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Occurrence of Virulence and Antibiotic Resistance Genes in Aeromonas Species Isolated from Diverse Sources

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Abstract
Occurrence of transferable or infectious diseases caused by multidrug resistant and inherent virulence properties is of great medical importance, thus becoming a serious or major community and public health concern. The aim of this study was to determine the virulence and antibiotic resistance gene of *Aeromonas* species isolated from diverse sources. The presence of virulence genes was detected in seven species of Aeromonas such as *A. hydrophilla, A. caviae, A. veronii, A. simiae, A. punctata, A. finlandiensis* and *Plesiomonas shigelloides* isolated from diverse sources. The detected virulence factors via polymerase chain reaction using specific primers include heat stable cytotonic enterotocin (AST), heat labile cytotonic enterotoxin, (ALT), cytotoxic enterotoxin (ACT), DNA adenine methyl transferase (DAM), enolase and haemolysin (HlyA) gene, while antibiotic resistance gene of Tet M was amplified at the amplicon size of 200 base pair. This is an indication that these species of *Aeromonas* are considered to be potential pathogens.
Keywords: *Aeromonas* species, Antibiotic resistance, virulent properties, diverse sources

1. Introduction

Aeromonas species are emerging as an important human pathogen because of suspected food-borne outbreaks (Hanninen *et al*., 1997). Increased incidence of *Aeromonas* isolation from patients with gastroenteritis and traveler's diarrhea (Yamada *et al*., 1997) is subject of public concern. It was once considered as an opportunistic pathogen in immunocompromised humans, now implicated as the etiologic agent involving immunocompetent individuals of all age groups (Janda and Abbott 1998).

In some research, these bacteria are always isolated repeatedly from diarrhea stools than from normal or control groups (Nishikawa and Kishi, 1988). It is therefore scientifically established that aeromonads can cause up to about thirteen percent (13%) of the described gastroenteritis cases in the United States of America (Kingombe *et al*., 1999).

In spite of the linkage between virulence factors possess by aeromonads and drinking-water, there is growing confirmation that species isolated from the environment commonly belong to various groups of Aeromonas that are related to gastroenteritis. A little similarity exists between the strains or the species from stools and that of drinking-water (Kuhn *et al*., 1997). Chiefly of a true is *A. caviae*, which was the dominant species in the water and the stool. It has been reported that drinking-water supplies are accountable for the increased incidence of Aeromonas-linked gastro-infections (Ghanem and Eraki, 1993).

Filler *et al*. (2000) reported diarrhea-associated acute renal failure in an infant, which was caused by a hemolytic-producing strain of *A. sobria*. In general, *Aeromonas* bacteremia occurs in patients with liver cirrhosis or malignancy (Ko *et al*., 2000). These Gram-negative, non–spore forming, rod-shaped facultative anaerobic bacilli are generally motile by polar flagella (Michael *et al*., 2000; Villari *et al.*, 2003) and grow over a wide range of temperature (0-40^{0}C) (Cheesbough, 2005).

A. hydrophila, A. caviae, and *A. veronii* were among the fourteen species of *Aeromonas* identified up to date and have been involved in various human infections (Chopra and Houston, 1999). Explicit importance is drawn by *A. hydrophila* from a public health perspective because of its involvement in food borne gastroenteritis and various opportunistic infections in immunodeficient patients (Panangala *et al*., 2007). *Aeromonas hydrophila* infection and resistance to antimicrobial are increasing worldwide because of their link with acute and chronic wound infections, septicemia and gastroenteritis (Abdelraouf *et al*., 2011).

Aeromonas species resistance to regularly used antibiotics is developing problem in man and fish. Resistance levels of the Genus *Aeromonas are on increase*, especially to β-lactam antibiotics (Rowe-Magnus *et al.,* 2002). Therefore, it is pertinent to investigate whether the virulence and antibiotic resistance genes displayed by these organisms is genetically linked. This work was centered on determination of antibiotics resistance and virulence genes of *Aeromonas* species isolated from different sources.

