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An improved microscopic method for the rapid diagnosis of emerging microsporidian parasite, *Enterocytozoon hepatopenaei* in shrimp farms

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***Penaeus* (*Litopenaeus*) *vannamei* shrimp samples (hepatopancreas and faeces) collected from grow-out farms were evaluated for the presence of newly emerged microsporidian parasite *Enterocytozoon hepatopenaei* by concentration techniques using either water–ether sedimentation or Sheather's sugar solution for spore recovery and subsequent microscopic detection by modified trichrome stain (Ryan-blue method). This improved selective staining protocol can easily resolve the identification of microsporidian spores in hepatopancreatic tubules compared to conventional stains. This method enables differential diagnosis of microsporidian spores by a characteristic staining pattern of pinkish-red, often with a belt-like diagonal stripe seen in the middle of the spore and a halo of unstained area at one end which could be easily distinguished from similarly staining particles or debris.**

Keywords: *Enterocytozoon hepatopenaei*, microsporidia, trichrome staining, shrimp aquaculture.

MICROSPORIDIA are obligate, intracellular, spore-forming endoparasites known to infect a wide range of eukaryotic hosts, both terrestrial and aquatic. To date, approximately

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187 genera and over 1300 species of microsporidia have been described as parasites infecting a wide range of vertebrate and invertebrate hosts, among which almost half infect aquatic species[1,2]. The parasite develops within the cytoplasm of the host cell through nuclear proliferation and spore formation (sporogony), though certain genera are known to undergo similar development within the host nucleoplasm as well. Several microsporidians have been reported as pathogens of penaeid shrimp as well as finfish[2]. Recently, a new species of microsporidian *Enterocytozoon hepatopenaei* (EHP) has been reported to cause hepatopancreatic microsporidiosis in penaeid shrimp in Asian countries like China, Indonesia, Malaysia, Vietnam and Thailand[3–5]. EHP infections have been reported in both black tiger shrimp *Penaeus monodon* and Pacific white leg shrimp *Penaeus* (*Litopenaeus*) *vannamei* causing severe economic loss to shrimp aquaculture[6]. The parasite has been confirmed in *P. vannamei* in India[7] during 2014, leading to detailed studies in farmed shrimp[8]. The horizontal transmission of the parasite occurs between hosts through ingestion of spores. It infects the epithelial cells of the shrimp hepatopancreatic tubules and is reported to be associated with severe growth retardation and/or white faeces syndrome in farmed shrimp, and reduced production[6].

There are no specific clinical signs associated with EHP infection. Currently, active surveillance of this infection is being done by employing techniques like histology, *in situ* hybridization and PCR. However, these techniques are time-consuming, expensive and need specialized laboratory facilities. The pathogen can also be detected by light microscopy of hepatopancreas or faecal smears. This is based on the presence of typical spores that are very small (1.1×0.7 μm) and are sometimes produced only in small numbers, even in heavily infected shrimp[6]. Poor resolution of EHP developmental stages and/or spore in tissues by traditional H&E staining has been reported[6,9]. The problem is compounded when dealing with very low infection, which again may require concentration techniques before attempting staining. Development of a modified trichrome staining method constituted a significant improvement for the routine screening of human microsporidiosis[10,11].

Here we present an improved microscopic method by a concentration techniques used in conventional parasitology followed by modified trichrome staining (Ryan-blue method) for the detection of spores of EHP in faeces or hepatopancreas. Further, precise localization of spores in hepatopancreas tissue sections has also been studied.

P. vannamei samples were collected from different culture ponds of shrimp farms ($n = 30$) located in the east coast of India (Tamil Nadu and Andhra Pradesh) during August 2014–December 2015. Majority of these farms experienced size variation/stunted growth and low-grade mortality, and in some cases had white faecal strings floating on the water surface during 45–90 days of culture period. Shrimps collected from individual ponds using cast net were transported back to the laboratory over ice for detailed studies using microscopy and molecular-based microsporidian detection methods. For histological study, shrimps were fixed immediately by the injection of Davidson's fixative in the hepatopancreas and abdominal segments, and further processed by routine histological techniques in the laboratory. A small portion of hepatopancreas and intestinal contents (faeces) was dissected out and applied on a slide, and a thin squash preparation was made by placing a cover glass with slight pressure. Similarly, thin smears were allowed to dry, fixed in methanol for 15 min and stained with Giemsa or 5% neutral buffered formalin for 15 min, and stained with hematoxylin and eosin (H&E). Both fresh wet mounts and stained smears were observed under a microscope (Nikon Eclipse 200, Japan).

