Original Article

New diagnostic methods of parasitic infections in freshwater Fishes

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ABSTRACT

Freshwater fishes are, doubtlessly, highly exposed to be infected by the parasitic infections which cause lots of economic losses. Accurate, early and fast diagnosis and controlling these parasitic diseases are of great importance. Nowadays, traditional methods in diagnosis are no more effective in the semi-intensive and intensive ways of culturing fish. Quick and easy spread of the infection from a district to another, even international spread is done with no boundaries. Traditional diagnosis may be fast, but it depends mainly on either naked eye remaking or wet mount examined under light microscope. The biggest disadvantage of this method is that it depends mainly on the expert that may be wrong or right. Therefore, using recent methods for diagnosis of fish parasitic diseases have become more accurate, faster and safer to prevent and control these parasitic diseases that prevailed in wild and cultured domestic or imported fishes. These recent methods include light microscopes to examine histopathological slides stained with histochemical stains, electron microscopes to examine tiny details of specific parasites, immunological tests that depend on antigen-antibody reactions, and molecular techniques which depend on DNA sequencing after fractionation and amplification of the parasites. The conventional methods for diagnosis of parasitic infections is considered the cornerstone for the identification of the parasites. While, non-conventional methods for diagnosis of parasitic infections should be applied. Molecular techniques and scanning electron microscope are considered new trends in the diagnosis of parasitic infections. Further studies are needed to investigate the strategy of diagnosis and control of parasitic infections of freshwater fishes.

Keywords: Freshwater fishes, Molecular techniques, scanning electron microscope

Introduction

In Egypt, fish diseases affect the fish production especially parasitic infections which are about 80% of the fish diseases [1]. This record is attributed to the longtime warm climate and weather of the characteristic sustenance of reproduction of the intermediate hosts for parasitic life cycle [2]. Parasitic diseases can affect the wild and cultured freshwater fishes directly (mortality rate) or indirectly (morbidity rate) causing increased economic costs [3, 4]. Parasitic infections cause morbidity with the signs of absence of reflex, off food, rates of conversion becoming less, prolonging the period of growth that causes more expenses [5, 6]. Some of zoonotic parasites affect marketability for their expensive or undesirable appearance [7, 8]. The delay in diagnosis and misdiagnosis of the parasitic infections may cause mass mortalities specially in cultured freshwater fishes [9]. The rapid and accurate diagnosis, therapy help to avoid indiscriminate use of chemo-therapeutics, and the case history will be useful during examining samples for diagnosis [10]. Light microscope is commonly used in the diagnosis of almost all parasitic fish infections directly seen by scraping the infested fish or examining the histopathological slides which should be stained by specific stains which have been developed to find parasites [11]. Electron Microscopy is used to observe the surface level changes in the parasites at higher magnification [12]. Agar gel precipitation, agglutination, ELISA, Dot ELISA, latex agglutination and fluorescent antibody test can be considered as rapid and accurate methods of laboratory diagnosis of some parasitic diseases [13]. The increased sensitivity and specificity of pathogen detection are solved by molecular techniques: restriction enzyme digestion, polymerase probe hybridization, chain reaction, in sit hybridization, and microarray [14]. The pathogens can be detected from fish without any signs, so the disease outbreak could be controlled because molecular diagnostic techniques have become faster and more sensitive than conventional diagnostic techniques [15].

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The traditional methods for diagnosis of parasitic infections in freshwater fishes:

The most common conventional way for diagnosis of a parasitic fish infection is isolation and identification of the infected organisms by clinical pictures, and directly visualization under light microscope (wet mount or stained parasites) [10]. Encysted parasites can also be examined under the microscope to interpret histopathological slides and stained microorganisms [7]. One of the important steps in the evaluation of the parasitic infection process is the laboratory diagnosis. In spite of that, specific parasites can be undetected due to their migration from host during handling and examination. However, the possibility of infection is not included in demonstrating or recording a parasite [16].

Identification of parasitic infections still depends on the experience of microscopists, whereas the taxonomies depend on taxonomists [10].

