

Antimicrobial Susceptibility Of Some Members Of Enterobacteriaceae Isolated From Salad Vegetables In Calabar

Iyonawan Elvis ODIGIE¹, Bolaji David AKINBO², Adedeji David ATERE³, Nosakhare Lawrence IDEMUDIA⁴, Anne ASUQUO¹

¹Medical microbiology/Parasitology Unit, Department of Medical Laboratory Sciences, University of Calabar, Calabar, Nigeria.

Email: odigie4luv@yahoo.com; Phone number: +2348036347042

²Haematology Unit, Department of Medical Laboratory Science, Achievers University, Owo, Nigeria.

Email: davidkings0384@gmail.com; Phone number: +2347030857729

³Chemical pathology Unit, Department of Medical Laboratory Science, Achievers University, Owo, Nigeria.

Email: ateread@gmail.com; Phone number: +2348039501172

⁴Department of Medical microbiology, University of Benin Teaching Hospital, Benin City, Nigeria.

Email: lawnosa@gmail.com; Phone number: +2348039258854

Abstract: There is potential for the normal faecal flora of humans to be augmented by antibiotic resistant strains of bacteria acquired in the course of eating fresh uncooked vegetable salads. The purpose of this study was to find out whether colonization of fresh vegetable by antibiotic resistant bacteria contributes to this resistance. One hundred vegetable samples were studied, of which fifty samples were washed and fifty of the samples unwashed. From these samples, 105 different strains of bacteria belonging to the family Enterobacteriaceae were isolated, Klebsiella, Proteus and Escherichia coli species were rare. The 105 isolated were investigated for susceptibility to common antibiotics. Peflacin and Ciprofloxacin were the most effective. Resistance to the drugs was found only in 3.15% for each of the drugs. Four percent of the isolates were resistant to tarivid while 9.45% were resistant to augmentin. Percentage resistant of 13.65, 18.9 and 25.2 was recorded for gentamicin, septrin and ampicillin respectively. Consequently, bacteria from vegetables are not responsible for the high prevalence of resistant Enterobacteriaceae in Faecal flora in Calabar.

[Iyonawan Elvis ODIGIE, Bolaji David AKINBO, Adedeji David ATERE, Nosakhare Lawrence IDEMUDIA, Anne ASUQUO. **Antimicrobial Susceptibility Of Some Members Of Enterobacteriaceae Isolated From Salad Vegetables In Calabar.** *N Y Sci J* 2015;8(2):31-36]. (ISSN: 1554-0200). <http://www.sciencepub.net/newyork>. 6

Keywords: Vegetables Salad, Enterobacteriaceae, Antibiotic resistant, Faecal

Introduction

Enterobacteriaceae are a large heterogeneous group of Gram negative rods whose natural habitat is the intestinal tract of humans and animals. The family includes many general (*Escherichia*, *Shigella*, *Salmonella*, *Enterobacter*, *Serratia*, *Proteus* and others). Some enteric organism e.g. *E. coli* is part of the normal flora and incidentally causes disease, while others like *Salmonella* and *Shigella* are regular pathogens. Enterobacteriaceae are facultative anaerobes, ferment a wide range of carbohydrates, possess a variety of toxin and virulent factors. (Jawetz *et al.*, 2010).

Members of the family, Enterobacteriaceae contaminates raw vegetables through the use of untreated animal manure as fertilizers, (Locking *et al.*, 2001) and the use untreated waste water for irrigation (Tacheuchi *et al.*, 2001). Outbreaks of infection associated with consumption of raw vegetables have occurred in high frequency during past few decades, several factors have contributed to this increase, including changes in agronomic and processing practice (Beauchat and Rhu, 2002). Salads and

vegetables may be contaminated with campylobacter, (Evan *et al.*, 2003). Tomatoes, cantaloupes and sprouts have been linked with out-breaks of salmonellosis, (Zheng *et al.*, 2013). An outbreak of illness caused by *E. coli* have been associated with raddish sprouts, lettuce, apple cider and Melon, (Aruscavage *et al.*, 2008). Antibiotic resistant strains of Enterococcus have been isolated from fresh produce, (Lynette and Jaykus, 2004). And it has been well documented that water and food are possible vehicles of resistant strain transmission to human intestinal flora (Witte, 2000). Resistance transfer within the gastro-intestinal tract is possible; hence, if food is highly contaminated with resistant bacteria, it could be an important source of resistance among intestinal flora (Corpet, 1988).

