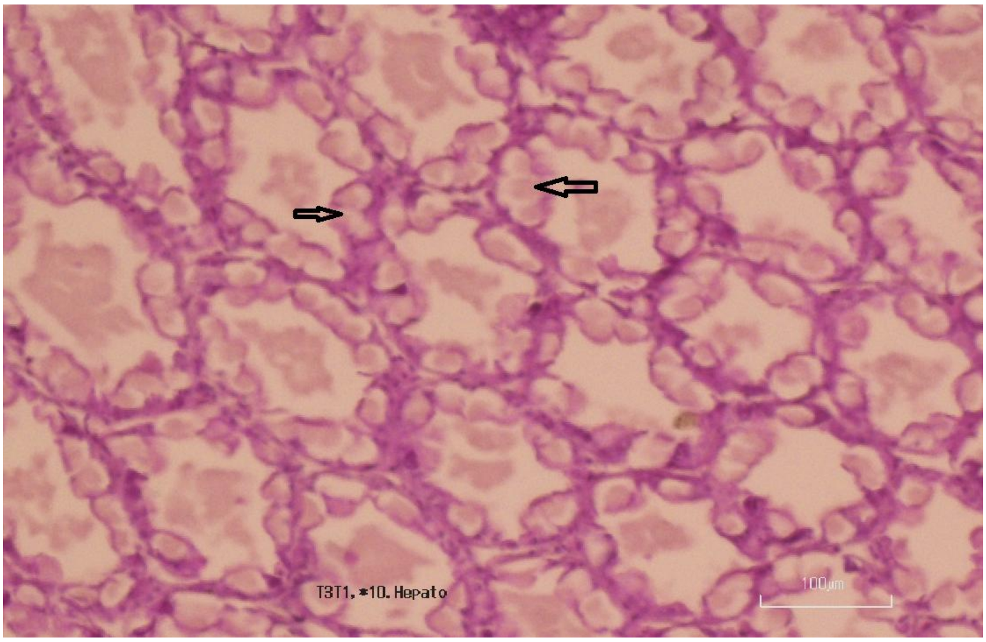

Figure 4: Abundance vacuolation forms within the hepatopancreas tissue section at the salinity of 50 ppt, H & E, ×10

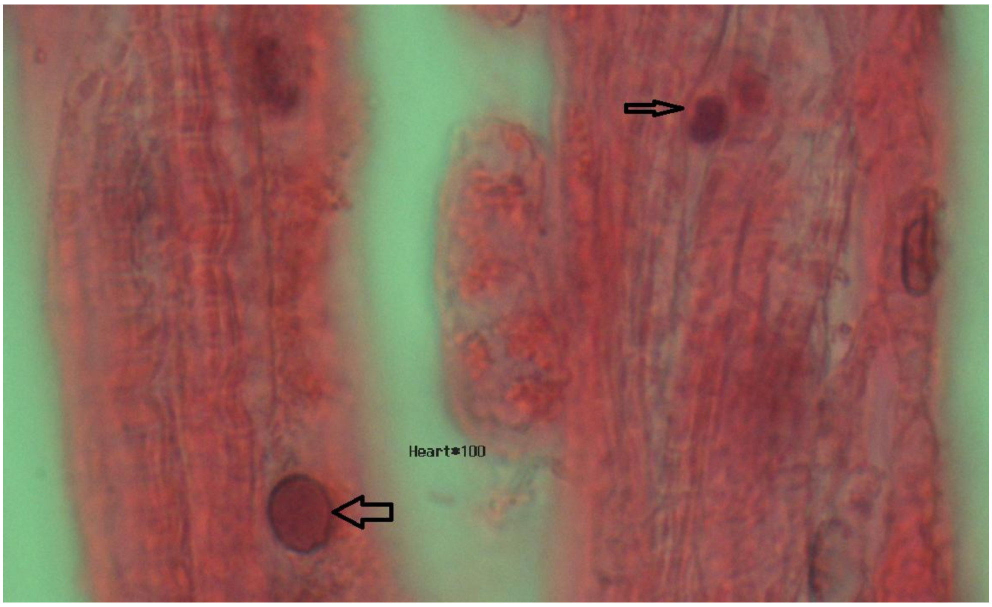

Figure 5: Heart muscle tissue of the shrimp, *P. vannamei* shows (big arrow) a marginated nucleus with light basophilic inclusion body (last phase) and a lesser one (thin arrow), H & E, ×100

