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A Step by Step Guide On Diagnosis of Freshwater Fishes in Nigeria Using Molecular Techniques and Its PCR Analysis: A Review

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ABSTRACT: *Pathogens can be detected from asymptomatic fish using molecular diagnostic methods so disease outbreak could be prevented. Several years ago, molecular techniques have been employed to diagnose fish diseases. These methods include the polymerase chain reaction (PCR), restriction enzyme digestion, probe hybridization, in situ hybridization, and microarray. This paper reviews a step by step guide for carrying out molecular diagnosis of fish disease. However, the application of molecular methods as a routine tool in a diagnostic laboratory in areas where relevant literatures is scarce is important for the adoption of these methods*.

KEYWORD: Molecular methods, fish disease, molecular diagnosis, aquatic environment, climate change.

INTRODUCTION

Molecular methods can be used to increase sensitivity and specificity of pathogen detection. These techniques include polymerase chain reaction (PCR), in-situ hybridization, micro-array among others. Since molecular methods are more sensitive than the conventional diagnostic techniques, pathogens can be detected from asymptomatic fish, so outbreak of disease could be prevented, thus; antibiotic treatment can be reduced (Ilhan and Ilknur, 2003). Several years ago, great advances have been made in understanding the molecular biology of fish pathogens and their hosts, and molecular biology has become a routine tool in search for improved methods of diagnosis and control of fish diseases. The detection of nucleic acid molecules has demonstrated its usefulness in detection and diagnosis of fish diseases (Ilhan and Ilknur, 2003).

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Impact of fish disease to aquaculture

Freshwater fish have been model hosts in the study of the community and evolutionary ecology of parasites for the last few decades. However, species identification is one of the main challenges in freshwater fish parasites. These parasites are usually small, soft bodied with few morphological characters. For instance, tapeworms are obligate internal parasites of vertebrates that display a wide range of body forms, life history and host associations (Ilhan and Ilknur, 2003).In an ideal environmental condition, healthy fish without lesion can carry pathogens capable of creating an avenue for the spread of diseases in the fish populations. However, disease may become eminent only when stressful conditions occur. In intensive aquaculture systems, the risk of stress may increase with a significant proportion of the stock infected. Therefore, it is imperative to detect pathogen from carrier fish for an effective fish disease control, since most often, the prevalence of diseases may change depending on factors such as seasons and temperature (Plumb, 1999).

Centuries ago, fish has been one of the main foods for humans and is still an important part of the diet in many countries (Leisner *et al.,* 2001). One of the advantages of fish as food source to man is because it is easily digested and has a high nutritional and health benefits (Leisner *et al.,* 2001). Fishes are important natural resources to humans world over, most especially in the rural areas (Adeleye, 1992), where fishes are hunt for both commercial and subsistence purposes.

IMPACT OF CLIMATE CHANGE ON AQUATIC ECOSYSTEMS

Many years ago, there has been a global report of diseases affecting aquatic organisms of different taxa (Harvell *et al.*, 1999). Climate change has affected the aquatic ecosystem, thus; making it susceptible to many anthropogenic disturbances such as pollution, habitat destruction and overfishing. Global warming has led to increased pathogen development and survival rates, disease transmission and host susceptibility (Harvell *et al.,* 2002).

However, environmental conditions play a pivotal role not only in the transmission of pathogens, but also as risk factors for the occurrences of clinical diseases. Unlike mammals that regulate their internal environments (homeostasis), most fishes are cold blooded (poikilotherms), with little or no ability to regulate their body temperature (Yael *et al.,* 2020).

In situations such as this, both the microbes and the host are physiologically tied to the environment they live in with an optimum temperature range for their survival, and extended periods beyond the optimal range could result in death (Harvell *et al.,* 2002).Aquaculture production has dramatically increased; fish consumption is largely dependent on fisheries (FAO, 1999). Wild fish plays an important ecological and economic role in the ecosystem as a major protein source for humans (Noga, 2010).

There are varieties of aquatic pathogens from aquaculture is well documented, there is still lack of baseline information with regards to pathogenic agents and their prevalence in wild fish population

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(Lafferty, 2015). Although aquaculture is a fast growing industry for the production of high protein-sources foods, his growth is accompanied by concerns from both public and private sectors (FAO, 2016), because fish production is commonly associated with serious environmental impacts such as water pollution, transmission of pathogens and changes in temperature (Fernandez-Gonzalez *et al.*, 2017).

