

New trends in diagnosis of parasitic infections in marine fishes

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Abstract: Parasitic diseases of fish are very important in aquaculture as it was the basis for all outbreaks of fish. It is very important to defiantly diagnose and control of the parasitic diseases of fish to avoid great economical losses in aquaculture. So it is very important to discover new techniques for diagnosis of fish parasitic infections. So that the aim of the present review was to throw light on the new trends of diagnosis of the parasitic diseases of marine fishes. Recent methods for diagnosis illustrated in the present review are immunodiagnostic techniques depends on antigen –antibody reaction, the methods includes; agar gel precipitacion test, Agglutination test, ELISA, Dot ELISA, fluorescent antibody technique and immunohistochemistry. Developments in molecular biology have led to a rapid growth in new methods for diagnosing fish parasitic infections. Techniques of major significance include polymerase chain reaction (PCR) for specific accurate diagnosis of fish parasites. In addition, scanning and transmission electron microscope which considered developed method for identification of fish parasites depending on computerized digital image analysis.

[Hussien A.M. Osman and Mona S. Zaki. **New trends in diagnosis of parasitic infections in marine fishes.** *Stem Cell* 2017;8(4):125-133]. ISSN: 1945-4570 (print); ISSN: 1945-4732 (online). <http://www.sciencepub.net/stem>. 21. doi:[10.7537/marsscj080417.21](https://doi.org/10.7537/marsscj080417.21).

Key words: diagnosis, parasitic diseases, marine fishes, immunodiagnostic, PCR, Scan electron microscope.

1. Introduction:

Parasitic diseases form about 80% of fish diseases. This might be because of the long time of warm climate and wealth of characteristic sustenance and also the accessibility of the main intermediate hosts like Cyclops, Mollusca and parasites. The parasite is the premise of all outbreaks of fish that took after and showed by optional disease with microorganisms or organisms prompting serious monetary misfortunes spoke to as high morbidity and mortality (Eissa,2002).

Parasitic diseases can affect on aquaculture systems in a number of methods which will determine their economic cost (Sommerville, 1998; Lagrue and Pouline,2015). Mortality has evident costs determined by the size and age of the fish, but, more often, parasite infection causes morbidity and loss of appetite with a resultant waste of food, reduced food conversion efficiency and specific growth rate, which over a grow-out period in a population of fish, may account for a noteworthy proportion of the profits (Trujillo-Gonzalez, *et al.*,2015).

In some cases, parasite infections might be zoonotic, or reduce the market value sector esteem inferable from their expansive size or unaesthetic appearance, and this has been known not dismissal of whole loads of fish even where there was no detectable effect on the welfare of the fish; other parasitic diseases may influence brood stock quality (Santoro *et al.*, 2013; Timi and Mackenzie, 2015).

A common mistake of fish culturists is misdiagnosing infection problems and treating their

diseased fish with the wrong drug or chemical. At the point when the substance doesn't work, they will attempt another, then another. Selecting the wrong treatment in light of misdiagnosis is a waste of time and money and might be more hindering to the fish than no treatment by any stretch of the imagination. The greater part of fish parasites must be distinguished by the utilization of a microscope. In the event that the microscope, or the individual utilizing it has no past experience, the determination is troublesome and faulty (Noga, 2010).

Molecular techniques can be used to solve that type of problems and increase sensitivity and specificity of pathogen detection. These techniques include polymerase chain reaction (PCR), restriction enzyme digestion, probe hybridization, in situ hybridization, and microarray. Since molecular diagnostic techniques are faster and more sensitive than conventional diagnostic techniques, pathogens can be detected from fish without any symptoms, so disease outbreak could be controlled. Thus, antibiotic and antiparasitic treatment can be reduced and pollution of the environment may be eliminated (Stokes *et al.*,1999; Hutson *et al.*, 2012; Muktar *et al.*,2016).

Thus, the present review was aimed to throw the light on the new trends of diagnosis of marine fish parasitic infections like molecular techniques for the detection and identification of parasitic fish pathogens. Also transmission and scan electron microscope.

The specified ability (available techniques) clarifies why, as showed below, for most parasitic

diseases the diagnosis is simply taking into account basic strategies (Clinical signs, macroscopical examination (PM) and microscopical examination) furthermore histopathology. Subsequently, the likelihood of ignoring or misidentification of a few parasites can't be rejected (Noga,2000). Efforts should be done to enhance the specialized level of indicative research centers, keeping in mind the end goal to get more exact and affirmed conclusion and confirmed diagnosis of parasitic infection in fish (Woo,1995).

