Microbiology Research Journal International

31(3): 31-39, 2021; Article no.MRJI.67457
ISSN: 2456-7043
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

Presence and Virulence Potential of *Aeromonas hydrophila* in Selected Water Sources for Household Consumption in Makurdi, Benue State

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2021/v31i330303
Editor(s):
(1) Prof. Lidija Kozacinski, University of Zagreb, Croatia, Balkans.
Reviewers:
(1) Merlin Sanicas, Aix-Marseille University, France.
(2) David Muli Kavili, Kenyatta University, Kenya.
Complete Peer review History: http://www.sdiarticle4.com/review-history/67457

Original Research Article

Received 18 February 2021
Accepted 23 April 2021
Published 10 May 2021

ABSTRACT

This study was conducted to investigate the prevalence of *Aeromonas hydrophila* in selected drinking water sources in Makurdi. A total of 100 water samples (Tap, river, stream, well, pond and borehole water) were collected from different locations in Makurdi. Isolation and identification of the organism was performed using standard microbiological techniques. Further confirmation of the isolates as *Aeromonas hydrophila* was carried out using the Microbact 24E detection kit and polymerase chain reaction (PCR). *A. hydrophila was* detected in 12 (12%) out of the 100 samples; 6.67%, 8.82%, 7.14%, 25%, 30% and 20% of tap, well, borehole, river, pond and stream water samples respectively. The highest isolation rate of *A. hydrophila* (30%) was from pond water. All *A. hydrophila* isolated exhibited heamolysin, protease and lipase activity. The findings of this study revealed that treated and untreated drinking water sources in Makurdi are contaminated with potentially virulent *A. hydrophila* strains which may pose a health risk to consumers. Therefore, basic water treatment should be applied to drinking water to reduce public health threat posed by this finding.

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Keywords: Aeromonas hydrophila; drinking water; virulence factors.

1. INTRODUCTION

Aeromonas hydrophila and related species are Gram-negative short rods, facultatively anaerobic, non-sporing, oxidase positive bacteria [1]. Due to its ubiquitous nature, *A. hydrophila* is found in many foods and have been isolated from dairy products, meat and poultry, seafood, vegetables and fresh water [2,3]. *Aeromonas hydrophila* has been incriminated as the main cause of *Aeromonas* associated human diseases beside *A. sobria* and *A. caviae* [4]. It has been reported to be responsible for intestinal and extra intestinal diseases ranging from relatively mild illnesses such as gastroenteritis and wound infections to life-threatening conditions such as septicemia, hemolytic-uremic syndrome and necrotizing fasciitis in humans [5]; also peritonitis and pneumonia [6]. Reports show that a greater risk of infection occurs in young children, elderly people and immune compromised patients [7]. Virulence in *A. hydrophila* is multifactorial, with disease resulting from the production of various virulent factors including haemolytic, cytotoxic and enterotoxigenic properties [8].

A. hydrophila has received much attention both as an emerging human pathogen and as an indicator of pollution associated with several aquatic environments including lakes, rivers, well, pond and chlorinated water sources that are considered to significantly impact public health [9,10]. In a study reported by Figueras and Ashbolt [11], *Aeromonas* was more prevalent than *Salmonella* or *E.coli* in patients with diarrhea in Nigeria. Globally, there is a problem in detection and monitoring of microbial pathogens in drinking water. Some developing countries have low drinking water quality due to inefficient common surveillance tools for waterborne pathogens [9,12]. According to Krovacek et al. [13], outbreaks of food –borne infections caused by *Aeromonas* are infrequently reported. However, a large part of the unidentified food borne infections and outbreaks may probably be caused by organisms such as *A. hydrophila* which are not routinely tested for identifying the etiology of food poisoning. *Aeromonas hydrophila* has been reported to be implicated in several foodborne outbreaks [3,9,10,13,14]. Reports indicate that the most common source of *Aeromonas* outbreaks has been water supplies, with *A. hydrophila* frequently isolated from surface and subterranean waters and also water distribution systems of treated and untreated drinking water, exposing the consumer to health risks [13,15]. Although the frequency of *Aeromonas* diarrhea is about 1.62% infections per million people, with high mortality in children [11], its occurrence in water and foods should not be neglected.

