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Isolation and Characterization of Aeromonas Species Isolated from Food and Diarrhoeagenic Stool in Ibadan Metropolis, Nigeria

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Abstract
Aeromonas species has been associated with gastroenteritis which resulted in about 1.3 million deaths of children less than 5 years as of 2008. The ability of *Aeromonas* species to survive in chlorinated water when they are in biofilms couple with antibiotics resistance and virulence toxins produce call for continuous monitoring of food and diarrhoeagenic stool for *Aeromonas* species. Cabbage (100), tap water (100) and diarreagenic stool (100) were randomly collected in Ibadan metropolis and analysed for the presence of *Aeromonas* species using standard method. The isolated *Aeromonas* species were identified using classical method and confirmed by PCR using 16S rRNA primmer and Random amplified polymorphic DNA (RAPD) analysis. Survival of *Aeromonas* species in different chlorine concentrations was determined using standard method. Antibiotics susceptibility profiles of the isolates were determine using disc diffusion technique. Data were analysed using one-way ANOVA at $\alpha_{0.05}$. Two hundred and twenty nine isolates of Aeromonas were obtained and identified as *Aeromonas veronii* (23), *Aeromonas caviae* (29), *Aeromonas hydrophilla* (52), *Aeromonas popoff* (15), *Aeromonas trota* (2), *Aeromona schubertii* (3), *Aeromonas jandaei* (16), *Aeromonas media* (10), *Aeromonas salmonicida* (28), *Aeromonas bestiarum* (10) *Aeromonas sobriae* (20) and *A. aquariorum* (21). The PCR confirms 10 species as Aeromonas, however RAPD differentiates *Aeromonas* species and confirms 60 to 100% genetic similarities indicating endemic infection. *Aeromonas* species was able to survive in most of chlorine concentrations used in this work. All isolates were 100% resistance to ampicillin, amikacin (85%), gentamycin, rifampicin, penicillin, nitrofurantoin (80% each) and nalidixic acid (75%). High multiple antibiotics resistance was observed among *Aeromonas* species with Minimum inhibitory concentration ranged from 16-512µg/mL for ofloxacin, ciprofloxaxin and tetracycline. *Aeromonas* species obtained from Cabbage, tap water and diarrhoeagenic stool are multidrug resistance confirming food and water transmission thereby calls for potent therapeutic measures and good manufacturer practices are recommended.
Keywords: *Aeromonas spp*, multiple antibiotics resistance, Cabbage, water, Diarrhoeagenic stool.

1. Introduction

Aeromonas species are gram-negative rods, oxidase-producing organism that grow on MacConkey agar. They are facultative anaerobes and ferment carbohydrates (Harris *et al*. 1985), nonspore forming bacteria that are autochthonous and widely distributed in aquatic environments (Daskalov 2006). They grow optimally at temperature ranges of between 22°C and 35°C, but growth can also occur at 0–45°C in a few species (Ghenghesh *et al*. 2008). Some species, such as *A. salmonicida*, do not grow at 35°C (Carnahan & Joseph, 2005). All Aeromonas resist pH ranges of 4.5 to 5.5 (Isonhood & Drake 2002). Growth optimally in sodium chloride concentration range of 0 to 4% and could not survive in 6% sodium chloride (Harris *et al*. 1985).

In nature, aeromonads are found in food (Nishikawa & Kishi1 988) as well as treated drinking or raw sewage water, fresh vegetables and domestic animals. *Aeromonas* species are known as causative agents of a wide spectrum of diseases in man and animals and some motile species are becoming food and waterborne pathogens of increasing importance (Ansari *et al*. 2011).

Aeromonas species have the intrinsic ability to grow in water distribution systems, especially in biofilms, where they are unaffected by chlorination (Chauret *et al*. 2001). The occurrence of *Aeromonas* species in the aquatic environment has been recognized as a potential health risk, and some countries have embraced aeromonad counts as indicator of water quality (Borchardt *et al*. 2003).

Common clinical manifestations of *Aeromonas* infections are bacteremia, diarrhea and soft-tissue infections (Figueras 2005). Aeromonas species have been involved in human infections and produce different kinds of extracellular hydrolytic enzymes such as hemolysins and cytotoxins (Chopra & Houston 1999), enterotoxins, proteases, leukocidin, phospholipases, arylamidases, esterases, amylase, elastase, deoxyribonuclease, chitinase, peptidases, and lipase (Seethalakshmi *et al*. 2010).

An outbreak of *Aeromonas* species had been reported in many parts of the world such as Califonia in 1988 and a college in Xingyi City, China in 2012 (Qian Zhang *et al*. 2012). *Aeromonas* species are rapidly becoming resistant to commonly used drugs, thus posing a serious risk to community health at large (Overman & Janda 199). However, there is need for continuous monitoring of food and surveillance of patient's diarrheogenic

stool to establish the source of human infection and risk assessment, to reach a decision. Therefore, the aim of this work was to isolate, characterize, determine the antibiotics resistance pattern of *Aeromonas* species isolated from divers sources.

2. Materials and Methods:

2.1 Sample collection

In this research work, 300 samples (100 samples each) were randomly collected from stool, Cabbage (*Brassica oleracea*) and Tap water from various centers in Ibadan Metropolis. Stool samples were collected in wide mouth screw capped bottle from patients with complaint of diarrhoea submitted to the UCH Ibadan Microbiology laboratory, during the year 2013-2014 and was defined as 3 or more loose stools per day and transported to Ogun State College of Health Technology, Microbiology Laboratory Ilese Ijebu in an insulated icebox with ice packs (under refrigeration) and processed after one to two hours of collection. Cabbage was also collected from five major markets in Ibadan metropolis that makes up the City. The major markets are Bodija, Oje, Apata market, Orita Challenge and Monantan market.

