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Polymerase chain reaction fails to detect *Listeria*species from wild and cultured Nile Tilapia (*Oreochromis niloticus*) caught from a large freshwater lake in Zambia

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Abstract

Fish have been identified as suitable vehicles for transmitting Listeria species to humans. Therefore, the thrust of this study was to determine possible Listeria species likely to circulate in freshly caught Nile tilapia (Oreochromis niloticus) in a large freshwater body. Accordingly, 150 Nile tilapia were sampled on landing. Of this number, half (n=75) were wild caught, while the other half (n=75) were from cage culture farms. All the sampled tilapia fish were independently caught from the same lake. Skin surfaces and gills were the only points sampled to restrict sites swabbed to those in contact with the environment. Swabs were processed using standard bacteriological culturing and identification tests. Only 2% (3/150) of the samples on culture and biochemical tests were presumptively Listeria. However, on Polymerase Chain Reaction (PCR), none yielded any noticeable results. None-detection of Listeria species on PCR may, in part, indicate the absence or undetectable levels in freshwater bodies. The detection of presumptive Listeria species phenotypically indicates possible contaminants other than Listeria. Our study has inherent limitations since it restricted itself to identifying contamination based on the fish's growth environment. We recommend determining the pathogen by incorporating enteric organs or whole fish analysis to increase isolation rates based on the current results.

Introduction

Listeriosis is a severe foodborne disease caused by *L. monocytogenes*, a gram-positive bacterium belonging to the phylum *firmicutes* [1]. Fish and fish products contribute about 5.5% to 12.1% of confirmed listeriosis cases in the United States [2]. Previous studies [3-5] reported significant variations of 0.55% to 38% in the prevalence of *Listeria* species in raw fish samples collected from fish carcasses and fish markets in Nigeria, Iran, and India. In Africa, disease outbreaks linked to the consumption of specific foods are underreported, compared to water-related, because of inadequate water supply or treatment in many regions.

Most countries in the Southern part of Africa, including Zambia, do not differentiate between foodborne and waterborne diseases (outbreaks). *L. monocytegenes* is naturally present in the environment and tolerate a broad range of environmental stressors. In water and soil environments, *L. monocytegenes* integrates into multispecies biofilms. Its sturdiness allows *L. monocytegenes* to survive in refrigeration food production environments making it a significant food production concern. Raw fish contaminated with *L. monocytegenes* pose a public health concern mainly when inadequately heat treated or served as ready to eat food.

Listeria species identification involves culture methods based on selective enrichment followed by plating and characterization of single isolates. Rapid molecular tests, like Polymerase Chain Reaction (PCR), are available now. For instance, the housekeeping *prs* gene for the genus *Listeria* which encodes phosphoribosyl pyrophosphate synthase [6]. Although the *prs* gene does not differentiate different *Listeria* species, it nevertheless acts as an indicator of the genus *Listeria* in samples [6]. The most common tilapia species farmed in cages is *Oreochrome niloticus*. Due to its ease and cost-effectiveness, tilapia cage culture has boosted freshwater fish farming and economic interest in tropical countries. Tilapia is among the world's most frequently consumed fish types, and its farming improves food security, including income generation. Cultivation practices have raised concerns that pathogenic bacteria can contaminate the waters and confer a risk of foodborne disease. However, knowledge of the prevalence of *Listeria* species in tilapia caught from a large freshwater lake in Zambia is limited.

Therefore, this study aimed to determine the presence of *Listeria* species circulating in freshly caught tilapia from a large freshwater lake in Zambia using PCR.

Methods

Study design: a cross-sectional study design was conducted between the periods of February to April 2021.

Study settings: Siavonga district is located along lake Kariba on the North shore in the Southern Province of Zambia, 16° 32' South of the equator and 28° 44' East. Most fish produced comes from aquaculture cage farms. The Siavonga district was chosen for the study due to its large number of aquaculture cage farms and capture fisheries. All the sites we selected for sampling the owners had to consent and fish availability during the study period. At capture, fisheries sampling was at three of the six recorded major sites along lake Kariba in the Siavonga district. These we coded as sites 1, 2, and 3. Out of 15 aquaculture cage farms in the Siavonga Town Council, only three gave consent for sampling. The coding for the sites was 4, 5, and 6 (Figure 1).