Given its health risks on individuals, the World Health Organization guidelines for drinking water quality have added *Aeromonas* to the list of potential human pathogens, and public water systems are now required to report the presence of *Aeromonas* through the Consumer Confidence Report Rule [16]. With the majority of virulent *Aeromonas* strains belonging to *A. hydrophila* HG1, regular monitoring of drinking water sources and a reliable identification of this strains is necessary to establish the risk associated with its presence in water sources [9,17].

With the wide spread nature of virulent and antibiotic resistant strains of *A. hydrophila* in drinking water sources, which exposes consumers to health risks, [2,7], it is important to assess the prevalence of *A. hydrophila* in drinking water sources in Makurdi, especially untreated water sources consumed by low income earners and rural dwellers.

The main objective of this study was to determine the presence of *A. hydrophila* in selected drinking water sources in Makurdi, and its virulence potential.

2. MATERIALS AND METHODS

2.1 Sample Collection

A total of 100 drinking water samples of 10 ml each were randomly collected aseptically in sterile Bijou bottles from borehole, river, tap, pond, stream and well in selected locations in Makurdi, Benue State, Nigeria (7.7322^{o}N, 8.5391^{o}E). These were conveyed to the laboratory within 3 h for analyses.

2.2 Isolation of *Aeromonas* spp. from Water

An aliquot of 1ml of each water sample was added in 9 ml Nutrient broth for enrichment and incubated at 37^{o}C for 24 h. A loopful from the enrichment broth was then plated on starch-ampicilin agar (SA agar) and incubated at 37^{o}C for 24 h. After the incubation period, plates were observed for growth consistent with *Aeromonas*

(yellow coloured colonies) and four colonies were randomly selected from each plate. The isolates were sub-cultured on Starch agar and incubated at 37^{o}C for 24 h repeatedly to obtain pure cultures [18]. These were then stored on nutrient agar slants in the refrigerator for further identification.

2.3 Phenotypical Characterisation of Isolates

Presumptive *Aeromonas* colonies were identified by standard physiological and biochemical tests according to Harrigan and McCance [19], Cowan [20] and Bergey's Manual of Systematic Bacteriology [21]. The isolates were differentiated on the basis of their cultural and morphological characteristics such as growth size and shape, elevation and pigmentation. They were then subjected to various biochemical tests including Gram reaction, Indole, Vogues Proskauer and Methyl red test, Oxidase, Catalase, motility test, citrate test, nitrate reduction, ammonia production tests, fermentation of sugars, gelatin hydrolysis and hydrogen sulphide production. Growth in different conditions such as pH, NaCl concentration and temperature was also tested. The isolates were further characterised using the Microbact 24E.

2.4 Molecular Identification of *Aeromonas hydrophila*

2.4.1 *Aeromonas hydrophila* DNA extraction

Presumptive *A. hydrophila* cultures were grown in trypticase soy broth (TSB) at 37°C for 18 h to obtain young culture prior to the extraction of genomic DNA. DNA extraction was carried out according to the procedure described by Hussain et al. [22]. Briefly, 1 ml of the cell suspension was centrifuged at 10,000 × g for 10 min at 4 °C and the cell pellet mixed with 600 µl of guanidine hydrochloride buffer (pH 8.0), incubated at room temperature for 30 min and again centrifuged at 10,000 × g for 10 min at 4°C. A 500 µl portion of the supernatant was transferred into another tube and mixed with 100% ice cold ethanol and centrifuged at 13,000 × g for 20 min at 4 °C. The supernatant was discarded and the pellet washed with 95%, and then 90% ethanol followed by centrifugation at 10,000 × g for 10 min at 4 °C. The pellet was then resuspended in 50 µl of molecular grade water, quantified in a Biophotometer (Eppendorf AG, Hamburg, Germany) and then stored at −20 °C to be used as PCR template.