2.2 Culture media

The culture media used include *Aeromonas agar* Blood agar, and Macchonkey agar and Alkaline peptone water. All the media were prepared according to the manufacturer's instructions. The powdered media was weighed using a Microwa Swiss electric weighing machine (7730 model) into 1-litre Erlenmeyer flask, 500ml of distilled water was added and homogenized in water bath for l0 minutes to completely dissolve the components. The media was sterilized in an autoclave at 121 ^{0}C for 15 minutes. It was cooled to about 45^{0}C, dispensed into Petri dishes, allowed to solidify and was dried in the oven at 37^{0}C for 15 minutes before use.

2.3 Isolation of *Aeromonas* spp

Isolation of *Aeromonas* species was carried out using the method of Nzeako *et al*. (2002) and Jatau & Yakubu (2004). About one gram/1mL of each sample (Cabbage, Tap water and Stool) were serially diluted in alkaline peptone water (Oxoid, PH 9.0) and inoculated in Aeromonas agar, blood agar (Palumbo *et al*. 1985), Mac Conkey Agar (Merck), and incubated at 35ºC for 48 hours. At the end of the incubation period of 24 h, the plates were observed for bacterial growth and representative colonies (green colonies with opaque centre and yellow colonies) were randomly selected. Isolates were sub-cultured on nutrient agar and incubated at 37^{0}C for 24 h repeatedly to get pure cultures.

2.4 Phenotypic Characterization of Isolates

The isolates were initially differentiated on the basis of their cultural and cellular morphological such as growth type, shape, elevation, size, pigmentation and consistency by employing macroscopic and microscopic, after which they were subjected to various biochemical tests using oxidase, catalase, esculin hydrolysis, the Voges-Proskauer (VP) reaction, gas from glucose, growth on mannitol and inositol. The *Aeromonas* isolates were further characterized phenotypically using the Microbact 24E

2.5 Phenotypic characterization using Microbac kits:

2.5.1 *Preparation of inoculums*

Two to three colonies of pure cultures of *Aeromonas* isolates were picked from an 18 h culture on blood agar plate and emulsified in 5mls of sterile saline solution (0.85%). This was thoroughly mixed to get a homogenous suspension.

2.5.2 *Strips preparation, inoculation and reading of the test strip*

Incubation box was prepared by adding 10ml of distilled water into the honeycombed well of the tray to create a humid atmosphere. The strips were removed from their packaging and separated into 4 smaller strips which were placed in incubation tray. Pure 18h old suspension of *Aeromonas* species was inoculated with the aid of sterile Pasteur pipette into the tubes by tilting the incubation box slightly forwards. The formation of the bubbles was avoided by placing the tip of the pipette against the side of the cupules and minerals oil was added so as to maintain anaerobic conditions, when the tube and the cupules were to be completely filled up, formation of a concave or convex meniscus was avoided. After this, it was incubated, at 35^{o}C for 18h. The strips were read at the end of incubation period. Complete fermentation showed yellow colouration as Positive (+) while Purple colour fermentation as negative (-). The results obtained from the tests carried out were used to phenotypically identify the organisms by reference to Microbact data base and Bagey's Manual of Systematic Identification Manual (Sneath *et al*. 1986).

2.6 Molecular Characterization of *Aeromonas* species

2.6.1 *DNA extraction*

Two colonies of each bacterial culture was suspended in 200 uL of sterile water and boiled at 100^0C for 10 min. This was followed by centrifugation at 8,000 rpm for 10 min. The resulting supernatant was transferred into a new Eppendorf tube for subsequent use as a DNA sample. Each DNA sample was assessed for yield in ng/uL and quality based on OD260/280 ratio. DNA was also diluted in distilled to a concentration range of 50 – 100 ng/uL for PCR assays**.**

2.6.2 *16S rRNA gene amplification*

The 16S rRNA gene was amplified using Primers 59-AGAGTTTGATCATGGCTCAG-39 (forward) and 59-GGTTACCTTGTTACGACTT-39 (reverse) according to Borrel *et al.* (1997). The PCR reaction is a 20-uL volume reaction, comprising x1 PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.2 mM (each) deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP (Promega Corp, Germany), 2 U of Taq I DNA polymerase (Promega Corp Germany), 30 pmol each of the primers and 1 uL of DNA template. PCRs were performed under the following conditions: denaturation at 93°C for 3 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. After the final cycle, extension at 72°C was allowed for 10 min. Also included in every set of PCR reactions was a negative control to which no template DNA was added. The PCR products were electrophoresed on 2% agarose gel pre-stained with ethidium bromide (0.5ug/mL) and viewed under UV light using a UV transilluminator with the DNA bands sized by extrapolation based on mobility of 100 bp DNA markers co-electrophoresed.

2.7 Random amplified polymorphic DNA analysis of *Aeromonas* species

Random amplified polymorphic DNA analysis was performed on 32 isolates using whole-cell DNA as template to determine the genetic relationships of isolates according to Campbell *et al.* (2000) using two different primers to obtain a banding pattern representative of the whole genome of an Aeromonas species DNA sample. The primer sequences were sequence 272 (5′-AGCGGGCCAA-3′) and sequence 208 (5′-ACGGCCGACC-3′). The PCR reaction was a 20 uL reaction that consisted of X 1 PCR buffer (50mM Tris-HCl, 50 mM KCl, pH 8.3), 2.5mM MgCl2, 250 micromolar of each dNTP, 20 picomole of each primer sequence, 1.5 U of Taq polymerase (Promega, Germany) and 50 – 100 ng genomic DNA template. Each primer was used for PCR in separate reactions and each PCR assay was run in duplicate for reproducibility. The amplification reaction was carried in a Biorad 37^0 C thermal cycler under the following amplification conditions: 1 cycle of DNA denaturation at 95^0C for 5 mins, followed by 35 cycles of DNA denaturation at 95^0C for 30s, primer annealing at 36^0C for 45s and primer extension at 72^0C for 60 s. Finally was a primer extension step at 72^0C for 7 min. The resulting PCR products were stored at 4^0C prior to electrophoresis (Campbell *et al*. 2000).