Antimicrobial Susceptibility Patterns of KPC-producing or CTX- M producing enterobacteriaceae was conducted between 2007 and 2007 at JMI Laboratories USA. CTX-M carrying isolates were highly susceptible to imipenem, meropenem and tigecycline whereas KPC- producing isolates were highly resistant to all antimicrobials tested except polymyxin B and tigecycline (Gales *et al.*, 2012). Also,

in a work conducted in tertiary care units in Gujara, shows that all antibiotics used in the study as monotherapy did not demonstrated statistically significant patterns on the Enterobacteriaceae studied, with the exception of Imipenen and meropenem while high levels or resistance to aztreonam was observed (Patel, 2000). Most Gram negative organisms are susceptible to agents such as cephalosporins, aminoglycosides, cotrimazole, ampicillin, ciprofloxacin (Cheesbrough, 2004).

The indiscriminate use of antibiotics in enhancing in farm animals may lead to the evolution of resistance by directly selecting for drug resistant pathogens (Smith et al., 2005). Multi-drug resistant salmonella was disseminated worldwide in the 1980's through contaminated feed made out of farmed fish that had been fed routine antibiotics (Angulo and Griffin, 2000). The ability of an organism to produce the enzyme β -lactamase increases the rate of resistance of such organisms to the β -lactam antibiotics. In work conducted in Brooklyn, Extended spectrum β -lactamases were endemic and spread between hospitals (Guillermo et al., 2000). Most Enterobacteriaceae become resistant to tetracycline through mechanisms such as enzymatic inactivations of tetracycline, efflux pump, and ribosomal protection (Viera et al., 2007). Enterobacteriaceae becomes resistant through vertical transfer of inherited mutations from previous generations and genetic recombination of DNA by horizontal genetic exchange, (WHO, 2010).

Few studies exist, that examine the prevalence of resistance among Gram negative organisms from vegetables. (Halmiton-Miller and Shah, 2001), although results are conflicting. The author characterized Enterobacteriaceae from salad vegetables and found a high degree of resistance to Ampicillin as well as narrow expanded spectrum Cephalosporins. A study conducted in Finland show that Enterobacteriaceae isolated from vegetables were highly susceptible to the antibiotics studies and multi-drug resistant strains were identified. In this study, Enterobacteriaceae from fresh vegetables (salad) isolated in Calabar would be screened and the significance of the results obtained would be fully discussed.

Materials and methods

Sample collection

Fifty samples, each of carrot, cabbage, lettuce, cucumber and onion were bought randomly from open markets in Calabar- Watt, Goldie, Akim, Edim otop and Marian markets between July-October 2010. These vegetables were collected from stalls in cellophane bags without the hand of the sampler touching it.

Processing of samples

12g each of sample from the same source were weighed, two samples were collected from each source, and one was washed before chopping, while the other was not washed. Both samples were placed in a conical flask, each containing 50ml of brain-heart infusion broth. These were incubated at 37°C overnight. Samples were properly washed with water with the sampler gloved, this was followed by washing with vinegar, before chopping on a foil paper surface that is heat sterilized (Bunsen flame) with a heat sterilized knife.

Isolation of bacteria:

After 16-24 hours of incubation, the broth was plated out in MacConkey agar, and incubated for 16-24 hours. Colonies obtained were purified on cysteine lactose electrolyte deficient (CLED)

Morphology of isolates:

Microscopic examination of isolates on the plates was carried out to obtain their colonial morphology. Specifically the colour, elevation, edge and consistency were observed and recorded. The Gram reaction of each isolate was determined using Gram staining method. Motility of the organisms was also determined by direct wet mount. This was done by placing a drop of an overnight broth culture inoculated with the test organism on a slide, a cover slip was carefully layered on this and examined for motile organisms using X10 and X40 objectives of the microscope.