A. Traditional methods for diagnosis of parasitic fish infections:

- 1-Case history.
- 2-Clinical signs.
- 3-Macroscopical examination (PM lesions).
- 4-Microscopical examination of fresh samples.
- 5-Isolation and identification.
- 6-Histopathological examination.

Identification of parasitic infections still, as generally, depend on experienced microscopists and customary taxonomy is crucial in the investigation of parasitology (Monis, 1999). Nevertheless, new methods can make vital commitments to the finding, scientific categorization and screening for parasitic infections (Gasser, 1999).

B. New trends in the diagnosis of parasitic fish infections:

- 1-Immunological Diagnosis.
- 2- Molecular diagnosis.
- 3-Transmission and Scan electron microscopy (SEM).

1-Immunological Diagnosis:

Advantages of immunological diagnosis:

Antigen-Antibody reactions are extremely specifically and sensitive. These form the basis of immunodiagnosics. These tools are utilized for the qualitative and quantitative evaluation of the pathogens and the defensive antibodies. These tests can be utilized at the ranch level without the help of the tools (Ndao, 2009).

Along these lines, in the advancement of diagnostic procedures in aquaculture, antibody- based (protein-based) immunodiagnosis which plays an essential role. This strategy has the favorable position over other traditional techniques in that it can distinguish sub-clinical/inert/ carrier state of infection and can determine the antigenic differences (Ndao, 2009).

This method is generally quicker and more specific and sensitive. Further, change of ordinary immunodiagnostic methods has brought about the movement of monoclonal antibody- based systems and this has expanded the fineness of detection and has permitted contemplating the pathogenesis of infections. Nevertheless, the specificity of antibodies also confines their helpfulness since real antigens are

not protected among life stages of specific pathogens. Despite the fact that there is an arrangement of polyclonal and monoclonal antibodies-based diagnostics ready for different aquatic animal pathogens (Bartholomew *et al.*, 1998).

1. Agar Gel Precipitation Test,
2. Agglutination Test,
3. ELISA,
4. Dot ELISA,
5. Fluorescent Antibody Test.
6. Immunohistochemistry.

1.1. Agar Gel Precipitation Test

In this test, immune response and present antigens are put in wells in agar plates and permitted to diffuse toward each other. The immune response is put in a center well and antigens (specific or non-specific) are set in encompassing wells. At the point when an immune response and its specific antigen meet each other at the correct concentration, the precipitate will form a clear white line between the two wells. This band is known as a precipitation line Bailey and Graham (1996) (Fig. 1).

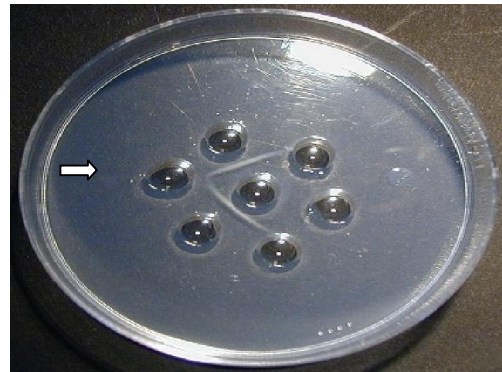


Fig 1: Showing agar gel precipitation test (arrow) (google).

1.2. Agglutination Test

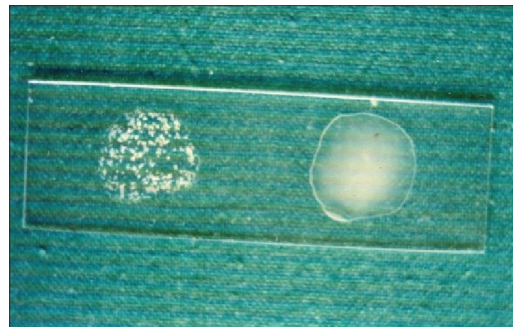


Fig 2: Showing agglutination test antigen-antibody reaction (+ result arrow) (google).

