



RESEARCH ARTICLE

Molecular Detection of Aeromonas Hydrophila from Frozen Fish; a Food Quality and Safety Concern

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ARTICLE INFO	ABSTRACT
Received: Apr 15, 2023	Aeromonas hydrophila is a pathogenic bacterium affecting fish that when ingested by humans, induces gastroenteritis. This research aimed to evaluate the occurrence of A. hydrophila from five different frozen fish brands labeled A-E marketed from five different stalls labeled 1-5, in Kano metropolis, Kano State, Nigeria. The first three brands A, B, and C were local brands while C and D were of foreign origin. A total of 500 samples, 100 from each brand were purchased from retail stalls as frozen fish and processed for the isolation and biochemical identification of A. hydrophila. Polymerase chain reaction (PCR) using specific ahh1 primer was employed to confirm the identity of A. hydrophila. Overall, 48% (n=240) of the samples were positive for A. hydrophila with incidences of 12% (n=60), 10% (n=50), 14% (n=70), 6% (n=30) and 6% (n=30) for A, B, C, D, and E brands respectively. Out of 240 samples, 20% (n=48), 21.25% (n=51), 19.17% (n=46), 19.58% (n=47) and 20% (n=48) were detected from stalls 1, 2, 3, 4 and 5 respectively. The PCR detection reveals the presence of ahh1 gene having 130 bp size. Presence of A. hydrophila in fish and fish products is a potential for spoilage and infection due to the organism.
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INTRODUCTION

Aeromonas hydrophila is psychrophilic gram-negative, non-motile, oxidase positive, facultative anaerobic rod belonging to the family Aeromonadaceae. Aeromonas is ranked among significant foodborne pathogens, basically due to the fact that it is capable of growing in cool temperature (Kirov, 2001; Hussain et al., 2014). Various types of foods including seafoods were reported to harbour Aeromonas hydrophila (Devlieghere et al., 2000; Simmons and Gibson, 2012; Suendarti et al., 2023).

It is well known that A. hydrophila is distributed in marine ecosystem such as marine fish and retailed outlets of sea foods. It is a microbiota of various fish species (Yogananth et al., 2000; Hussain et al., 2014) A. hydrophila and A. sobria have been frequently found to be associated septicemic haemorrhage in poikilotherms such as fish, amphibians and reptiles (Janda and Abbott, 2010). Such Aeromonad primary pathogens genera are also considered pathogenic associated with severe diarrhoea in immunocompromised patients (Chopra and Houston 1999). Several studies revealed A.

hydrophila (HG1), *A. sobria* (HG8) and *A. caviae* (HG4) as commonest strains linked to the diseases of human intestines (Borrell et al., 1997; Hussain et al., 2014; Rahman et al., 2024). This study aimed at determining the occurrence of *A. hydrophila* from local and foreign brands of fish and from different stalls using PCR assay targeting the amplification of *ahh1* gene.

METHODOLOGY

Sampling

Fish specimen was purchased from retailer shops of Kano metropolis, Kano State Nigeria in sterile whirl-pack bag. These specimens were readily transported to the laboratory for analysis.

Sample processing and *A. hydrophila* identification

A total of 25 grams of fish specimen was homogenized in 225 ml buffered peptone water (BPW) (Oxoid, Hampshire, UK) for 1.00 min at 230 rpm and subjected to incubation. One milliliter of the homogenized sample was dispensed into 10 ml tryptic soy broth. Incubation followed at 25°C for 24 h. Colonies were subjected to Gram staining and biochemical tests such as oxidase, Voges-Proskauer, indole production, β -hemolysis, motility, catalase, H₂S and gas production for presumptive identification. Stock cultures were maintained in a broth medium supplemented with glycerol at -20°C (Cagatay and Sen, 2014; Alotaibi et al., 2023).

Transport of Culture for PCR Analysis

Samples presumptively identified as *A. hydrophila* were prepared in skimmed milk agar and transported to the Center of Excellence for Food Safety Research, Faculty of Food Science and Technology, University Putra Malaysia, 43400, Serdang, Selangor, Malaysia for PCR analysis. Upon arrival, samples were sub cultured on nutrient broth (Oxoid, UK) for DNA isolation.

DNA Extraction

The genomic DNA of *A. hydrophila* presumptive isolate was extracted using of EZ-10 Spin Column Bacterial Genomic DNA Miniprep Kit (BS 423 Bio Basic, Canada). About 1 mL of a 24 hour's old culture of *A. hydrophila* in Luria Bertani (LB) broth was spun in an Eppendorf tube at 2,500 x g for 5 minutes at room temperature. The supernatant was removed and the cell pellets were washed twice with PBS buffer (Thermo scientific, Waltham, Massachusetts, USA). This was then followed by another resuspension of cells in 200 μ L Tris-EDTA (TE) buffer and the addition of 400 μ L of digestion solution. The resulting solution was mixed well and 3 μ L of Proteinase K solution (2mg/150 μ L) was added to the sample and then incubation for five minutes at 55 °C. In addition, absolute ethanol (260 μ L) was dispensed to the sample. The entire sample was transferred to EZ-10 spin column put into collection tube of 2.0 mL capacity; centrifuged at 8,000 x g (10,000 rpm) for two minutes.

