E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2018; 6(1): 19-26

www.fisheriesjournal.com
Received: 09-11-2017
Accepted: 10-12-2017

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Aetiological studies on mixed infection of Abdominal segment deformity disease (ASDD) and *Enterocytozoon hepatopenaei* (EHP) in cultured *Litopenaeus vannamei*

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Abstract
Samples of cultured *Litopenaeus vannamei* showing symptoms of Abdominal Segment Deformity Disease (ASDD) and simultaneously infected by *Enterocytozoon hepatopenaei* (EHP) collected during the period 2013-2014 from culture ponds of Andhra Pradesh, India were subjected to histopathological, microbiological, molecular and Transmission Electron Microscopic (TEM) studies. Histopathological studies revealed changes such as excessive vacuolization in the gills, sloughing off epithelial layers in the gut, disintegration and lacunae formation in the muscle fibres, degeneration of hepatopancreatic tubules besides haemocytic infiltration into the inter tubular spaces. Spores of *Enterocytozoon hepatopenaei* (EHP) were found inside the B-cells of hepatopancreas. All the samples were found positive for EHP but IHHNV 50% only. *Vibrio harveyi, V. alginolyticus* and *V. vulnificus* were found as associated bacteria. TEM studies confirmed 20-25 nm virus like particles in gill and muscle tissue. This constitutes the first report from India for the mixed infection of ASDD and EHP in cultured *L. vannamei*.

Keywords: Shrimp, *Litopenaeus vannamei*, ASDD, histopathology, Microsporidean, *Enterocytozoon hepatopenaei*, *Vibrio*

1. Introduction
The culture of *Litopenaeus vannamei*, introduced in India in the year 2008 has proved to be a very successful venture, almost replaced *P. monodon* culture. Despite their success, diseases like White Spot Disease (WSD), Black Gill Disease, White feces disease, White Muscle Disease, Infectious hypodermal and hematopoietic necrosis disease (IHHNV) and ASDD are severely affecting the culture of *L. vannamei* in many areas in India and Andhra Pradesh in particular. Some of them are reaching epidemic proportions and causing severe economic loss. It is estimated that 60% of disease losses in shrimp culture has been caused by viral pathogens and 20% by bacterial pathogens [1]. Over the years there is an increasing trend in the economic loss due to diseases.

The diseases are caused mainly due to poor management and unhealthy environmental conditions prevailing in the pond. Under conditions of high density, aquatic species are subjected to high stress condition, increasing the incidence of diseases, consequently decreased production [2]. Stress is mainly responsible for manifestation of disease [3] [4] and often induced by changes in water quality parameters such as temperature, oxygen, salinity and ammonium. All these conditions not only cause epidemics but also favour the introduction of new diseases. Detailed scientific investigations dealing with various diseases of cultured *L. vannamei* from different culture ponds are necessary to prevent any epidemics and severe losses to the culture.

A more recent disease of *L. vannamei* in Asia is abdominal segment deformity disease (ASDD), possibly caused by a yet unknown local virus [5]. It was first reported in cultured *L. vannamei* in Thailand, Malaysia and Indonesia [5] [6]. The affected shrimp shows deformed abdominal segments accompanied by muscle necrosis and degeneration. The economic loss due to Abdominal segment deformity disease (ASDD) of cultured white leg shrimp, *Litopenaeus vannamei* causes economic loss of approximately 10% in affected ponds because of the unsightliness of distorted abdominal muscles. *E. hepatopenaei* was reported from *P. monodon* by [7]. and in *L. vannamei* by [8].

2. Materials and Methods

Shrimps showing the symptoms of ASDD with retarded growth were collected (360 No's) during the period 2012 - 2015 from 10 culture ponds of Srikakulam and East Godavari Districts of Andhra Pradesh, India characterized by distorted abdominal segments and with hardened muscle tissues (Fig 1&2).

Fig 1: Healthy *L. vannamei*

Fig 2: Infected shrimps collected from culture ponds

2.1 Histopathological studies

The diseased shrimps were fixed in Davidson's fixative (DF) following the method Bell and Lightner [9] at the farm site itself and brought to the laboratory. Following fixation various organs (Gills, hepatopancreas, gut, cephalothorax and muscle tissues) were separated and cut into small pieces and dehydrated in different grades of ethyl alcohol, cleared in Xylene and embedded in paraffin wax and bees wax (1: 1 ratio) at 60 ^{0}C. These blocks were trimmed and (Leica RM 2125) 5 µm thick Sections were cut and stained using Mayer's Haematoxylin- Eosin (H&E) and later mounted in Canada balsam for microscopic observation.