2. Materials and methods

2.1 *Microorganisms*

The *Aeromonas* species used in this work had been previously characterized as described by Adegoke and Ogunbanwo (2016). The organisms were resuscitated using Aeromonas Agar and maintained on a slant at 4^{0}C. Twenty four hour old cultures were used for various experiment carried out in this work except where otherwise

stated.

2.2 *Detection of tetracycline (Tet M) resistance gene in Aeromonas species*
Polymerase chain reaction was carried out to amplify the Tet M gene of the bacteria using the primer pair Tet-F (5′- GGTGCAGAAGGACCAGGCACAGAT -3’. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25p Mol of each primer (Jena Bioscience, Germany), 2 unit of Hot FIRE Pol DNA polymerase (Solis Biodyne), Proof reading Enzyme, 5µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in a Peltier thermal cycler (PTC100) (MJ Research Series) for an initial denaturation of 95°C for 15 minutes, followed by 30 amplification cycles of 30 seconds at 95°C; 1 minute at 50°C and 1 minute 30 Seconds at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight markers.

2.3 *Molecular screening of virulence properties of Aeromonas species*
Polymerase chain reaction was carried out to amplify the virulence genes such as *cytotoxic heat enterotoxin* (act) gene, cytotoxic heat stable enterotoxin genes (Ast), enolase gene, DNA adenine methyl transfers genes (DAM) and enolase gene of the bacteria using different primers as shown in Table 2.1. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 25pMol of each primer (Jena Bioscience, Germany), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in a Peltier thermal cycler (PTC100) (M J Research Series) for an initial denaturation of 95°C for 15 minutes, followed by 30 amplification cycles of 30 seconds at 95°C; 1 minute at 50°C and 1 minute 30 Seconds at 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethnidium bromide staining. 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight markers.

Table 2.1: List of primers used in PCR identification of virulence genes of *Aeromonas* species

Target genes	Primers used	Forward sequencing	Amplicon size (bp)	Reference
Dam	dam-	F 5′ATGAAAAAAACACGCGCTTTTTAAAATGG-3’ R (5′-TCAGCCGAGTGGCGCCAGTTCGGCGTCG-3′)	1000	Erova *et al.,* 2006
Ast	ast-	F 5′-ATGCACGCACGTACCGCCATG-3’ R (5′-GGACTTTTTCACCGCAGCGGGTT-3′).	1000	Sha *et al*., 2002
Act	act-	F 5′-ATGCAAAAACTAAAAATAACTGGC-3’ R (5′ TTATTGATTGGCTGCTGGCTGCACGCT-3′).	1200	Chopra *et al.*, 1993
Enolase	enol-	F 5′-ATGTCCAAGATCGTTAAAGTGAT3’ R (5′-TTAAGCCTGGTTCTTCACTTCTT3)	1000	Sha *et al*., 2003
HylA	hylA-	F 5′-ATGAGTTTTGCCGATAGTTTATTTTTCCTGA -3’ R (5′-TTACGATTCCTGAGCGGGCTTGTCGGCCGGCGTG -3′)	1000	Erova *et al*., 2006

3. Results

Different virulent genes possessed by *Aeromonas* species observed in this research were heat stable cytotonic enterotocin (AST), heat labile cytotonic enterotoxin, (ALT), cytotoxic enterotoxin (ACT), DNA adenine methyl transferase (DAM), enolase and haemolysin (HlyA) gene and antibiotics resistance gene Tet M.

The detection of tetracycline resistance genes (Tet M) in *Aeromonas* species is shown in plate 1. All the species of *Aeromonas* possessed a Tet M gene, which is depicted at 200 base pair amplicon size.

