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Characterization of antibiogram susceptibility profile of *Vibrio* species isolated from fresh vegetables

Etinosa O. Igbinosa[1*] and Emmanuel E.O. Odjadjare[2]

[1]Applied Microbial Processes & Environmental Health Research Group, Department of Microbiology, Faculty of Life Sciences, University of Benin, PMB 1154, Benin City, Nigeria
[2]Department of Basic Sciences, Benson Idahosa University, PMB 1100, Benin City, Nigeria

***Corresponding author email**: etinosa.igbinosa@uniben.edu

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ABSTRACT: Fresh vegetables water leaf *(Talinum traingnlare),* pumpkin leaf *(Telfairia occidentalis)* and scent leaf *(Ocinum gratissimum)* collected from urban markets in Benin City, Nigeria, were investigated for the presence of *Vibrio* species using standard microbiological techniques. The antibiotic susceptibility profiles of the isolates against a panel of 13 antibiotics, was carried out according to the method described by The National Committee for Clinical Laboratory Standards (NCCCLS). The average vibrios population density in the vegetables ranged from $1.9 \times 10^5 \pm 0.04$ - $3.7 \times 10^5 \pm 0.32$ cfu/g; $1.9 \times l0^5 \pm 0.04$ - $3.6 \times 10^5 \pm 0.30$ cfu/g and $1.0 \times 10^5 \pm 0.07$ - $3.7 \times 10^5 \pm 0.32$ cfu/g for water leaf, pumpkin leaf and scent leaf respectively. The isolates were identified as *Vibrio alginolyticus*, *V. campellii*, *V. cholerae*, *V. damsel*, *V. fischeri, V. harveyi*, *V. mimicus*, *V. parahaemolyticus* and *V. splendidus*. The resistance profile of the isolates to the test antibiotics, ranged from 0 to 44%; with rocephin being the most effective antibiotic and ciprofloxacin and augmentin being the least active drugs. Although the isolates were sensitive to many of the test antibiotics, the study demonstrated that there is growing resistance among *Vibrio* species isolated from fresh vegetables to important antibiotics like ciprofloxacin, and augmentin. It is therefore imperative that stakeholders, especially vegetable farmers are enlightened on the need to apply best practices in the growth of fresh vegetables in order to reverse the growing trend of antimicrobial resistance and avert a possible public health disaster.

Keywords: antimicrobial agents, antibiotic profiling, fresh vegetables, *Vibrio* species

Introduction

Vibrio species are Gram-negative, motile, facultative anaerobic, oxidase positive, non-spore forming, polar flagellated, curve rod shaped bacteria (Ceccarelli *et al*., 2013; Zhang and Orth, 2013; Velazquez-Roman *et al*., 2014; Wu *et al*., 2014). Several species which include *Vibrio cholerae, V. fischeri, V. halioticoli, V. metschnikovii, V. harveyi, V. parahaemolyticus* and *V. alginolyticus* have been reported to cause food-borne infection, usually associated with eating undercooked seafood (Su and Liu, 2007; Guoxiang *et al*., 2009). However, recent food-borne outbreaks throughout the world have been intensively linked to consumption of fresh fruits, vegetables and unpasteurized juices (Tunung *et al*., 2012). The United States Centers for Disease Control and Prevention (CDC) reported that while infections due to *Shigella*, *Listeria*, *Escherichia coli* O157:H7 and *Salmonella* decreased dramatically in 2005, infections due to *Vibrio* increased (Chang *et al*., 2011).

Previous findings from Okafor *et al*. (2003) reported the presence of *Escherichia coli, Vibrio* spp. and *Salmonella* spp., in raw vegetables harvested from soils irrigated with contaminated streams in Nigeria. Other microorganisms found in vegetables had also been reported (Chai *et al*., 2008; Learn-Han *et al*., 2009). Some food-borne disease outbreaks have been reported to be due to field contamination before these fresh vegetables are even harvested (Traore *et al*., 2014). Although epidemiological data on the incidence of food-borne diseases are inadequate, and the outbreak often not investigated, the recurrent episodes of food-borne illnesses with symptoms of gastrointestinal distress like diarrhea, vomiting, abdominal cramp and nausea has remained a major cause of mortality and morbidity in Nigeria (Nweze, 2010). More than 90 % of the cases of food poisoning each year are caused by *Staphylococcus aureus, Salmonella, Clostridium perfringes, Clostridium botulinum, Campylobacter, Vibrio parahaemolyticus, Bacillus cereus* and enteropathogenic *E. coli* (De Boer and Beuner, 2011). Moreover, food-borne outbreaks in developed and developing nations have been consistently linked to consumption of fresh vegetables and fruits.