Molecular tools for diagnosing fish disease

The utilization of molecular methods has brought new insights to different biomedical areas. However, molecular parasitology laboratory specializes in the use of molecular methods for investigations on the field of host-parasite interactions (Shayan *et al.,* 2007). Other aspects of the use of molecular methods include the characterization and determination of species, sub-species and strain of generated mutations during the treatment of anthelminthic animals (Le Jambre *et al.*, 1999). The efficiency of DNA extraction methods for any biological sample is determined through DNA quality and recovery rate. Consequently, the extraction of DNA with high quality and quantity is a key step in the genetic analysis (Shayan *et al.,* 2007).

Several DNA extraction methods have been employed in the preparation of DNA from various organisms, ranging from the isolation of genomic DNA from small amounts of biological materials such as single worms or blood smears is not always applicable using the traditional DNA isolation methods that is based on phenol/chloroform/isoamyl alcohol, which is one of the most utilized methods for DNA extraction in developing countries of the world (Shayan *et al.,* 2007).

Fish disease diagnosis

Fish disease management and assessment is a major concern to commercial aquaculturists. However, the ability to identify the presence or absence of a pathogen in fish would be of significant economic benefits. If the concentration of the infectious organism is determined in the fish or the water environment, the changes in abundance of these organisms could be monitored as well. However, developing a system that could access the carrier state of fish accurately within an area that harbours a disease causing organism would help in developing management programs (Ilhan and Ilknur, 2003).

Several advances have been made in improving the sensitivity and specificity of diagnosinf fish disease. In molecular methods, a typical DNA is extracted from the sample and probed by DNA hybridization and analyzed using a restriction fragment length polymorphism (RFLP). Moreover, DNA is amplified using the polymerase chain reaction (PCR), which employs specific primers for diagnostic sequences. The polymerase chain reaction (PCR) is hybridized with specific oligoprobes or non-specific primers used in producing random amplified polymorphic DNA (RAPD) (Prichard, 1997 and McKeand, 1998).

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Detection and Labeling of Nucleic Acids

Radioisotopes were once the trend several years ago, but in the safety interest of the researcher, other methods were adopted. A variety of detection and labeling methods presently can provide a dynamic system suitable for any application, beginning from dot blots to in situ hybridization. (Tijssen, 1993). These include labeling with biotin or digoyygenin and detection by antibody binding coupled with fluorescent, chemiluminescent or colorimetric detection methods (Gonzalez *et al.*, 1997).

Molecular Analysis

DNA Extraction

The extraction of DNA will be made possible using the procedures below:

- Pipette 50µl Protease (or proteinase k) into the bottom of a 1.5ml micro-centrifuge tube.
- Add 200µl samples to the micro-centrifuge tube. About 200µl of whole blood, plasma, serum, buffy coat, or body fluids, or about 5 x 106 lymphocytes in 200µl PBS.
- Add 200µl Lysis Buffer to the sample. Mix by vortexing for about 15 seconds to yield a homogenous mixture.
- Incubate the mixture at 75^0C for 10 minutes to allow DNA yield reach a maximum after lysis for about 10 minutes at 75^0C.
- Centrifuge the 1.5 ml micro-centrifuge tube briefly to remove drops from the inside of the lid.
- Add 250µl ethanol (96 – 100%) to the sample, and mix again by pulse-vortexing for about 15 seconds. After mixing, the 1.5 ml micro-centrifuge tube, briefly centrifuged to remove drops from the inside of the lid.
- Carefully apply the mixture obtained to the Mini spin column (in a 2 ml collection tube) without wetting the rim.
- Close the cap and centrifuged at 12,000 x g (8000 rpm) for 1 minute.
- Place the Mini spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.
- Open the Mini spin column carefully and add 500µl de-ionized solution without wetting the rim.
- Close the cap and centrifuged at full speed 12,000 x g for 1 miunte.
- Place the spin column in a new 2 ml collection tube and discard the old collection tube containing the filtrate.
- Centrifuged at 14, 000 g for 3 minutes at room temperature to remove the residual ethanol.
- Place the spin column in a clean 1.5 ml micro-centrifuge tube, and discard the collection tube containing the filtrate.
- Carefully open the Mini spin column and add 200µl Ethanol Buffer.
- Incubate at room temperature (72^0C) for 1minute and then centrifuged at 14, 000 x g for 1 minute.

