Int J Pharm Bio Sci 2015 July; 6(3): (B) 951 - 965

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

DIAGNOSIS OF PARASITIC DISEASES IN BIVALVE MOLLUSCS

R.CAROLINE JEBA* AND S.ANITHA

Department of Bio-technology, Dr.M.G.R.Educational And Research Institute University, Maduravoyal, Chennai, India.

ABSTRACT

The class bivalves belongs to the phylum mollusc. The Class Bivalves with about 7500 species, includes animals with two shell valves such as mussels, oysters, scallops clams etc. Mollusc resources are very much useful to coastal communities and others in the different ways and forms. Efforts have to be made to conserve and protect this resources for the sustainable development of coastal ecosystem and the dependent population. Many molluscs are a great source of food for man in many parts of world. Molluscs are eaten by humans. Many bivalve molluscs are dangerous to humans to avoid this project has been done by identifying the major parasites affecting the bivalve molluscs which also helps to develop the mollusc population by treating the infected molluscs. This was screened by conventional microscopic method, histopathological technique and by molecular method (PCR).

KEYWORDS: **Bivalve molluscs, diseases, humans, diagnostic methods**

R.CAROLINE JEBA
Department of Bio-technology, Dr.M.G.R.Educational And Research Institute University, Maduravoyal, Chennai, India.

***Corresponding author**

INTRODUCTION

Aquaculture, also known as aqua farming, is the farming of aquatic organisms such as fish, crustaceans, molluscs and aquatic plants *Klinger et al., 2012*[1]. Aquaculture involves cultivating fresh water and salt water populations under controlled conditions. Mari culture refers to aquaculture practiced in marine environments and in underwater habitats. Aqua cultured shellfish include various oyster, mussel and clam species. These bivalves are filter feeders which rely on ambient primary production rather than inputs of fish or other feed. The phylum Mollusc is one of the largest, most diverse and important groups in the animal kingdom. There are over 50,000 described species in the phylum and about 30,000 of these are found in the sea *Barnes,1988*[2]. The class bivalves belongs to the phylum mollusc. The Class Bivalves with about 7500 species, includes animals with two shell valves such as mussels, oysters, scallops clams etc.

Bivalves

Bivalves are characterized by external covering that has two parts hinged shell that contains a soft bodied invertebrate. Like fish, bivalve molluscs breathe through their gills. An internal organ called mantle secretes calcium carbonate so that as the inner invertebrate grows, the outer shell provides a roomier home. Two mantle lobes that secrete two shell valves, hinged dorsally, cover the body organs. Two muscles, the anterior and posterior adductors, control the opening and closing of the shell valves. The exploitation of filter feeding made it possible for bivalves to colonise a wide variety of habitats that had hitherto been inaccessible to their protuberance ancestors. In bivalves where the two shell valves are the same shape, such as mussels and clams, the valves are drawn together by an anterior and posterior adductor muscle. When these are relaxed the shell is opened by the elasticity of the ligament. Contraction of the adductor muscles closes the shell. When a bivalve dies these muscles can no longer contract and the ligament force the shell open. A dead bivalve always has gaping shell.In bivalves with dissimilar valves, e.g. oysters and scallops; there is a single centrally placed adductor muscle. This performs the same function as the two adductors in mussels and clams. Mussels have been extensively used to assess environmental contamination. Radio nucleotides and metals such as uranium, vanadium and lead are highly concentrated in the water gets accumulated in the outer periostracal layer of the shell.