2.4.2 PCR amplification

The amplification of the *A. hydrophila* specific gene was employed for the confirmation of *A. hydrophila* isolates. The sequences of the pairs of primer (AHH1) were forward 5'-GCC GAG CGC CCA GAA GGT GAG TT-3' and reverse 5'-GAG CGG CTG GAT GCG GTT GT-3' with the estimated amplicon size of 130 bp. The reaction mixture (25 µl) for the PCR amplification consisted of 1 µl of the genomic DNA, 10 µM each of the forward and reverse primers (1 µl), 12.5 µl of 2x PCR BIO Taq Mix Red (PCR Biosystems Ltd, London, UK) and 9.5 µl ultra pure water. Amplification was done in a thermal cycler (Kyratec SuperCycler Thermal Cycler, Australia). The PCR conditions for the *A. hydrophila* AHH1 gene identification included initial denaturation at 94°C for 3 mins, then the 45 cycles consisting of denaturation at 94°C for 60 s, annealing at 57°C for 60 s and an extension at 72°C for 90 s. The final step of extension was at 72°C for 3 mins and held at 4^{o}C until collection [22,23].

2.4.3 Agarose gel electrophoresis for visualization of the PCR products

The PCR products were analyzed using agarose gel electrophoresis (Bio-Rad Powerpac 300) in 1.5% agarose pre-stained with 0.5 mg of ethidium bromide per ml and were visualized and recorded by Gel Doc (GMV20-Model). The amplicon sizes of the products were measured using Gelpilot 100bp DNA ladder (Qiagen, Hilden, Germany).

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2.7 Detection of Virulence

2.7.1 Detection of lipase production

The presence of extracellular lipases was determined using the method described by Abd-El-Malek [4]. Each serially diluted isolate was plated on phenol red agar (prepared by incorporating phenol red (0.01% w/v), olive oil (0.1% v/v), $CaCl_2$ (0.1% w/v) and agar (2% w/v), and incubated at 37^{0}C for 24 h. Positive lipase activity was confirmed by observing the formation of a precipitate with yellow colouration around the colonies. An uninoculated plate served as a control.

2.7.2 Detection of protease production

The skimmed milk agar was used for this test. It was prepared by adding 1% (w/v) skimmed milk to the appropriate agar as described by Harrigan and McCance [19]. The media was sterilized by autoclaving at 121^{0}C for 15 mins. The warm media was dispensed into sterile Petri dishes and allowed to solidify. The plates were inoculated by streaking the respective isolates across the plates. Uninoculated plates served as control. At the end of the incubation, a clear zone around the line of streaking indicated casein hydrolysis due to the activity of the protease enzyme.

2.7.3 Detection of haemolysin production

Haemolytic activity was detected by the plate method. Each isolate was streaked onto 5% sheep blood agar plates and incubated at 37°C for 24 h. The presence of a clear zone around the colonies was taken as positive for haemolysin production [10].

3. RESULTS AND DISCUSSION

3.1 Isolation and Characterisation of *Aeromonas hydrophila* from Selected Water Sources for Household Consumption

Twelve presumptive *A. hydrophila* strains isolated from fresh water sources in Makurdi were identified to species level using their

physiological and biochemical characteristics. *Aeromonas hydrophila* showed yellow to honey coloured colonies of 2-3mm diameter. Flooding the plates with lugol's iodine showed a clear zone of hydrolysed starch surrounding the colonies against a black agar background [7,18,24].

Table 1 shows the morphological and biochemical test results for identification of presumptive *A. hydrophila* isolated from selected drinking water sources in Makurdi. All isolates were Gram negative, rod shaped, oxidase and catalase positive. The biochemical tests result (Table 1) obtained from Microbact 24E was used to identify the isolates with reference to Microbact data base. These characteristics were in conformity with the documentations of Adegoke and Ogunbanwo [10], Cowan [20] and Bergey's manual [21]. Reports from previous researchers have shown that *Aeromonas* identification is not always conclusive without molecular identification as some species display heterogenous biochemical characteristics [25]. Therefore, the isolates were further confirmed using Polymerase Chain Reaction (PCR) technique. Twelve *A. hydrophila* were identified.