2.7.1 *Analysis of RAPD fingerprints*

The amplification products were electrophoresed in 1.5% agarose gel in Tris-borate buffer. The resulting RAPD bands were scored as 1 for presence and 0 for absence. Genomic DNA samples producing the same banding pattern of less than 3 polymorphic band differences were assigned the same RAPD type. Each RAPD type was also represented as a cluster in the dendogram construct. Computer analyses were carried out using the GelCompar II software (version 3.0; Applied Maths, Kortrijk, Belgium). Similarity between fingerprints was calculated with the Dice coefficient. Cluster analysis was performed using the Neighbour joining tree. The similarity levels between the profiles generated is expressed as percentage similarity and presented as a dendrogram (Campbell *et al*. 2000).

2.8 Aeromonas Identification

The isolates were identified based on the results of the various biochemical tests using Bergey's Manual of Systematic Bacteriology.

2.9 Influence of different Chlorine concentrations on the survival of *Aeromonas* species

The species of Aeromonas used for this work include: *Aeromonas hydrophila, Aeromonas caviae, Aeromonas veronii, Aeromonas sobria, Aeromonas trota, Aeromonas media, Aeromonas acquoriorum, Aeromonas bestiarum, Aeromonas salmonicida, Aeromonas jandaei, Aeromonas popoffi* and *Aeromonas schubertii.* A fresh plate of the organisms under study was prepared on Nutrient agar. 0.1 mL of an over-night culture (3.2×10^7) of the test organisms was inoculated into alkaline peptone water containing different concentration of chlorine (0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg l^{-1}) and incubated aerobically at 37^0C for 18h.The turbidity was observed after 18h and then Serial dilution were made and appropriate dilution was plated out by pour plates technique and incubated at 37^0C for 18 h and bacterial colony was then counted and recorded (American Public Health Association 1980).

2.10 Phenotypic screening of the virulence properties

2.10.1 ***Screening for Lipase Production***

The presence of extracellular lipases was determined using tween-calcium method according to Michelim et al. (2005), each isolate was inoculated into Trypticase soy agar (TSA) with 1% tween 20 (v/v). The plates were incubated at 30ºC for 24h and positive lipase activity was confirmed by observing the formation of a precipitate around the colonies. Uninoculated plate served as a control.

2.10.2 ***Screening for 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 listed above (Harrigan & Mccance 1966). The media was sterilized by autoclaving at 110^{o}C for 10 minutes in a water bath. On cooling, they were dispensed into sterile Petri- dishes and then left to solidify. The plates were inoculated by streaking the respective isolates across the plates. Un-inoculated 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.

2.10.3 ***Capsule stains***

This was done on cultures grown on alkaline peptone water containing 1% D-glucose. Bacteria were suspended in India ink (50%), dried, fixed in methanol, and counterstained with carbol fuchsin for 2 min. Capsules were deemed to be present if the clear zones around the bacteria exceeded 1/3 of the diameter of the red-colored bacteria when examined by oil immersion lens (Lo *et al*. 2001).

2.10.4 ***Heamolysis***

Haemolytic activity was detected by the plate method. Each strain was streaked onto 10% sheep blood agar plates and incubated at 37°C for 24 h. Hemolysis was considered positive when a clear zone with alpha- or beta-hemolysis was detected around the colony (Brenden & Janda 1987).

2.10.5 ***Flagella staining***

This was done by culturing the isolates on alkaline peptone water containing 1% D glucose, smear was made from the young cultures, the slide was flooded with Leif son stain after appearance of shinny film stream of water wash was made. The slide was treated with 1% methylene blue air dry and observes under oil immersion oil. The flagella appeared red and the bacteria cell appeared blue in colour **(Rabaan *et al*. 2001).**

2.11 Antibiotic susceptibility profile

Mueller Hinton Agar was prepared and allowed to gel. The isolate with an initial microbial load of 6.0 x 10^5 c.f.u/ml equivalent to 0.5 McFarland standard suspensions were used to inoculate the plates which was allowed to cover the entire surface of the Mueller Hinton Agar and left for few seconds. Antibiotic discs were positioned on the agar surface and the plates were incubated at 37^{0}C for 24h. Thereafter, the zones of inhibition were determined and interpreted using CLSI breakpoint as standard zones depending on the antibiotics used (CLSI 2011), as sensitive/ intermediate sensitive/ resistant.

3.Results and Discussion

3.1 Occurrence and Biochemical characterization of *Aeromonas* species

The occurrence of *Aeromonas* species in the Cabbage, tap water and diarrhoeagenic stool is presented in Table 1. Three hundred samples were collected in all, out of which two hundred and twenty nine were positive for the *Aeromonas* species with Cabbage having the highest occurrence of *Aeromonas* (86 (28.66%) followed by tap water while diarrhoeagenic stool recorded the least (64 (21.33%). *Aeromonas* species are gradually becoming a subject of growing interest due to its pathogenic effects on humans, aquatic organisms and its contribution to food spoilage (Rahman *et al*. 2007). It has been reported that the incidence of different species of *Aeromonas* isolated from food or clinical cases in relation to their virulence properties vary greatly to the geographical region. The main source of infection is thought to be water and foods (Kirov 1993). A reliable identification of isolates, especially in foods and water is becoming more and more popular and this is necessary to establish the risk associated with their distribution in nature (Maria Elena *et al*. 2013).