The purification and concentration of microsporidian spores from hepatopancreas or faecal samples were performed either by floatation using Sheather's sugar solution or sedimentation by water–ether method[10,11], followed by microsporidia-specific staining using modified trichrome stain[12] with minor modifications. Briefly, 2 ml of diethyl ether was added to 10 ml coarse-filtered suspension of faecal or hepatopancreas homogenate, and vortexed for 20 s and then centrifuged (2000 rpm, 5 min). Both the fat and supernatant layers were discarded, and the pellet was resuspended in 10 ml of distilled water and centrifuged. This washing procedure was repeated twice and the pellet was resuspended in 500 μl of distilled water.

Purified spores were also observed under microscope using agar-layer method described for myxozoans[13], with slight modification. Briefly, 1.5% agarose solution was spread onto a pre-heated glass slide to form a 1 mm thick uniform layer. A small drop (15–20 μl) of spore suspension was spread on a circular cover glass which was inverted gently to form a hanging drop, and carefully placed over the agarose layer and observed under a phase contrast microscope (Nikon-Eclipse E200, Japan). A thin smear of purified spores was also stained by conventional Giemsa and modified trichrome stain (Ryan-blue method) for microsporidians. Briefly, trichrome stain solution was prepared by mixing 6 g of Chromotrope 2R (Sigma, USA), 0.5 g of aniline blue (Sigma, USA), and 0.25 g of phosphotungstic acid (SRL Laboratories) with 2 ml glacial acetic acid. After the mixture was allowed to stand for 30 min, 100 ml of distilled water was added and pH was adjusted to 2.5 with 1 M hydrochloric acid. Following fixation for 10 min, the slides were stained in trichrome-blue for 90 min, rinsed for 10 s in acid alcohol (0.45% acetic acid in 90% ethanol) and then for 10 s in 95% ethanol. The slides were then processed through two changes of 95% ethanol for 5 min each, followed by 100% ethanol for 10 min and then transferred into xylene for 10 min. The stained slides were mounted in DPX and