The test is utilized to determine unknown antigens; blood with the unknown antigen is blended

with a known antibody and regardless of whether agglutination happens determine the antigen; utilized as a part of tissue appropriating and blood grouping and diagnose. In the direct agglutination test, serum is added to the cells that have the surface self-Ag to be tested (**Hudson and Hay,1989**) (Fig. 2).

1.3. ELISA

The motivation behind an ELISA is to determine whether a specific protein is available in a specimen and assuming this is the case, how much. There are two primary minor departure from this strategy: you can determine the amount of antibody in a specimen, or you can determine the amount of protein is bound by an immune response. The uniqueness is whether you are attempting to evaluate an antibody or some other protein (**Hudson and Hay,1989; Rohde, 2002**).

In this illustration, we will utilize an ELISA to determine the amount of a specific antibody agent is available in the blood test. ELISAs are performed in 96-well plates which give high throughput results. The base of every well is enveloped with a protein which will join to the antibody you need to quantify. Entire Whole blood is permitted to clot and the cells are centrifuged out to come about the unmistakable serum with antibodies (called essential antibodies). The serum is incubated in a wall, and every well contains a different serum. A positive control serum and a negative control serum would be included among the 96 samples being tested. After a time, the serum is removed and weakly adherent antibodies are washed off with a series of buffer washing (**Hudson and Hay,1989**).

The parasite-particular IgG antibody test utilizing the ELISA strategy has been produced in Korea since 1982, patients from hospitals across the nation are alluded the clinics for diagnosis and observing of four tissues attacking parasitic diseases. Their study was led to determine the prevalence of IgG antibody against *C. sinensis* *Paragonimus westermani* *T. solium* metacestode, and sparganum by ELISA (**Lee and Hall, 2009**).

1.4. DOT ELISA

The main difference between the regular ELISA and the dot-ELISA represents as the surface used to bind the antigen of choice. In the dot-ELISA, the plastic plate is replaced by a nitrocellulose or other paper membrane onto which a small amount of sample volume is applied. The choice of binding matrix greatly improved the specificity and sensitivity of the assay by reducing the binding of nonspecific proteins usually observed when plastic binding matrixes are used (**Pappas et al., 1998**).

The advantages of this procedure incorporate its usability, its speed, and the simplicity of result understanding. It is quick, and financially savvy and all the more significantly, can be utilized as a part of

the field. For all these reasons, the Dot-ELISA has been is still widely utilized as a part of the identification of human and animal parasitic diseases, including amebiasis, babesiosis, fascioliasis, cutaneous and instinctive leishmaniasis, cysticercosis, echinococcosis, schistosomiasis, toxocaraiasis, toxoplasmosis, trichinosis, and trypanosomiasis. In the most recent couple of years, distributed studies have shown the utilization of the dot-ELISA for the diagnosis of *Fasciola gigantic*, *Haemonchus contortus*, *Theileria equi*, *Trypanosoma cruzi* (**Kumar et al., 2008**), and *Trypanosoma brucei* (**Courtioux et al., 2005**).

1.5. Fluorescent Antibody Test

It is a research center test that utilizations antibodies labeled with fluorescent color that can be used to reveal the presence of microorganisms. This strategy produces a clear detection of antigens utilizing the fluorescently marked antigen-specific antibodies. Since detection of the antigen in a substrate of test (cell smear, liquid or patient-immunized culture medium) is the objective (**Hudson and Hay,1989**) (Fig.3).

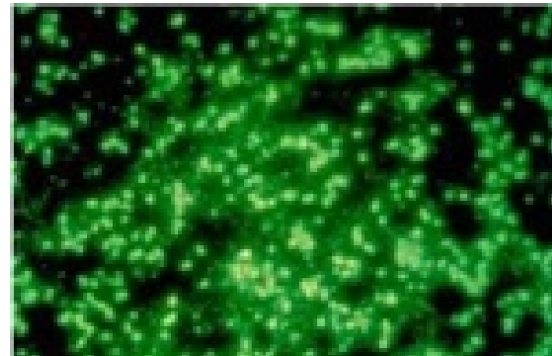


Fig 3: Showing specific antibody attached to florescent dye (antibody binds to antigens) Using a fluorescent microscope (+ resultant arrow) (google).

1.6. Immunohistochemistry

Immunohistochemical strategy used to distinguish specific pathogens in tissue fragments. Immunohistochemical recoloring strategies have been produced for the recognition of infections.