2.2 Microbiological Studies

The haemolymph (0.2 ml) was drawn with the help of sterile, small insulin syringes from the ventral sinus of diseased shrimps and placed in test tubes containing Nutrient Broth (2 ml) for enrichment. After enrichment (24 hrs), known quantity of enriched samples were pour plated onto Zobell's Marine Agar (ZMA) and Thiosulphate Citrate Bile salts Sucrose (TCBS) agar media respectively for the estimation of total bacterial count and total Vibrio count. Those colonies which appeared dominant and distinct were picked and streaked onto another petridish for obtaining pure culture. Pure cultures thus obtained were preserved on slants under a layer of liquid paraffin for further analysis.

Various morphological and biochemical characterization tests were carried out following standard Bergey's Manual of Systematic Bacteriology [10]. for the identification of the bacterial isolates. Virulence studies were carried out in order to fulfil Koch's postulates by injecting the bacterial isolates at a concentration of 3 x 10^7 cfu / g to healthy juvenile shrimps following the method of Liu *et al*., (1996) [11].

2.3 Antibiotic sensitivity tests

Selected bacterial isolates (S1, S2, S3) having the high virulence were tested for their sensitivity to standard antibiotics namely erythromycin, streptomycin, furazolidone, ampicillin, kannamycin, tetracycline, penicillin, gentamycin, chloramphenicol, ciprofloxacin, oxytetracycline and norfloxacin. Young cultures of 18 hrs. age were spread evenly over Mueller Hinton Agar (MHA) dishes and the antibiotic discs (Himedia, Bangalore) were placed over the dish carefully and incubated overnight at 32^{0}C. Upon incubation, the inhibition zones obtained were measured with Kirbybauer scale [12].

2.4 Molecular studies

Gills and Hepatopancreas (HP) of the infected shrimp were fixed in 95% ethyl alcohol for conducting polymerase chain reaction (PCR) studies. 30-50 mg of tissue from each diseased shrimp was taken and thoroughly washed with distilled water. Total genomic DNA was extracted by using C-TAB method [13]. PCR was performed for the nucleic acid extracted from gill tissue to detect the presence of White Spot Virus (WSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) and Hepatopancreatic tissue for Monodon Baculo Virus (MBV), Hepatopancreatic parvovirus (HPV) and *Enterocytozoon hepatopenaei* (EHP).

2.5 Transmission Electron Microscopic (TEM) studies

Tissues of ASDD infected shrimps (Hepatopancreas, Haemolymph, Gill and Gut) were cut to a size of 1 mm^3 with a sharp blade and fixed in 2.5% Glutaraldehyde for 2 - 24 hours at 4ºC followed by washing with 0.1 M Phosphate buffer and post fixation in 1% OsO_4 (Osmium tetroxide) 2 hours at 4ºC. Then, the tissues are thoroughly dehydrated in different concentrations of acetone. Clearing was done with Toluene (30 min x 2) and finally infiltrated and embedded into a resin and made into hard blocks that allow thin sectioning by ultra microtome. The area to be examined under the TEM was selected after observation under the light microscope. These ultrathin sections were stained by double staining method using uranyl acetate and lead citrate. These sections were finally mounted on copper grid for TEM observations.

3. Results

3.1 Histopathological studies

3.1.1 Hepatopancreas (HP): cells in the hepatopancreatic tubules showed hypertrophy and degeneration (Fig.3 & 4). There were acidophilic granular inclusions in the cytoplasm of the tubular epithelial cells (Fig.5). Excessive vacuolisation was also noticed in the tubular epithelial cells (Fig.6). There was severe haemocytic infiltration and granuloma in the inter tubular spaces (Fig. 7,8). Microsporidian spores inside B cells of the hepatopancreatic tubule granular inclusions in epithelial cells were also observed (Fig. 9 & 10). Various stages of microsporidian cysts were also seen in the HP cells (Fig. 11&12).

3.1.2 Gills: Gills showed lesser degenerative changes when compared to other tissues like HP and gut. Hypertrophy of the pillar cells with reduced lacunae was observed occasionally. Degeneration of cuticular layers surrounding the gill filaments was also noticed (Fig. 13&14).

3.1.3 Muscle: The muscle fibres of the deformed abdominal segments were distorted (Fig. 15), with granulomas (Fig.16), with more no. of empty spaces / lesions between them (Fig. 17). Muscle fibres were more disorganised with broken filaments besides infiltration of haemocytes into the tissue (Fig. 18).