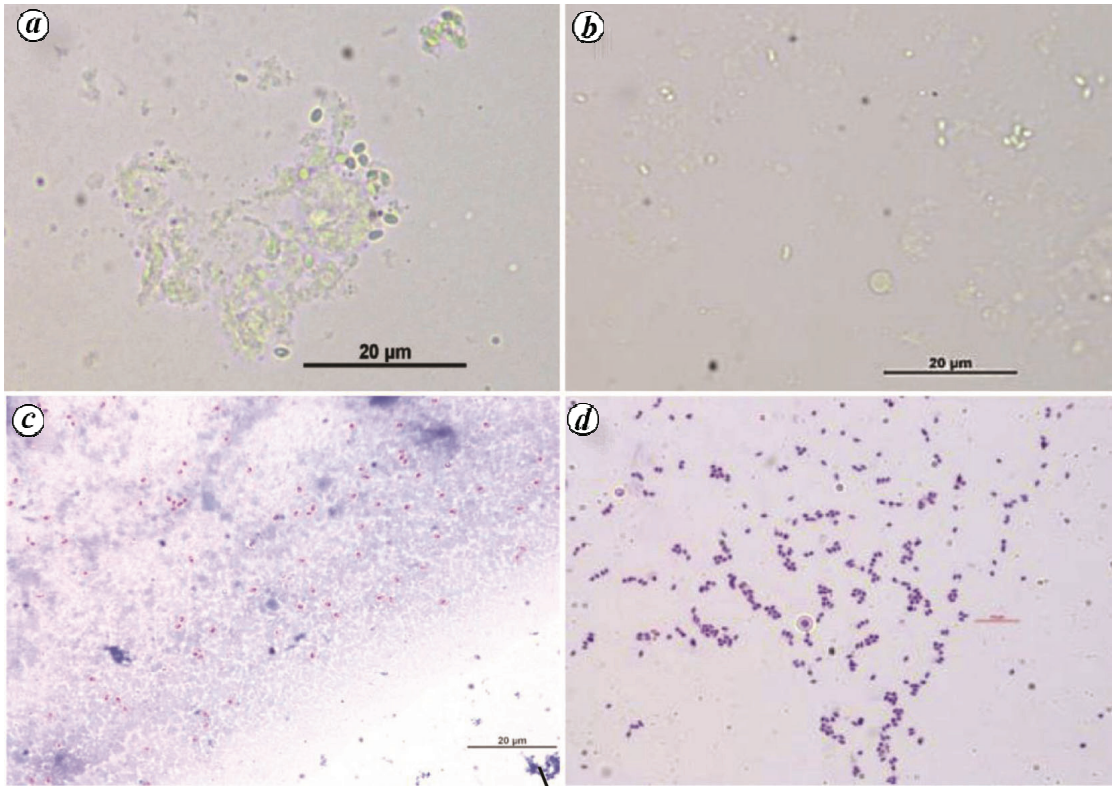

Figure 1. *Enterocytozoon hepatopenaei* (EHP) spores in the white faecal samples on: ***a***, wet mounting; ***b***, agar layer method; ***c***, modified trichrome; ***d***, Giemsa staining.

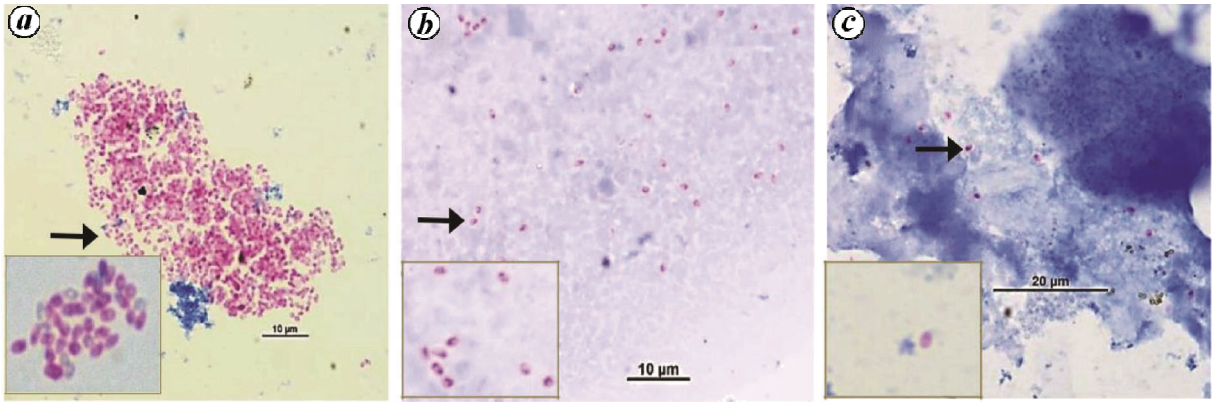

Figure 2. Spores of EHP from shrimp faeces after formalin–ether sedimentation, stained by modified trichrome staining: (***a***, heavy; ***b***, medium; ***c***, low infection). Spores (arrows) are ellipsoidal staining pinkish-red with a small polar or central non-staining zone. (Inset) Enlarged view.

observed under a microscope for detailed studies on microsporidian spores.

For histopathological studies, sagittal sections of the head portion (cephalothorax) of shrimp were processed by routine histological method[14] using tissue embedding system (Leica Microsystems, Germany) and sectioning (4–5 µm) by semi-automatic microtome (Leica RM 2245, Germany). Portions of the intestine, gills and pleopods were also included. The tissue sections were also stained by routine H&E and finally mounted in DPX. A similar set of slides was also stained using Chromotrope 2R-based modified trichrome-blue as described above, and observed under oil immersion objective. A positive reference sample of fish microsporidian (*Glugea* sp.) smear and tissue cyst (xenoma) was used for studying comparative staining characteristics by H&E and modified trichrome stain. Digital images were taken using a digital camera (Nikon DS-Fi2) using Nikon NIS-Elements Imaging software suite (version F 4.30.01).