Fig. 1 shows amplified Polymerase Chain Reaction products of *A. hydrophila* strains on Ethidium bromide stained agarose gel (1.5%). PCR identification gives a reliable identification of *A. hydrophila* isolates [26]. PCR products of 130 bp amplicon size were obtained from the reaction synonymous with identification of *A. hydrophila*. This result agrees with biochemical tests using Microbact 24E. Similar PCR products were reported for *A. hydrophila* isolated from various natural and treated water sources [27,28,29].

3.2 Prevalence of *Aeromonas hydrophila* in Selected Water Sources for Household Consumption in Makurdi

The results on Table 2 show the presence of *A. hydrophila* in selected water sources for household consumption in Makurdi. *Aeromonas hydrophila* was found in at least one water sample from all the water sources assessed with higher rates of occurrence in stream, river and pond water. These are drinking water sources used mostly by rural dwellers without treatment. This may have a potential risk on residents of these areas as most of the people living in this areas use this water for domestic activities and for drinking especially during the dry season. Similar reports were made by earlier researchers in other parts of the world [17,30,31,32,33], that *A. hydrophila* is a pathogen associated with water from ponds, rivers, lakes, borehole or groundwater, surface water and chlorinated water. The presence of this pathogenic organism can pose severe health risks to consumers, children and immune compromised individuals in particular, being a primary pathogen in cases of acute diarrhea [22,34].

The lowest occurrence of the organism was observed in tap water. Koksal et al. [35] and Scoaris et al. [36] reported similar low presence of *A. hydrophila* in tap water sources. This is likely due to treatment given to the water, especially chlorination, which can reduce the number of microorganisms in water. *A. hydrophila* in tap water may occur due to low levels of residual chlorine, contamination through broken water pipes, stagnant piped water and presence of organic matter [11,37]. The presence of *A. hydrophila* in tap water confirms its possibility as a vehicle for transmission of the organism.

3.3 Virulence Factors of *A. hydrophila* from Selected Water Sources for Household Consumption in Makurdi

The identified *A. hydrophila* strains were tested for various virulence factors including haemolysin, proteases and lipase production. Results on Table 3 revealed the virulence of *A. hydrophila* strains from the selected water sources. All the *A. hydrophila* strains exhibited haemolytic, protease and lipase activity which has been reported as the most common virulence factors in motile *aeromonads* [32]. Similar virulent factors in *A. hydrophila* were reported by other researchers [10,15,26]. Haemolysins are a group of multifunctional enzymes that play a vital role in *A. hydrophila* pathogenesis. Ahh1 is the most abundant of the strains of *A. hydrophila* that produce many widely distributed haemolysins. The presence of this gene is a strong evidence of pathogenic potential of *A. hydrophila* isolates [22,23,38]. The production of these virulence properties have been known to contribute to pathogenesis and disease in humans [8,38]. The pathogenesis is that of a toxicoinfection with symptoms including fever, diarrhoea and abdominal pain [39]. Senderovich et al. [34] reported similar virulence in *A. hydrophila* isolated from diarrhea patients. More attention is required to ensure safety of consumers of water from such sources because a high rate of virulent *A. hydrophila* occurrence can lead to waterborne outbreak [40].

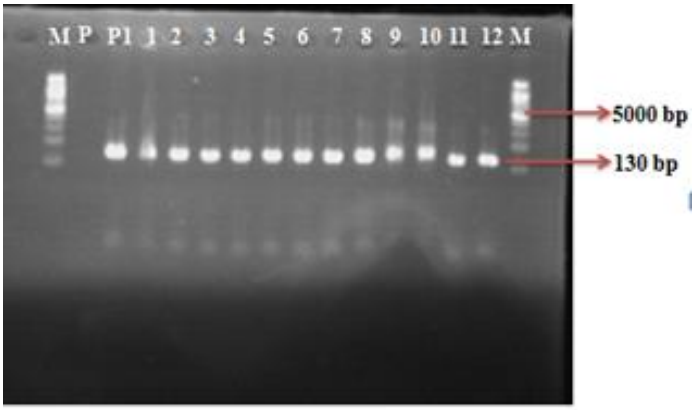

Fig. 1. Ethidium bromide stained agarose gel (1.5%) showing amplified PCR products of *Aeromonas hydrophila strains*