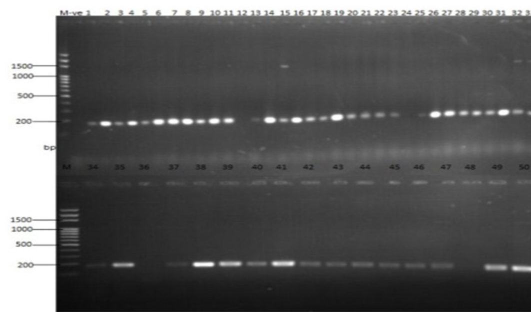

Plate 1: Tet M antibiotic resistance gene of *Aeromonas* species at the amplicon size of 200 bp.
Key: Lane M = Molecular marker, Lane –Negative = –Negative Control., Lane 1 = positive control; Lane 2-50 = *Aeromonas* species.

Molecular amplification of cytotoxic enterotoxin gene (ACT) is shown in Plate 2. Only two species of *Aeromonas* possessed this gene and was amplified at the amplicon size of 1200bp. These species are *A. hdrophila* and *A. caviae*. Molecular detection of Heat labile cytotonic enterotoxin gene (ALT) is shown in Plate 3. The species of *Aeromonas* amplified are *A. hydrophila, A. caviae, A. veronii* and *A. punctate* at lane 2, 6, 7, 10 and 11 with the amplicon size of 500 base pair. Plate 4 shows molecular detection of Heat stable cytotonic enterotoxin (AST) gene present in nine species of *Aeromonas* at the amplicon size of 1000bp. The species of *Aeromonas* possessed AST gene include *A. hydrophila, A. caviae, A. veronii, A. punctate, A. simiae* and *Plessiomonas shigelloides* which was amplified at lane 3, 7, 8, 9, 10, 11, 12, 17, 18, 25 and 35.

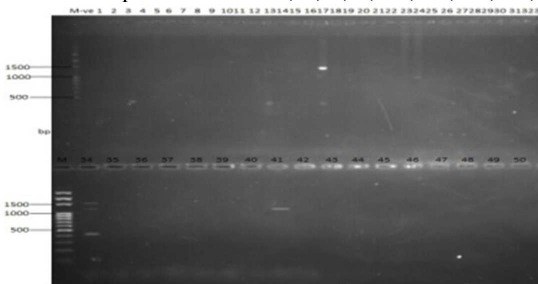

Plate 2: Cytotoxic enterotoxin (ACT) gene of *Aeromonas* species at amplicon size of 1200bp
Key: Lane M -- Molecular marker, Lane –Negative Control., Lane 1-50 *Aeromonas* species.

Plate 3: Heat labile cytotonic enterotoxin gene (ALT) of *Aeromonas* species at the amplicon size of 500bp
Key: Lane M -- Molecular marker, Lane –Negative Control, Lane 1-50 *Aeromonas* species.

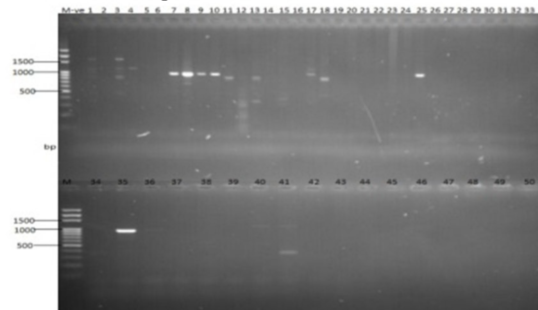

Plate 4: Heat stable cytotonic enterotoxin gene (AST) of *Aeromonas* species
Key: Lane M = Molecular marker, Lane –Negative = Control, Lane 1-50 = *Aeromonas* species Amplicon size at 1000bp.

Plate 5 shows the molecular detection of enolase gene in *Aeromonas* species. The species of *Aeromonas* with

this virulence gene are *A. hydrophila, A. caviae, A. veronii, A. punctate* and *Plessiomonas shigelloides*. They were all amplified at lane 3, 11, 17, 18, and 34 at the amplicon size of 1300bp. Plate 6 shows molecular amplification of DNA adenine methyl transferase (DAM) gene. Only two species of *Aeromonas* namely *Aeromonas hydophila* and *Aeromonas caviae* had this gene. This was amplified at lane 17 and 34 with the amplicon size of 900bp.