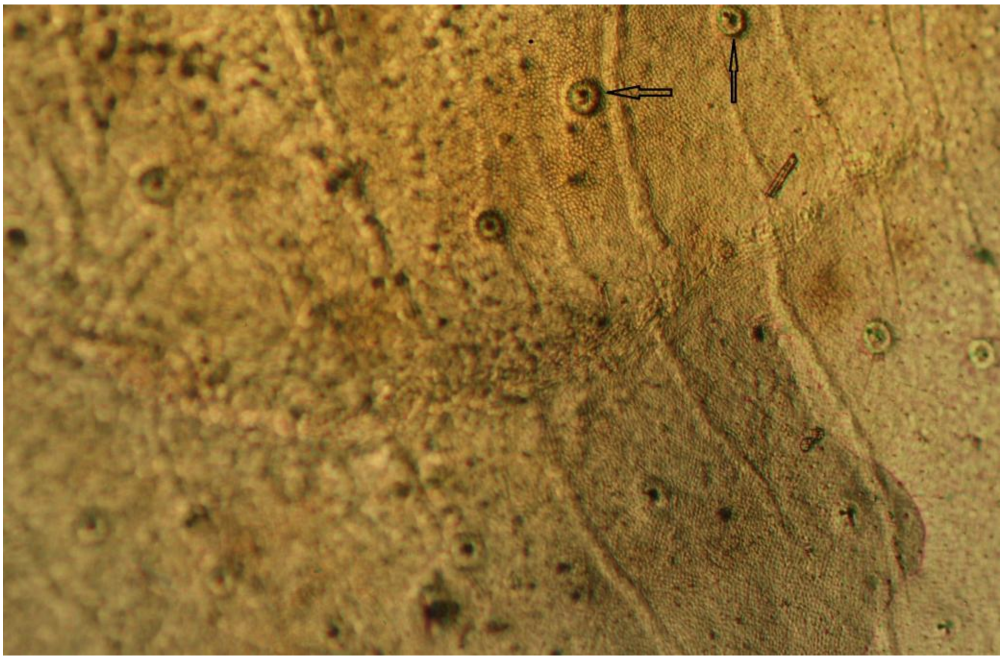

Figure 6: Concentric circles on epidermis of carapace in acute mortality. Such our result just observed under Light Microscope, wet mount, ×4

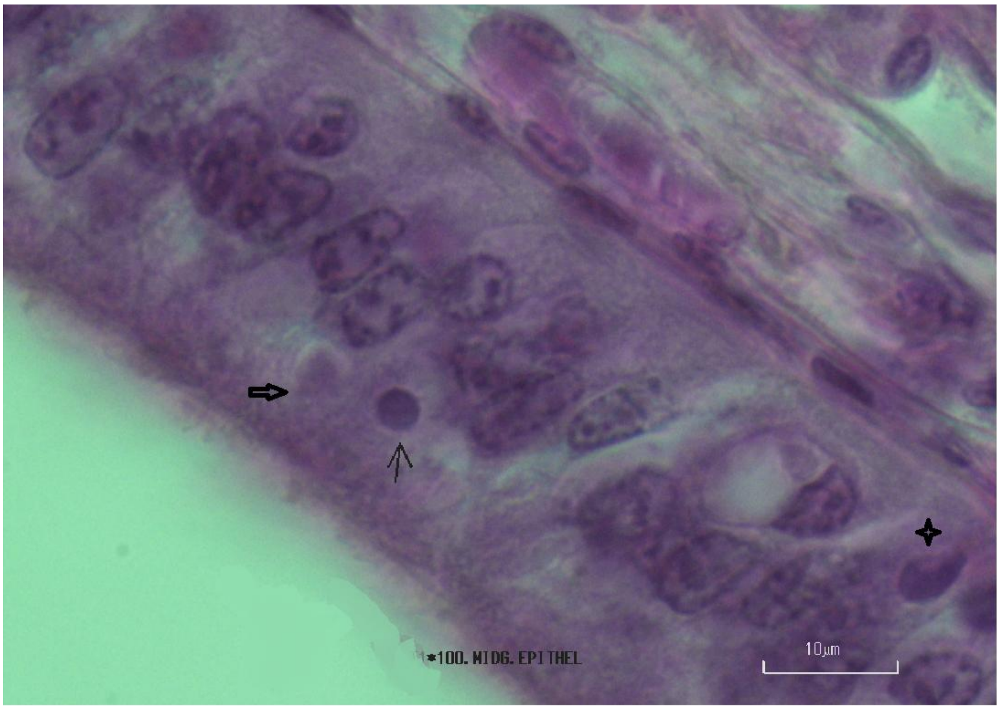

Figure 7: Hollow arrow shows a cowdry type inclusion body (early stage) with light surrounding halo, asterisk and arrow also shows developed early stage in the epithelial cells of midgut, H & E, ×100