The discard of the flow-through in collection tube was done prior to the addition of 500 μ L of wash solution. Resultant mixture was also subjected to spinning at 8,000 x g (10,000 rpm) for 2 minutes. This step was repeated with discard of the flow-through. To completely remove residue of Wash Solution, the sample was spun at 8,000 x g (10,000 rpm) for an additional minute. The EZ-10 column containing the sample was placed in a clean and sterile 1.5 mL Eppendorf tube and 50 μ L of Elution Buffer was added directly into the central membrane of the column and incubated at 37 °C for 2 minutes to increase the DNA recovery yield. This was then followed by spinning at 8,000 x g (10,000 rpm) for 2 minutes for eluting the DNA from the column. The Genomic DNA quality was assessed by analytical 0.7% agarose gel.

Amplification of the PCR products

The PCR products of the specific gene for the identification of *A. hydrophila* were amplified using *A. hydrophila* 16S rRNA specific primer (*ahh1*). The sequences of the primer (*ahh1*) pairs include

forward primer 5'-GCC GAG CGC CCA GAA GGT GAG TT-3'; reverse primer 5'-GAG CGG CTG GAT GCG GTT GT-3' having an estimate of 130 bp amplicons (Wang et al., 2003). The volume (50 µL) for the PCR mixture to amplify the gene include 2 µL genomic DNA, 10 µM primers (both forward and reverse) at 2 µL, then 25 µL of master mix (PCR Biosystems Ltd, London, UK) and 19 µL sterile deionized water. The mixture was amplified in thermal cycler (Kyrathec Super Cycler Thermal Cycler, Australia).

Amplification conditions for the PCR include initial denaturation at 94°C for 3 minutes, 45 cycle of final denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min 30s. The final extension at 72°C for 3 minutes and the holding temperature of 4 °C (Wang et al., 2003; Hussain et al., 2014).

Visualization of the PCR Products Using Agarose Gel Electrophoresis

Electrophoresis using agarose gel was conducted on the products of the PCR using 1.0% agarose with 0.5 mg EtBr (ethidium bromide) for visualization. The electrophoretic gel was illuminated and visualized using Gel Documentation (GMV20-Model). Size of the amplicons of the PCR products was assessed with 100bp marker (Qiagen, Germany).

RESULTS

The results of the incidence of *A. hydrophila* from different brands of frozen fish purchased from different stalls are tabulated below. It can be observed in Table 1 that stall 2 had the highest percentage of occurrence of 21.25% (n=51), followed by stalls 1 and 5 with 20% (n=48) occurrence each. Finally, stalls 4 and 3 with 19.58% (n=47) and 19.17% (n=46) respectively.

Table 1: Occurrence of *A. hydrophila* in fish purchased from stalls in Kano metropolis

Stall	No. of Positive Samples for stall	Percentage positive (%)
1	48	20.00
2	51	21.25
3	46	19.17
4	47	19.58
5	48	20.00
Total	240	100

The incidence of *A. hydrophilic* in five different brands of frozen fish sold in Kano metropolis is displayed in Table 2. Out of a total of 500 samples, 240 were positive for *A. hydrophila*. However, out of 100 samples of each brand, the positives were 60, 50, 70, 30 and 30 samples were positive for *A. hydrophila* for A, B, C, D and E brands respectively. These made the percentages of the total positive samples as 25, 21, 29, 12.5 and 12.5% respectively.

Table 2: Occurrence of *A. hydrophila* in various brands of frozen fish sold in Kano metropolis

Brand	No. positive for <i>A. hydrophila</i>	Percentage positive (%)
A	60	25
B	50	21
C	70	29

D	30	12.5
E	30	12.5
Total	240	100

Figure one shows the PCR products of the representative strains of the isolated *A. hydrophila* from various frozen fish brands marketed in Kano metropolis. The reference strains have also been depicted in the figure.