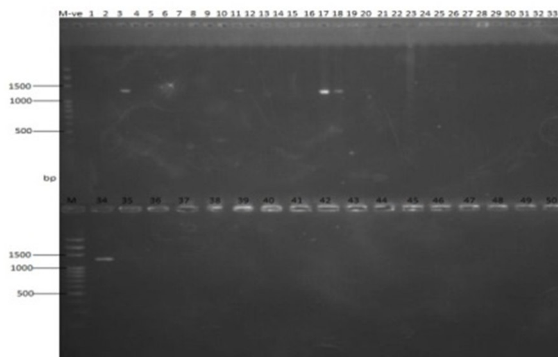

Plate 5: Enolase gene (Enolase) of *Aeromonas* species at amplicon size of 1300 bp
Key: Lane M = Molecular marker, Lane –Negative = Control, Lane 1-50 = *Aeromonas* species

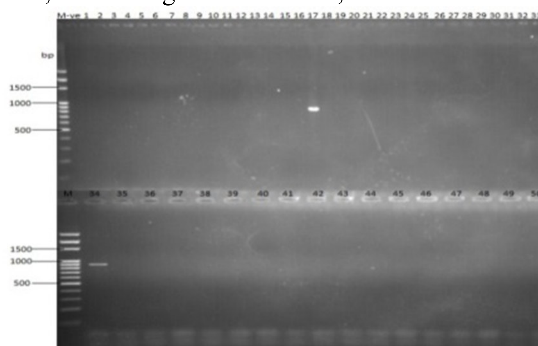

Plate 6: DNA adenine methyl transferase (Dam) gene of *Aeromonas* species
Key: Lane M = Molecular marker, Lane –Negative = Control, Lane 1-50 = *Aeromonas* species.
The molecular detection of haemolysin A (hylA) gene at the amplicon size of 1200bp is shown in Plate 3.7. Two species of *Aeromonas* possessed this gene as shown in Lane 17 and 34 for *A. hydrophila* and *A. veronii.*

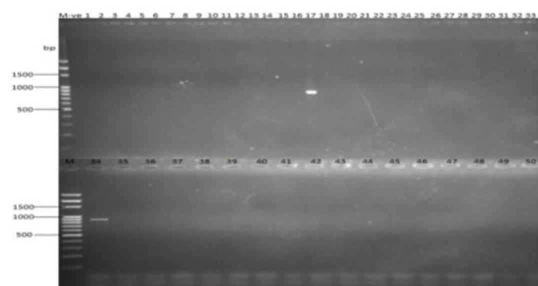

Plate 7: DNA adenine methyl transferase (Dam) gene of *Aeromonas* species
Key: Lane M = Molecular marker, Lane –Negative = Control, Lane 1-50 = *Aeromonas* species

4. Discussion

The detection of resistance tet M gene from *Aeromonas* species isolated from diverse sources in this study is a serious clinical problem in the treatment and containment of diseases cause by Aeromonads. This work aligned with the finding of Sinha *et al*. (2004) who reported the resistance of *Aeromonas hydrophila* isolated from water to various antibiotics such as ampicillin, nalidixic, methicillin and cefazoline which is genetically mediated. Various authors have documented the prevalence and transmission of multi-drug resistance genes among clinical and food isolates (Henriques *et al.,* 2006; Rahman *et al*., 2009). The majority of the pathogens isolated in this study were multidrug resistant to antibiotics. Resistance could be due to gene transfer between bacteria in a vertical, horizontal, transformation or by transduction and conjugation. Therefore, a gene for antibiotic resistance which had evolved from natural selection may be due to many antibiotic resistance genes reside on the plasmid that facilitates their transfer (Aubry-Damon and Courvalin 2003). As a result of this resistance, it can lead to

modification of composition of microorganism populations, leading to other illnesses and allow micro-organisms that are naturally resistant to the antibiotic to increase in number. Cecilia-Jernberg, (2010) pointed out that the significance of this could be possibly life-threatening. The continuing presence of resistance genes in human gut bacteria vividly increases the possibility of them being transferred to and exploited by harmful bacteria that pass through the gut. This could lessen the success of future antibiotic treatments and potential new strains of antibiotic-resistant bacteria could emerge (Cecilia-Jernberg 2010).

