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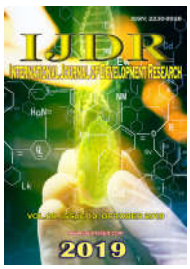

RESEARCH ARTICLE OPEN ACCESS

RESISTANCE PROFILE OF CONTAMINATING ORGANISMS ISOLATED FROM READY -TO-EAT VEGETABLE SALAD FROM SELECTED EATERIES IN A UNIVERSITY SETTING

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ABSTRACT

In recent years, salad has become a very popular component of menu served at birthdays, wedding parties and at home; they are also sold in fast food centers in most major cities in Nigeria. Reports of unverified rampant cases of gastroenteritis following consumption of meals served with fresh vegetable salads have become serious public health concern which necessitate the need for this investigation. Ready to eat salad samples were investigated for pathogenic bacteria and the strains isolated were subjected to biochemical and morphological test for identification. The isolates were subjected to antibiotic susceptibility test using standard antibiotics disks following the Clinical and Laboratory Standards Institute guidelines. The Multiple antibiotic resistance index was determined. The isolates were also subjected to investigation to determine the resistance factors using the QIAGEN plasmid purificationmini kit. A total of nine bacteria were isolated from the three samples of salad isolated and identified namely *Staphylococcus aureus*, *Salmonella typhi, Shigella dysenteriae, Escherichia coli, Proteus mirabilis, Enterobacter cloacae, Micrococcus luteus, Streptococcus can is* and *Pseudomonas aeruginosa,* The total viable count of to the isolates ranged from $2.6X10^5$cfu/mlto $8.1x10^5$cfu/ml. The total coliform count ranged from 1.23 $X10^5$cfu/ml to $7.4X10^5$ cfu/ml. Percentage resistance was 86% for *S. aureus*; 93% for *S.typhi;* 50% for *Shigella dysenteriae;* 57% for *E.coli;* 71% for *Proteus mirabilis*; 57% for *Enterobacter* spp; 43% for *Micrococcus* spp; 71% for *Streptococcus pygenes* and 64% for *Pseudomonas aeruginosa*.The MAR index ranged between 0.43 for *Micrococcus* spp and 0.93 for *Salmonellatyphi* showing a high degree of multiple drug resistance (MDR). The plasmid profile of the resistant bacteria showed *Salmonella typhi, Shigella dysenteriae* and *Escherichia* coli had plasmid borne resistant factors while *Staphylococcus aureus* had chromosomal borne resistance factor. The result of this work emphasized danger of multidrug resistant pathogens in ready to eat unprocessed food.

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INTRODUCTION

Antibiotics, also known as antibacterial, are substances that destroy or slow down the growth of bacteria. Antibiotics (Penicillin) was first discovered by Alexander Flemings in 1928. Almost immediately Flemings also predicted the development of antibiotic resistance (Gould, 2016). Antibiotic resistance (AR) in bacteria is one of the biggest challenges facing humanity and health workers in particular (Milan, 2018) and plasmids play a very important role in the dissemination of antibiotic resistance (AR) among human pathogens.

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AR has become alarming as more and more bacteria are becoming resistant to common antibiotics making them to become multi drug-resistant bacteria. It can be transferred to the bacteria that cause human diseases even if the bacteria are not related. One of the means by which bacteria develop resistance is through plasmid mediated transfer of resistance (Li *et al.,* 2019). Plasmids are small, circular, auxiliary, dispensable chromosome DNA strands. In general, they exist separately from and are replicated independently of the main bacterial chromosome, although the majority of replication functions are provided by the host cell. They do not accommodate any of the set of core genes needed by the cell for basic growth and multiplication, but rather carry genes that may be useful periodically to enable the cell to exploit

particular environmental situations, for example survive and thrive in the presence of a potentially lethal antibiotic. Hence, plasmids carry a considerable variety of genes, including those that confer antibiotic resistance and resistance to a number of toxic heavy metals, such as mercury, cadmium and silver, those that provide enzymes that expand the nutritional ability of the cell, virulence determinants that permit invasion of and survival in animal systems and functions that enhance the capacity to repair DNA damage (Stanisich, 1988). Most Plasmids have the ability to be transferred within and between species and can therefore be acquired from other bacteria. This property makes resistance due to plasmid much more threatening than resistance due to chromosomal mutation in terms of spread of antibiotics resistance (Hugo and Russel, 2004). Approximately 80% of antibiotics in the U.S. are used by the agricultural industry, mostly for food production. Ready-to-eat foods, such as dairy products and fresh produce, do not undergo a "kill" step such as cooking during preparation, so antibiotic-resistant bacteria can either be directly consumed or can contaminate kitchen surfaces or other foods (ASM, 2017).Transmission of Antibiotic resistant (AMR) bacteria (AMRB) to humans and to the human gastro intestinal tract is of concern due to either direct infections or the possibility of horizontal gene exchange with other potentially pathogenic members of the gut microbiota favored by the high cell densities found in the gut (Haug *et al.*, 2011; Collignon *et al*., 2016).