Biochemical test and identification of isolates

Isolates were gram stained and tested for oxidase and catalase production. The ability of each isolate to ferment glucose was tested. Gram negative rods that were oxidase negative, glucose fermenting and catalase positive were considered to be in the family of enterobacteriaceae and were included in the work. Further identifications were based on motility, indole, urease production and Kligler iron agar (KIA) reactions.

Antimicrobial sensitivity test.

The modified Kirby Bauer disc diffusion method on nutrient agar was used (Ochie and Kolhatkar, 2000). Inoculum was standardized by inoculating a loopful of an overnight culture of test organism into 5ml of nutrient broth (peptone water) and incubate for 4-6 hours to achieve the same turbidity as 0.5 MacFarland standards. A sterile swab stick was dipped into the standardized inoculum suspension and used to streak nutrient agar plate and allowed to dry. Routinely used antibiotics such as Tarivid (5ug), Peflacin (5ug), ciprofloxacin (5ug), Augumentin (30ug), Gentamicin (10ug), Septrin (30ug) and Ampicillin (10ug), were aseptically placed on the nutrient agar plate streaked with test organism and incubated overnight. Zones of inhibition of the different antibiotics were measured and recorded.

Result

A total of hundred samples were collected from the markets in Calabar.

Table 1 shows the sources of the samples and the numbers collected from each source. A total of 204 isolates were recovered of which 105 belonged to the family enterobacteriaceae. Thirty seven isolates belonging to the family enterobacteriaceae were isolated from specimens washed with water and vinegar, while 68 isolates were recovered from the unwashed samples; the most frequent isolates were *Klebsiella* species, *proteus* species, and *Escherichia coli* species.

(Table 2), four samples, yielded no bacteria. These included (2 washed onions), 1 washed (cucumber) and 1 unwashed (onion), while two samples from Akim market did not yield growth of enterobacteriaceae.

Antimicrobial Resistance

The 105 enterobacteriaceae isolated from this study were investigated for sensitivity to common antibiotics. Of the 7 antimicrobials tested, peflacin and ciprofloxacin were the most effective. Resistance to the drugs was found only in 3 isolates (3.15%). Four (4.2%) isolates were resistant to tarivid while 9 (9.45%) isolates were resistant to augmentin. Percentage resistance of 13.65, 18.9 and 25.2 was recorded for gentamicin, spetrin and ampicillin respectively. A total of 18 isolates from watt, 40 from Marian, 18 from Goldie, 19 from Akim and 26 from Edim-otop markets. The isolates were tested against common antibiotics clinically used in Calabar.

Table (3), shows the distribution of enterobacteriaceae according to various markets. A total of 37 isolates were recovered from washed samples of which 7 were from watt, 9 from Marian, 4 from Goldie, 7 from Akim and 10 from Edim-otop while a total of 68 isolates were recovered from unwashed samples of which 11 of them were from watt, 22 from Marian, 10 from Goldie, 12 from Akim and 16 from Edim-otop markets. The isolates were tested against common antibiotics clinically used in Calabar.

Table (4), shows the frequency of occurrence of resistant isolates from the various markets under study. Of the total of 11 isolates from Watt market tested, 1 showed resistance to ciprofloxacin, and Augmentin, while 3 isolates showed resistance to septrin, ampicillin and gentamicin respectively. Percentage resistance from Watt market was 11.55%. The percentage resistance from Marian market was 12.6% of which isolate was resistant to tarvid, peflacin, augmentin, and septrin, while four isolate was resistant to Gentamicin and ampicillin. The percentage resistance from Goldie market was 26.25% of which one isolates were resistant to Gentamicin, and ampicillin. The percentage resistance from Goldie market was 26.25% of which one isolate was resistant to tarivid, and ciprofloxacin, 2 isolates resisted peflacin, 3 isolates were resistant to augmentin and Gentamicin, while 6 isolates resisted spetrin and 9 resisted ampicillin.