Table 1: One way ANOVA & multi-comparison test to compare Epigastric hematopoietic spheroid values between treatments after exposure

Parameter		Mean	Std. Error	Sig.
Spheroid	T1	10.50	1.14	.784
	T2	10.00	1.05	
	T3	9.66	0.61	

T=Treatment,

According to table 2 and figure 8, the mortality at the salinity of 30 ppt began at 72 h after inoculation as well as the salinity of 40 ppt but at 50 ppt it started after 50 h. The minimum and maximum count of the mortality at the salinity of 30, 40 and 50 ppt were 3.5, 8.5; 0.5, 4.5 and 1.5, 7.5, respectively. No mortality was observed in the untreated control groups of 30, 40 and 50 ppt during the experiment.

Table 2: Mortality count in distinct hours after inoculation in different salinities (*n=30*)

	Hours post inoculation																		
S	36	50	54	56	60	72	82	92	96	102	110	114	120	126	138	142	152	160	170
S30 PPT	0	0	0	0	3	3.5	4.5	8.5	6.5	4									
S40 PPT	0	0	0	0	1.5	1.5	2.5	4.5	4	1.5	3	3	1	3.5	2	0.5	0	0.5	1
S50 PPT	0	7.5	3.5	2.5	4.5	3.5	5	2	1.5										

S=Salinity

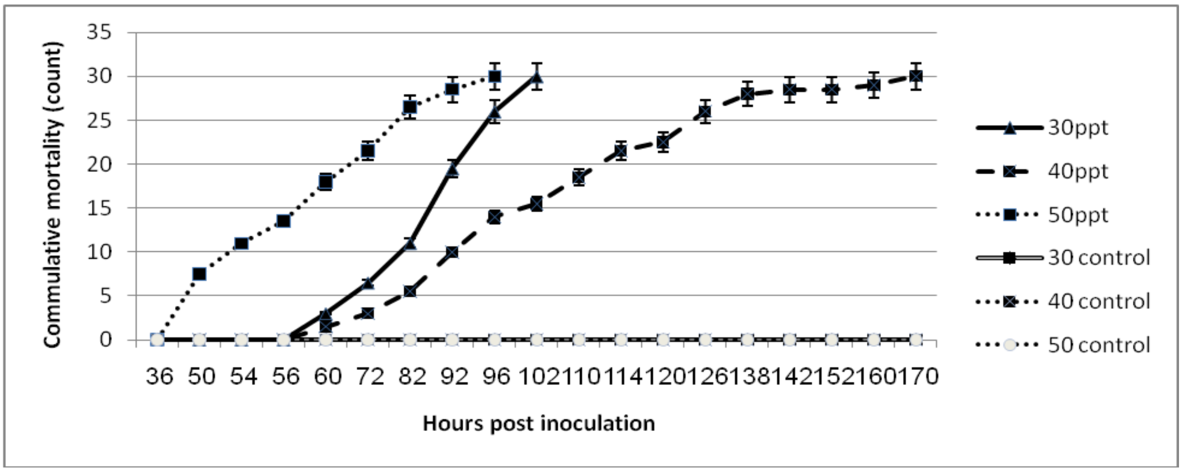

Figure 8: Commulative mortality of each group after distinct hours post inoculation

The results of the Chi-Square statistics in the Kruskal-wallis test in table 3 showed there are significant differences between the treated groups in defined hours post inoculation ($p<0.05$). The significant values related to the differences between paired groups calculated by Mann-Whitney test are given in table 4. Table 5 shows One-way ANOVA and multi-comparison test to compare THC, TPP and various DHC between treatments after 1 h post-mass mortality in each treatment. According to the results of table 5 there was at least a significant difference among the THC mean value ±Std error in the treatments 30 ppt, 40 ppt and 50 ppt ($p<0.01$) which were respectively equal to 5.90± 0.24, *n=6;* 9.93±0.34, *n=6;* 6.70±0.47, *n=6.* The value of DHC (LGC) as percent± SEM through the mentioned treatments were 26.40± 2.04, *n=6;* 19.06± 1.63, *n=6;* 31.73± 2.11, *n=6*, for the DHC (SGC) were 61.46± 1.88, *n=6;* 69.18± 1.86, *n=6;* 56.41± 2.33, *n=6*, for the DHC (HC) were 12.10±0.62, n=6; 11.41±1.37, *n=6;* 11.85±0.49, respectively. The values of the TPP as mean ($\mu g.\mu l^{-1}$) ± Std error among the treatments 1, 2 and 3 were 60.73±6.94, *n=6;* 97.31±4.49, *n=6;* 63.85±5.22, *n=6*, respectively. Table 6 shows equality of means in THC and TPP between the treatments and the controls (untreated groups). According to the table 6, there was a significant difference ($p< 0.05$) between the treatments (Treatment

