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Isolation and Molecular Characterization of *Streptococcus iniae* from African Catfish (*Clarias gariepinus*) from Selected Markets in Federal Capital Territory, Abuja, Nigeria

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Abstract

Fish plays an important role in the human diet. *Streptococcus iniae* is an opportunistic zoonotic pathogen and causes serious infections in humans, often through transmission of *Streptococcus iniae*-contaminated fish. The risk of zoonotic transmission to humans highlights the need to evaluate *Streptococcus iniae* from African catfish (*Clarias gariepinus*) in the Federal Capital Territory, Abuja. To achieve these goals, four hundred (400) samples of *Clarias gariepinus* organs namely liver, kidney, intestine, gills, heart and skin were purchased from selected markets (Wuse, Garki, Kado fish market, Gwagwalada, Zuba, Kubwa and Duste Allah). Detection of *Streptococcus iniae* was carried out using standard method on tryptic soy agar. Isolates were further identified using conventional biochemical tests: Gram's staining, catalase, oxidase, hemolytic zone on 5% sheep blood agar, hydrolysis of starch and fermentation of mannitol, ribose, xylose, sorbitol, glucose, inulin, lactose, arginine, arabinose, inositol, raffinose, rhamnose. Forty-three 43 (10.8%) suspected isolates of *Streptococci* were subjected to species specific polymerase chain reaction for amplification at 377 bp and all of the isolates turned out negative. It is therefore suggested that more investigative studies become necessary to determine the presence of this pathogen in different fish species in Nigeria.

Keywords

Clarias gariepinus, Organs, *Streptococcus iniae*, PCR

1. Background

Aquaculture has become a worldwide economically important industry which requires continuing research with scientific and technical developments and innovations [1]. *Streptococcus iniae* (synonym: *S. shiloi*) was first described in captive Amazon freshwater dolphins (*Inia geoffrensis*) [2]. Subsequently, it was found to cause disease in multiple economically important fish species throughout the world, such as Nile tilapia *Oreochromis niloticus* [3]. Streptococcosis is primarily caused by the Gram-positive bacteria *Streptococcus iniae, Streptococcus agalactiae, Streptococcus dysgalactiae*, and *S. ictaluri*. *Streptococcus iniae* emerged as a major pathogen of farmed and wild fishes in the 1990-2000's and more recently, it has also been identified as a potential zoonotic pathogen, with at least 25 confirmed cases of human infections caused by *S. iniae* to date [4]-[6]. Infection of *Streptococcus iniae* in fish results in septicemia with the clinical signs of erratic swimming, loss of equilibrium, unilateral or bilateral exophthalmia, eye opacity, ascites and hemorrhage of the internal organs and base of the fins and darkening of the skin with a pale liver and enlarged spleen [6]-[10].

Streptococcus iniae and *Streptococcus agalactiae* are both human pathogens [11] [12]. *Streptococcus iniae* infections in humans occur due to direct contact with a diseased or dead fish and indirect contact with contaminated water resulting in the development of bacteraemic cellulitis, with occasional localisation in other organs or joints cellulite, endocarditis, meningitis, severe systemic infections, and rarely death in human [13] [14]. The disease is responsible for significant economic losses in the world aquaculture industry resulting in economic losses estimated at US $150 million annually [15]. It is generally assumed that streptococcosis has a worldwide distribution, having been described in fishes from Europe, the Americas, the Middle East, throughout Asia and Australia [6] [16] [17]. Due to *Streptococcus* phenotypic diversity, it is not possible to be identified accurately, so it is essential to search for accurate diagnostic methods to apply the effective treatment [18]. Identification of *Streptococcus* is highly specific using molecular tool such as Polymerase chain reaction [19] [20]. Currently *S. iniae* is not listed in the databases of the most commonly used rapid or automated identification systems, including the RAPID Strep strip, the Vitek system, API 20 STREP system, or the ATB Expression system [4] [5], thereby the use of one of these systems in isolation may result in misidentification or a reading of "unidentified" [4] [5] [21]. To date, infections of *S. iniae* have been found in several fish species including, flounder (*Paralichthys olivaceus*) [22], red porgy (*Pagrus pagrus*, L.) [23], Nile tilapia (*Oreochromis niloticus*) [24], mandarin fish (*Siniperca chuatsi*) [25], golden pompano (*Trachinotus ovatus*) [26], and Adriatic sturgeon (*Acipenser naccarii*) [27]. Antibiotic resistance is a worldwide public health problem that continues to grow due to improper use and abuse of antibiotics in both human and veterinary medicine, it is an important cause of disease and death [28]. Antibiotic resistance in fish shows the chance of transfer of AMR genes to humans, and the difficulty in controlling diseases [29], which leads to their limi-