Vegetable salad is a very common food accompaniment in Nigeria. The vegetables that usually make up this recipe include tomatoes, cucumber, carrots, green chili, cabbage and lettuce. They are sold in almost every market, and can be seen hawked around by traders (Abdullahi *et al.,* 2010).Fruits and vegetables have been identified as significant sources of pathogens and chemical contaminants (Uzeh *et al*., 2009). As a result, environmental and food microbiologists have continued to identify and suggest control measures for hazards at all stages in the supply chain (Johngen, 2005). Vegetables are exposed to microbial contamination through contact with soil, dust and water during harvest or postharvest handling. Raw vegetables when used in salad preparation without sufficient washing, make salads unfit for human consumption (WHO, 2002).Bacteria involved in salad contamination include but not limited to *E.coli, Enterobacter*, *Salmonella* sp., *Shigella* sp and *Pseudomonas aeruginosa* (Tambekar, 2006). Aim of this research work is to elaborate on the risk of exposure of humans to AMRB (antimicrobial resistant bacteria) through ready to eat food at sale points or retail stores, to help optimise AMR surveillance schemes, in particular reference to systematic AMR surveillance at food retail stores, point of sale especially agricultural produce and also the genetic investigation into AMRB.

MATERIALS AND METHODS

Sampling: Fresh vegetables salad (ready-to-eat salads) were bought from three (3) selling points around Achievers University, Owo. Each sample (300-400 g) was properly identified and labelled, placed separately in a sterile plastic bag and transported to the laboratory in an ice box within 2-4 hour of collection, where they were prepared for bacteriological examination. Sample type, source and other relevant data were recorded for each sample. All tests were carried out at the school Microbiology laboratory.

Preparation of sample: Twenty five grams of vegetables salad was weighed using a weighing balance (S. Mettler) and 225 ml peptone water (0.1%) was added to a blender jar capacity 500 ml and was mixed for 2 minutes. Ten-fold dilutions was prepared under aseptic conditions from each sample using 0.1% peptone water as diluents, this results in a dilution of 10^{-1} ,decimal dilutions of 10^{-2}, 10^{-3} and 10^{-4}will then be prepared by serial dilution. A 9ml portion of peptone water was added into each test-tubes and 1ml of the sample homogenate was dispensed into the first test-tube (10^{-1}) using a syringe. The solution was mixed gently and properly. Further dilution was carried out on the 4 test tubes.

Standardization of inocula: One percent (1%) of solution of sulphuric acid was prepared and mixed properly. Also, 1% solution of barium chloride was prepared by dissolving 0.5g of dehydrated barium chloride ($Bacl_2$ H_2O) in 50ml of distilled water. A 0.5ml of aliquot of barium chloride solution was added to 99.5ml of sulphuric acid solution and mixed together. The solution was then transferred into a capped tube for further use at temperature of 4^{o}C (Cheesebrough, 2000).

Total plate count method: Using separate sterile pipettes, decimal dilutions of 10^{-2}, 10^{-3}, and 10^{-4} were prepared and more as appropriate, of vegetable salads by transferring 1ml of previously diluted sample to 9ml of diluents. All dilutions were shaken vigorously to allow for even dispersal. One ml of each dilution was pipetted into separate, duplicate, and appropriately marked Petri dishes. Prepared nutrient agar was dispensed aseptically into the inoculated plates. The plates were incubated at 35^{o}C for 24-32 h. Colonies were counted using a colony counter (J-3) and the total aerobic microorganism were calculated per gram.

Gram staining: Isolated strains were subjected to Gram Staining using standard procedure as described by Cheesebrough (2000).

Biochemical identification and characterization of isolated bacteria: The various bacteria colonies were identified based on their colonial, morphological and biochemical characteristics. Including catalase, oxidase, Sugar fermentation and Coagulase tests.

Antibiogram of isolated bacteria: The M100 of the CLSI (2019) standard was used to interpret the result of the antibiotic susceptibility test. Isolated bacteria were subjected to antibiotics susceptibility test using the disk diffusion methods of Kiry-Bauer *et al.,* (1996). Standard antibiotic disk including; septrin (30µg), sparfloxacin (30µg), gentamicin (30µg), Augmentin (30µg), chloranphenicol (30µg), ciprofloxacin (30µg), amoxilin (30µg), pefloxacin (30µg), tarivid (30µg), streptomycin (30µg), ampiclox (30µg), zinnacef (30µg), erythromycin (30µg), amoxacilin (30µg), rocophin (30µg) were used.

Multiple Resistance Index: The MAR index for the resistant bacteria isolates was determined according to the procedure described by Krumperman (1983). This is essentially to determine the degree of bacterial resistance to antibiotics. The indices were determined by dividing the number of antibiotics to which the organism were resistant to (a) by the number of the antibiotics tested (b), Resistance to three or more antibiotics is taken as MAR and MAR greater that 0.2

indicates a high risk source of contamination where antibiotics are often used.