Pathogen antigen is limited by an antibody agent raised against the infection and subsequent identification steps result in a coloured item (colored antigen parasite-bacteria or virus) that can be inspected by light microscopy. Immunohistochemistry used for conclusion of marine fish parasites. Rabbit immune response was increased vas *Sphaerospora dicentrarchi*, a tissue parasite of Sea bass. Light and electron immune-histological recoloring were utilized to decide the specificity (**Smis et al.,1989**).

In light immunohistochemistry the polar capsules and valves of the *S. dicentrarchi* spores showed up unequivocally stained, though formative stages were most certainly not. Electron microscopic histochemistry revealed an extreme marking in valves and some developmental stages. The cross - response was seen with all the myxosporean parasites examined, even with those having a place with other genera. Polar capsules of all the myxosporean species with the exception of *Polysporoplasma sparis* were the main structures stained by the polyclonal antibody (Smis *et al.*,1989).

These perceptions could uncover the presence of moderated antigenic epitopes in polar capsules of various Myxosporea (Muñoz *et al.*, 1998). The enteric myxozoan parasites *Enteromyxum leei* (Diamant, Lom et Dyková) and *Enteromyxum scophthalmi* Palenzuela, Redondo et Álvarez-Pellitero are in charge of high weight reduction in infected fish, which prompts subchronic disease and low death rates in gilthead Sea bream (GSB), *Sparus aurata* L., and to high death rates in turbot, *Psetta maxima* (L.). The detection of initial parasite stages in histological sections is particularly difficult, however can be streamlined by method for specific antibodies. Rabbit polyclonal antibodies (pAbs) were raised against *E. scophthalmi* and *E. Lee*, and direct protein connected immunosorbent examine (ELISA) and immunohistochemistry were utilized to portray their affectability and specificity (Estensoro *et al.*, 2014).

2. Molecular techniques in diagnosing fish parasitic infections:

Great advances have been made in improving the sensitivity and specificity of diagnosis of bacterial, viral, and parasitic fish diseases. In the molecular techniques, typically, DNA is extracted from the sample which can be probed by DNA hybridization and analyzed by restriction fragment length polymorphism (RFLP). More commonly, DNA is amplified by the polymerase chain reaction (PCR) using specific primers for diagnostic sequences (MacKenzie and Abaunza, 2013).

Techniques of molecular diagnosis of parasitic fish infections:

2.1. Polymerase chain reaction (PCR):

Polymerase chain reaction is a technique for amplifying a specific region of DNA, defined by a set of two "primers" at which DNA synthesis is initiated by a thermostable DNA polymerase. Usually, at least a million-fold increase of a specific section of a DNA molecule can be realized and the PCR product can be detected by gel electrophoresis. The regions amplified are usually between 150-3,000 base pairs (bp) in length (Muldrew, 2009; Timi and Mackenzie, 2015) (Fig.4).

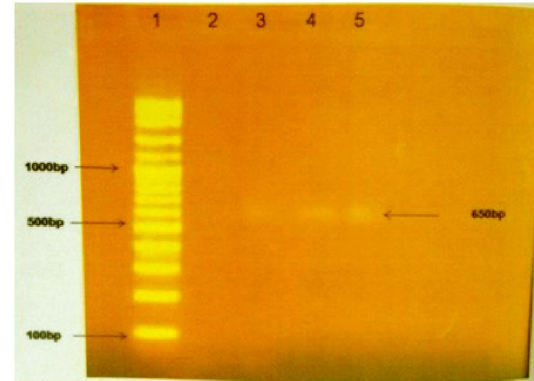


Fig.4: Showing Arepresentaive gel displaying the ssrDNA analysis of ITS-region from individual adult specimens of *Diplectanum* spp. 3,4 and 5 at 650 bp. Lane 1 represents the 100bp DNA ladder as a marker (bp) (El-Raziky, 2016).

Primer design is important to get the best conceivable sensitivity and specificity. The reaction incorporates template DNA that might be in different structures, from a simple tissue lysate to purified DNA, preliminaries, polymerase protein to catalyze formation of new duplicates of DNA, and nucleotides to form the new copies (Timi and Mackenzie, 2015).