Virulence properties of *Aeromonas* species such as cytotoxic enterotoxin (ACT), heat labile cytotonic enterotoxin (ALT), heat stable cytotonic enterotoxin (AST) and DNA adenine methyl transferase (Dam) were investigated molecularly. The molecular result through PCR product showed the presence of some of these virulence properties in the *Aeromonas* species in this research work. This is in agreement with the work of Albert *et al*. (2000) who reported a significant correlation between heat stable cytotonic enterotoxin (Ast) genes and diarrhea in children habouring such strains. According to Grim *et al*. (2013) virulence factors such as cytotoxic enterotoxin and heat labile cytotonic enterotoxin in *Aeromonas hydrophila* often enhance pathogenicity in both the humans and animals infected with such pathogens.

In this study, the identification of different types of virulence factor of the seven species of *Aeromonas* namely *A. hydrophila, A. caviae, A. veronii, A. punctate, A. simiae, A. finlandiensis* and *Plessiomonas shigelloides* from cabbage and water is an indication of its potential to cause severe diseases in man most especially diarrheal. This study is in agreement with the work of Sha *et al*. (2002) and Sha *et al*. (2003) where differential expression of various types of virulence genes such as enolase and enterotoxin was carried out in *Aeromonas hydrophila* induced gastroenteritis.

The detection of virulence genes such as ALT, AST and ACT in this work from the food (cabbage and water) is suggestive of evidence of water-to-human transmission and could be very fatal to human health which agreed with the findings of Bijay *et al*. (2010) who isolated various virulent genes as mentioned above from water and clinical samples.

Heat stable cytotonic enterotoxin gene (AST) was detected in *A. hydrophila, A. caviae, A. veronii, A. punctate, A. simiae* and *Plessiomonas shigelloides*. According to Chopra *et al.* (1996) and Sha *et al*. (2002) cytotonic enterotoxin (AST) gene was identified in a diarrheal isolate that induced fluid secretion in the ligated small intestinal loops of animals. Furthermore, the cytotoxic enterotoxin (ACT) gene was found to be present only in *A. hdrophila* and *A. caviae*. This finding was in agreement with the work of Mohammad and Hassan (2004) and Burk *et al*. (1981) where intra-gastric administration of Aeromonas culture supernatants to suckling mice was carried out and identified different types of virulence factors as mentioned above. Heat labile cytotonic enterotoxin (ALT) gene was also detected in this study and the species of *Aeromonas* found with this gene are *A. hydrophila, A. caviae, A. veronii, A. punctate* and *A. simiae,* this finding aligned with the work of Bijay *et al*. (2012) who identified heat labile cytotonic enterotoxin gene from a species of Aeromonas in diarrheagenic stool .

Dam and hlyA genes were all identified from *Aeromonas* species isolated from cabbage, water and diarrheagenic stool in this work and were in agreement with the work of Bijay *et al*. (2012) who worked on water and clinical samples with the conclusion of evidence of water-to-human transmission.

It was discovered that multiple virulence genes were present in *Aeromonas hydrophila* which agreed with the finding of Sen and Rodgers (2004) who pointed out from the study conducted on food samples and drinking water that a single species of *Aeromonas* had multiple genes and other species had at least one virulence gene.

5. Conclusion

This research work has identified a number of virulent factors which portrait *Aeromonas* species as pathogenic organism. Seven species *of Aeromonas* with virulence properties were isolated from diverse sources such as pipe-borne water, cabbage and diarrheagenic stool indicative of food-water transmission, which could pose a health challenge to populace especially in the developing nations.

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