It has been proven that antibiotic therapy can reduce the duration and severity of symptoms of *Vibrio* infections in severe cases; however bacterial resistance to antibiotics has become an emerging medical issue threatening the public health due to the wide availability of antibiotics and sometimes misuse of drugs without proper prescription. Previous studies have shown that streptomycin, rifampicin, kanamycin, tetracycline and polymixin B were active against *Vibrio* spp. Ottaviani *et al*. (2001) showed that *V. parahaemolyticus* were resistant to penicillin, carbenicillin, ampicillin, cephalotin, kanamycin and rifampicin (Zulkifl *et al*., 2009). Zulkifl *et al*. (2009) also reported the resistance of *V. parahaemolyticus* towards tetracycline. The growing problems with antimicrobial drug resistance are beginning to erode our antibiotics' capacity to combat antibiotic resistance and thus limiting therapeutic options to present-day clinicians (Zulkifl *et al*., 2009). Hence, surveillance of antimicrobial resistance is indispensable for empirical treatment of infections and important in preventing the spread of antimicrobial resistant microorganisms (Letchumanan *et al*., 2015). The aim of this study therefore, was to determine the antibiotics profile of *Vibrio* species isolated from fresh vegetables in urban markets located in Benin City, Nigeria.

Materials and Methods

Samples collection

Fresh vegetable samples such as water leaf (*Talinum traingulare*), pumpkin leaf (*Telfairia occidentalis*) and scent leaf (*Ocinum gratissimum*) were bought from Oba market, New Benin market and Uselu market in Benin City, Nigeria and placed separately in a sterile polyethylene bag and taken immediately to the laboratory. A total of 90 samples (30 for each vegetable) were randomly purchased from the markets.

Sample processing/enumeration and isolation of vibrios

One gram (1 g) of each sample was homogenized in 9 mL of sterile distilled water to make the stock culture. The stock culture was diluted serially to the order 10^{-5} dilutions. Dilutions were plated on Thiosulfate Citrate Bile salt Sucrose (TCBS) agar (HiMedia, India) for total aerobic plate count using pour plate technique. Plates were incubated at 35 ± 2 °C for 24 h. Bacterial colony count was expressed as colony forming unit per gram (Cfu/g). Bacterial isolates were characterized and identified by visual observation of the cultural characteristics (such as colour, transparency, shape, elevation and moisture/dryness) of the colonies which were further confirmed by morphological/microscopic and biochemical reactions. Colonies of the mixed bacterial culture plates were purified by plating on freshly prepared TCBS agar plates. Plates were incubated at 35 ± 2 °C for 24 - 48 h. Pure isolates were inoculated in nutrient agar slants and stored in a refrigerator for antibiotics profiling.

Antibiotic profiling

Susceptibility of the *Vibrio* isolates to antibiotics was determined by the disc diffusion tests based on the guidelines of the National Committee for Clinical Laboratory Standards (NCCCLS, 2001).

Disc diffusion method

Vibrio isolates were grown in nutrient agar and incubated at 35 ± 2 °C for 24 h. The cultures were evenly streaked on Mueller-Hinton (Merck, Germany) agar plates using sterile swab before placing antibiotics discs. Impregnated Antibiotics discs of 6 mm in diameter containing 12 different types of antimicrobial substance including pefloxacin (10 µg), gentamycin (10 µg), ampiclox (30 µg), amoxicillin (30 µg), ceftriaxone (30 µg), ciprofloxacin (10 µg), streptomycin (30 µg), erythromycin (30 µg), cotrimoxazole (30 µg), chloramphenicol (30 µg), sparfloxacin (10 µg), augmetin (30 µg) were placed on the culture plates and incubated at 35 ± 2 °C for 24

h. After incubation, the diameter zone inhibition was measured and compared with the interpretative chart from BBL (Sensi - Disc Antimicrobial Susceptibility Test Discs: Approved Standard, 1996) to determine the sensitivity profile of the isolates to the antibiotics.