During each round of the thermocycling response, the template DNA is denatured, primers anneal to their complementary locales and polymerase compound catalyzes the expansion of nucleotides to the end of every preliminary, along these lines making new duplicates of the objective district in each round. Hypothetically, the expansion in measure of item after each round will be geometric. Reverse transcriptase polymerase chain reaction (RT-PCR) is used to detect specific mRNA and determine levels of gene expression (Koo and Jaykus, 2000; Foltz *et al.*,2009).

In fact this method is so touchy to empower quantitation of RNA from a single cell. In the course of the most recent quite a while, the improvement of novel sciences and instrumentation stages, enabling detection of PCR products on a constant premise has prompted far reaching appropriation of ongoing RT-PCR as the technique for decision for quantitative changes in quality expression. The affectability and specificity accomplished in a very much composed RT-PCR makes it a perfect device for use in the reconnaissance and checking of undercover infections (Altinok and Kurt, 2003; El-Raziky, 2016).

2.2. Multiplex PCR:

Novel advancements, for example, outline of PCR conditions that can identify several pathogens at one time in a multiplex response will enhance time and cost-productivity of this system, countering one of the significant contentions against the selection of these methods as standard. In multiplex PCR more

than one target sequence can be enhanced by including more than one sets of primers in the reaction (Williams *et al.*, 1999).

Multiplex PCR can possibly give significant investment funds of time and exertion inside the lab without trading off test utility. Since its presentation, multiplex PCR has been effectively connected in numerous regions of nucleic acid diagnostics, including quality erasure investigation (Chamberlain *et al.*, 1989), quantitative examination (Rithidech *et al.*, 1997), and RNA location. In the field of irresistible fish disease, the strategy has been appeared to be a significant technique for recognizable proof of infections, microscopic organisms, growths and parasites in the same time (Zou, 1997; Cunningham, 2002).

2.3. Restriction enzyme digestion:

The common use for restriction enzymes is to generate a "fingerprint" of a particular DNA molecule. Because of the sequence specificity of restriction enzymes, these enzymes can cut DNA into discrete fragments which can be separated by gel electrophoresis. This model of DNA fragments make a "DNA fingerprint" and each DNA molecule has its own fingerprint. Other restriction enzymes can be used to further characterization of a particular DNA molecule. The location of these restriction enzyme sites on the DNA molecule can be compiled to create a restriction enzyme map (Grizzle *et al.*, 2002) (Fig.5).

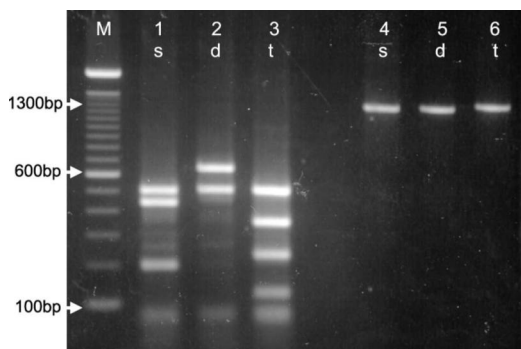


Fig. 5: Showing PCR-amplified internal transcribed spacer region of ribosomal RNA genes from *Gyrodactylus salaris* (lanes 1 and 4), *G. derjavini* (lanes 2 and 5) and *G. truttae* (lanes 3 and 6), undigested (lanes 4– 6) and digested with restriction enzyme *Sau3AI* (lanes 1 – 3) to reveal species-specific RFLP (Cunningham, 1997).

Molecular systematics majorly affect some taxes and it will enthusiasm to perceive how they overlay traditional taxonomy. Molecular systems generally are more valuable for Parasitology than the immunodiagnostic methods, e.g. IFAT and mAB probes, for reasons of effortlessness, affectability and accuracy (Holzer *et al.*, 2007; El-Raziky, 2016)..

Different protozoan and metazoan parasites are critical issues in fish societies. The quantity of nucleic acid systems accessible for their recognition and recognizable proof is similarly substantial (Gasser, 1999).

To date, detection and identification of parasites in aquaculture have concentrated on the ribosomal RNA (rRNA) gene array, the ribosomal DNA or rDNA. These genes are ideal targets for diagnosis for several reasons; they occur in tandem array along the chromosome and this multi -copy arrangement provides multiple targets for primer or probe binding (Waters and McCutchan, 1990).