tation and developing laws and legislations for antimicrobial use in aquaculture [30]. Limitations are facing the farmers for choosing the antimicrobial agent hence, only three antimicrobials; oxytetracycline (Terramycin® 200), florfenicol (Aquaflor®) and ormethoprim and sulfadimethoxine (Romet30) are approved by the United States Food and Drug Administration (USFDA) as feed additives in aquaculture sector [31]. Florfenicol has been used as medicated feed for control. This study therefore aimed to molecularly isolate and identify *Streptococcus iniae* from African catfish *(Clarias gariepinus*) sold in selected wet markets in FCT, Abuja.

2. Materials and Methods

2.1. Ethical Approval

This present study was authorized by the animal ethics committee of Ahmadu Bello University Zaria. Ethical clearance was sought from Committee on animal use and care.

Approval No. ABUCAUC/2024/011.

2.2. Study Area and Design

This study was conducted in the Federal Capital Territory (FCT), Abuja which was formed in 1976, from parts of Nasarawa, Niger and Kogi States. The territory is located just north of the confluence of Niger and Benue rivers. It is bordered by Niger State to the West and North, Kaduna to the Northwest, Nassarawa to the East and South and Kogi to the Southwest. Abuja has an estimated human population of 1,405,201 according to 2006 census [32]. It lies between latitude 8.25 and 9.20 North of the equator, and longitude 6.45 and 7.39 East of Greenwich Meridian. Abuja is geographically located in the center of the country. The Federal Capital Territory has a land mass of approximately 7315 km^2 of which the actual city occupies 275.3 km^2. It is situated within the savannah region with moderate climatic conditions.

A cross-sectional epidemiological study method was adopted in the study. Firstly, three (3) area councils namely, Gwagwalada, Bwari and Abuja municipal area council were selected using simple random sampling by balloting. Secondly two major markets were selected from Gwagwalada, Bwari and three from Abuja municipal area councils. A total of 400 samples of the liver, gills, kidney, intestine, heart and skin were harvest from *Clarias gariepinus* purchased from seven different major markets namely Gwagwalada (62), Zuba (34), Garki (56), Wuse (28), Kado fish market (85), Duste Alhaji market, (56) and Kubwa (79) using proportionate distribution method. All organs were harvest aseptically and analyzed in the Bacterial Zoonoses Laboratory Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, Kaduna state.

2.3. Phenotypic Characterization of Bacterial Isolates

Bacteriological examination, prior to dissection and collection of swab specimen

the fish was stunned. The skin samples were obtained by gentling rubbing the sterile swab stick over the length of the fish and processed in 5 ml of tryptic soya broth. Thereafter, an alcohol-soaked wipes was used to swabbed through the length of the fish to eliminate the chance of bacterial contamination and using a sterile knife the fish was cut through the vent and one portion of the kidneys, liver, intestines, heart and gills were obtained, weighed using a weighing balance and homogenized following the descriptions of [33]. The homogenate was pre-enriched on five mL of tryptic soy broth (Difco, Detroit, MI, USA) and incubated for 2 - 3 h at 27˚C, and using a sterile inoculating wire loop a loopful of the broth culture was streaked on tryptic soy agar supplemented with defibrinated sheep blood (5 drops) and incubated for 24 h at 28˚C. For selective isolation of *Streptococcus iniae*, individual colonies were re-cultured on tryptic soy agar (TSA) to ensure purity and stored at −70˚C in tryptic soy broth (TSB) containing 10% (v/v) glycerol for further identification by standard methods [15].