1, T1; T2; T3) and untreated groups for the THC and for the TPP, too. The amounts of THC and TPP before and after challenging are given in table 6. Three types of hemocytes including Large Granular Cell (LGC), Small Granular cell (SGC) and Hyaline Cell (HC) were observed in differentiated hemocyte counts (Fig. 10).

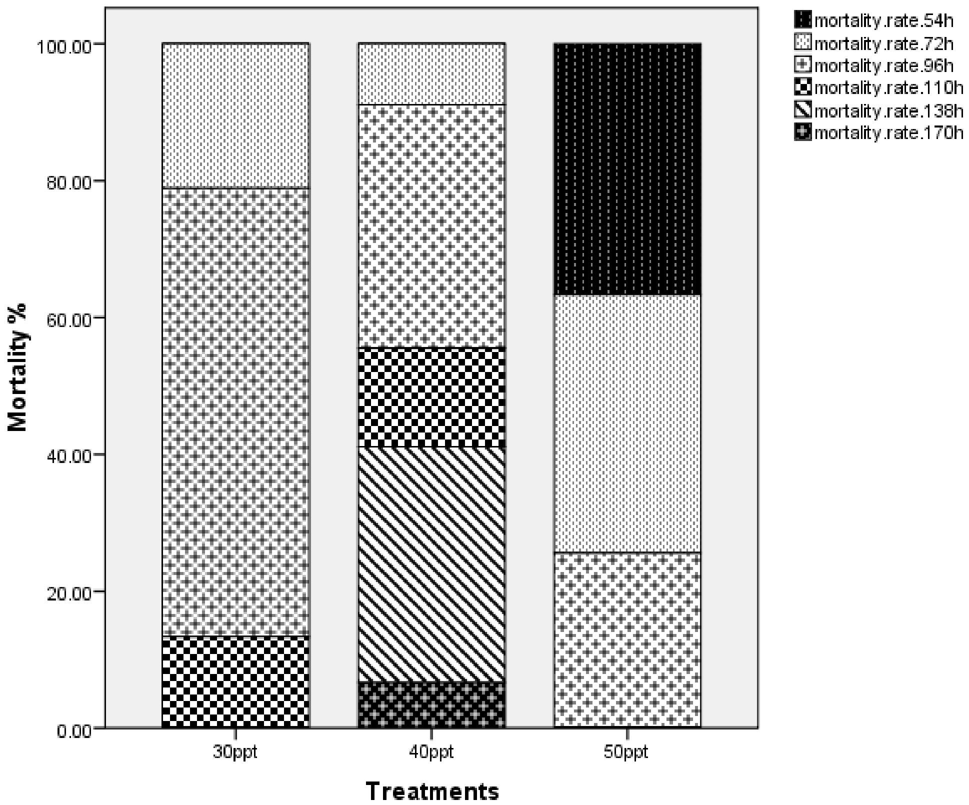

Figure 9: Mortality percent of each group after distinct hour post inoculation

Table 3: Mean ranks of mortality for each treatment in distinct hours, value and significance of the differences between groups

	Tr.	*n*	M.r.54h	M.r.72h	M.r.96h	M.r.110h	M.r.138h	M.r.170h
Ranks	1	3	3.50	5.00	8.00	6.17	3.50	3.50
	2	3	3.50	2.00	5.00	6.83	8.00	8.00
	3	3	8.00	8.00	2.00	2.00	3.50	3.50
Chi-Square			7.623	7.322	7.32	5.93	7.62	7.62
Asymp. Sig.			0.022	0.025	0.02	0.05	0.02	0.02

Tr. =Treatment, M.r. = Mortality rate

Table 4: The significant value of Mann-Whitney related to differences between paired groups (*n=3*)