Table 1: Occurrence of *Aeromonas* spp. from food samples and diarroeagenic stool

Sources	No. of Samples Collected	No. Positive for Aeromonas
Stool	100	64 (21.33%)
Cabbage	100	86 (28.66%)
Tap water	100	79 (26.33%)
Total	**300**	**229 (76.33%)**

The biochemical characterization of the *Aeromonas* species and sugar fermentation is shown in table 2. All the isolates were oxidase, catalase, urease, citrate, and gelatin positive. Esculin hydrolysis was negative except for *A. caviae* and *A. hydrophila* that were positive. It was revealed that all the species utilized lysine with the exception of *A. cariae* and conversely all the species did not utilize ornithine with the exception of *A. veronii*. All the isolates fermented glucose, inositol and adonitol as their main sources of carbon but without gas

production. *A. popoffi* and *A. hydrophila* were lactose and arabinose fermenters. *A. caviae, A. sobria, A trota, A. bestiarum* and *A. hydrophila* fermented rhamnose and *A. acquoriorum, A. cariae, A. sobria, A. veronii* and *A. hydrophila* were sucrose fermenter. *A. bestiarum, A. hydrophila* and *A. media* fermented arabinose. All the isolates obtained in this work were Thio citrate bile salt sucrose negative, catalase positive and motile with the exception of *Aeromonas salmonicida*. All with the exception of *A. cariae* and *A. media* did not hydrolyse esculin while majority of the isolates showed heamolysis on blood agar. This is in line with the findings of Abbott *et al.* (2003) and Niamah (2012).

Table 2: **Biochemical Characterisation of *Aeromonas* species**

probable identiy	10ugAmp	Carbenicilin	TCBS	HAEMOLYSIS	MOTILITY	COLISTIN	CATALASE	ESCULIN HYDROLYSIS	Glu	Man	Xyl	Ino	Sor	Rha	Suc	Lac	Ara	Ado	Raf	Sal	Citrate	Urea	Indole	Vp	H2s	Oxidase	Gelatin	lysine	Ortnithine	Onpg	Malonate	Arginine
A. acquarirum	R	R	-	-	+	R	+	-	+	-	+	+	-	-	+	-	-	+	-	-	+	+	+	-	-	+	+	+	-	-	-	+
A. cariae	R	R	-	+	+	S	+	+	+	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	+	-	-	-	-	+
A. sobriae	R	R	-	-	+	S	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	-
A. veronii	R	R	-	+	+	R	+	-	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
A. trota	R	R	-	+	+	S	+	-	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	-	+	+	+	+	-	-	-	+
A. schbertii	R	R	-	+	+	R	+	-	+	+	+	+	+	-	-	-	-	+	-	+	+	+	+	-	+	+	+	+	-	+	-	+
A. jandaei	R	R	-	+	+	R	+	-	+	-	-	+	+	-	-	-	-	+	-	+	+	+	+	+	-	+	+	+	-	-	-	+
A. popoffi	R	R	-	+	+	S	+	-	+	+	+	+	+	-	-	+	-	+	-	+	+	+	+	-	+	+	+	+	-	+	-	+
A. bestrarum	R	R	-	+	+	R	+	-	+	+	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
A. salmonicida	R	R	-	+	+	R	+	-	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+
A. hydrophillia	R	R	-	+	+	R	+	-	+	-	-	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
A. media	R	R	-	_	+	S	+	+	+	-	+	+	-	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+

Key: glu-glucose, man-Manitol, Xyl-Xylose, Ino- Inositol,Sor-Sorbitol,Rha-Rhamnose,Suc-Sucrose,Lac-Lactose,Ara-Arabinose,Ado-Adonitol,Raf-Raffinose,Sal-Salicin,Cit-Citrate,Ure- Urea,Ind-Indole,Vp-Voges proskauer,H_2S-Hydrogen sulphide,Oxid-Oxidase,Gel-Gelatin,Lys-Lysine,Orthn-Ornithine,Onpg-O-NitrophenylBdgalactopyranoside,Malo-Malonate,Arg-Arginine, R=≤ 14mm, S=≥ 20mm, (–) Negative reaction, (+) Positive reaction

3.2 Molecular characterization of *Aeromonas* species

The isolates identities were further confirmed by molecular techniques by means of PCR analysis targeting 800 bp amplicon of the 16S rRNA gene for *Aeromonas* species (Plate 1)**.** The RAPD finger print generated 1 – 20 bands ranging from 50 bp – 850 bp (Plate 2). The banding pattern produced reproducible result when repeated with primer sequence 208 only. Dendogram analysis assigned the RAPD fingerprints into 10 clusters (i.e. RAPD types) of 3 – 8 isolates per cluster (Fig 1). The genetic similarity among the clusters ranged from 60% - 100%. *Aeromona* species isolates from Cabbage, tap water and diarrheogenic stool were seen in the same cluster signifying cross contamination of heterogeneous transmission. Cluster patterns further indicated that no disease outbreak occurred in the study area but suggested endemic transmission, including food to human transmission and transmission via the faecal-oral route.

The convectional identification scheme based on biochemical characteristics were not enough as pointed out by Janda (2001). Conventional method used in this work was able to differentiate the isolates into twelve species whereas molecular method differentiated the isolates into ten species. This shows the superiority of molecular identification over the routine or the conventional method. This work agrees with the findings of Martinez *et al*. (1992) who reported the superiority by 16S r RNA and RAPD tool in differentiating Aeromonas into different species.