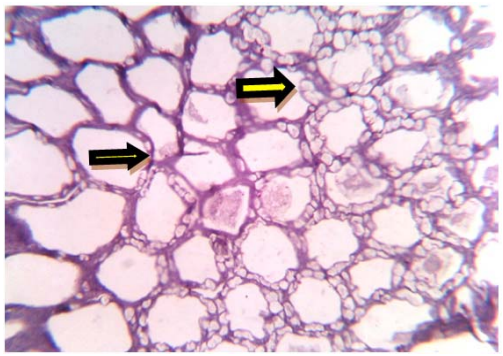

Fig 3: Section of HP showing hypertrophied cells and emptying of the tubules (100 X)

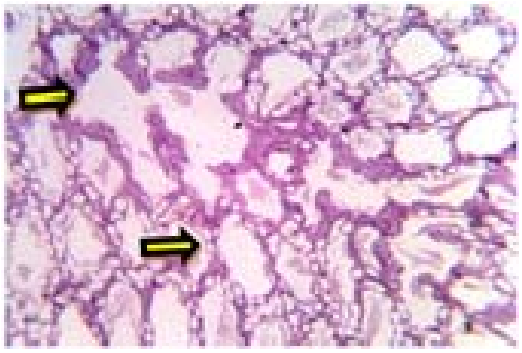

Fig 4: Section of HP showing tubular degeneration and merging of the tubules (100 X)

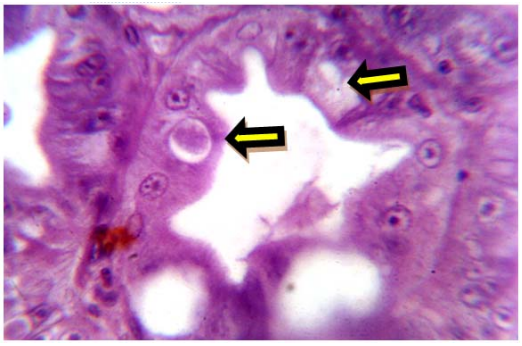

Fig 5: Section of HP showing acidophilic, granular inclusions in the cytoplasm of tubule epithelial cells (400 X)

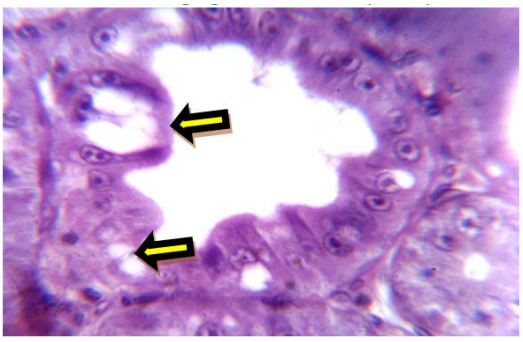

Fig 6: Section of HP tubule showing the process of vacuolization (400 X)

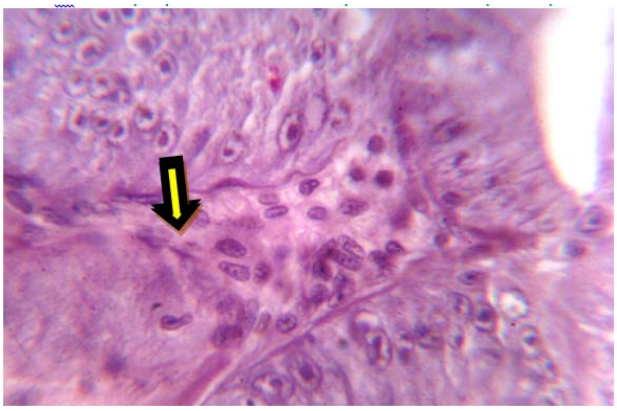

Fig 7: Section of HP showing the Haemocytic infiltration (400X)

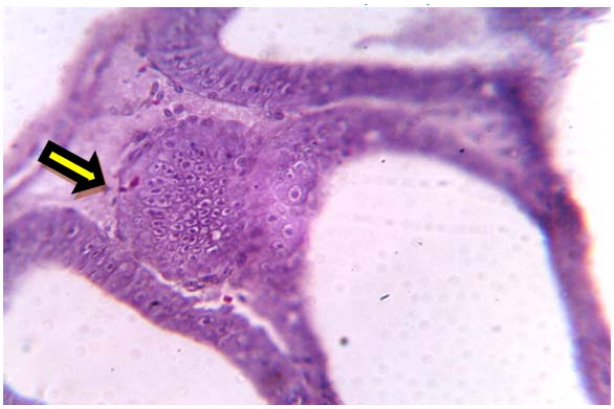

Fig 8: Section of HP showing the Granuloma (400 X)