2.4. Biochemical Identification

Presumptive colonies were tested for gram's reaction based on colonial morphology and phenotypic identification bases on biochemical tests such as catalase, oxidases, motility, Voges Proskauer, indole and hemolytic reaction on blood agar base (Merck, Germany) supplemented with 5% defibrinated sheep blood [15]. The CAMP reaction of the isolates was determined using the conventional diffusion test [34]. Bacterial growth on bile-esculin agar (40% bile) was tested using the method of [35]. Starch hydrolysis using the method of [36]. Isolates of suspected *Streptococcus iniae* were subjected to fermentation and sugar utilization for the identification and species differentiation of isolates using glucose, salicin, sucrose, ribose, mannitol, trehalose, arabinose, inulin, lactose and sorbitol [36].

2.5. Identification of the Isolates by Molecular Methods

2.5.1. DNA Extraction

DNA extraction was performed using suspension of isolated colonies prepared in sterile distilled water following protocol of bacterial DNA extraction kit (DNA_{zol}^{R} BD, USA). Two to three well isolated colonies of suspected *Streptococcus iniae* isolates were suspended in 0.5 ml autoclaved distilled water. One ml of DNAzol was added in bacterial suspension. The mixture was vortexed vigorously for 15 - 20 seconds and stored at room temperature for 5 minutes then centrifuged for 1 minute at 8000 g. A volume of 0.4 ml of isopropanol was added to the lysate for precipitation of DNA. The precipitated DNA was sedimented by centrifugation at 6000 g for 5 minutes, supernatant was removed and 0.5 ml of DNAzol was added to the DNA pellet. The DNA pellet was vortexed until it was completely dispersed. Then it was centrifuged at 6000 g for 5 minutes. The supernatant was removed and washed, the DNA pellet was mixed with 1 ml of 75% chilled ethanol. Again, it was centrifuged at 7000 g for 5 minutes. The ethanol wash was decanted and stored in the eppendrof tube vertically for 15 minutes to evaporate any residual

ethanol. The DNA pellet was then dissolved in 50 ul molecular grade water. The purity and quantity of genomic DNA in each sample was evaluated by measuring optical densities at 260 and 280 nm wavelengths. The DNA concentration of each sample was adjusted to 50 ng/µl for PCR.

2.5.2. PCR ASSAY

PCR reactions were carried out targeting the intergenic space of *Streptotococcus iniae* 16S rDNA using primers specific for *Streptococcus iniae*, as described by [37] as presented in Table 1. The optimized PCR parameters that were used for species-specific primer were set at 35 cycles of denaturation at 94˚C for 1 minute, primer annealing at 60˚C for 1 minute, followed by initial extension at 72˚C for 1 minute, and a final extension at 72˚C for 10 minutes in an automated thermal cycler (PTC-100; Bio-Rad™). The amplified products were then resolved by electrophoresis in 1.5% agarose gel at 100 V for 60 minutes. Gel was stained with ethidium bromide solution and documentation was done using the Gel Doc system.

Table 1. Primers set for specific amplification of *Streptococcus iniae.*

Gene	Primers sequence	Amplicon BP Size	Reference
SP-1 R	5'GAAAATAGGAAAGAGACGCAGTGTC-3'	377	[37]
SP-2 F	5'-CCTTATTTCCAGTCTTTCGACCTTC-3'		

3. Results

Table 2. Frequency of presumptive *Streptococcus iniae* from the three Area Councils of the FCT, Abuja.

Sample Types	No. of *S. iniae*	Percentage %
Skin swab	7	16.3
Liver	4	9.3
Kidney	3	6.9
Heart	9	20.9
Intestine	2	4.7
Gills	18	41.8
Total	43	100

Four hundred samples collected from various markets in the Federal Capital Territory, consisting of the skin, liver, kidney, heart, intestine and gills in the study area, 43 suspected streptococci were isolated and biochemically identified from *Clarias gariepinus* representing 10.8% (Table 2). The isolates obtained were further sought for molecular characterization using a species-specific PCR assay uti-

lizing 16S rRNA to discriminate whether they are true isolates of *S. iniae* by amplifying at 377 bp fragment. The results following amplification turned out to be negative as shown in Figure 1 (Lanes 2 - 10 only shown out of the 43 isolates tested which were all negative).