The genes contain highly conserved regions, notably at the termini, which enable the use of 'universal' primers that will amplify certain genes from almost any eukaryote or prokaryote (Medlin *et al.*, 1988). There are also more variable regions of sequence within the genes and spacers. These have been found to vary between groups and species of parasite, enabling design of methodology that will separate organisms at the appropriate taxonomic level (Kent *et al.*, 1998).

So useful are the ribosomal RNA genes that the term 'riboprinting' has been coined for the technique of restriction enzyme digestion of PCR amplified rDNA that results in a 'fingerprint' pattern that enables identification of species or strains (Xiao and Desser, 2000; Dominik *et al.*, 2010; Liu *et al.*, 2011).

Metazoan parasites, for example, the louse, are regularly promptly obvious and recognized, however some groups are more dangerous. More than 400 types of the monogenean class *Gyrodactylus* have been depicted (Williams and Jones, 1994). Of these, one, *G. salaris*, is a harmful pathogen of Atlantic salmon, in charge of virtual killing of this profitable fish in numerous Norwegian waterways (Mo, 1994).

Exceptional shield measures are set up to shield other vulnerable salmon populaces from disease, which can be effortlessly spread with developments of fish. Successful operation of these measures depends on exact discovery of *Gyrodactylus* species, the vast majority of which are totally innocuous. Distinguishing proof by examination of the attachment organs under high power light microscopy requires incredible experience and expertise (El-Raziky, 2016).

These methods exploit sequence differences in rRNA genes and spacers in the design of species-specific DNA probes (Cunningham *et al.*, 1995 a, b) and RFLP (Cunningham, 1997) applied to DNA amplified by PCR. Similar variations have now been discovered that detect *G. salaris* and *G. thymalli*, two species that have previously been virtually indistinguishable. Regions of the rRNA genes have been found to provide reliable markers for a large

number of *Gyrodactylus* species.

About other metazoan fish parasites, the anisakid nematodes have gotten consideration because of their capacity to infect humans (Adams *et al.*, 1998). The internal deciphered spacer of the rRNA array has been broke down and PCR combined with RFLP or single-strand confirmation used to distinguish species and help further investigation of these parasites (D'Amelio *et al.*, 2000).

The detection and study of protozoan parasites offers greater challenges for molecular diagnostics due to their small size and endoparasitic nature. The problem includes detection and is not just a question of identifying a parasite that is known to be there because it can be seen under low-power magnification or with the naked eye. So this work has centred on the rRNA genes (Kent *et al.*, 2000).

Great progress has been made in the study of the Myxozoa (Bartholomew, 1998). Of these, attention has centred on *Myxobolus cerebralis* and PKX, the causative agents of salmonid whirling disease and proliferative kidney disease, respectively. Sequences from the rRNA genes have been analysed to study these parasites at the levels of genera and isolate (Salim and Desser, 2000). Sequences from the small subunit rRNA gene confirmed that PKX is indeed a myxosporean (Saulnier *et al.*, 1999). rRNA sequences have now been used to study the life cycle, host range and alternating stages of these parasites (Xiao and Desser, 2000). The final identification of PKX as *Tetracapsula renicola* (Kent *et al.*, 2000) is a notable success of molecular approaches. Of great interest for diagnosticians is the development of probes, also based on small subunit rRNA sequences, that can be used in nonradioactive in situ hybridisation to detect these parasites in host tissue sections (Antonio *et al.*, 1998; Morris *et al.*, 1999). In addition to ribosomal RNA studies, analysis of PKX antigen genes has begun (Saulnier *et al.*, 1996), providing further targets for study, especially examination of immunogenicity.

Several myxozoan infections are noteworthy problems in North American salmonid creation and can possibly bring about comparable pathology in European aquaculture, in spite of the fact that not in the blink of an eye managed under Community enactment. A basic test for the presence of *Ceratomyxa shasta* is given by PCR amplification of parts of the little subunit rRNA gene (Palenzuela *et al.*, 1999 a, b) *Kudoa thyrsites*, which is additionally found in Europe (Barja and Toranzo, 1993), is not horribly noticeable but rather might be distinguished microscopically (Brocklebank, 1997). Taxonomic investigation of this organism has started, utilizing rRNA qualities (Hervio *et al.*, 1997).

The microsporidia *Nucleospora salmonis* and *Loma salmonae* have additionally been examined in North America. By, amplification and examination of rRNA genes and spacers give demonstrative PCR measures that can be connected to early stage diseases (Barlough *et al.*, 1995; Docker *et al.*, 1997) and to produce data for further investigation of the organism (Nilsen, 2000).