This requires considerable need apart from being time-consuming and labor-intensive, and all the previous methods should be limited [17].

Light microscopies examine stained fish tissue sections. Immuno-histochemical staining methods have been developed to detect parasites and their reactions. A coloured product that can be visualized by light microscopy indicates that there is an antigen which is localized by an antibody raised against the parasites and subsequent detection steps. Immuno-histochemical strategies used to distinguish specific pathogens in fragments of tissue. Immuno-histochemical recoloring strategies have been produced for the recognition of the infections. The detection of initial parasite stages in histological sections is particularly difficult, however can be streamlined by methods for specific antibodies [18]. Parasitic gill infections and musculature have encysted metacecaria in Nile tilapia [19].

Protozoal gill infection of a goldfish, white spot disease in common carp and monogentic trematod parasite particles in-between the secondary gill filaments in the primary gill lamellae with hyperplasia, and congestion of gills blood vessels and larvae encapsulated nematode embedded in Abdominal musculature of Epinephelus chlorostigma fish [10].

New trends in diagnosis of parasitic freshwater fish's infections.

Nevertheless, new methods can make accurate and rapid diagnosis by various advanced technologies to find scientific categorization and screening parasitic infections such as electron microscopy [12].

Those include:

1. The electron microscopy diagnosis:

Electron microscopes are broadly classified into transmission electron microscopy to observe changes in the tissues at higher magnification and scanning electron microscope to observe the surface level changes in the parasites at a higher magnification [12].

Parasites were collected and prepared for scanning by the electron microscope are as follows: Living specimens were kept for 30 minutes in the refrigerator before fixation in 4% gluteraldehyde solution at 4°C for (48 hrs according to [20]. Then, the specimens were washed with Cacodylate buffer and post fixed for 4 hrs. With aqueous osmium tetroxide (OsO4), dehydrated through acetone, were dried in Polaron Equip., E300 critical point drying apparatus using liquid CO2, mounted on aluminum stub with double phase sticker. The specimens were then coated with gold palladium in an E5000 sputter coating unit (Polaron Equip.) coating unit, and examined in a Joel T330 scan-electron microscope operating at 20 Kev. Scanning Electron Microscopy to Necocucullanusneocucul tap worm parasite was detected in Characid freshwater fish (Bryconguatemalensis) [21].

2. Immunological Diagnosis:

Antigen - Antibody reactions are considered basic immunodiagnostic method (highly specific and sensitive). This method is applied for estimation of the pathogens or protective antibodies. In addition, the steps of evolution of disease diagnosis in aquaculture, antibody-based immune diagnosis play a sensitive role [22].

This method is quick, highly sensitive and specific detecting latent/sub-clinical/carrier infection. It can distinguish the antigenic differences [23].

The antibody-based techniques either monoclonal which are specific diagnostic tools or more accurate in the detection has allowed studying the pathogenesis of the infections or, polyclonal which are not protected in the life stages of specific pathogens. There are arrangements of polyclonal and monoclonal antibodies-based diagnostics ready for different aquatic animal pathogens [24].

2.1. Agar Gel Precipitation Test.

In this test, available antigens and antibodies are put in wells in agar plates and permitted to diffuse toward one another. The immune response happens in a center well, and the antigens are placed in encompassing wells. When an immune response and specific antigen meet each other with proper concentrations, the precipitate will form a seen white line between the two wells. This line is called a precipitin line [25]. This test is specific. The disadvantage of this test is that it needs a specific antigen for each parasite.

2.2. Agglutination Test.

Unknown antigens will be discovered by using the agglutination test; the blood is mixed with the unknown antigen with known anti-bodies. Regardless of whether agglutination occurs or not, that helps to determine the antigens. In direct agglutination test, the serum is to be added to a suspension to cells that have the surface self-Ag to be tested [26]. This test is rapid. The disadvantage of the test is that it is less accurate and nonspecific.