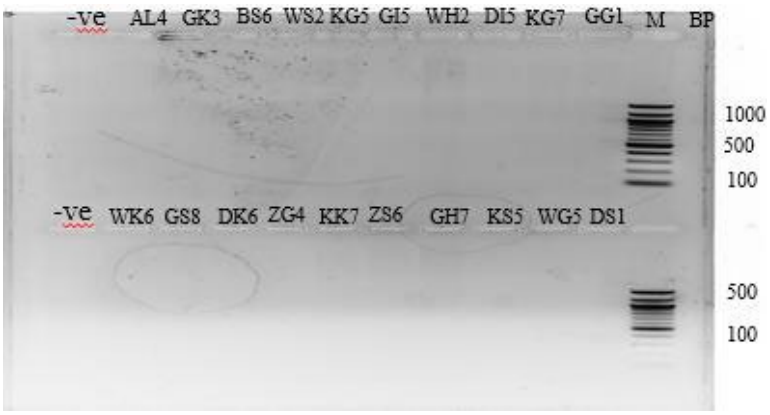

Figure 1. Gel Electrophoresis of *S. iniae* amplification. Lane 1: M (Molecular weight marker) Lane (2 - 10) test isolates, lame 11 –ve control.

4. Discussion

Streptococcus iniae is a major pathogen in aquaculture and poses a notable public health risk. In this study, forty-three presumptive isolates of *S. iniae* were analyzed using species-specific polymerase chain reaction (PCR), but all tested negative. This outcome contrasts with earlier reports [38] that documented the isolation of *S. iniae* from African catfish fingerlings reared in commercial farms in Akure, Nigeria.

The absence of *Streptococcus iniae* in *Clarias gariepinus* may suggest a low zoonotic potential for bacteraemic cellulitis. While such infections can occasionally localize in other organs or joints, leading to conditions such as cellulitis, endocarditis, meningitis, severe systemic disease, and, in rare cases, death in humans, these outcomes appear unlikely in the study area.

The observed difference may be due to variations in isolation methods, as their isolates were not confirmed through molecular detection. Reliance on biochemical characterization for identifying *Streptococcus iniae* has been questioned, given the frequent occurrence of misidentification [5] [39] [40]. The identification of *Streptococcus iniae* using the API20 Strep kit and Rapid ID 32 Strep is unreliable, owing to inadequate phenotypic testing and the absence of *S. iniae* in their reference databases [41]. As stated by [4] [5] [21]. Using any of these systems in isolation may result in identification errors or yield an "unidentified" outcome. Nevertheless, *Streptococcus iniae* has been successfully isolated from various fish species by other researchers, including hybrid tilapia [7], rainbow trout [42], Gilthead sea bream [43], barramundi [36], Snapper [44], (Nile tilapia) [6] [15] [37], European sea bass [45]-[47]. The inability to detect *Streptococcus iniae* from this present study could be associated with host species, geographic location, environ-

mental factors such as water temperature and salinity [48]-[51]. Many Gram-positive lactic acid bacteria such as streptococci, enterococci, lactococci share sugar fermentation traits with *S. iniae*. Because of this overlap, biochemical tests alone can misidentify *S. iniae*, therefore molecular methods are needed for accurate identification [52]. The primers set used in this study was previously used by [37] in the Identification and Genetic Characterization of *Streptococcus iniae* Strains Isolated from Diseased Fish in China. Although *Streptococcus iniae* is a pathogen capable of infecting multiple fish species, the susceptibility of a given species may vary when exposed to *S. iniae* strains originating from different host species [53]. To the best of our knowledge, this study represents the first attempt to isolate *Streptococcus iniae* in Nigeria using molecular tools. The negative results obtained suggest that molecular epidemiological approaches may be required to confirm the presence of this pathogen in *Clarias gariepinus* and other fish species within the country.

In conclusion, aquaculture is among the fastest-growing industries in Nigeria and globally. Consequently, molecular epidemiology may be essential to confirm the presence of this pathogen in fish. Further research is required to assess the significance of this emerging pathogen in various fish species, including tilapia, within Nigeria.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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