OYSTERS

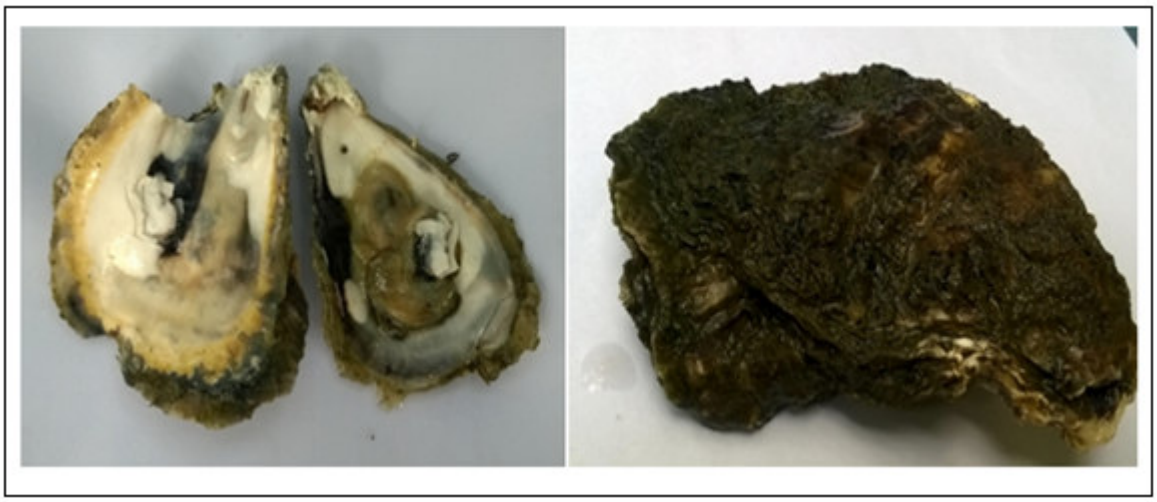

Figure 1
Edible oyster of species Crassostrea rivularis

Oysters belongs to the family “Ostreoidae” .This family includes edible oysters which mainly belong to the genera Ostrea,Crassostrea,Ostreola and Saccostrea,pearl oysters of genera. Thirteen species of edible oysters have been reported from India among which, four species viz., Crassostrea gryphoides, C. rivularis, C. madrasensis and Saccostrea cucullata are of commercial value. C gryphoides and C. rivularis are mostly found in the North-Western coast of India. Crassostrea madrasensis enjoys a wider distribution along the South-West and East coasts of India and it is found in almost all the estuaries and backwaters.

CLAMS

Figure2
Edible clam of species Katelysia spp

A number of clam species belonging to the families Arcidae, Veneridae, Corbiculidae, Tridacnidae, Solenidae, Mesodesmatidae, Tellinidae and Donacidae are exploited along the Indian coast. Venerid clams are most sought after in the clam fisheries of India and three genera , namely Meretrix, Paphia and Katelysia are important.Clams belongs to the family “Unioniade”.Clams can live in the marine and fresh water. They have smooth, shiny hard shells. They lack heads but most can react to changes in light. Many edible clams are roughly oval shaped or triangular; however razor clams have an elongated parallel sided shell. On the inside of each valve are two muscle scars, the attachment points for the large posterior adductor muscle and the much reduced anterior adductor muscle *Audemard et al., 2006*[3]. Their population status or structure, physiology, behaviour or the level of contamination with elements or compounds can indicate the state of contamination status of the ecosystem *Chapman,A.D, 2009*[4]. Bivalve molluscs have a wide use such as a protein source, raw materials in cement and pharma industries. Occurrence of diseases in bivalves would affect its production *Comps et al.,1979*[5]. Against this parasitic, bacterial and viral diseases, this project was formulated with an objective to screen for parasitic diseases. Off all the diseases, parasitic diseases have not been reported widely in India. Against this parasitic, bacterial and viral diseases, this project was formulated with an objective to screen for parasitic diseases. Off all the diseases, parasitic diseases have not been reported widely in India[6].

Figure 3
Green mussels species Perna viridis

The few number of mussel species belonging to the family 'mytilidae' found in the Indian coasts are *Margaritifera margaritifera, Perna viridis* (green mussels)

MATERIALS AND METHODS

MATERIALS

Samples

The Bivalve samples were collected from the Pulicat backwaters near ponneri, Thiruvallur district, Tamil nadu. Samples were brought in live condition to the lab and maintained in aerated troughs. The details of the bivalves samples collected for the study is tabulated as follows.

Table 1
Details of the bivalve samples used in the study

Sample code	Sample details
CP1/15	Clam gill
CP2/15	Clam mantle
CP3/15	Clam heart
CP4/15	Clam foot
OP1/15	Oyster gill
OP2/15	Oyster mantle
OP3/15	Oyster heart
OP4/15	Oyster digestive gland
MP1/15	Mussel digestive gland

Chemicals and molecular biologicals used in the study

1. Ethyl alcohol

Fixing of samples was done either with ethyl alcohol or Davidson's fixative. DNA extraction kit was used with the samples fixed in 70% alcohol (70% ethyl alcohol, 30ml distilled water).

2. Davidson's fixative

Davidson's fixative solution used for fixing the samples for histopathology can be prepared by mixing the following ingredients (total, 900 ml) in an appropriate size container and mixing well *OIE 2000*[7].

a. 300 mL distilled water
b. 200 mL formalin (37% formaldehyde)

c. 100 mL acetic acid
d. 300 mL 95% alcohol

3. DNA Extraction chemicals

Commercially available DNA extraction kit was used to perform the DNA extraction of required parts from the samples collected for the study (QIAGEN DNA extraction kit, USA)

4. PCR chemicals

v.a. PCR Primers

Primers used for the diagnosis of the diseases of bivalves are listed in table 2. The primers included self designed and published primers.