4. Advantages of Molecular Methods

Give high sensitivity and rapidity of diagnosis. Principal advantage of molecular diagnostic methods is in the detection of non-cultivable pathogens; DNA amplification can assist in detecting the pathogens (parasite) that are present in low numbers (handling a tiny volume of specimen). Can be used to detect potential infection and thereby identify the (carrier hosts) of infection that is expressive in epizootiology. Can be used to differentiate antigenically similar pathogens (El-Raziky, 2016).

2.5. Disadvantages of Molecular methods:

Molecular methods are cost-intensive procedures. These tests cannot detect unsuspected pathogen. Molecular methods will have difficulty in detecting new pathogens as the exclusive use of these would overlook such infections (Singh, 1997).

3. Scanning and transmission electron microscopy:

Morphology-based strategies are still to a great degree utilized, for example, SEM and TEM (scanning and transmission electron microscopy) (Fig. 6, A, B and D) and are key for deciding parasitic protozoa and other little histozoic stages of parasites (Stadtländer, 2007).

Basic morphology is supported by:

1-Specific staining techniques, e.g. chaetotaxy (Shinn *et al.*, 1998),

and newer technologies such as:

2-Confocal microscopy (Arafa *et al.*, 2007) and

3- Image analysis.

Advancements in image analysis have made it conceivable to examine digitally forms to determine the key elements. This has been most developed during the time spent the recognition of *Gyrodactylus salaris* from other salmonid types of the class (Shinn *et al.*, 1998). The key attributes of types of the gyrodactylids are the size, shape, number of the hard parts, the sclerites, and copulatory organs (Fig. 6, C) and these help promptly to computerized picture examination. Late advancements have demonstrated that these can be semi-computerized, accordingly easing the requirement for authority taxonomists (Harris *et al.*, 2008) (Fig. 6).

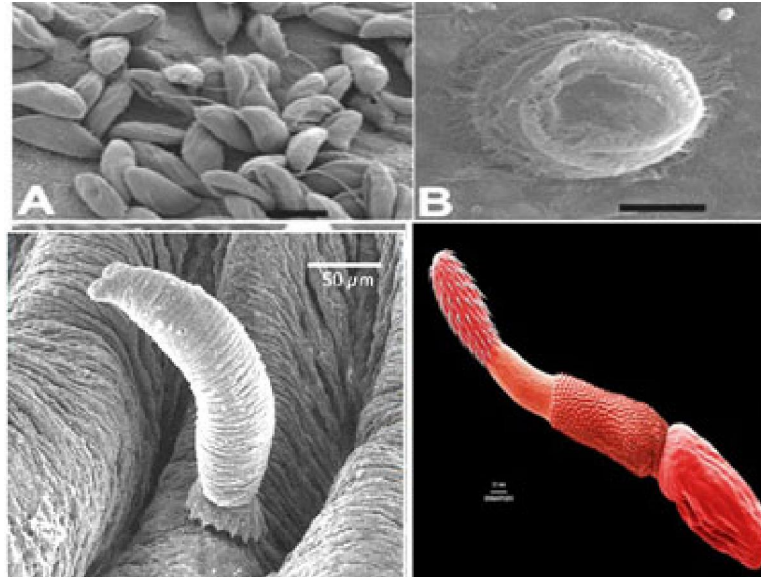


Fig. 6: Showing (A) *ichthyobodo necator* on dorsal fin of rainbow trout. (Zoological Museum, University of Copenhagen, Denmark), (B) Showing *trichodina sp.* on caudal fin of rainbow trout. Scale bar 20 μm . (Courtesy of Dr J. Bresciani, University of Copenhagen, Denmark), (C) SEM of *gyrodactylus* attached to skin of Seabass fish (google) (<http://www.fish-as-pets.com/2007/09/monogenean-parasites-marine-fish.html>) and (D) SEM of *Acanthocephalan proboscis* (google).

Conclusions:

Traditional methods for diagnosis of parasitic diseases should be displayed as it is considered the stone corner of identification of parasites of marine fishes. Confirmative diagnosis using recent advances such as molecular diagnosis (PCR, Multiplex PCR and Restriction enzyme digestion) and scanning electron microscope should be carried out.

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