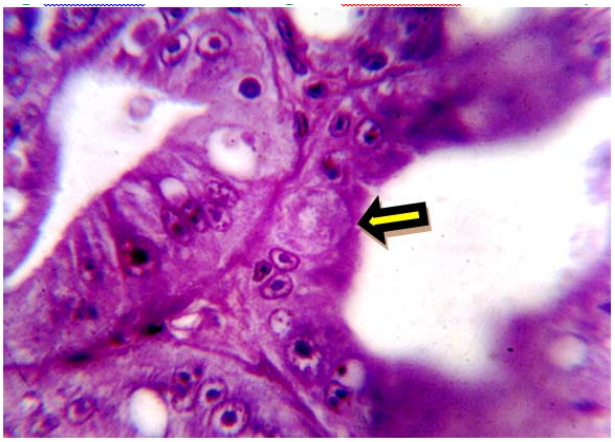

Fig 9: Section of HP showing developing microsporidian in the B – cell of the hepatic tubule (400 X)

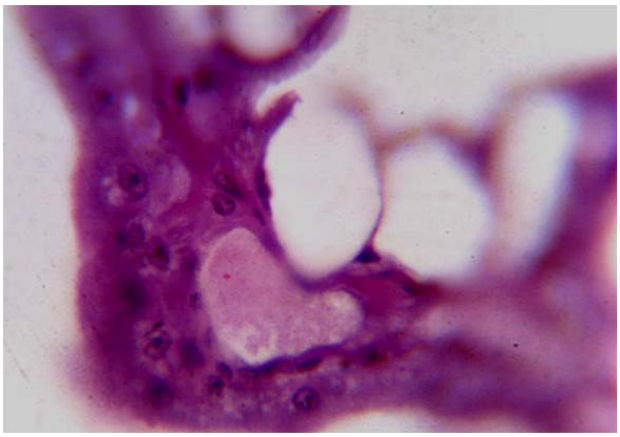

Fig 10: Section of HP showing the developing spores within the cyst (400 X)

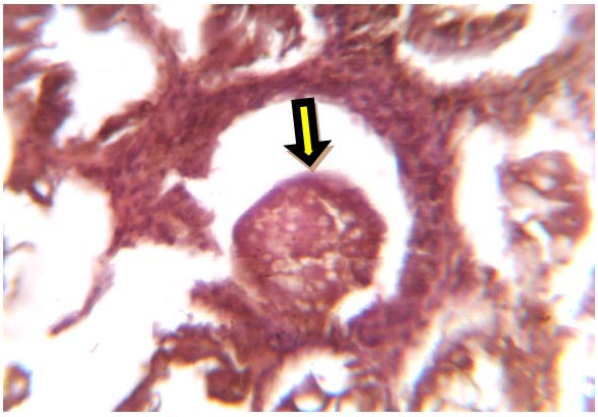

Fig 11: Section of HP showing developing spores (400X)

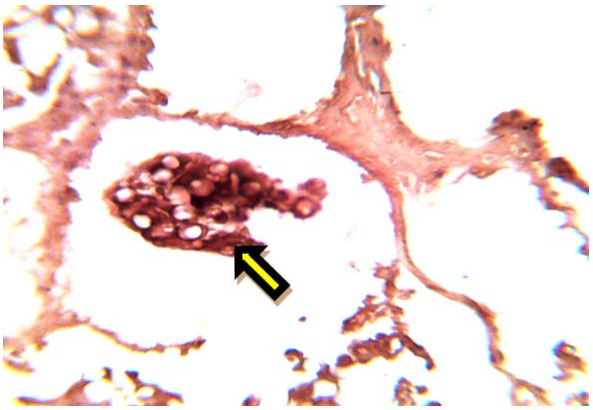

Fig 12: Section of HP showing a developed spore mass (400 X)

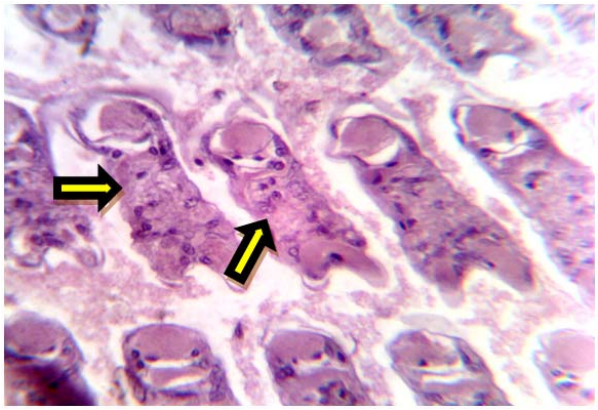

Fig 13: Section of gill showing filaments with hypertrophied Pillar cells with lesser number of lacunae (100 X)