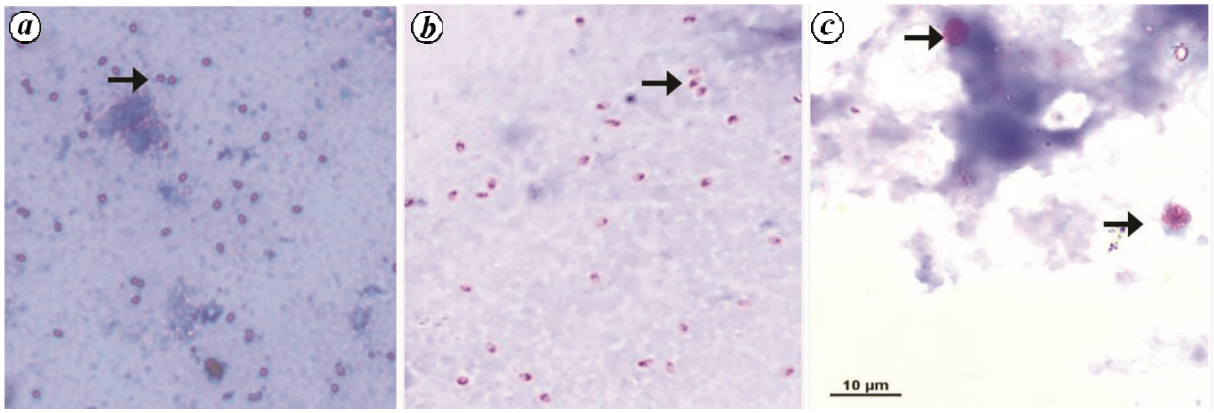

Figure 3. Wet smears from *Penaeus vannamei* hepatopancreas showing EHP spores and presumptive developmental stages packed with EHP spore-like structures. ***a***, Stained with Giemsa; ***b***, Modified trichrome stain; ***c***, Packed spore-like bodies in vesicles – trichrome stain (1000×).

EHP spores were distinctly visualized by light microscopy of thin smear preparations of faeces and hepatopancreas. Spores were ovoid and refractile measuring approximately 1 µm, but not discernable even after Giemsa staining due to the large quantity of faecal and tissue debris from the samples. However, the detection enhanced considerably in case of concentration of spores by differential sedimentation or floatation techniques (Figure 1). The number of microsporidian spores detected in concentrated samples ranged from fifty to several hundreds per slide, and the ease of detection of microsporidian spores by modified trichrome stain (Ryan-blue method) was mainly due to colour contrast of the spores than those stained by Giemsa or H&E staining. This technique stains the spores as well as spore wall a bright pinkish-red in different species of microsporidians, often with a belt-like stripe, which also stains pinkish-red in the middle of the spore and a halo of unstained area at one end (Figures 2 and 3). These combined techniques of spore concentration followed by staining may be adequate for the differential diagnosis of clinically important microsporidiosis in shrimp. The H&E and trichrome staining of shrimp hepatopancreas also revealed suspected presumptive developmental stages and spore-like bodies, mostly in the distal end of the hepatopancreatic tubules. However, spores were evident in sections stained by modified trichrome method (Figure 4). The staining may also be useful for qualitative and quantitative assessment (Figure 2) of the parasite load in shrimp samples (faeces, hepatopancreas, etc.) and tissue localization of developmental stages in histological sections of hepatopancreas (Figure 4). The positive reference sample of fish microsporidian (*Glugea* sp.) smear and tissue cyst (xenoma) was also stained by trichrome stain for comparative study of staining characteristics (Figure 5). Though PCR and *in situ* hybridization techniques are preferred for routine diagnosis[3,4,15], they have limitations like cross-reactivity of primers with DNA from closely related microsporidians and assessing the severity of infection in shrimps. Hence, it would seem prudent to monitor the shrimp farms using conventional staining techniques for viable spores, quantum of parasitic load and to study parasite biology.

Light microscopic examination of the stained clinical smears, especially the faecal samples, is an inexpensive method of diagnosing microsporidian infections, even though it does not allow identification of microsporidia to the species level. The most widely used staining technique is trichrome staining method or its modifications. The modified trichrome staining method[12,16] constituted a major improvement in detection of microsporidian infection in faeces or other tissues that could resolve pink-coloured microsporidian spores stained by Chromotrope 2R against bluish[12] or greenish[16] background, and detect low and heavy infections with ease using light microscopy.