	Hours post inoculation				
Treatments	54	72	96	110	138
01:02	1	0.04*	0.05*	0.03*	0.03*
01:03	0.03*	0.04*	0.03*	1	1
02:03	0.03*	0.04*	0.03*	0.03*	0.03*

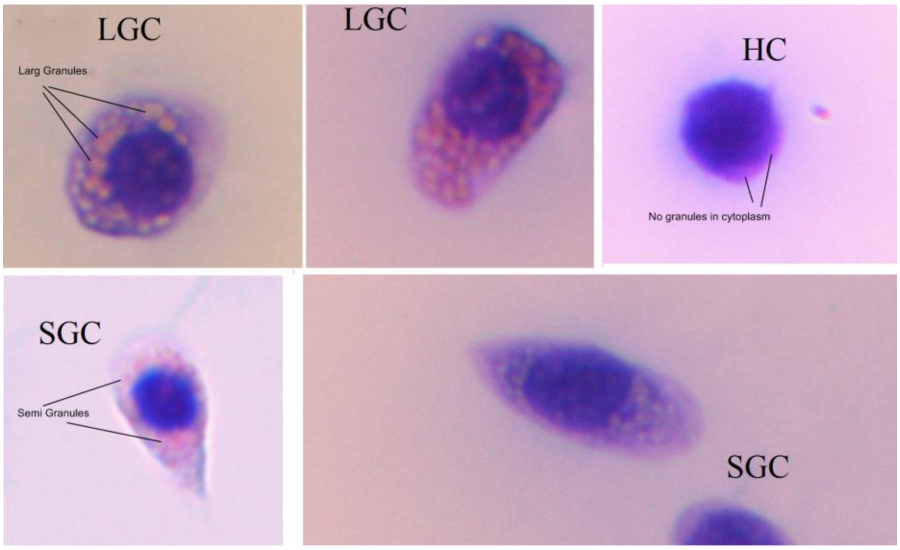

Figure 10: Differentiated Hemocyte Count shows three types of hemocytes, LGC, SGC and HC. New method of MGG staining ×100

Table 5: One way ANOVA & multi-comparison test to compare THC, TPP & variant DHC between treatments after 1 h post mass mortality

Parameter		Mean	Std. Error	Sig.
THC($\times 10^5$)	1	5.90^a	0.24	.000
	2	9.93^{ab}	0.34	
	3	6.70^b	0.47	
DHC.LGC%	1	26.40	2.04	.001
	2	19.06^a	1.63	
	3	31.73^a	2.11	
DHC.SGC%	1	61.46	1.88	.002
	2	69.18^a	1.86	
	3	56.41^a	2.33	
DHC.HC%	1	12.10	0.62	.868
	2	11.41	1.37	
	3	11.85	0.49	
TPP(µg.µl^{-1})	1	60.73^a	6.94	.001
	2	97.31^{ab}	4.49	
	3	63.85^b	5.22	

Same superscripts show no significant difference in the same main rows. T=Treatment, LGC=Large Granular cell, SGC=Small Granular Cell,HC=Hyaline Cell

Table 6: T- test for equality of means in THC and TPP between treatments and controls

Parameter		Leven's test sig.	Mean	Std. Error	t-test Sig.
THC.T1	T1	.478	5.90^a	.24	0.00
	control		7.40^a	.35	
THC.T2	T2	.683	9.93^a	.34	0.00
	control		12.30^a	.27	
THC.T3	T3	.187	6.70^a	.47	0.00
	control		8.71^a	.23	
TPP.T1	T1	.055	60.73^a	6.94	0.00
	control		182.34^a	23.64	
TPP.T2	T2	.300	97.31^a	4.49	0.01
	control		130.48^a	9.91	
TPP.T3	T3	.028	63.85^a	5.22	0.01
	control		180.35^a	30.20	

Same superscripts show no significant difference in the same main rows.