Specific public health attention has not been fully drawn into the role of *Aeromonas* spp as causative organisms of diarrhea and other gastroenteritis in Nigeria. Aeromonas species outbreak of diarrhea and other gastroenteritis has been reported in a college, Xingyi City Guizhou, China (2012), Lebanon, Israel and Califonia (Qian Zhang *et al*. 2012). However its presence in foods such as Cabbage (*Brassica oleracea*) and water as it was carried out in this study represents a potential risk for the development of gastroenteritis. *Aeromonas hydrophila* was the predominant species isolated from diarrhoeagenic stool in this study. This aligns with the finding of Khalifa *et al.* (1999) in Tripolis Libya. High level of *A. hydrophila*, *A. sobria* and *A. cariae* were equally isolated from the stool suggesting their recent inclusion as causative agent of gastroenteritis (Namdari & Bottone 1990).

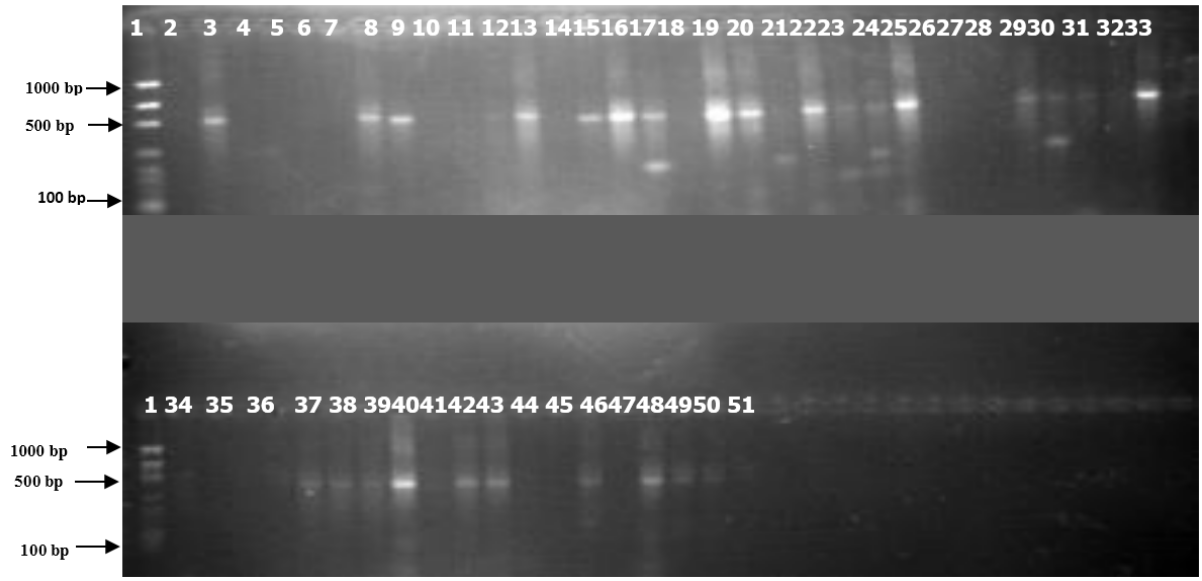

Plate 1: Amplification of the variable region of the 16S rRNA gene of *Aeromonas* spp
Lane1- marker, 3 control - *A. caviae, 4-51* - Sample numbers

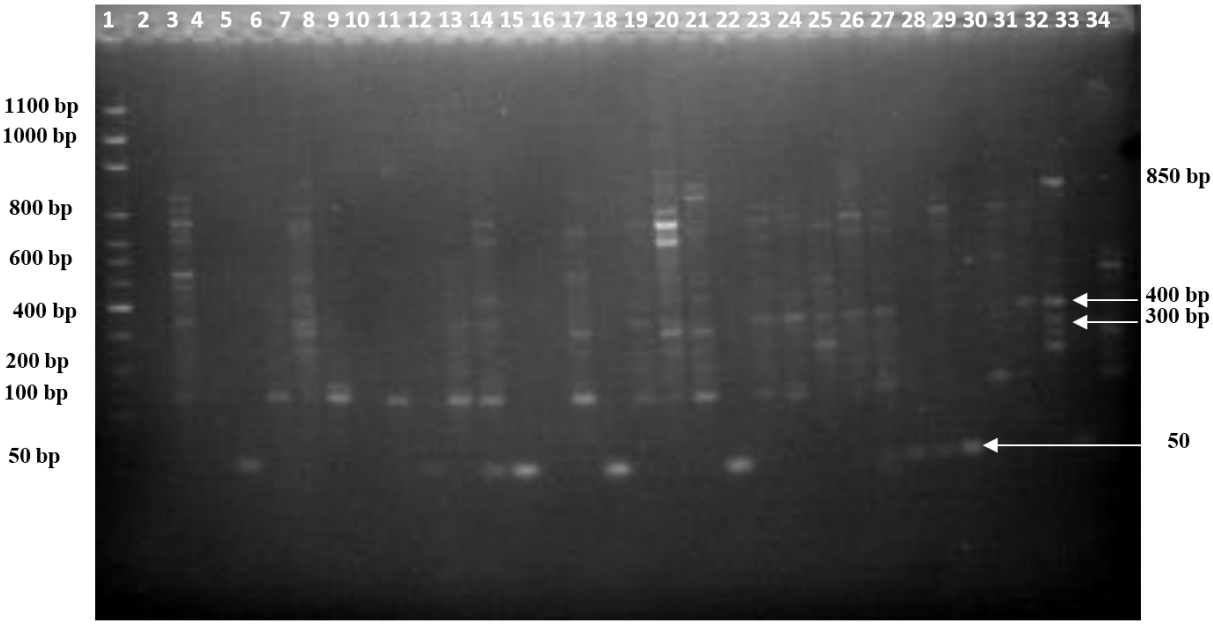

Plate 2: RAPD analysis of *Aeromonas* spp isolated from Cabbage, tap water and diarrhoegenic stool
Lane1-marker, 3 control - *A. caviae, 4-34* Sample numbers