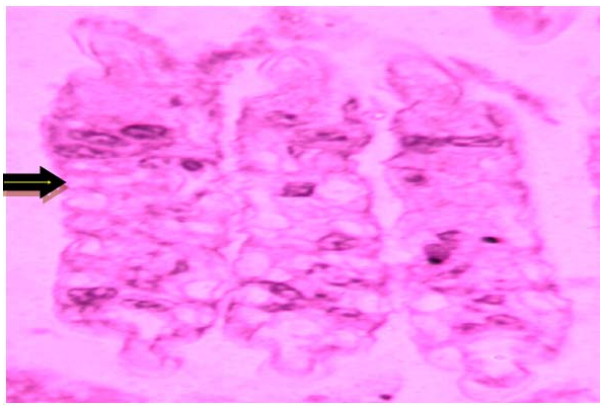

Fig 14: Enlarged view of gill showing hypertrophied pillar cells and degenerating cuticular layers at the distal parts (100 X)

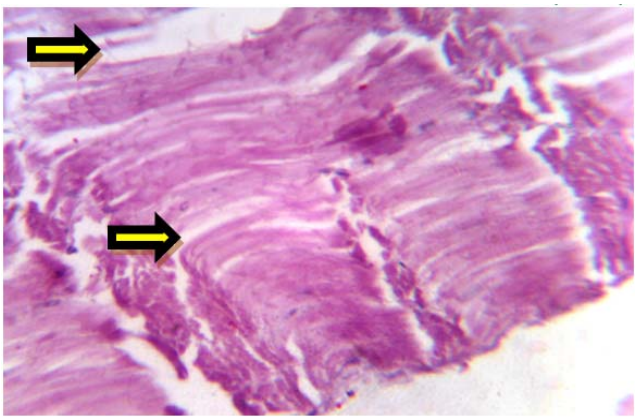

Fig 15: Section of muscle tissue showing distorted muscle filaments with lesions (100 X)

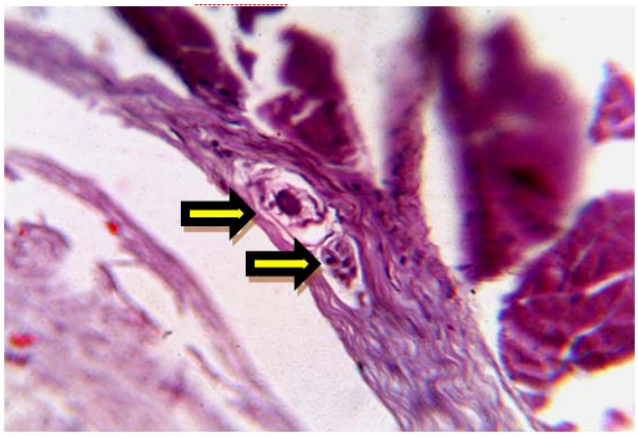

Fig 16: Section of muscle tissue showing Granulomas (400 X)

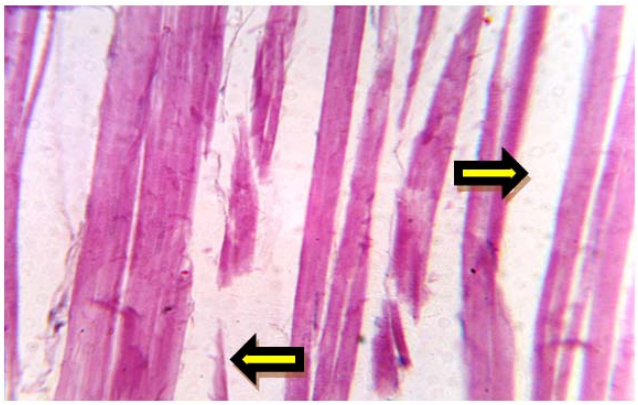

Fig 17: L.S. of muscle tissue showing loss of myofibrils to produce empty spaces, along with breaking filaments(100 X)

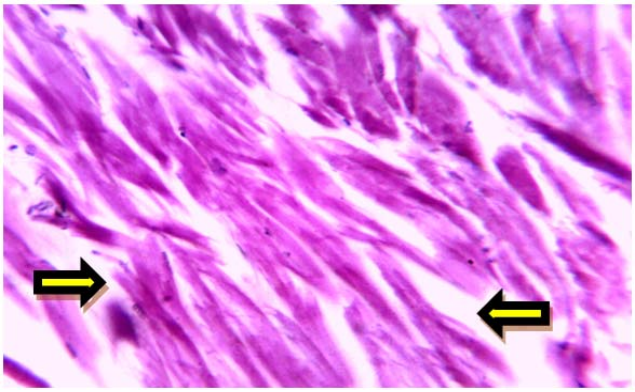

Fig 18: L.S. of muscle tissue showing highly disorganized muscle fibres (100 X)