In the present study, we used aniline blue as the counter stain. It was found to be stable, considerably specific, but exhibited varying sensitivity depending on the degree of infection in samples (Figure 2). As a reference standard, we have compared the staining characteristics of a related fish microsporidian, *Glugea* sp. (Figure 5). The study also highlights the possibility of enumeration of spores in the sample to assess the quantification and level of infection. It has been reported that EHP produces less number of spores even in heavily infected shrimp[6] and hence the concentration protocol from faecal sample could enhance the detection and trichrome staining-improved differentiation and resolution of spores. The protocol was found to be simple, fast and could be used for routine diagnosis and monitoring of EHP in faecal strings and hepatopancreas of shrimp.

Although this method will stain microsporidia, other bacteria, some yeast cells and feed debris may also tend to stain pink to red; the shapes and sizes of the various components may be helpful in differentiating the microsporidia from surrounding materials. This technique, however, is time-consuming and requires more than 2 h, which is still fast compared to the PCR method of detection. Nevertheless, the trichrome-blue stain offers a single method for the detection of microsporidian in a wide

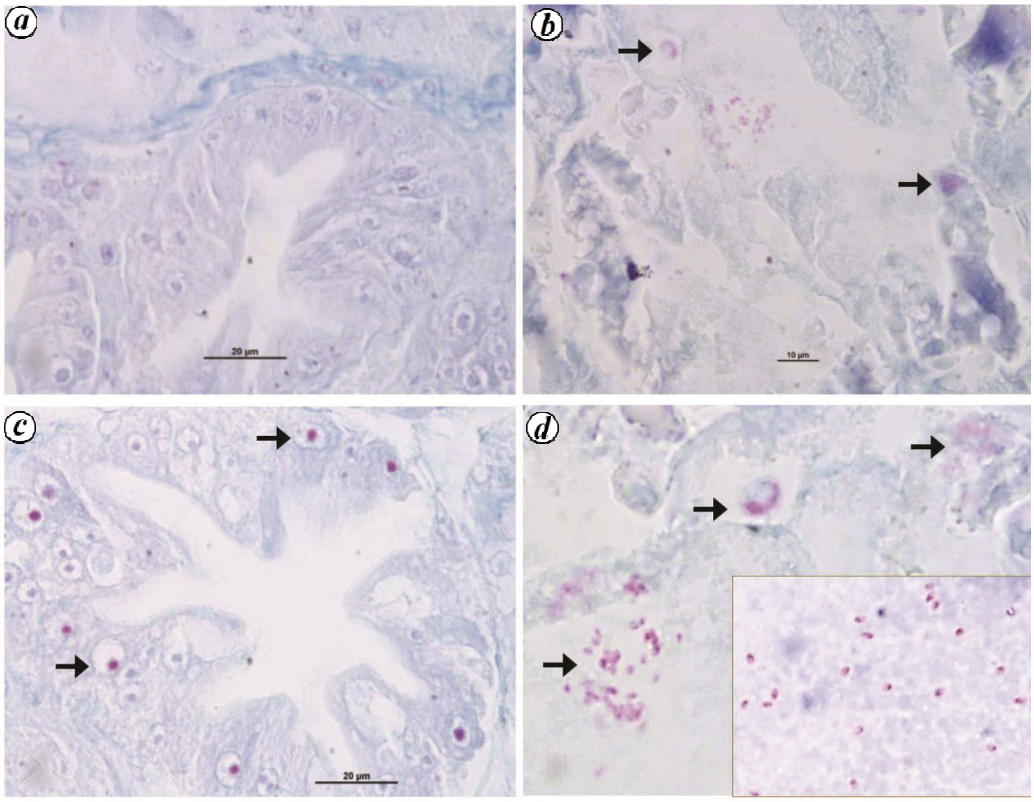

Figure 4. Histological sections of *P. vannamei* hepatopancreas showing presumptive developmental stages. ***a***, Normal hepatopancreas. ***b*** and ***c***, Developmental stages of EHP in hepatopancreatic tubular epithelium. ***d***, Note the destruction and degeneration of hepatopancreatic tubular epithelium and spore-like bodies stained using modified trichrome method (1000×). (Inset) Purified spore stained using trichrome method.