Table2

PCR Primers for emerging diseases used for screening the samples

Primer code		Primer sequence	Product size	Reference
Perkinsosis	(F) PerkITS-85	(5'-CCG-CTT-TGT-TTG-GAT-CCC-3')	703bp	*Villalba et al., 1993*[8]
	(R) PerkITS-750	(5'-ACA-TCA-GGC-CTT-CTA-ATG-ATG-3')		
Bonamiosis	(F)Bo	(CATTTAATTGGTCGGGCGCG)	300bp	*Corbeil et al.,* 2006[9]
	(R)Boas	(CTGATCGTCTTC)		
Marteiliosis	(F)Pr4	(5'-CCG-CAC-ACG-TTG-TTC-TTC-TTC-ACT-CC-3)	266bp	*Berthe et al.*, 2000[10.]
	(R)Pr5	(5'-CTC-GCG-AGT-TTC-GAC-AGA-CG-3')		

v.b. PCR Reaction Mix (PCR master mix)

Commercial PCR master mix with optimized concentration if bases, buffer and Mgcl2 was used in the study (GeNei™ master mix, Bangalore, India).

v.c. DNA marker

Commercially available 100bp molecular marker was also separated parallely to compare the PCR amplified product (medoxbio molecular marker, Chennai, India).

v.d. Agarose

Molecular biology grade Agarose gel was prepared in TBE buffer with 4 µl ethidium bromide for gel electrophoresis.

v.e. Tris Borate EDTA (TBE) Buffer (pH-8.2)

Stock Solution (10X)

Tris.Hcl - 108.0g
Boric acid – 55.0g
Disodium EDTA – 9.3g
Distilled Water to make 1000ml

Working solution (1X)

TBE buffer 10X – 100ml
Distilled water – 900ml

v.f. Gel loading buffer

6X gel loading buffer (commercially available, Bangalore Genei, Bangalore, India).

v.g. Gel documentation

The amplified product in gel were observed under UV and documented using a gel documentation unit(BioRad).

METHODS

Collection and fixing of sample

The bivalve samples were collected from the pulicat backwaters near Ponneri, thiruvallur district, Tamil nadu. Samples were brought in live condition to the lab and maintained in aerated troughs.

Maintenance of the sample

Bivalve samples were maintained with good aeration; adequate feeding and maintenance of water quality parameters at ideal levels in the wet lab of state referral laboratory, Tamilnadu Fisheries university (TNFU), Madhavaram.

Figure 4
Maintanence of fixative samples in the laboratory

Screening methods followed for diagnosis of parasitic diseases

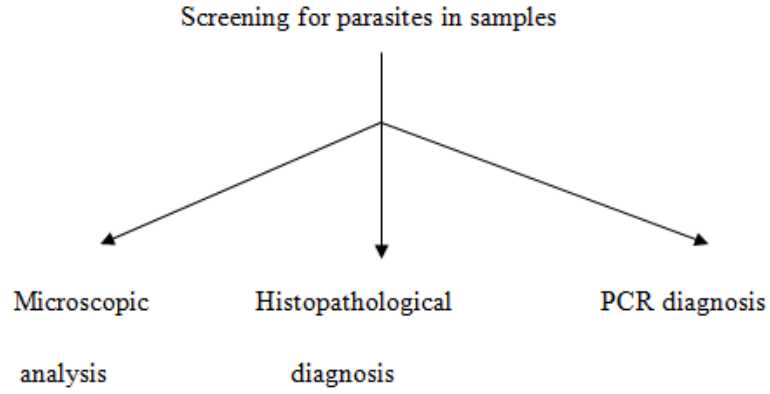

Table 3
Methodologies followed for screening of parasitic diseases

Disease	Methodology followed			
	Wet mount	Tissue imprints	Histopathology	PCR
Perkinsosis		✓	✓	✓
Bonamiosis			✓	✓
Marteiliosis		✓		✓

a)Microscopic examination
The live molluscs were opened carefully and the organs of interest were excised. Adequates of the organs were stained followed standard methods and observed by microscopic method.

a)Smear
A drop of haemolymph was placed on a glass slide and smear.Obervations are made at 100-400x after staning in lugol's iodine.

b)Fixed stained tissue
The target tissues of the bivalve samples were smeared in a slide using Lugol's iodine stain.

c)RFTM for perkinsosis

b)Histopathology diagnosis of samples for Perkinsosis, Bonamiosis

Samples are fixed with Davidson's fixative for carrying out the histopathological study for Perkinsosis and Bonamiosis. The diseases were identified by histopathology and they were documented for the result analysis.

c) PCR diagnosis for parasitic diseases Perkinsosis, Bonamiosis, Marteiliosis

Bivalve samples were screened by PCR using the primers. Primers were selected for the diagnosis of the diseases of bivalve listed in the study (table 4). The primers included were self designed and published primers. PCR standard protocols were followed in case of self designed primers and protocols based on the published papers in case of published primers.

5. DNA Extraction

Organs such as gill, mantle, heart, foot and digestive gland were exercised from the bivalve sample were used for the extraction as the target organ of the pathogens of interest vary. The extracted DNA was coded and stored in a deep freezer (-70°C) for the further study. Specific extracted DNA part was selected for each disease and the target organ varies for every disease.