2.3. Enzyme Linked Immunosorbent Assay (ELISA).

ELISA will be used to decide the amount of specific antibodies' agents available in blood tests which are accomplished in 96-well plates that give valuable throughout results. The bottom of every well is enveloped with a protein which will join to the antibody you need to quantify. The whole blood is permitted to the clot, and the cells are centrifuged out to come about the serum that cannot be misunderstood with antibodies which are incubated in a well, and every well contains a different serum. Negative and positive control serum will be remarked through the 96 samples. After a short time, the serum will be removed, and weakly adherent antibodies are washed off with a series of buffer washing [27, 28]. Channel catfishes, Ictalurus punctatus**,** are eluded from the clinic for diagnosis and observing tissues attaching parasites. This study was done to determine the prevalence of IgG antibodies against Ichthyophtherious multiphaluiis cysts by ELISA [29]. The advantages of this test are that it needs little concentration of Antibodies and it is an early diagnostic technique. While, its disadvantage is that it needs a reader.

2.4. DOT- ELISA.

Dot-ELISA is broadly used as an immunological tool in researches. The difference between the regular ELISA and dot-ELISA is represented as the surface used to bind the antigen of choice. In dot-ELISA, the plastic plate is replaced by a nitrocellulose. The choice of binding matrix greatly improved the specificity and sensitivity of the assay by reducing the binding of non-specific proteins which was usually observed [30].

The advantages of this procedure is that it is quick, and its results are easily read. Some studies have shown the utilization of this test for the diagnosis of Trypanosoma cruzi and Trypanosoma brucei [31].

2.5. Latex Agglutination Test.

This test is a laboratory method to check certain antibodies or antigens in a variety of body fluids including blood. Babesias is a blood parasite in Nile tilapia, and catfish infections yield acceptable results by [32].

2.6. Fluorescent Antibody Test.

This test is for utilizations of antibodies tagged with dye, and used to make the presence of micro-organisms apparent. The antigens are detected using fluorescently marked antigen-specific antibodies of some protozoal parasites [33]. M. rotundus can be diagnosed in the skin mucus of the infected fish by using MAb 2D12 [34].

3. Molecular diagnosis.

These techniques are faster and much more sensitive than other methods that are used to diagnose fish infections. In these techniques, DNA is taken from the sample that can be examined by DNA analysis and hybridization by the restriction fragment length polymorphism (RFLP), and amplified by polymerase chain reaction (PCR) using specific primers for diagnostic sequences. By using the PCR-restriction fragment length polymorphism (RFLP) method, various closely related nematodes can be differentiated on their banding pattern in agarose gel [35].

The techniques of molecular diagnosis of parasitic fish infections are:

3.1. Polymerase Chain Reaction (PCR).

Polymer as a chain reaction is a technique to amplify 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 increases of a specific part of a DNA molecule can be realized and the PCR product can be found by gel electrophoresis [36].

Primers are important to get the best sensitivity and specificity [8]. Agarose gel electrophoresis shows the results of Gyrodactylus anguillae PCR products and M, 100 bp molecular weight marker (bp) [37].

In the most recent research, the improvement of novel sciences, enabling detection of PCR products on a constant premise has prompted far reaching appropriation of ongoing RT-PCR as a method for decision and quantitative changes in quality expression [14].

3.2. MultiplexPCR.

These techniques make it possible to produce considerable savings of time and effort in the laboratory without compromising test. Since its introduction, it has been applied successfully in many parts of nucleic acid diagnostics, including analysis of gene deletion, quantitative and RNA detection [38].

In the field of parasitic fish diseases, the strategy was seen to be a significant technique for recognizing the infections, microscopic organisms, growths and parasites in the same time [39].

In the field of fish, Cryptocaryon irritatans parasites have been discovered by Multiplex PCR. The amplified PCR product size of both DNA samples A and B were of 200 basic pairs [40]. Adult specimens of Diplectanum species were isolated from the skin of sea bass for standardization of the PCR technique3,4 and 5 at 650 bp. Lane 1 represents the 100bp) [14].