Sample size and sampling: sampling was done through the collection of fish to be sampled based on the two main fishing types found in the Siavonga district: aquaculture cage farms and capture fisheries. To get equal variation, we collected one hundred and fifty tilapia swabs from capture fisheries (n=75) and aquaculture cage farms (n=75). Swab samples from each fish came from the exterior surface and gills, independently sampled at the landing stage. We used random sampling at both the capture fisheries and the aquaculture cage farms to pick the individual tilapia for swabbing from the involved sites.

Sample collection and processing: we used a standard procedure for collecting swab samples from the fish, briefly, a sterile metal template to pre-mark a $5cm^2$x $5cm^2$ area for swabbing. After swabbing the fish with a moist cotton swab, the swab was inserted back into a screw-cap tube containing Amies transport media. We recorded metadata such as farmed or wild-caught, collection site, and collection date. Sample storage was at -4°C before transportation, usually within 72hrs after sampling; we took it to the Microbiology laboratory at the School of the Veterinary Medicine University of Zambia. Samples were assigned laboratory codes upon receipt. Immediately after arrival, the swabs from the tubes were transferred into 9ml of pre-enriched broth (Oxoid) and incubated at 37°C for 48hrs. During the cultivation and identification of *Listeria* species, the *L. monocytogenes* strain ATCC19118 we used as a positive control.

Isolation and identification of *Listeria* species: the protocol for the isolation of *Listeria* species is previously described by Mpundu P *et al*. [7]. Briefly described as follows; firstly, a fish sample was inoculated into 9ml of pre-enriched broth (Oxoid) and incubated at 37°C for 24hrs. Later 1ml of pre-enriched broth (Oxoid) was transferred into 9ml of Fraser broth (Oxoid) (enriched with *Listeria* selective supplement) and vortexed for 1min, followed by incubation at 37°C for 48hrs. A loop full of the *Listeria* enriched culture was inoculated onto the surface of *Listeria* selective agar (Oxoid), incubated at 37°C for 48hrs, and observed for colonies with morphology typical for *Listeria* species, i.e., green-blue color, with a possible greenish sheen. Presumptive *Listeria* species colonies were sub-cultured on Nutrient Agar (Oxoid) incubated at 37°C for 24hrs to obtain pure colonies. The quality of the agar plates was tested by cultivating the *L. monocytegenes* strain ATCC19118. We performed standard biochemical tests on the purified presumptive *Listeria* species: Gram staining, catalase, sulphur indole motility, oxidase, urease, Voges Proskauer, and methyl-red tests.

Deoxyribonucleic acid (DNA) extraction and polymerase chain reaction identification of *Listeria*: polymerase chain reaction assays were performed on presumptive *Listeria* isolates to verify their identity. Chromosomal

deoxyribonucleic (DNA) was isolated from the isolates grown on nutrient agar using the ZYMO Research quick DNA miniprep kit, following the manufacturer's instructions. For PCR detection of *Listeria* species, a partial 370bp fragment of the *prs* gene was targeted by using the primer pair *prs*-F (5'-GCT GAA GAG ATT GCG AAA GAA G-3') *prs*-R (5'-CAA AGA AAC CTT GGA TTT GCG G-3') [6]. PCR was under the following conditions initial denaturation at 94°C for 1 min, 30 cycles at 94°C for 45 s, 53°C for 45 s, 72°C for 2 min, and final extension step at 72°C for only 5 min. Finally, the PCR product was separated on a 1.5% agarose gel stained with ethidium bromide to visualize a potential 370 bp band. We used the *L. monocytogenes* strain ATCC19118 for quality control.