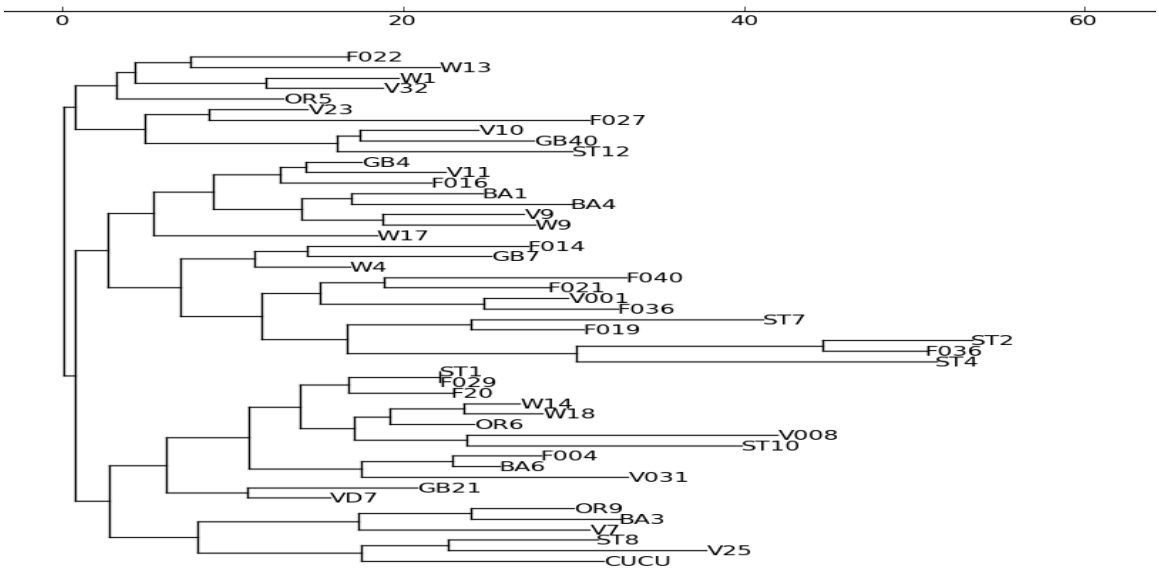

Fig. 1: Dendrogram depicting the relationship among *Aeromonas* spp

Interpretation

Genetic differences 0 – 40%

Genetic similarity 60 -100%

Implication = endemic infection (no outbreak)

Food to human transmission

3.3 Influence of difference chlorine concentration on the survival of *Aeromonas* species

Table 3 shows the survival of *Aeromonas* species at different concentration of chlorine. At 0.01 mg l^{-1} of chlorine concentration *Aeromonas jandaei and Aeromonas hydrophila* recorded the highest growth of $1.0x10^6$ cfu/ml while *Aeromonas caviae* had the least growth ($2.1x10^5$cfu/ml). At 0.1 mg l^{-1} of chlorine concentration *Aeromonas popoffi and Aeromonas salmonicida* had the least growth ($1.4 x10^5$ cfu/ml) while *Aeromonas veronii* had the highest growth of $2.7x10^5$ cfu/ml. At 0.4 mg l^{-1} of chlorine concentration the growth of some of *Aeromonas* species reduced drastically while *Aeromonas caviae*, *Aeromonas schubertii, Aeromonas trota* and *Aeromonas jandaei* did not grow at all and the highest growth was observed in *Aeromonas hydrophila, Aeromonas salmonicida* and *Aeromonas bestiarum* with $2.0x10^2$ cfu/ml each while the least growth was recorded in *Aeromonas sobria, Aeromonas media* and *Aeromonas acquoriorum* with $1.0x10^2$ cfu/ml. *Aeromonas* species was able to survive in most of chlorine concentrations used in this work. Metel *et al*. (2002) isolated *Aeromonas* species from drinking tap water samples in the city of Denizli in Turkey while Midilli (1998) also reported the occurrence of *Aeromonas hydrophila* from tap water.

Table 3: Survival of *Aeromonas* species in different Chlorine concentrations

Isolates	Chlorine Concentrations mg l^{-1}					
	0.01	0.1	0.2	0.3	0.4	0.5
Aeromonas hydrophila	$1.0x10^6$	$1.7x10^5$	$1.5x10^4$	$1.3x10^3$	$2.0x10^2$	-
A. caviae	$2.1 x10^5$	$1.8x10^4$	$1.4x10^3$	$2.0x10^2$	-	-
A. veroni	$1.9x10^6$	$2.7x10^5$	$1.5x10^4$	$3.0x10^2$	$1.2x10^2$	-
A. sobria	$1.6x10^6$	$1.8x10^5$	$2.6x10^4$	$1.4x10^3$	$1.0x10^2$	-
A. schubertii	$1.9x10^6$	$1.6x10^5$	$1.5x10^4$	$1.3x10^3$	-	-
A. trota	$1.8x10^5$	$1.5x10^4$	$1.6x10^3$	$2.0x10^2$	-	-
A. salmonicida	$1.0x10^6$	$1.4x10^5$	$1.5x10^4$	$1.5x10^3$	$2.0x10^2$	-
A. media	$1.1x10^5$	$1.6x10^4$	$1.5x10^3$	$1.4x10^2$	$1.0x10^2$	-
A. popoffi	$1.9x10^6$	$1.4x10^5$	$1.4x10^4$	$1.3x10^3$	$1.1x10^3$	-
A. jandaei	$1.0x10^6$	$1.6x10^5$	$2.1x10^3$	$1.0x10^2$	-	-
A. acquoriorum	$1.6x10^6$	$1.7x10^4$	$1.5x10^3$	$2.3x10^2$	$1.0x10^2$	-
A. bestiarum	$1.7x10^5$	$1.5x10^4$	$2. 6x10^3$	$1. 3x10^3$	$2.0x10^2$	-