Results

Vibrio species isolated from the vegetables include *Vibrio alginolyticus*, *V. campellii*, *V. cholera, V. damsel*, *V. fischeri*, *V. harveyi*, *V. mimicus*, *V. parahaemolyticus* and *V. splenndidus.* Table1 shows the total population density (cfu/g) in the fresh vegetable samples. It was observed that *Vibrio cholerae* had the highest microbial count of $3.7 \times l0^5 \pm 0.32$ cfu/g (water leaf); while the lowest count ($1.0 \times l0^5 \pm 0.07$ cfu/g) was observed amongst *V. alginolyticus* isolates (scent leaf) and strains of *V. splenndidus* (pumpkin leaf). The total mean vibrios count in the various vegetables ranged from $1.9 \times 10^5 \pm 0.04$ - $3.7 \times l0^5 \pm 0.32$ cfu/g (water leaf); $1.9 \times l0^5 \pm 0.04$ - $3.6 \times l0^5 \pm 0.30$ cfu/g (pumpkin leaf) and $l.0 \times l0^5 \pm 0.07$ - $3.7 \times l0^5 \pm 0.32$ cfu/g (scent leaf).

Table 2 shows the antibiotic sensitivity pattern of the *Vibrio* species isolates. All the *Vibrio* spp., except *V. alginolyticus* were susceptible to pefloxacin and sparfloxacin. However *Vibrio* species were slightly resistant to other antibiotics but 4 different species showed the highest resistance to augmentin and ciprofloxacin.

Table 1: Total population density of vibrios recovered from fresh vegetable samples

Vibrio isolates	WL	PL	SL
	cfu/g		
V. alginolyticus	$2.5 \times 10^5 \pm 0.14$	$1.7 \times 10^5 \pm 0.15$	$1.0 \times 10^5 \pm 0.07$
V. campellii	$3.5 \times 10^5 \pm 0.27$	$3.6 \times 10^5 \pm 0.30$	$2.1 \times 10^5 \pm 0.22$
V. cholerae	$3.7 \times 10^5 \pm 0.32$	$3.1 \times 10^5 \pm 0.25$	$3.7 \times 10^5 \pm 0.32$
V. damsel	$1.7 \times 10^5 \pm 0.5$	$2.2 \times 10^5 \pm 0.23$	$2.9 \times 10^5 \pm 0.17$
V. fischeri	$1.8 \times 10^5 \pm 0.1$	$1.9 \times 10^5 \pm 0.04$	$1.6 \times 10^5 \pm 0.16$
V. harveyi	$1.9 \times 10^5 \pm 0.04$	$1.0 \times 10^5 \pm 0.07$	$1.4 \times 10^5 \pm 0.13$
V. mimicus	$1.5 \times 10^5 \pm 0.1$	$1.8 \times 10^5 \pm 0.1$	$2.3 \times 10^5 \pm 0.09$
V. parahaemolyticus	$2.1 \times 10^5 \pm 0.18$	$1.6 \times 10^5 \pm 0.16$	$2.0 \times 10^5 \pm 0.14$
V. splenndidus	$1.4 \times 10^5 \pm 0.13$	$1.4 \times 10^5 \pm 0.13$	$1.6 \times 10^5 \pm 0.16$

Legend: WL = Water leaf, PL = Pumpkin leaf, SL = Scent leaf

Discussion

The high population density and type of *Vibrio* spp isolated in this study indicates that the fresh vegetables under study were of very poor microbial quality and thus of serious public health concerns. *V. cholerae* for example is notorious for cholera in humans causing approximately 1.4 million illness and 600 deaths annually in the United States (Okafor *et al*., 2003; Traore *et al*., 2014). The situation is even more delicate because preparations of vegetables usually do not require extensive heat treatment that would otherwise have eliminated the potential pathogens (Adeyemi *et al*., 2008); hence, it is important to thoroughly wash such vegetables in waters containing appropriate food grade antibacterial chemicals to significantly reduce the microbial load before consumption.

Broad spectrum antibiotics were used in this study for determination of the antibiotic susceptibility profile of the *Vibrio* isolates. Ceftriaxone was observed to be the most effective antibiotic in the treatment of all the *Vibrio* spp., as all the test isolates showed sensitivity to the antibiotic. Tunung *et al*. (2012) reported that 93.48 % of the isolated strains of *V. parahaemolyticus* from vegetables were resistant to nalidixic acid while 4.35 % were resistant to imipinem. In the present study, all the isolated *Vibrio* spp. except *V. alginolyticus* was susceptible to pefloxacin and sparfloxacin (Table 2). Bacteria may be impervious to several drugs simultaneously or drugs of the same family. For instance, the resistance of *Vibrio* species to β-lactam antibiotics has been reported. All the *Vibrio* spp. tested in this work were susceptible to ampiclox with the exception of *V. cholerae* and *V. parahaemolyticus* while all but *V. alginolyticus* and *V. splenndidus* were susceptible to amoxicillin (Table 2).