6. Preparation of PCR mixture

Reagents	Quantity/concentration required/reaction
PCR master mix	22 µl(1x)
Forward primer	1 µl(30 picomoles/reaction)
Reverse primer	1 µl(30 picomoles/reaction)
DNA of target organ	1 µl(50mg)
Total	25 µl

7. *Agarose gel electrophoresis*

The amplified products from first step were separated by agarose gel electrophoresis. 2% agarose gel was prepared in 1X tris-borate EDTA buffer (TBE buffer) added with 4 µl ethidium bromide around 250ml was poured on the agarose gel electrophoresis apparatus.4 µl of PCR amplified product were loaded into preformed wells of agarose gel.2 µl of 100bp molecular weight DNA ladder was also added in the gel comparing the size of the PCR products. Gel electrophoresis was carried out at 100 volts for 30min. After the run,the gel was viewed under UV gel documentation system. The results were documented, transferred and stored in the computer.

8. PCR Amplification

PCR amplification of the target DNA was performed. Briefly 22µl of Master mix,1µl each of Forward and Reverse primer,1µl of extracted sample DNA were mixed and loaded in the PCR thermal cycler.4µl of PCR amplified products were added in gel for separation of DNA.Commercially available 100bp molecular weight marker was also separated parallel to separate the size of PCR amplification products.The amplified products of gel were observed under UV and documented using a gel documentation unit.

RESULTS

I. Microscopic examination of parasites in bivalve molluscs

a)Perkinsosis

Smear technique

Presence of spherical cells 2-15µm in diameter with a large vacuole and an eccentric displaced nucleus. This indicates the presence of Perkinsus sp.

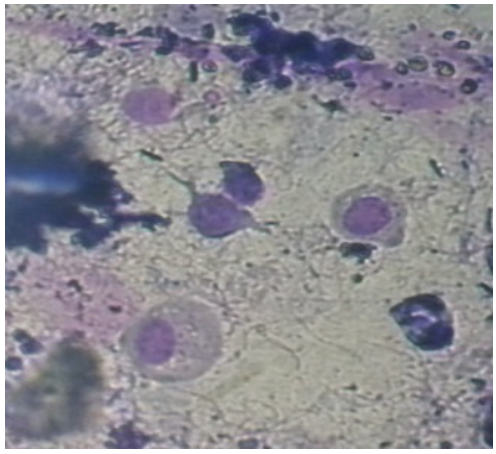

Figure 5
Adductor muscle of Oyster Showing eccentric displaced nucleus

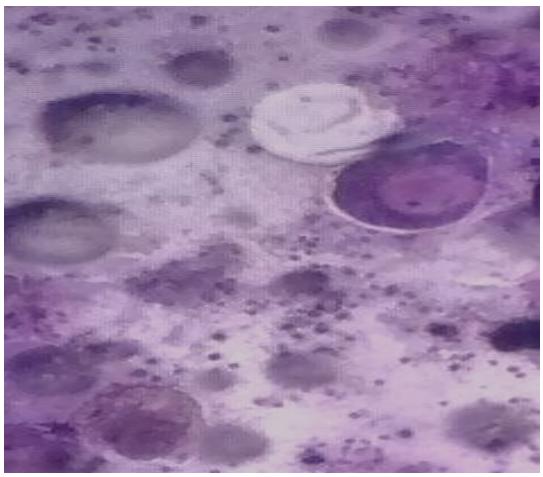

Figure 6
Adductor muscle of Clam Showing eccentric displaced nucleus

Tissue imprints

The target tissues of the samples oyster and clams were smeared in a slide using Lugol's iodine stain. This shows the presence of Trophozoites in the given samples.