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PCR Experiment

- Place the template DNA in a mixture containing the four DNA nucleotides, pair of primers flanking the target sequence, Taq DNA polymerase, buffer and water.
- Heat the mixture at 94^0C to separate (denaturation) the hydrogen bonds holding the two stands of double-stranded DNA molecules together.
- Cool the mixture down to 50 – 60^0C, and allow the primers to anneal to specific target on the single stranded DNA molecules.
- Raise the temperature again to 74^0C to enable the Taq DNA polymerase attach to one end of each primer and synthesizes new strand of DNA complementary to the template DNA molecules.
- Raise the temperature back to 94^0C to denature the double-stranded DNA into one strand of the original molecule and one new strand of DNA into single strands.
- This will lead to a second cycle of denaturation-annealing-synthesis at the end of which there will be eight DNA strands.
- Repeat the cycle 25 times to enable the double stranded molecule convert to 50 million new double-stranded molecules, with each one of them being a copy of the starting molecule delineated by the annealing sites of the two primers.
- Perform the PCR reaction in a thermo-cycler machine, which is programmable heating block that cycle between melting, annealing and polymerization temperatures.

PCR Procedure

- Label the PCR tubes for samples and controls.
- Thaw the PCR reagents and prepared PCR reaction mix.
- Aliquote the reaction mix in an individual PCR reaction tube.
- Add the template i.e. sample/ control in the appropriate labeled tube.
- Add 1µl template to each tube to achieve 25µl total reaction volume.
- Open the PCR machine's software and edit parameters e.g. run ID, user ID, sample ID's, sample volume and cycling conditions according to desired protocol.
- Place the sample tubes in the thermal cycler
- Close the lid and the program will run simultaneously.
- On completing the PCR, remove the tubes from the thermal cycler and proceed for agarose gel electrophoresis or other downstream application. Otherwise, store the PCR products at -20^0C.

Gel Electrophoresis Procedure

- Weigh 1.5g of agarose and dissolve in 100ml of 0.5X TBE buffer by heating using microwave oven and swirl flask to mix until clear solution is obtained.
- Dissolve the agarose completely and cool to ~500C.

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- Add 5ml of 20,000X RedSafe into the agarose solution and mix thoroughly or use an alternative method of staining agents such as GelRed.
- Prepare the gel tray using a comb with enough wells for number of PCR reactions and two wells for the markers.
- Pour the gel solution into the gel tray carefully to avoid bubbles trapping in the gel and allow cooling for ~30 minutes.
- Carefully remove the comb and rinse immediately with water.
- Place the tray in a tank containing 0.5X TBE buffer just enough to submerge gel.
- Pipette 5µl of 5X orange G loading dye onto a parafilm paper for each PCR reaction.
- Mix 25µl of PCR reaction with 5µl of 5X orange G loading dye (5:1 proportion) on the parafilm paper and load separately into each well.
- Once all samples have been loaded, load the first and last empty well on the gel with 10 -12 µl of ladder and cover the lid of the gel tank.
- Connect the leads to their respective sockets (black to black, and red to red) on gel tank to the power supply.
- Electrophores sampless at 4 – 10V per cm distance between electrodes for the desired length of time.
- Turn off the power pack before removing the gel tray from the tank and DNA bands will be visualized on the UV transilluminator in a dark room (chamber).
- Capture the photograph of the gel picture using a gel documentation system.

CONCLUSION AND RECOMMENDATION

In conclusion, molecular tools are increasingly relevant to fish diseases. However, sequencing the complete genome of pathogens allows great advances in studying the biology, and improving diagnosis and as well as control of pathogens. Conversely, using new methods of analyzing polymorphism in nucleic acids improves specificity, sensitivity and speed of diagnosis and offer ways of examining the genotype and phenotype of various pathogens.

Molecular methods aids epidemiological studies, as well as identify causes of disease outbreak or the presence of pathogens. It is therefore recommended that molecular tools can be a routine tool in the search for improved methods of diagnosis and control of fish pathogens and the epidemiology of infected fishes. However, in Nigeria, the application of these methods on a routine basis in diagnostic laboratories is few. It is time to apply these methods in the diagnosis of diseases in aquaculture.

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