Data analysis: all the obtained data from the tilapia fish carcasses were entered in the excel datasheet and imported to Stata version 15 (Stata cop, College Station, Texas, U.S.A). The primary outcome was the presence of the *Listeria* species contamination from tilapia fish swabs. We calculated the prevalence of presumptive *Listeria* species contamination as the proportion of the total tilapia fish swabs collected that were contaminated phenotypically.

Ethics: approval of ethics was acquired from Excellence in Research Ethics Committee reference no. (Ref.no.2020-Jan-006). We sought permission from Siavonga Town Council and maintained the confidentiality of all sampling sites involved throughout this study.

Results

Of the 150 tilapia samples collected from captured fisheries and aquaculture cage farms, 2.0% (3/150) were phenotypically indicative of *Listeria* species contamination. None of the presumptive *Listeria* isolates were *prs* positive. However, the positive control samples gave the expected 370 bp on the agarose gel, which secured reliability. The highest prevalence of presumptive *Listeria* isolates occurrence was found in samples from capture fisheries 2.7% (2/75) (95% CI: -1.048 - 6.453), aquaculture cage farms 1.3% (1/75) (95% CI: 1. 284 - 3.916) (Table 1).

Discussion

The present study determined the prevalence of *Listeria* species contamination of tilapia from lake Kariba in the Siavonga district in Zambia. In the present study, none of the surface swab samples collected from fresh tilapia carcasses belonged to the genus *Listeria* on PCR. To the best of our proof, the present study is the first that investigated *Listeria* species contamination in tilapia from a large freshwater lake in Zambia. A similar study in India showed that *Listeria* species surface swabs had 72.4% of tropical fish and fishing environments [8]. The significant difference deduces from the differences in the sampled fish; Jeyasekaran and others collected sample swabs from fish markets and fish processing units. Onjong and others conducted a study on the microbiological safety of fresh tilapia in value chains, and a 2% prevalence of *L. monocytegenes* sampled at landing was recorded [9]. The differences in isolation with this present study deduction are from the sampled tilapia fish characteristics and techniques used in the isolation method. This present study utilized the *prs* gene to confirm the *Listeria* genus, a housekeeping gene found in all *Listeria* species. At the same time, Onjong and co-workers used VITEK 2 system version 0.8.01 biomerieux, Inc. Hazelwood, MO. These two methods may yield different results due to differences in sensitivity [10]. Further, this study only collected swabs at the landing stage without multiple processes.

Human activities occurring near sampled water bodies may thus influence the level of *Listeria* contamination in the fish. In the Siavonga district, cattle and crop farming are rare, and most people solely depend on fishing for a living. The low level of *Listeria* might partly be due to the lack of

extensive farming activities in this area. The none-detection of *Listeria* contamination on PCR observed in the present study could also be explained because sampling was at the primary production stage. Furthermore, sampling was from a single lake, where environmental factors are assumed to be similar, previous studies reporting a higher prevalence of *Listeria* species mainly sampled fish from different water bodies.

Climatic conditions, mainly rainfall, contribute to bacterial contamination of surface water. The collection of samples was during the rainy season, when the probability of isolating *Listeria* species is usually higher than during the dry season, as reported in other studies. However, Hansen and others (2006) failed to find significant seasonal variations of *L. monocytegenes* levels in water from freshwater fish farms [11]. The principle limitation of this study is the low sample size. However, some studies have used less than ten (10) samples of fish from freshwater and have been able to isolate *Listeria* species. Additionally, the other limiting factor may link to the approach used. Including entire fish or the enteric contents would have increased the likelihood of isolating *Listeria*. On the other hand, large freshwater bodies have a practical dilution effect. Another insidious factor that we might have inadvertently ignored may have significantly affected the final results.

Conclusion

Results from the study intimate the likelihood of low levels of *Listeria* species in wild-captured and cultured tilapia from lake Kariba in the Siavonga district of Zambia. However, conducting further studies across different seasons is recommended. Additionally, there is need to repeat the same study with an increased sample size and inclusion of enteric organs and whole fish analysis to increase isolation rates.