3.2 Microbiological Studies

The Total Bacterial Count (TBC) and the Total Vibrio Count (TVC) in the diseased shrimps ranged from 1.5 x 10^4 to 2.4 x 10^6 CFU /ml and 1.2 x 10^3 to 2.5 x 10^4 CFU/ml respectively (Table. No.**1**). Out of 36 isolates, those bacterial isolates (S 1, S 2 and S 3) which showed maximum virulence against juvenile shrimp, subjected to taxonomical characterization tests (Table. No. 2) were identified as *V. harveyi, V. alginolyticus* and *V. fluvialis.*

3.3 Antibiotic sensitivity studies

This study showed that, all the three isolates were highly sensitive to Ciprofloxacin with maximum inhibition zones of 18, 36 and 36 mm for S 1, S 2 and S 3 respectively. Also, all these three isolates, S 1, S 2 and S 3 were resistant to Ampicillin and S 1 and S 3 were also found resistant to Penicillin G. S1 isolate showed better sensitivity to Chloramphenicol, Streptomycin and Ciprofloxacin among others. Similarly, S2 isolate showed better sensitivity to Ciprofloxacin, Oxytetracycline, Streptomycin among others and S3 isolate showed better sensitivity to Ciprofloxacin, Chloramphenicol and Tetracycline among others (Table No. 3).

3.4 Molecular Studies

All the 36 samples isolated from the ASDD affected shrimps were found negative to WSSV, MBV and HPV but 50% were positive for IHHNV. However, all the samples were found positive for the microsporidian *E. hepatopenaei*.

Table 1: Average Total Bacterial Counts (TBC) and Total Vibrio Counts (TVC) of ASDD affected shrimps

Sample No.	TBC(cfu/ml)	TVC (cfu/ml)	Sample No.	TBC(cfu/ml)	TVC(cfu/ml)
1	1.9 x 10^4	1.2 x 10^3	19	2.4 x 10^5	2.6 x 10^3
2	3.5 x 10^5	1.5 x 10^3	20	2.5 x 10^4	1.5 x 10^3
3	2.0 x 10^5	1.7 x 10^3	21	1.8 x 10^5	3.5 x 10^3
4	2.4 x 10^6	2.0 x 10^4	22	2.4 x 10^4	1.4 x 10^3
5	2.0 x 10^4	1.2 x 10^3	23	1.9 x 10^5	1.2 x 10^4
6	2.4 x 10^5	1.5 x 10^3	24	2.4 x 10^6	2.5 x 10^4
7	4.8 x 10^4	1.4 x 10^3	25	2.0 x 10^5	2.6 x 10^3
8	5.6 x 10^5	2.5 x 10^3	26	2.3 x 10^4	1.4 x 10^3
9	8.9 x 10^4	2.0 x 10^3	27	2.4 x 10^5	1.2 x 10^3
10	4.5 x 10^4	1.2 x 10^3	28	2.0 x 10^4	1.2 x 10^3
11	3.6 x 10^5	3.6 x 10^3	29	3.0 x 10^4	1.8 x 10^3
12	3.7 x 10^5	2.5 x 10^3	30	2.4 x 10^5	2.4 x 10^3
13	3.5 x 10^4	1.8 x 10^3	31	3.5 x 10^5	2.3 x 10^3
14	4.2 x 10^5	3.2 x 10^4	32	2.8 x 10^4	1.4 x 10^3
15	6.5 x 10^4	3.6 x 10^3	33	2.8 x 10^5	3.6 x 10^3
16	2.5 x 10^4	1.5 x 10^3	34	2.4 x 10^4	1.2 x 10^3
17	2.4 x 10^5	2.0 x 10^4	35	3.5 x 10^5	2.0 x 10^3
18	3.2 x 10^5	1.8 x 10^3	36	2.4 x 10^5	2.5 x 10^3

Table 2: Taxonomical characterisation tests conducted for the three selected bacteri

Name of the test	Isolate S1 (*V. har*)	Isolate S2 (*V. alg*)	Isolate S3 (*V. fluv.*)
Gram's staining	-	-	-
Shape	Rod	Rod	Rod
Motility	+	+	+
Oxidase	+	+	+
Catalase	+	+	+
O/F test	F	F	F
Acid production from glucose	+	+	+
NaCl tolerance test			
2%	+	+	+
4%	+	+	+
6%	+	+	+
8%	+	+	+
10%	+	+	+
Temperature tolerance test			
4 ^{0}C	-	-	-
20 ^{0}C	+	+	+
30 ^{0}C	+	+	+
40 ^{0}C	+	+	+
Decarboxylation of amino acids			
Arginine	-	-	+
Ornithine	+	+	-
Lysine	+	+	-
MRVP test	+	+	+
VP test	-	+	-
Indole test	+	+	-
Starch hydrolysis	+	+	-
Urea hydrolysis	+	+	+