Table 2: Antibiotic sensitivity pattern of *Vibrio* species isolated from fresh vegetables

Antibiotics	*Vibrio* solates									Percentage Resistance
	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8	V_9	
Pefloxacin (10 µg)	R	S	S	S	S	S	S	S	S	11 %
Gentamycin (10 µg)	S	S	S	S	R	S	R	S	S	22 %
Ampiclox (30 µg)	S	S	R	S	S	S	S	R	S	22 %
Amoxicillin (30 µg)	R	S	S	S	S	S	S	S	R	22 %
Ceftriaxone (30 µg)	S	S	S	S	S	S	S	S	S	0 %
Ciprofloxacin (10 µg)	S	S	S	R	R	R	R	S	S	44 %
Streptomycin (30 µg)	R	S	S	S	S	S	S	S	R	22 %
Erythromycin (30 µg)	S	S	R	S	S	R	S	S	S	22 %
Cotrimoxazole (30 µg)	S	S	S	R	S	S	S	R	S	22 %
Chloramphenicol (30 µg)	S	S	S	S	S	S	R	S	R	22 %
Sparfloxacin (10 µg)	R	S	S	S	S	S	S	S	S	11 %
Augmetin (30 µg)	S	R	S	R	S	R	R	S	S	44 %

Legend: V_1 =*V. alginolyticus*, V_2 = *V. campellii,* V_3 = *V. cholera*, V_4 = *V.damsel*, V_5= *V. fischeri*, V_6= *V. harveyi*, V_7 = *V. mimicus*, V_8 = *V. parahaemolyticus*, V_9 = *V. splenndidus*, S = susceptibility, R = Resistance

V. campellii, *V.damsel*, *V. harveyi*, *V. mimicus* was resistant to augmentin and similarly *V. damsel*, *V. fischeri*, *V. harveyi*, *V. mimicus* was not sensitive to ciprofloxacin. This result is similar to the observation of Adeyemi *et al*. (2008) who reported that halophylic *Vibrio* species were resistant to amoxicillin, augmentin, chloramphenicol and nitroforantoin. Such species also showed multiple resistance patterns to gentamycin, nitrofurantoin, tetracycline, augmentin, chloramphenicol, amoxycilin, ofloxacin, cotrimoxazole, ceftriazone, and ciprofloxacin (Adeyemi *et al*., 2008). In the present study, *V. parahaemolyticus* showed drug resistance to ampiclox, and cotrimoxazole (Table 2). This is in agreement with Zulkifl *et al*. (2009) report on multi-drug resistance of *V. parahaemolyticus* isolated from raw foods.

Foods contaminated with antibiotic resistant bacteria is a threat to public health as the antibiotic resistant determinants may be transferred to other bacteria of clinical significance, and *V. parahaemolyticus* is a candidate vehicle for such transfer because of its diversity and also because it can survive in the gastrointestinal tract of both humans and animals (Zulkifl *et al*., 2009). The occurrence of multi-drug resistant strains of bacteria in the environment could be an indication of excessive usage of antibiotics in the fields (Letchumanan *et al*., 2015). Antimicrobial agents have been employed to treat bacterial infections of fruits, vegetables and ornamental plants and the most extensively used antibiotics on plants is streptomycin (Kummerer, 2009). Many scientists have researched on the possibility for a range of drugs to be absorbed from the rhizosphere by plants and have evaluated prospective importance of this exposure route in terms of human health (Kummerer *et al*., 2008). In particular, specific antibiotics are taken up by carrot and corn (Kummerer *et al*., 2008). Nevertheless, more work need to be done to elucidate the antimicrobial sensitivity of pathogenic *Vibrio* species so as to determine a more proper and effectual treatment of *Vibrio* diseases in humans and animals (Zulkifl *et al*., 2009).

Conclusion and recommendation

This study has provided new insights into the diversity and antibiotic susceptibility profiles of *Vibrio* species in fresh vegetable samples collected in various markets in Benin City, Nigeria. Most of the antibiotics used in this study were effective against *Vibrio* species and could be potential candidates for the treatment of infections caused by *Vibrio* species. However, the study also demonstrates an increasing resistance of *Vibrio* spp., to important broad spectrum antibiotics like ciprofloxacin and augmentin; indicating an emerging public health concern. There is therefore need for continuous monitoring of the antibiotic profile of *Vibrio* species and appropriate enlightenment programmes towards ensuring that vegetables to be eaten raw are properly washed before consumption in order to guarantee food safety and ultimately preserve the public health.

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