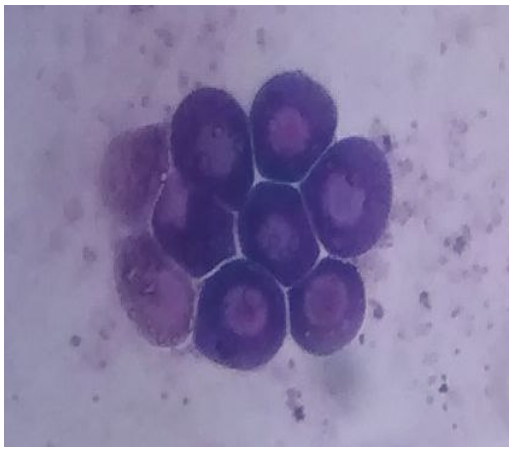

Figure 7
Gill-Oyster

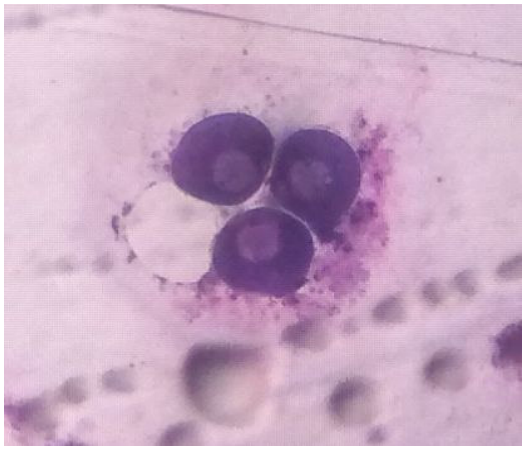

Figure-8
Mantle-oyster

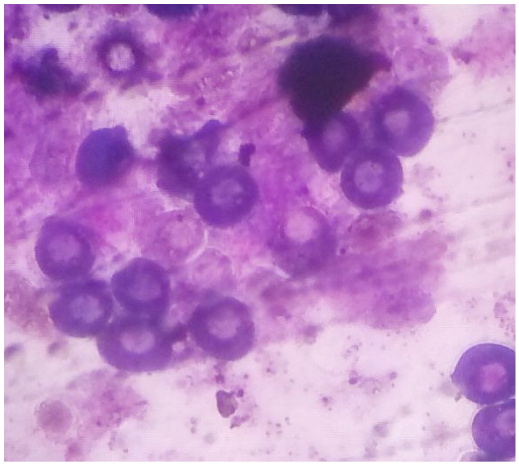

Figure9
Gill-Clam

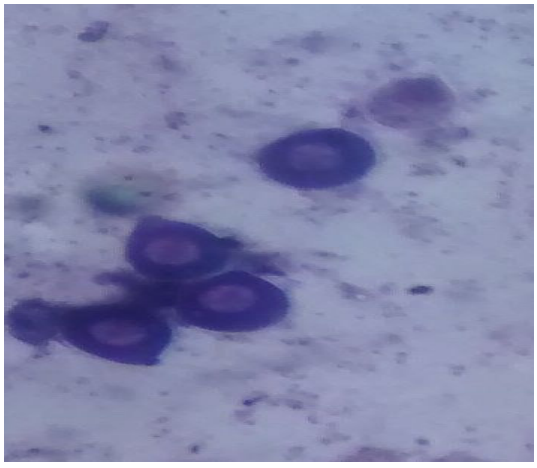

Figure 10
Mantle-Clam

RFTM Assay

Ray's fluid thioglycollate medium assay of the oyster and clam target tissues showed the enlarged blue black hypnospores characteristic of Perkinsus. The samples collected from the pulicat backwaters showing a prevalence of 100%. The hypnospores were circular in appearance.

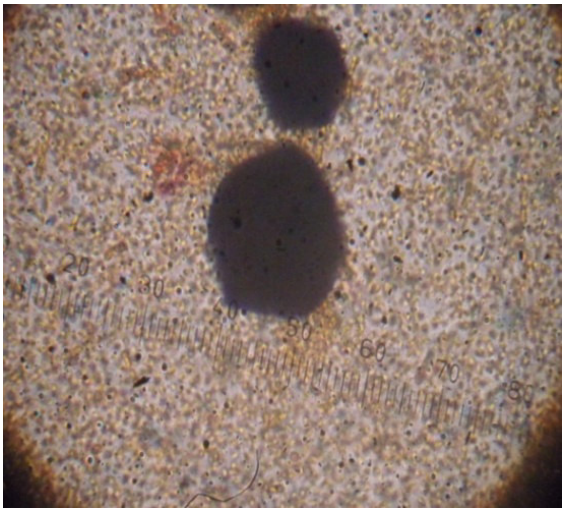

Figure 11
Magnified hypnospores

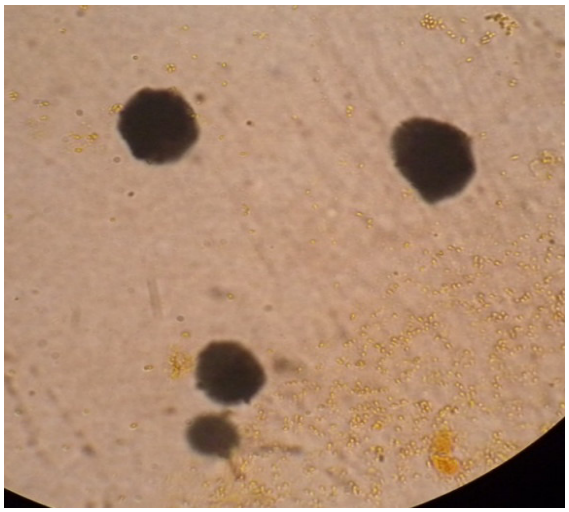

Figure 12
Magnified hypnospores

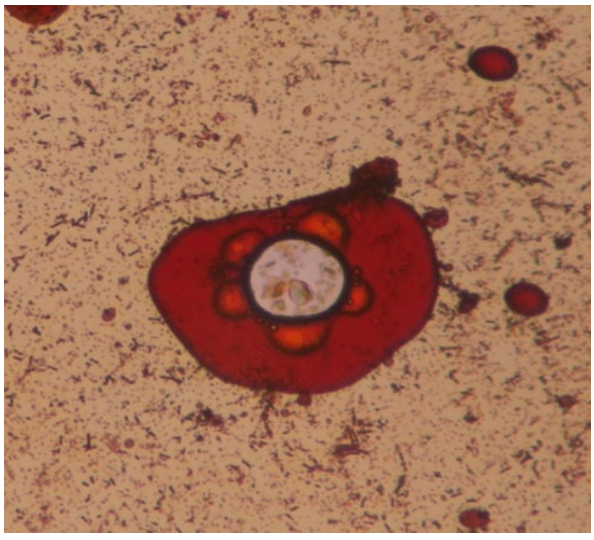

Figure 13
Signet ring appearance of hypnospore

b) Marteiliosis

Tissue imprints

The target tissues of the bivalve samples shows the presence of Pale halos around large, strongly Stained granules and, in larger cells, cell within cell arrangements are observed