Esculin hydrolysis	-	-	+
Gelatin liquefaction	-	+	+
Utilisation of carbohydrates			
L-Arabinose	+	-	+
Dextrose	+	+	+
Fructose	+	+	+
Lactose	-	-	-
Mannose	+	-	+
Galactose	+	+	+
Sucrose	+	+	+
Trehalose	+	+	+
Cellobiose	+	-	+
Melibiose	-	-	-
Salicin	+	-	+
Xylose	-	-	-
Citrate utilisation	+	+	+
Nitrate reduction	+	+	+
ONPG hydrolysis	+	-	+
Growth on TCBS	Y	Y	Y
Inhibition by 0/129 phosphate			
10 µg	R	R	R
150 µg	S	S	S
Luminescence	+	-	-

V. har. = Vibrio harveyi, V. alg. = V. alginolyticus, V. fluv. = V. fluvialis

Table 3: Antibiotic sensitivity tests of the isolates

Name of the Antibiotic	Diameter of Zone of inhibition (mm)		
	S 1	S 2	S 3
Norfloxacin	15	10	15
Ciprofloxacin	18	36	36
Oxytetracycline	15	24	15
Chloramphenicol	23	17	31
Gentamycin	14	16	16
Ampicillin	R	R	R
Kanamycin	11	21	16
Penicillin G	R	19	R
Tetracycline	17	17	18
Furazolidone	10	10	12
Erythromycin	12	21	15
Streptomycin	22	28	12

S1, S2, S3 = Bacterial isolates, R= Resistant

3.5 TEM Studies

Numerous virus – like, non-enveloped, icosahedral particles of 20-24 nm size were observed in the sarcoplasm of the muscle tissue (Fig. 19). Similar type of particles were also obtained in the cytoplasm of the pillar cells of the gills (Fig. 20).

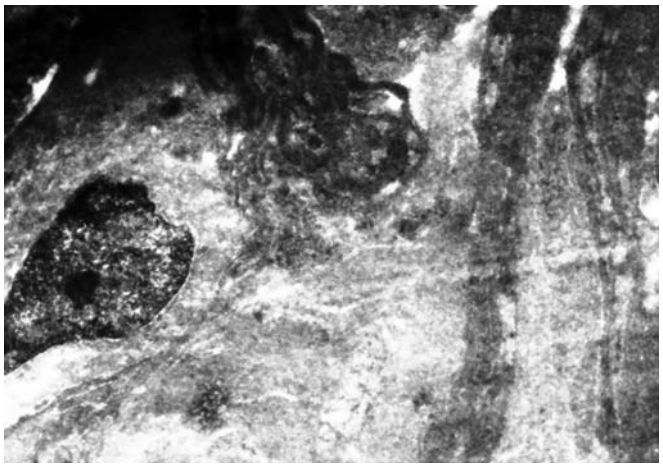

Fig 19: Muscle tissue

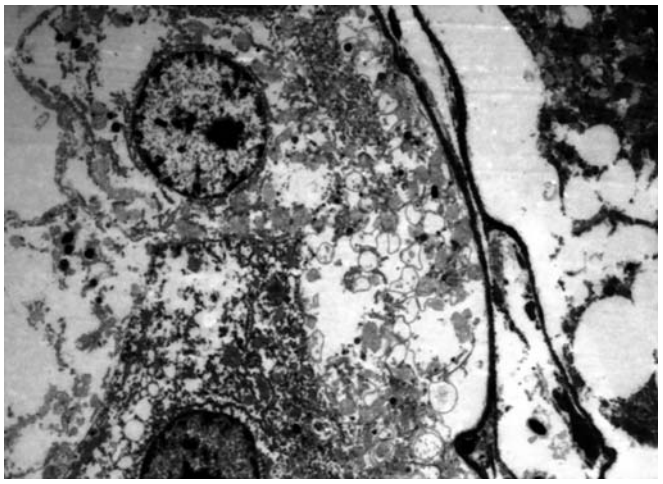

Fig 20: Virus particles in Gill

4. Discussion

Abdominal Segment Deformity Disease (ASDD) of shrimp is a relatively new disease reported from Thailand & Indonesia, since the introduction of *P. vannamei.* It is characterized by twisting and bulging of the abdominal segments of juvenile shrimp in grow-out ponds, sometimes accompanied by limited muscle whitening. Histopathological analysis of ASDD samples by Waraporn *et al.,* (2008) [6] revealed abdominal muscle necrosis and degeneration, with haemocytic infiltration. Flegel (2009) [5] found aggregation of hemocytes in the areas of distorted muscle tissue that occurs in the absence of basophilic cytoplasmic inclusions. In the present study complete degeneration and emptying of the tubules, excessive vacuolization was noticed in the tubular epithelial cells of hepatopancreas. There was severe haemocytic infiltration and granuloma in the inter tubular spaces. There were acidophilic granular inclusions in the cytoplasm of the tubular epithelial cells. Hypertrophy of the pillar cells in the gill filaments with reduced lacunae and degeneration of cuticular layers was also observed. Muscle fibres were more disorganised with broken muscle fibres, lesions, infiltration of haemocytes and also granulomas.