3.4 Antibiotics susceptibility pattern of *Aeromonas* species

Antibiotics susceptibility pattern of *Aeromonas* species from diverse sources is shown in Table 4. All the isolates were 100% resistance to ampicilin however; they were all resistance to oxacillin with the exception of *A. popoffi.* All the isolates with the exception of *A. veronii* and *A. popoffi* were resistance to chloramphenicol and amikacin.

A. salmonicida and *A. schubertii* were susceptible to augmentin while *A. schubertii, A. popoffi* and A. *hydrophilla* were susceptible to gentamycin. The most sensitive organism in this work was *A. popoffi.*

Susceptibility of *Aeromonas* species to different classes of antibiotics and each group that made up of the classes is presented in Table 5. The class of aminoglycosides is made up of amikacin, gentamycin, azithromycin and streptomycin. Amikacin had 85% resistance followed by gentamycin with 80% resistance, azithromycin and streptomycin had 70% and 75% resistance respectively. Quinolones as a class of antibiotics is made up of ofloxacin, ciprofloxacin, nalidixic acid and oxacillin. *Aeromonas* spp were 25% resistance to ofloxacin and ciprofloxacin while nalidixic acid and oxacillin had 75% resistance each. The beta lactam penicillin class of antibiotics is made up of ampicillin and penicillin. *Aeromonas* spp were 80% resistance to penicillin and 100% resistance to ampicillin. Also beta lactam inhibitor as a class of antibiotics is made up of augmentine which had 25% resistance to *Aeromonas* spp while chloraphenicol, tetracycline and nitrofuratoin had 55%, 70% and 80% resistance to *Aeromonas* spp respectively. However, *Aeromonas* spp were 80% resistance to rifampicin which belongs to rifamycin group.

All the isolates were resistance to ampicillin; this was in agreement with the work of Capilla *et al.* (2003). A very high percentage of *Aeromonas* species show resistance to ofloxacillin, azithromycin, chloraphenicol, gentamycin, nalidixic acid, amikacin, oxacillin, augmentine, penicillin, stremptomycin, tetracycline and rifampicin while they

Table 4: Antibiotics resistance pattern of A*eromonas* species isolated from diverse sources

Aeromonas species	Antibiotics														
	Ampicillin	Ofloxaxin	Azithromycin	Ciprofloxacilin	Chloraphenicol	Gentamycin	Nalidaxic acid	Amikacin	Oxacilin	Augmentine	Nitrofuratoin	Penicillin	Streptomycin	Tetracycline	Rifampicillin
A. acquorium	R(0)	R(8-9)	R(2-8)	R(3-13)	R(4-12)	R(3-9)	R(3-11)	R(3-10)	R(4-12)	R(3-9)	R(3-9)	R(4-12)	R(3-9)	R(2-9)	S(20-23)
A. caviae	R(01)	R(9-10)	R(3-9)	S(23-26)	R(5-9)	R(4-9)	R(4-10)	R(4-9)	R(4-9)	R(3-8)	R(4-11)	R(3-9)	S(20-23)	R(5-12)	R(5-9)
A. sobriae	R(0)	S(21-2 3)	R(3-8)	R(3-11)	R(3-12)	R(3-9)	R(3-9)	R(3-7)	R(4-10)	R(3-9)	R(3-11)	S(21-23)	S(21-23)	S(20-23)	R(2-10)
A. veronii	R(0)	R(4-11)	S 22-24)	S(23-24)	S(25-27)	R(5-11)	R(5-9)	S(20-22)	R(4-8)	R(2-9)	S(20-24)	S(20-21)	S(21-24)	R(3-9)	S(20-24)
A. trota	R(3-11)	R(4-9)	R(7-11)	R(8-11)	R(11-12)	R(3-6)	R(3-9)	R(2-9)	R(4-9)	R(2-8)	S(21-22)	R(5-9)	R(4-9)	R(3-7)	S(20-21)
A. schubentii	R(0)	S(21-22)	R(1-10)	R(4-9)	R(4-9)	S(22-25)	S(23-24)	R(3-9)	R(2-9)	S(21-25)	R(5-9)	R(6-9)	R(7-9)	R(61 0)	R(5-9)
A. jandaei	R(0)	R(3-10)	R(4-8)	R(2-7)	R(4-12)	R(2-11)	R(3-10)	R(3-13)	R(2-11)	R(2-9)	S(20-24)	R(5-10)	R(2-8)	R(3-9)	S(20-23)
A. popofii	R(0)	S(22-26)	S(23-25)	S(22-25)	S(23-26)	S(23-24)	S(23-25)	S(20-22)	S(21-24)	R(3-12)	S(21-22)	S(20-24)	R(3-11)	R(2-8)	R(3-9)
A. bestiarum	R(0)	R(3-9)	R(2-8)	S(21-25)	R(3-12)	R(4-11)	S(23-26)	R(2-12)	R(3-9)	R(4-9)	S(22-23)	R(2-8)	R(2-11)	R(2-9)	S(21-22)
A. salmonicida	R(0)	R(5-11)	R(3-11)	S(22-23)	R(4-8)	R(6-9)	R(4-9)	R(5-10)	R(3-12)	S(22-25)	R(2-9)	R(4-10)	R(5-12)	S(21-24)	R(5-12)
A. hydrophillia	R(0)	S(20-22)	S(23-26)	S(22-23)	R(4-10)	S(21-22)	S(22-24)	R(4-11)	R(5-8)	R(5-12)	S(20-23)	R(1-12)	R(3-8)	S(21-22)	R(2-9)
A. media	R(1)	R(3-8)	R(3-9)	R(3-9)	R(4-10)	R(2-9)	R(3-7)	R(3-11)	R(4-12)	R(3-9)	R(1-8)	S(20-22)	S(20-23)	R(2-9)	S(20-22)