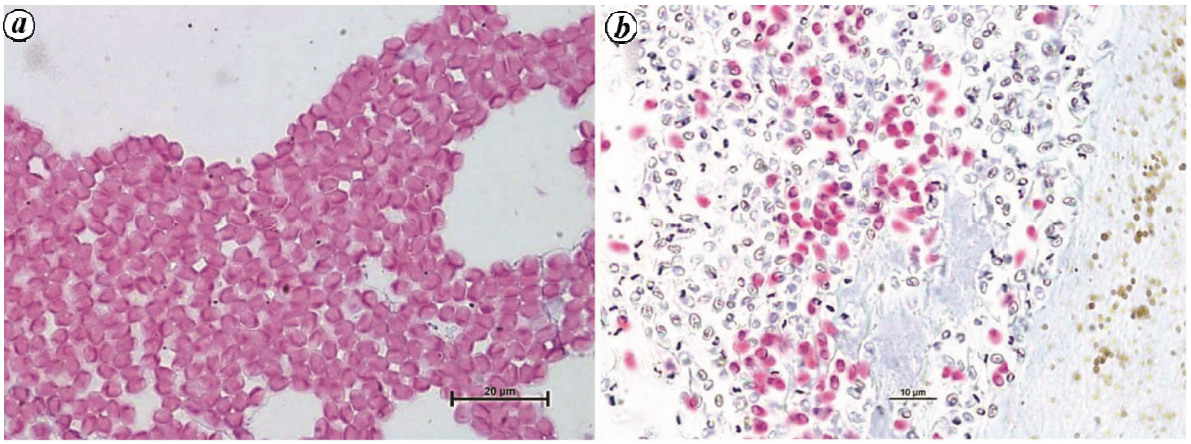

Figure 5. ***a***, Microsporidian *Glugea* sp. stained using modified trichrome method. ***b***, Histological section of the cyst (xenoma) of fish microsporidian *Glugea* sp. showing thick wall and stratified developmental stages and mature spores within the xenoma (1000×).

range of species, with no limitation of false positives as often observed in PCR. Further, this method serves as a practical means of non-invasive diagnostic tool, as in the case of specific pathogen-free broodstock of *P. vannamei* imported in the country for quarantine purpose, therapeutic trials and improving the ability to follow the infection pathways in shrimp farming. In conclusion, conventional microscopy could be used as a diagnostic tool for studying epidemiological distribution of parasites in shrimp farming using archived shrimp tissue samples. It can also help monitor shrimp farms for microsporidian infection and to study parasite life cycle and transmission dynamics in shrimp ponds.

Conflict of interest: The authors declare that they have no conflict of interest.

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Haematobiochemical parametrs of fishes of preferential nutritional habits

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This study compares the haematobiochemical parameters of fishes of different food habits, viz. *Labeo rohita* (herbivore), *Channa striatus* (carnivore), *Clarias batrachus* (omnivore) and *Cirrhinus reba* (detrivore). Statistical analyses reveal the significant differences in haematobiochemical parameters like haemoglobin, red blood cell, packed cell volume, mean corpuscular haemoglobin and while blood cells with respect to nutritional habits. The monocytes have been observed to vary significantly at $P < 0.001$ level, mean corpuscular volume deviates at $P < 0.05$, whereas both eosinophils and albumin show significant variation at $P < 0.01$ level. Haematobiochemical parameters have been regarded as a valuable tool for monitoring the health of fish.

Keywords: Freshwater fish, haematobiochemical parameters, nutritional habit, statistical analysis.

FISHES are the first successful aquatic vertebrates which are characterized by the presence of paired fins with fin rays and breathe through gills. These are a rich source of simple animal protein and omega three fatty acids which are highly digestible and absorbable by humans. The omega three fatty acids help in protecting the heart. Therefore, healthy and disease-free fish need to be consumed for human beings for better health. One of the difficulties in assessing the health status of natural fish population is the lack of reliable references in normal condition. Therefore, ichthyologists have developed interest in haematological studies. Moreover, these haematological parameters are influenced by many factors such as age, sex, dietary state and stress[1]. The variation in haematological parameters in relation to sex is observed in common carp *Cyprinus carpio*[2] and *Heterobranchus longifilis*[3]. Significant seasonal variation is recorded in *Mastacembelus armatus*[4]. Concentration of salt is reported to show significant increase in blood parameters in common carp[5]. Since the aquaculture industry is expanding, standardized methods are essential for monitoring the health status of fishes. The application of haematological indices in fish farming may enhance disease-free production by identifying early detection of stress and diseases[6]. This, in turn will contribute to more specific, timely and effective disease treatments in the future. Haematological

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