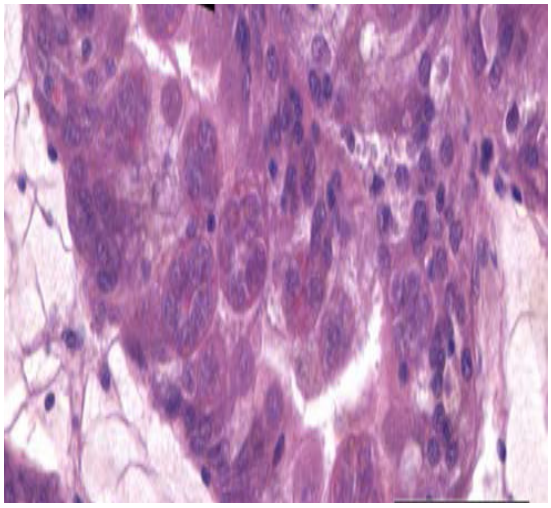

Figure 14
Pale halop rounds of oyster

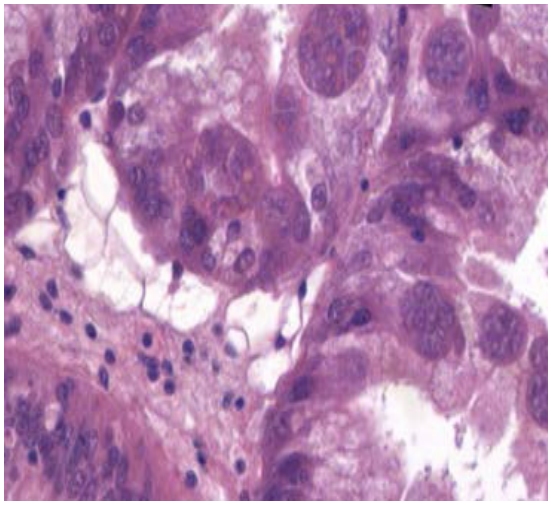

Figure 15
pale halo rounds of mussel

II. Histopathology

a)Perkinsosis

Perkinsus like organisms measuring 4.7µm to 7.3µm were observed in the histological preparations. However typical 'signet ring' stages of trophozoites were not observed in the histological preparations studied.

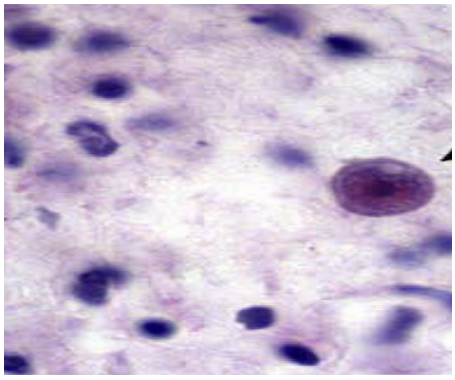

Figure 16
Trophozoites of oyster showing eccentric nucleus

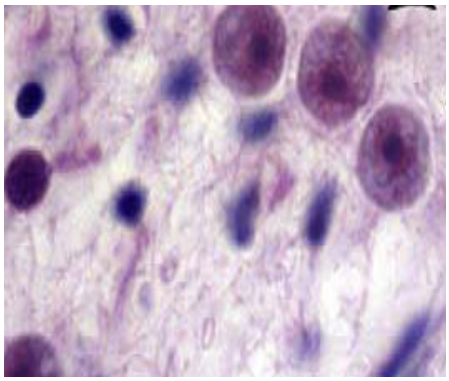

Figure 17
Trophozoites of clam showing eccentric nucleus

b) BONAMIOSIS

A positive result is the presence of parasites as very small cells of 2–5 µm wide within the Haemocytes