Key: Zone of inhibition: Sensitive ≤20mm, Intermediate between 15-19mm, Resistance Less than 14 mm.

were susceptible to ciprofloxacillin and nitrofuratoin, this result was in agreement with the findings of Jones *et al*. (1982) and Schmidt *et al*. (2001). Susceptibility of *Aeromonas* species to chloraphenicol, stremptomycin and amikacin (Schmidt *et al*. 2001) was totally contrary to the finding of this study because there was high resistance of *Aeromonas* spp to these antibiotics. More so due to uncontrolled use of antibiotics for the treatment of bacterial infection, *Aeromonas* species might have developed resistance towards several of these antibiotics and so portends danger in the antimicrobial therapy.

Table 5: Class of antibiotics and the percentage resistance

Class of antibodies	Group of antibiotics	no of isolates tested	No resistance	Percentage (%) resistance
Quinolone	Ofloxacilin	229	58	25
	Ciprofloxacilin	229	58	25
	Nalidixic acid	229	172	75
	Oxacillin	229	172	75
Aminoglycosides	Amikacin	229	195	85
	Gentamycin	229	184	80
	Azithromycin	229	161	70
	Streptomycin	229	172	75
B- Lactam penicillin	Ampicillin	229	229	100
	Penicillin	229	184	80
B-Lactam inhibitor	Augmentine	229	58	25
Tetracycline	Tetracycline	229	161	70
Chloramphenicols	Chloramphenicol	229	126	55
Nitrofuran	Nitrofuratoin	229	184	80
Rifamycin	Rifampicin	229	184	80

3.5 Phenotypic virulence properties of *Aeromonas* species

Table 6 shows the various phenotypic virulence properties of *Aeromonas* species identified in this research work. *Aeromonas hydrophila* (57.69%) were haemolysin producer and *A. bestiarum* had the least percentage of haemolysis (20%). *A. sobriae* produced the highest percentage of proteases (75%) while *A. jandaei* was the least protease producer (12.50%). *A. salmonicida* had the highest percentage of lipase (35.71%) while *A. sobriae* was the least lipase producer (15%). Researchers have identified a number of virulent factors which make *Aeromonas* species to be pathogenic. The presence of flagella has been associated to be a common feature of regular and persistent infection, in fact they are antigenic. Motility of *Aeromonas* species as confirmed in this research is feature linked with the invasive ability and is directly associated with enteric infection (Kirov *et al*. 2002. These virulence properties have been known to contribute to pathogenesis of diseases and infections in both human and fish (Chacon *et al*. 2003). However, none of these are capable of being responsible for all symptoms of diseases stages. Studies had also been shown that *Aeromonas* species isolated from water have different virulence factors that can cause diseases in man as well as in other animals (Nam & Joh 2007). Of all the species of *Aeromonas* isolated in this work, *Aeromonas hydrophila, Aeromonas caviae* and *Aeromonas veronii* are the most common species known to cause the major human infections and they account for more than 85% of all the clinical isolates (Joseph & Carnahan 2000).

Table 6: Phenotypic virulence properties of *Aeromonas* species isolated from diverse sources

***Aeromonas* spp**	**Haemolysis NO (%)**	**Proteases NO (%)**	**Capsule NO (%)**	**Flagella NO (%)**	**Lipase NO (%)**
A. hydropyila (52)	30 (57.69)	12 (23.07)	52 (100%)	52 (100%)	10 (19.23)
A. acquoriorum (21)	-	10 (47.61)	20 (95%)	16 76.2%)	8(38.09)
*A. sobria (*20)	-	15 (75)	15 (75%)	18 (90%)	3 (15)
A. veronii (23)	13 (56.53)	5 (21.73)	20 (86.9%)	23 (100%)	5 (21.73)
A. trota (2)	1 (50)	1 (50)	2 (100%)	(100%)	-
A. schubertii (3)	02 (66.66)	01 (33)	2 (66,6%)	2 (66%)	-
A. jandaei (16)	11 (68.75)	02 (12.50)	10 (62.5%)	10 (62.5%)	03 (18.75)
A. poppoffi (15)	-	10 (66.66)	12 (80%)	10 (66%)	05 (33.33)
A. bestiarum (10)	2 (20)	05 (50)	4 (40%)	2 (100%)	03 (30)
A. salmonicida(28)	-	14 (50)	15 (53.5%)	-	10 (35.71)
A. media (10)	-	06 (60)	8 (80%)	6 (60%)	04 (40)

Conclusions

This research work confirms the incidence of *Aeromonas* species that are multiple antibiotics resistances with virulence properties from diverse sources which indicates public health problem, hence stringent control in the use of antibiotics for chemotherapy of Aeromonas infections is suggested to reduce the organism's resistance to

frequently used antibiotics and a biothepeutc treatment is needed to avoid outbreak occurrence. Also for proper identification of *Aeromonas* species, the use of polyphasic (phenotypic and genotypic) techniques is recommended.

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