Table 1: Description of vegetable samples:

	Watt Market	Marian Market	Goldie Market	Akim Market	Edim otop Market
Carrot	2	2	2	2	2
Cabbage	2	3	2	1	2
Cucumber	2	2	2	2	2
Lecttuce	2	2	2	2	2
Onion	2	1	2	3	2
Total	10	10	10	10	10

Table 2: Types and Number of Bacteria Isolated From Specimens

Isolates	Washed specimens	Unwashed specimens
<i>Klebsiella species</i>	4	12
<i>Escherichia coli</i>	4	8
<i>Proteus species</i>	4	9
<i>Salmonella</i>	1	2
<i>Shigella</i>	3	5
<i>Serratia</i>	5	7
<i>Citrobacter species</i>	3	6
<i>Enterobacter species</i>	2	3
<i>Providencia species</i>	4	5
<i>Unidentified group</i>	6	11
Total	37	68

Table 3: A distribution of enterobacteriaceae according to markets

Isolates	Watt	Marian	Goldie	Akim	Edim-Otop
<i>Klebsiella species</i>	2	-	-	-	2
<i>Escherichia coli</i>	1	2	-	1	2
<i>Proteus species</i>	2	-	1	2	1
<i>Salmonella</i>	-	1	-	-	-
<i>Shigella</i>	1	-	2	-	2
<i>Serratia</i>	1	-	2	-	2
<i>Citrobacter species</i>	1	-	-	1	1
<i>Enterobacter species</i>	-	-	1	-	1
<i>Providencia species</i>	-	34	-	1	2
Unidentified group	-	-	1	-	1
Total	7	9	4	7	10

Table 4: Resistance frequency among all isolates

Antimicrobial	Watt	Marian	Goldie	Akim	Edim-Otop
Tarivid 5ug	-	1	1	-	2
Peflacin 5ug	-	1	2	-	-
Ciprofloxacin 5ug	1	-	1	-	1
Augumentin 30ug	1	1	3	3	1
Gentamicin 10ug	3	4	3	-	3
Seprin 30ug	3	1	6	4	4
Ampicilin	3	4	9	2	6
Total	11	12	25	9	17

The percentage resistance from Akim market was 9.45% of which 2 isolates were resistant to ampicillin, 3 were resistant to septrin. Isolates from Edim-otop market showed a percentage resistant of 17.85% of which 1 isolate was resistant to ciprofloxacin and augmentin, 2 were resistant to tarivid, 3 were resistant to gentamicin, 4 were resistant to spetrin and 6 were resistant to ampicillin. The overall resistance to quinolones among the 105 isolates tested from the five markets studied was as follows, four to tarivid, 3 were resistant to peflacin and ciprofloxacin. Among other drugs, a total 9% isolates were resistant to augumentin, 13 isolates were resistant to gentamicin, 18 isolates resisted septrin and 24 isolates were resistant to ampicillin.

Discussion and conclusion

The role of fresh vegetables with the overall framework of human exposure to drug resistant bacteria has until now been insufficiently investigated from public health perspective (Hamilton-Miller and Shah, 2001).

Many studies have focused on the contribution of antimicrobial resistance to the severity of hazards posed by foodborne pathogens. However, some specific measures have been adopted along the food chain to minimize the current trend in the acquisition of drug resistance among food pathogens such as Salmonella, (Pittout et al., 2008). The role of commensal bacteria as potential hazards both directly

as opportunistic organisms and indirectly as carries of resistance genes has only been partially explored to date, (Aleksun and Levy, 2006; Oluyeye et al., 2006).