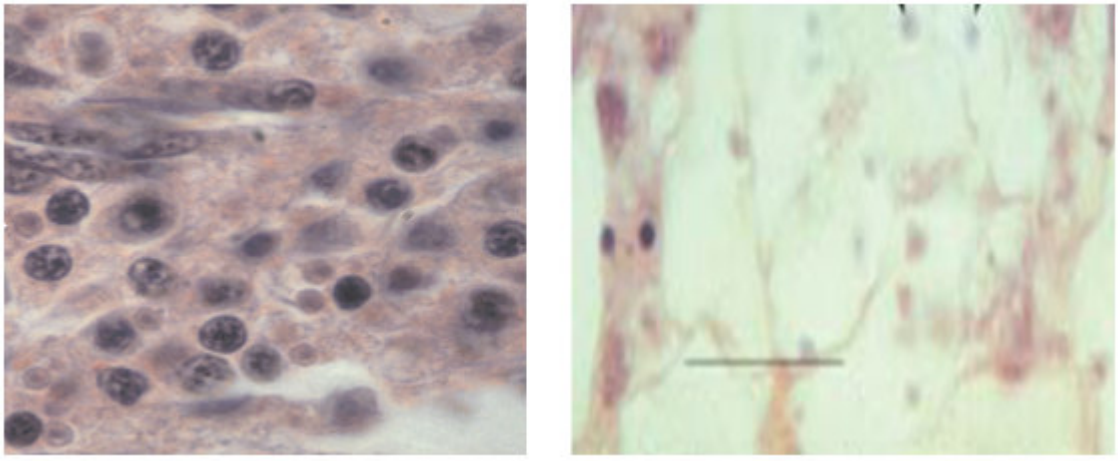

Figure 18 & 19
showing the Bonamia parasites within haemocytes. Both single nucleate forms and replicating binucleate forms.

III.PCR
a) Perkinsosis
PCR screening

PCR screening of the tissues using the perkinsus genus specific primers amplified the product specific to Perkinsus sp.(700bp) confirming the presence of perkinsus sp. Infection.

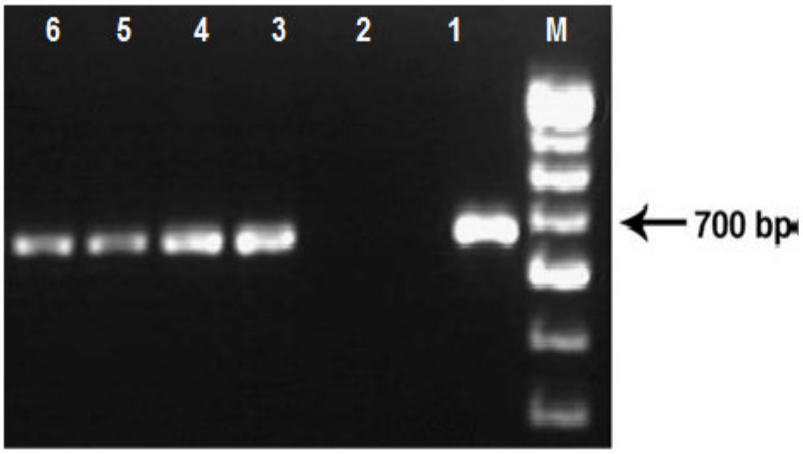

Figure 20

Lane-M -100bp molecular weight DNA marker,
Lane-1 –Positive control,
Lane-2 –Negative control,
Lane-3 –PCR product got amplified with positive template (oyster),
Lane-4 –PCR product got amplified with positive template (oyster),
Lane-5 –PCR product got amplified with positive template (clam),
Lane-6 –PCR product got amplified with positive template (clam)

b) Bonamiosis

PCR screening of the tissues using the bonamiosis genus specific primers amplified the product specific to Bonamiosis sp.(300bp) in oysters (Lane 1&2) confirming the presence of bonamiosis sp. infection and absence in clam (Lane 3&4).

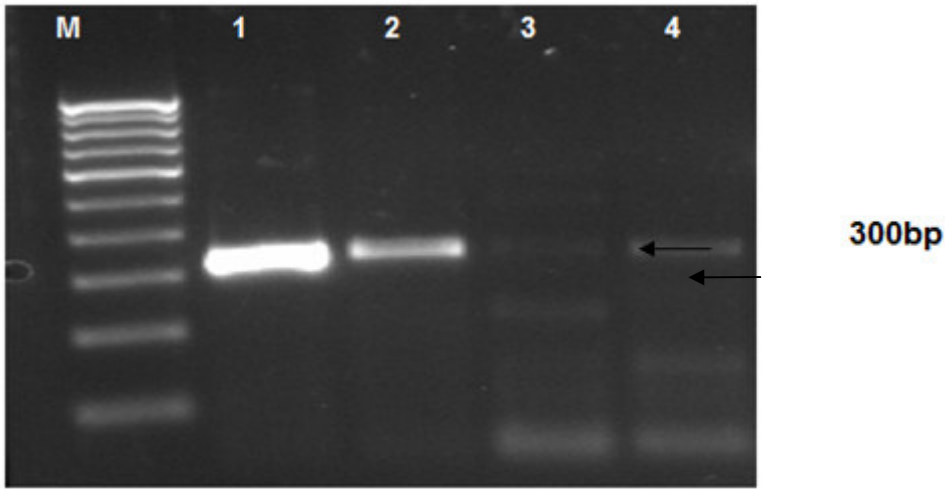

Figure 21

Lane-M – 100bp molecular weight DNA marker,
Lane-1 –PCR product got amplified with template DNA (oyster),
Lane-2 –PCR product got amplified with template DNA (oyster),
Lane-3 –PCR product got amplified with negative template (clam),
Lane-4 –PCR product got amplified with negative template (clam)

c) Marteiliosis

PCR screening of the tissues using the Marteiliosis genus specific primers amplified the product specific to Marteiliosis sp.(266bp) confirming the presence of Marteiliosis sp. infection.