What is known about this topic

- *The prevalence of Listeria species in raw beef and poultry ranges from 0.095 to 15%.*

What this study adds

- *To the best of our awareness, this is the first study that investigated Listeria species contamination in tilapia from a large freshwater lake in Zambia.*

Competing interests

The authors declare no competing interests.

Authors' contributions

Prudence Mpundu conceptualized the study and wrote the manuscript. Marina Elisabeth Aspholm and Musso Munyeme: validation of the molecular work protocol, and manuscript proofreading. John Bwalya Muma, Nawa Mukumbuta, and Walter Muleya contributed to manuscript editing and proofreading. All the authors have read and agreed to the final manuscript.

Table and figure

Table 1: presumptive *Listeria* species results in tilapia fish from captured fisheries and aquaculture cage farms from Kariba, a large freshwater lake, Zambia (n=150)

Figure 1: map of the Siavonga district showing the study areas

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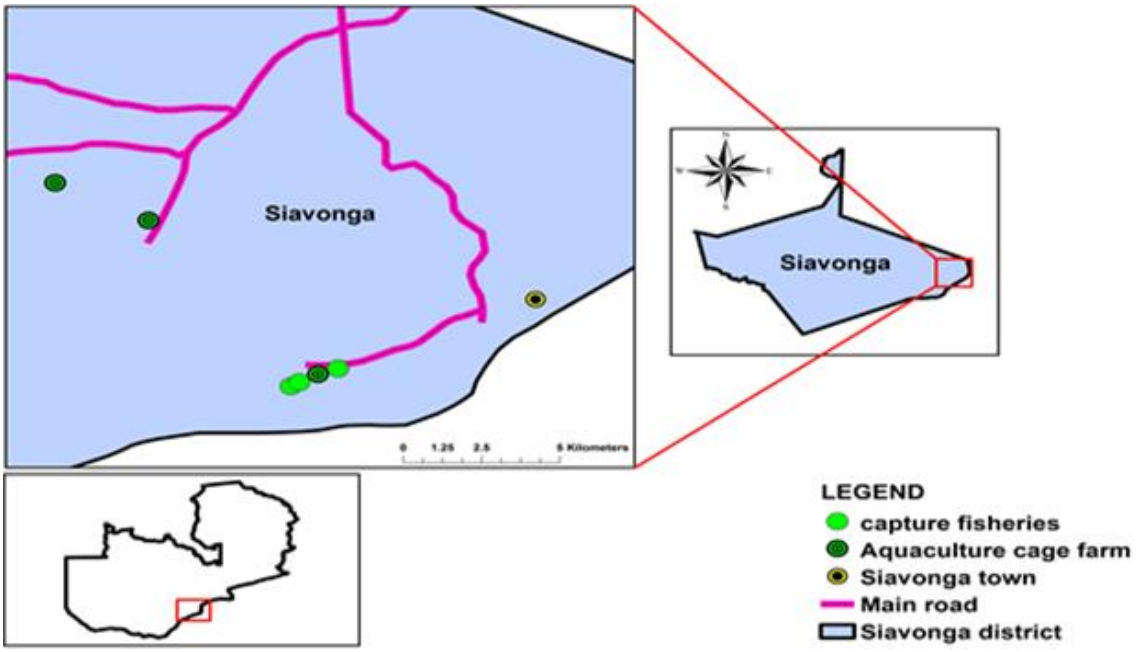
Table 1: presumptive *Listeria* species results in tilapia fish from captured fisheries and aquaculture cage farms from Kariba, a large freshwater lake, Zambia (*n*=150)				
Variable	**Number of samples**	**Presumptive *Listeria***	**Prevalence**	**95% CI**
Part swabbed				
Gills	75	2	2.66	0.048 - 6.453
Exterior surface	75	1	1.33	0.284 - 3.916
Source of the tilapia				
Capture fisheries	75	2	2.66	0.048 - 6.453
Aquaculture cage-farms	75	1	1.33	0.284 - 3.916



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