In this study, a total of 105 Enterobacteriaceae were isolated and investigated for their sensitivity to common antibiotics. Of which 7 antibiotics tested, the Quinolones (Peflacin and Ciprofloxacin) were most effective. Resistance to these drugs was found to be 3.15% each for the 105 isolates. Resistance to Tarivid was seen in 4.2% while 9.45% were resistant to Augumentin. The percentage resistance of 13.65, 18.9 and 25.2 was recorded for Gentamicin, Seprin and Ampicillin respectively. In a previous work carried out by Plano et al, (2009), 83.6% of the food sample tested positive. Gram negative bacteria resistant to one or more groups of antibiotics, with a great heterogeneity in the resistance pattern among the lactose fermenters and non-lactose fermenters. Moreover, both Gram negative groups, show frequencies as high as 97.8% to ampicillin and augumentin. The non-lactose fermenters were 28% resistant to more than 3 groups of antibiotics such as gentamicin, ampicillin and augumentin. The degree of resistance to ampicillin, gentamicin and augumentin in Plano's work far exceed that observed in this study.

In this study, 25.2% and 13.65 of isolates were resistant to ampicillin and gentamicin, this contrasted the work of Benzason et al, (2008), where 93.5% were resistant to ampicillin and only 2.8% were resistant to

gentamicin. Bezanson's work show isolates were susceptible to gentamicin and resistant to ampicillin, this could be the result of over prescription, and abuse of ampicillins by patients. Of a particular concern is the high prevalence of resistance to nalidixic acid which was the first quinolones discovered, this could be the result of over usage of nalidixic acid in the treatment of urinary tract infections, and literatures suggest that resistance to nalidixic acid determined by Disc diffusion method may be a reliable indicator of decreased susceptibility to ciprofloxacin, (Albayrak et al., 2004). In this study, samples from Marian, and Edim-otop markets showed the highest prevalence of Enterobacteriaceae with Klebsiella and Proteus species having the highest rate of occurrence.

In Osterblad et al, (1999), there was no resistance at all to gentamicin and ciprofloxacin while 15% resistance was recorded for ampicillin. The coliforms, klebsiella, Echerichia and citrobacter species were frequently isolated from vegetables in this study. This is in line with Machada et al, (2006), on microbiological quality of organic vegetables produced in soil treated with different types of manure and mineral fertilizer, where all isolates were fecal coliforms such as E. coli, Klebsiella, citrobacter and enterobacter. Enterobacteriaceae flora such as Enterobacter, proteus, Pseudomonas, Providencia and Serratia associated salad vegetables in this study, corroborate literature on the types of bacteria found in vegetables (Hamilton-Miller and Shah, 2001).

In this study, the effect of washing on bacteria load of each vegetable was also determined. The vegetables tested had a high content of the genera, Klebsiella, E.coli and Enterobacteriaceae. Washing with vinegar was effective in reducing the number of bacteria, but did not eliminate them. Pathogens such as salmonella and shigella were still isolated from vegetables after washing; though in a very low prevalence. These findings are public health concern as these organisms can contribute to outbreak of food borne disease. The washing method used in this study, reduced the bacteria load from 68% isolates in the unwashed specimens to 37% isolates in the washed specimens. These also corroborate literature on bacteria flora of fruits and vegetables in Lebanon and the effects of washing on the bacteria content, were washing procedure reduced bacteria load from 80% in unwashed samples to 30% after washing.

In conclusion, a very low frequency of antimicrobial resistance in Enterobacteriaceae isolated from vegetables in Calabar was observed. This contradicts previous studies which found high resistance levels (Levy et al., 1984). Due to the low frequency of resistance found in this work, Enterobacteriaceae isolated from fresh vegetables do not pose a great risk of public health hazards.

Recommendation

I recommend that in future studies, molecular typing method might usefully be employed to explore the inter-relationship of human and environmental populations of Enterobacteriaceae.

Corresponding Author:

Mr A. D. Atere,
Chemical Pathology Unit,
Department of Medical Laboratory Science,
Achievers University, Owo, Nigeria.
E-mail: ateread@gmail.com
Phone number: +2348039501172

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