3.3. Restriction enzyme digestion.

Restriction enzymes were used to create a "fingerprint" of particular DNA molecule. These enzymes can cut DNA into discrete fragments that can be portioned by gel electrophoresis because of the sequence specificity of restriction enzymes. This model of DNA fragments makes a "DNA fingerprint", and each DNA molecule has its own specific fingerprint. Other restriction enzymes can be used in more characterization of a particular DNA molecule. The location of these restriction enzymes is placed on the DNA molecules which can be compiled to create a restriction enzyme map, PCR-amplified internal transcribed spacer region of ribosomal RNA genes for identifying freshwater mussels (Unionoida) and parasitic glochidia larvae from the host fish gills [41].

Molecular systems generally are more valuable for Parasitological than the immunodiagnostic methods [14, 33].

3.4. DNA microarrays.

DNA microarrays are using DNA microarrays to detect the unique DNA sequences. DNA sample that hybridizes to a given place on the microarray can be detected by fluorescent array detection and the data analyzed by computer programs [42]. This tool is matured in density, sensitivity, lower cost, rapid detection, automation, and low background levels. Microarrays may provide a better choice for vast scale diagnostic testing and can survey a sample for a multitude of sequences simultaneously [38].

DNA microarrays are suitable for the simultaneous detection of 15 fish pathogens based on 16S ribosomal RNA polymorphisms [43].

The amplified product of Mayxobolus sp. sample A was likely by Mayxoboluscerebralis18S ribosomal RNA gene's partial and complete sequence and amplified product of Mayxobolus sp. [44]. Microarray analyses of the expression of genes in Japanese flounder Paralichthys olivaceus leucocytes to Neo heterobothrium hirame infection from monogene of a parasite were done by [45]. Furthermore, the time of analysis is short. DNA microarrays technology can be used in the future to diagnose fish diseases specially during the symptomatic period of diseases.

3.5. Loop Mediated Isothermal Amplification (LAMP).

Loop-mediated isothermal amplification (LAMP) is a new nucleic acid amplification method that amplifies DNA with high efficiency, specificity and rapidity under isothermal conditions [46, 47].

This technique was applied to parasitic pathogens, such as myxozoan parasite of Salmonid fish tetra capsuloides bryosolmonae [48].

3.6. Nested PCR.

In this method, two pairs of primers were used in two frequent runs of polymerase chain reaction. This protocol was 100 times at least more sensitive than serological methods depending on the magnetic bead enzyme immunoassay. For example, the detection of Maxybolus cerebralis from rain bowtrout (Oncorhynchus myknis) by [49].

The advantages of Molecular Methods:

Molecular techniques permit great advances to improve diagnosis and control of pathogens in aquaculture with more specificity, speed and sensitivity of diagnosis. Therefore, molecular tools should be configured as a routine technique in aquaculture and laboratories for improved methods of diagnosis and control of infectious fish diseases in laboratories.

The disadvantages of Molecular methods:

Molecular techniques procedures are of high cost, and cannot detect unsuspected pathogen specially, new pathogens which will be hardly detected by molecular methods.

The traditional methods of controlling freshwater parasitic infected fishes.

Classical methods for parasitic diseases treatment depend on antiparasitic chemical drugs with their drawbacks [50]. In addition, some considerations should be taken such as LD_{50} of drug of choice, method of application, sensitivity of host to chemotherapy [51]. A few chemicals, like malachite green are carcinogenic on fish and human, with long withdrawal time and remaining viability on fish substance. They are accumulated as hurtful residues in the fleshy fish and environments [52]. Some chemicals need low water temperature such as formalin [53], and the organic matters decrease the effect of pot. Permanganate [10].

Conclusion and Recommendations

1. Molecular tools and scanning electron microscopes are considered the excellent new trends in the diagnosis of the parasitic infections specially in imported fishes.
2. The conventional methods for diagnosis of parasitic infections are reconsidered the cornerstone for identification of parasites.
3. Proper management and nutrition should be kept in mind when managing parasitic disorders.
4. Traditional tools should not be ignored in the control of such problems.
5. The early diagnosis of parasitic freshwater fish infections is recommended as a magic solution to prevent freshwater parasitic fish diseases.

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