Lane M- 100bp DNA ladder (Gelpilot, Qiagen); Lane P- Negative reference strain (E. coli ATCC 35401); Lane P1- Positive reference strain (A. hydrophila ATCC 7966); Lanes 1 to 12 -A. hydrophila isolates: M39a, M39b, MK1, M22, AIE, A2A, A2C, A2E, BC1, B22, MX and MC

Table 1. Morphological and biochemical tests for identification of presumptive *Aeromonas hydrophila*

Isolates	M39a	M39l	MK1	M22	A1E	A2A	A2C	A2E	BC1	B22	MX	MC
Characteristics												
Gram reaction	-	-	-	-	-	-	-	-	-	-	-	-
Shape	rod	rod	rod	rod	rod	rod	Rod	rod	rod	rod	rod	rod
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate	+	+	+	+	+	+	+	+	+	+	+	+
Lysine	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine	-	-	-	-	-	-	-	-	-	-	-	-
H_2S	+	-	+	+	+	-	+	-	+	+	+	+
Indole	+	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-	-
V-P	+	+	-	+	+	+	-	+	+	+	+	+
Methyl red	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	-	+	-	+	+	+	-	-	+	+	+
Lactose	-	-	-	+	-	-	-	-	+	-	-	+
Arabinose	+	+	+	+	-	+	+	+	-	+	-	+
Inositol	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	+	-	+	+	-	+	+	+	-	-	+
Glucose	+	+	+G	+G	+	+	+G	+	+	+G	+G	+
Mannitol	-	+	+	-	+	+	-	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	-	+	-	+	+	-	+	+	+	-	-	+
ONPG	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	-	+	+	-	+	+	+	-	-	+
Growth at 37C	+	+	+	+	+	+	+	+	+	+	+	+
6.0% NaCl	-	-	-	-	-	-	-	-	-	-	-	-
pH 4	-	-	-	-	-	-	-	-	-	-	-	-

Key: + = positive, – = negative, G = gas

Table 2. Prevalence of *Aeromonas hydrophila* in selected water sources for household consumption in Makurdi

Water source	Number of samples collected	Number of samples positive for *A. hydrophila*
Tap	15	1(6.67)
Well	34	3(8.82)
River	8	2(25)
Pond	10	3(30)
Borehole	28	2(7.14)
Stream	5	1(20)

Values in parentheses denotes percentage

Table 3. Virulence characteristics of *Aeromonas hydrophila* from selected household water sources in Makurdi

Isolates	M39a	M39b	MK1	M22	A1E	A2A	A2C	A2E	BC1	B22	MX	MC
Virulence test												
Haemolysin activity	+	+	+	+	+	+	+	+	+	+	+	+
Protease activity	+	+	+	+	+	+	+	+	+	+	+	+
Lipase activity	+	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+

Source of A.hydrophila: M39a and M39b- River water; MK1 and MX –Borehole water; MC-Tap water; BC1-Stream water; M22,A1E and B22- Well water; A2A,A2C and A2E- pond water

4. CONCLUSION

This study indicates the presence of pathogenic *A. hydrophila* with virulence potential in stream, pond, river, well, borehole and tap water sources evaluated in Makurdi. This poses a major threat to public health since these sources of water are used for drinking and household chores. Therefore, there is a need to advocate for adequate basic water treatment/ purification such as boiling of water from these sources before drinking to reduce the health risks associated with *A. hydrophila* infections, especially in susceptible populations such as the immunosuppressed, children and elderly people.

DISCLAIMER

The research was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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