Discussion

Figure 2 shows the severity of the infections in whole treatments especially in treatment, 2 which demonstrates two sharp white bands of WSV loaded, that means the salinity of 30 ppt could be better media for multiplication of WSV. All the shrimp in the treatments of 30, 40 and 50 ppt were died in the durations of 42 h, 130 h and 46 h, respectively. Also the main mortality percent of the shrimp at the salinity of 30 ppt occurred 72 h after inoculation. This situation for the salinity of 40 ppt was observed 72 h and 138 h post inoculation and for the 50 ppt it was observed 54, 72 h and 96 h post inoculation (fig. 9). Some of the researchers who studied on the field of WSV believed that when the shrimp intramuscularly challenged with the WSV, the mortality occurred at even 6 hours post inoculation (Hameed et al., 2000; Yoganandhan et al., 2003; Carbajal-Sánchez et al., 2008). Dissimilar to our results that the main mortality occurred in 50 ppt, 50 h post inoculation and varied with other shrimp in 30 & 40 ppt with which mortality began in 60 hours after challenging, Carbajal- Sanchez et al. (2008) found in a short duration (lower than 60 h post inoculation) that no differences were observed in mortality count in *P. vannamei* in different salinities ranging from 25-50 ppt in exposure to IHHNV.

This mortality continued with a faster trend resulting in shorter survival durations at 30 ppt and followed by 50 ppt but the mortality trend in 40 ppt is slower and the survival duration is longer than those of 30 & 50 ppt. It was likely that the severity of the disease in treatments of 30 and 50 ppt (lower and greater value than the normal condition) were greater than the salinity of 40 ppt and shrimp in 40 ppt have more time to retrieve the injured tissues or resist relatively against the viruses. Similar to our results, in the case of salinity of lower levels, Carbajal-Sanchez et al. (2008) believed that lower salinity shows a severe trend of mortality in *L. vannamei* in comparison to the normal and greater levels normal condition. Low salinity may make shrimp susceptible to bacterial diseases (Hameed et al., 2000; Jiravanichpaisal et al., 2004) and high salinity can affect negatively on the growth of *L. vannamei* in challenging with IHHNV (Bray et al., 1994). Based on our results the shrimp reared in salinity of 50 ppt (high salinity level) were died faster (less than 3 days) in comparison to 40 & 30 ppt. No significant difference was observed between groups 1 & 2 in mortality count 54 h post inoculation ($p > 0.05$) but there are significant differences between groups 1 & 3 and also between 2 & 3 at the same hour after exposure ($p<0.05$). No significant differences were observed 72 hours post inoculation ($p>0.05$), similar to the result of 96 hours post inoculation. In 110 hours after exposure there are no significant differences between groups 1 & 2 and also between 2 & 3 ($p<0.05$) but no significant difference was observed between groups 1 & 3 ($p>0.05$). Finally, after 138 hours post inoculation, there are significant differences between groups 1 & 2 and also 2 & 3 ($p<0.05$) but no significant difference was observed between treatments 1 & 3 ($p>0.05$). In the other study which was done in Iran the growth rate of the shrimp, *L. vannamei,* which reared in salinity of 40 ppt, was greater than that of 30 ppt (Ramezani Fard, 2006). Lower salinity or hardness could lead shrimp to susceptibility to WSV (Tendencia et al., 2010). Based on our results, TPP is lower in the hemolymph of moribund shrimp in whole treatments in comparison to the corresponding control groups. Our observations proved this finding too because a significant increase in plasma volume of the moribund shrimp in comparison to the healthy ones was observed when collecting the hemolymph. Similar to our result, other researchers found TPP reduced post inoculation as well as THC (Jiang, 2010; Mohajeri et al., 2011) but THC after 48 hpi went up and reached the maximum at the level of 12×10^{6} ml^{-1} at 192 h post inoculation when the shrimp expose with *A. hydrophila* (Mohajeri et al., 2011). The result of TPP is in contradiction to another finding (Annies and Rosamma, 2007). Liu et al. (2006) showed that THC in the early hours post inoculation decrease, fluctuate after that and tend to return to normal level before inoculation but in contrary to the former study, other results such as (Jiang, 2010) and our result showed that THC decreased after inoculation in moribund shrimp. No significant differences were observed among the treatments ($p<0.05$) for THC, DHC (LGC), DHC (SGC) and TPP but at least a significant difference was observed among the treatments for DHC (HC), based on the results of table 5. Histopathological observations showed no pathological differences in varied treatments within the infected target tissues. Vacuolation features increased while the salinity reached to 50 ppt (figs 3, 4). No significant differences were observed between the amounts of spheroid formations in epigastric hematopoietic tissue in the shrimps exposed to different salinities. In contrary to our previous study (Kakoolaki et al., 2010a) which was done in the field of *P. indicus,* the current results showed the LGC of *L. vannamei* are more in quantity in comparison to that of former species. Finally, it is concluded that *L. vannamei* can tolerate different salinities but it can resist more against WSV while being reared in the salinity of 40 ppt in comparison to 30 and 50 ppt.

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