Microsporidian infection first reported in the hepatopancreas

of *P. monodon* from Thailand and was identified as a new genus designated as *Enterocytozoon hepatopenaei* by Tourtip, 2005 [14]. It is restricted to tubule epithelial cells of the hepatopancreas of *P. monodon*. More recently, another microsporidian closely resembling it in morphology and tissue preference was found in Thai-farmed *P. vannamei* exhibiting white feces syndrome (WFS). Various developmental stages of microsporidian cysts and spores inside B cells of the hepatopancreatic tubules as granular inclusions were also noticed. Few microsporidians have been shown to infect only the tubule epithelial cells of the crustacean hepatopancreas [15] [16] [17] [18] [19]. Rajendran *et al*., (2016) [20] reported for the first time of the emergence of EHP infection in cultured *P. vannamei* in India from the slow growing as well as WFS-infected animals, however they could not conclusively elucidate the association of EHP with these clinical signs through experimental infection trials. There was no retarded growth in ASDD infected shrimp as reported by Waraporn *et al*., (2008) [6] but the present study indicates that the retarded growth could be due to the damage caused by *E. hepatopenaei* in the hepatopancreas there by affecting the metabolism and ultimately growth.

Microbiological studies revealed the associated bacteria in the infected shrimps belonged to three species of *Vibrio* such as *V. harveyi, V. alginolyticus* and *V. fluvialis.* These bacteria could be regarded as secondary infecting agents besides the viral and Microsporideans in *L vannamei*. The three bacterial isolates were highly sensitive to Ciprofloxacin and showed maximum inhibition zones of 18, 36 and 36 mm for *V. harveyi, V. alginolyticus* and *V. fluvialis* repectively but were resistant to Ampicillin and PenicillinG.

Shrimp viruses such as infectious hypodermal and haematopoietic necrosis virus (IHHNV) and infectious myonecrosis virus (IMNV) previously known to cause physical deformity and muscle abnormality in shrimp were ruled out as causative agents of ASDD by negative findings using specific polymerase chain reaction (PCR) and in situ hybridization methods [6]. Majority of the ASDD affected shrimp showed the absence of recognizable shrimp pathogens by histopathology and also with the molecular tests. It is significant to note that all the samples were found positive to EHP but only 50% were positive to IHHNV.

The presence of a retroviral-like element that appeared to be causally linked to axonal degeneration leading to muscle atrophy and distorted abdominal segments in *L. vannamei* indicates the involvement of the virus in the disease [21].Since the agent appeared to be transmitted from broodstock to their offspring, it is recommended that precautionary strategy should focus on immediate management and monitoring of broodstock and if possible, long-term selection of stocks free of the element was described by Waraporn *et al*., 2013. The presence of non-enveloped viral-like particles (20-22 nm) in muscles, gills and ventral nerve cords were noticed in ASDD in *P. vannamei* by Waraporn *et al*., 2008 [6]. Flegel, (2009) [5] found that injection of membrane-filtered (0.22µm) tissue homogenates resulted ASDD in challenged shrimp, suggesting that it might be an infectious disease caused by a viral agent. Transmission electron microscopy revealed the presence of viral-like particles in affected muscles and in the ventral nerve cord [5]. The present TEM studies revealed that numerous virus-like, non-enveloped, icosahedral particles of 20-24 nm size in the sarcoplasm of the muscle tissue and also in the cytoplasm of the pillar cells of the gills. This is the first report of mixed infection of ASDD and *E. hepatopenaei* from India.

5. Conclusion

Dual infection of ASDD and EHP in cultured *L. vannamei* is first report from India. Major histopathological changes were observed in tissues like hepatopancreas, muscle, gills etc. virus-like particles of 20-24 nm size in the muscle and gills were observed. The brooders need to be thoroughly investigated for the presence of EHP infection and kits need to be developed for the identification of viral pathogen after its characterization. Strict quarantine measures besides best management practices need to be adopted for sustainable production by minimizing the disease outbreaks.

6. Acknowledgements

The authors are grateful to the University Grants Commission (UGC), Government of India, New Delhi for their financial assistance to carry out this research work through UGC-Major Research Project (UGC-MRP). The authors are also thankful to Dr. T.W. Flegel for his immense help in the investigations.

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