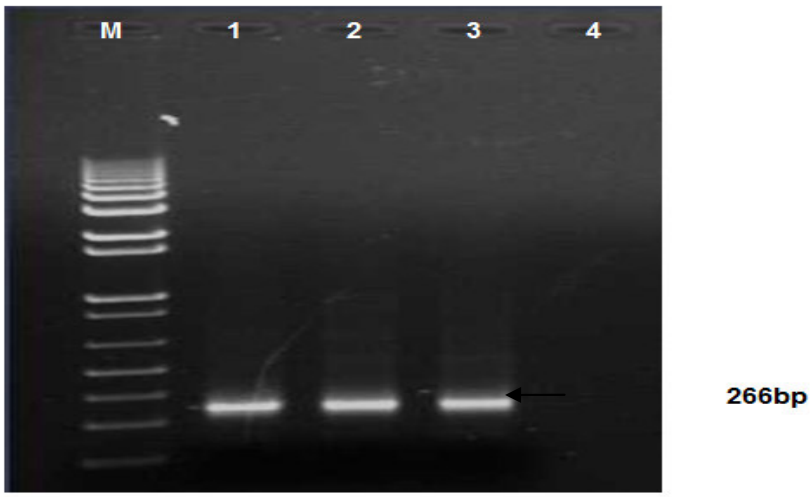

Figure 22

Lane-M - 1kb molecular weight DNA marker,
Lane-1 –PCR product got amplified template DNA (mussel),
Lane-2 –PCR product got amplified template DNA (mussel),
Lane-3 –PCR product got amplified template DNA (oyster)
Lane-4 –PCR product got amplified with negative template (oyster)

DISCUSSION

The result of the three bivalve samples namely oyster, clams and mussels shows the presence and absence of the particualr parasites which are perkinsosis, bonamiosis, marteiliosis. The tissue imprint technique for perkinsosis and marteiliosis shows the prevalence of those parasites in the target tissues of the samples. Wet mount is not applicable for all the three parasites for screening *OIE 2000*[6]. Although this tissue microscopic examination technique does not confirm the presence of the disease to 100% but also it is considered as the screening technique. The major confirmation techniques are the histopathology and PCR. Histopathological results confirms the presence of parasites. Polymerase Chain Reaction (PCR) result images confirms the presence of parasites namely perkinsosis, bonamiosis, marteiliosis in the bivalve samples. The above results and discussion confers that the bivalve samples were screened by microscopic and several methods would achieve the detectable presence of the parasites.

CONCLUSION

Thus bivalve samples are screened for parasites. Bivalves infected by the diseases are harmful to humans. This diagnostic methods are more useful in identifying the infected bivalves and those infected bivalves are immediately transfered to separate places to avoid the other bivalves from getting this infection. Transmission is direct from host to host. In conclusion, research work can further be initiated towards validating and standardizing this formulation for clinical and preclinical studies.

REFERENCES

1. Klinger,d.h. et al..Moving beyond the fished or farmed dichotomy.Marine policy.(2012)
2. Barnes,R.D. invertebrate zoology, 4th edn. W.b. Saunders co., Philadelphia. (1980)
3. Audemard, C., Calvo, l.M.R., Paynter, k.t., Reece, k.s.,Burreson, e.m.,. Real-time pcr investigation of parasite ecology: in situ determination of oyster parasite *perkinsus Marinus* transmission dynamics in lower chesapeake bay(2006)
4. Chapman A.D, numbers of living species in the world, second edition. (2009)
5. Comps, m., and Duthoit, j.l. Infections virales chez les huîtres *crassostrea angulata* (lmk) et *c. Gigas* (th.). *Haliotis* 8, 301-308, (1979)
6. Paynter,Bondad-reantaso Tourtip mari, puoltes, international journal of pharma and biosciences *Soc.* 36, 229–248 (2011).
7. Villalba a., Mourelle s.g., carballal m.j. & lopez m.cEffects of infection by the Protistan parasite *marteilia refringens* on the reproduction of cultured mussels *mytilus galloprovincialis* in Galicia (nw spain). *Dis. Aquat. Org.,* 17, 205–213, (1993)
8. Corbeil s, Arzul i, robert m, heasman m, cholet b et al. Molecular Characterization of an australian isolate of *bonamia exitiosa*. *Dis aquat org*;71,81-85.(2006)
9. Berthe F.C.J., Le roux F., Peyretaillade e., Peyret p., rodriguez d., Gouy m. & vivarès c.p. The existence of the phylum Paramyxea Desportes and Perkins, 1990 is validated by the phylogenetic analysis of the *Marteilia refringens* small subunit ribosomal RNA. *J. Euk. Microbiol*., 47 (3), 288–293. (2000).
10. Oie (office international des epizooties) diagnostic Manual for aquatic animal diseases, 3rd edn. Oie, paris, P 129–134.(2000)