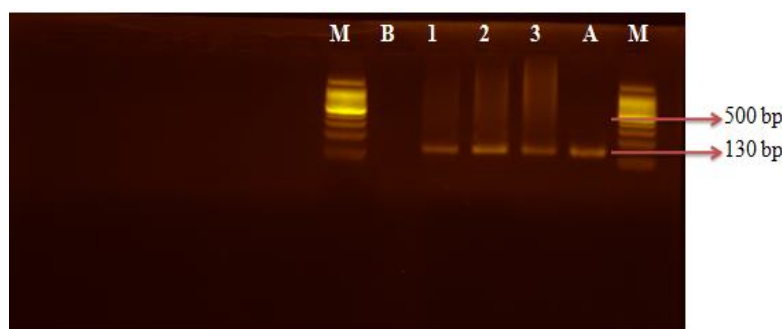


Figure 1: Agarose gel (1.5%) stained with ethidium bromide depicts amplified ahh1 gene of the *Aeromonas hydrophila* strains Lane: M, 100bp DNA ladder (Gelpilot, Qiagen); Lane A, Positive (*E. coli* ATCC 35401); Lane B, Negative (*E. coli* ATCC 35401); Lanes 1 to 3, representative *A. hydrophila* isolates.

DISCUSSION

Species of the *Aeromonas* possess high adaptation to moist or aquatic habitats and are labeled as a bacterial pathogen of animals and man. *Aeromonas* is a genus that is composed of above 20 species. Out of these, *A. hydrophila*, *A. caviae*, *A. media*, *A. veronii* bv. *Sobria*, *A. veronii* bv. *Veronii* *A. caviae* (synonymous with *A. punctata*) are especially of clinical importance as they are able to cause septicemia, gastroenteritis, and wound and soft tissue infections (Janda and Abbot, 2010). A set of virulent factors such as cytotoxic and hemolytic toxins and enterotoxin are produced by *Aeromonas* spp. Reviews have implied that few proportion of species of *Aeromonads* have really been disease-causing with their transmission hitherto via unidentified course. This indicates that further studies such as epidemiological and molecular are required (Von Graevenitz, 2007; Persson et al., 2015). Traditionally, identification of *Aeromonas* species is conducted by various tests of biochemicals. Although the tests seem to be inconclusive as there is a display by *Aeromonas* species various biochemical properties in comparison with methods of molecular analysis. Thus the accuracy of identification using biochemical analysis is relatively not absolutely reliable (Abbot et al., 2003; ØRMEN et al., 2005). Molecular species identification employed in this study corresponds with that employed by Hussain et al. (2014) and Persson et al. (2015).

The occurrence of *A. hydrophila*, in frozen fish obtained from different stalls in this study revealed that there was no significant difference ($P > 0.05$) in occurrence of the *A. hydrophila* in the stall. This is possible as all the stalls operate in the same manner of purchase and delivery of the item. The same food safety regulations might be adhered to by the marketers. However, the rate of the occurrence of *A. hydrophila* among various brands of the fish indicates a significant difference ($P < 0.05$) as the locally frozen fish had higher number of samples contaminated with *A. hydrophila* than their foreign counterparts. This may be attributed to the poor adherence to the food quality and safety regulations as enshrined by the relevant authorities (Amaami et al., 2017; Azanaw et al., 2019).

Detection of *A. hydrophila* from fish was similarly conducted by Hussain et al. (2014) in which about 16% percent detected were the species of *A. hydrophila*. In addition, Abbot et al. (2003) found about 37% of the samples of shellfish haboured *A. hydrophila*. Moreover, the survival of *A. hydrophila* in frozen fish is owing to their ability to withstand low temperature indicating that they are psychrophiles (Kirov, 2001; Hussain et al., 2014) as well as psychrotrophic (Simmons and Gibson, 2012).

Using the PCR, there was confirmation that 48% of the brands of the commercially sold frozen fishes were positive *A. hydrophila*. This is nearly similar to the 56% of commercially vended fresh fish containing *Aeromonas* spp. as assessed using 16S rRNA gene (Hussain et al., 2014). It was observed by Bin Kingombe et al. (2004) that 51 samples were MPCR positives for *Aeromonas* spp. from the 65 samples that contributed to nearly 78 % were detected. This is higher than obtained in this study. The results that contradict this obtained by the conventional biochemical tests were solved by PCR with regards to the strains of *A. hydrophila*. There were reports that biochemical tests sometimes lack specificity for some pathogenic bacterial strains (Park et al. 2003; Beaz-Hidalgo et al. 2010) which is not needed to be similar for all the species. In this study, the presence of hemolysin gene, *ahh1* PCR assay was performed. There was an early report that the production of hemolytic cytotoxin is considered a proof of the presence capability of *Aeromonas* spp. especially *A. hydrophila* (Wang et al. 2003).

CONCLUSION

A. hydrophila was found to have higher prevalence in locally made frozen fish than the foreign ones. Moreover, all stalls were found to have no significant difference in their composition of frozen fish contaminated with *A. hydrophila*. PCR conducted proved the confirmation of the *A. hydrophila* to support the biochemical typical of *A. hydrophila*. The prevalence of *A. hydrophila* in frozen fish indicates their psychrophilic nature and can lead to severe gastroenteritis due to the organisms if the fish are eaten raw or undercooked.

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