Isolation and characterization of resistance plasmids: Plasmid DNA was extracted from bacterial strains by using mini prep alkali lysis method (Birn Boim and Doly, 1979) with minor modifications. Briefly, it was diluted using twice the volumes of solutions II and III followed by a 15 min incubation on ice and number of phenol/chloroform/isoamyl alcohol (25:24:1) extractions. For plasmid extraction, bacteria were grown in Luria- Bertani (HiMedia, India) broth supplemented with 2% NaCl, with shaking. The strains were maintained as frozen stocks at –80°Cin marine broth (HiMedia, India) plus 20% (v/v) glycerol.

Plasmid Curing: Curing treatments were carried out using ethidium bromide (Molina-Aja *et al.,* 2002). An overnight culture of plasmid contained resistant bacteria strain (200 µl) was added into five different 5-ml cultures of LB broth containing 2% NaCl, previously adjusted to pH 7.5. Increasing concentrations of the curing agent were added to the five tubes over the range from 50 to 500 µg/ml. The cultures were then incubated overnight at 37°C under constant agitation and observed for growth. The cells from the culture tube that contains the highest concentration of curing agent permitting visible growth (usually in the range of 150-250 µg/ml) were serially diluted and plated on to Luria agar plates containing 2% NaCl and were grown up to single clones. These clones were tested for the antibiogram pattern, for the antibiotics to which they are originally resistant. Bacterial isolates, that showed change in the resistance pattern to the susceptible, were subjected for plasmid extraction.

RESULTS

Total Bacteria count: The total bacterial count for sample A (Salad from POS 1) (POS- point of sale)was $5.2x10^{-5}$cfu/ml, sample B(Salad from POS 2) was $8.1x10^{-5}$cfu/ml and sample C (Salad sample from POS 3) was $2.6x10^{-5}$cfu/ml. The total coliform count for sample A was $1.23x10^{-5}$cfu/ml, sample B was $2.4x10^{-5}$ and sample C was $7.4x10^{-5}$cfu/ml (Table 1)

Table 1. The total plate count of bacteria isolates

Sample	Total viable counts cfu/ml	Total coliform counts cfu/ml
A	5.2×10^{-5}	1.23×10^{-5}
B	8.1×10^{-5}	2.4×10^{-5}
C	2.6×10^{-5}	7.4×10^{-5}

Bacterial; isolates; After subjecting to morphological, cultural and biochemical tests and identification using the Bergey's manual, The isolated bacteria strains include; *Staphylococcus aureus, Salmonella typhi, Shigella dysenteriae, Escherichia coli, Proteus mirabilis, Enterobacter cloacae, Micrococcus luteus, Streptococcus canis and Pseudomonas aeruginosa.*

Antibiotic profile of test isolates: Table 2 presents the antibiotic profile of the test isolates using standard antibiotics. Zones of inhibition with values lower than 10mm are designated resistant while values ≥10 are designated as sensitive. *S. aureus* was resistant to all the antibiotics used except pefloxacin and streptomycin with a percentage resistance of 86. *S. typhi* was resistant to all antibiotics used except septrin, and erythromycin with a resistant factor of 93%. *S. dysenteriae* was resistant to seven of the fourteen antibiotics used which include chloramphenicol, sparfloxacin, augmentin, ampiclox, amoxacillin, Zinnacef, and rocophin with a resisance factor of 50%. However, *E.coli* was sensitive six of the fourteen antibiotics used including septrin, chloramphenicol, ciprofloxacin, gentamycin, pefrloxacin, and streptomycin with a percentage resistance of 57%. Similarly, *Proteus mirabilis* was sensitive to chloramphenicol, gentamycin, rocophin and erythromycin with percentage resistance of 71.

Enterobacter spp. was also sensitive to ciprofloxacin, pefloxacin, tarivid, amoxacillin and erythromycin with a resistance factor of 57%. On the other hand *Micrococcus* spp. was resistant to sparfloxacin, pefloxacin, tarivid, ampiclox, zinnacef and erythromycin with 57% resistance factor. *Streptococcus pyogenes* was sensitive to chloramphenicol, augmentin, pefloxacin, and streptomycin with 71% resistance factor and *Pseudomonas aeruginosa* was sensitive to ciproflaxin, augmentin, tarivid, amoxillin, and zinnacef with a percentage resistance of 64.

Multiple Antibiotic Resistance Index: Multiple Antibiotic Resistance (MAR) Index = a/b; where a represents the number of antibiotics to which the test isolate depicted resistance and b represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility. The MAR for *Staphylococcus aureus* was 0.86; MAR for *Salmonella typhi* was 0.93; MAR for *Shigella dysenteriae* was 0.5; mar FOR Escherichia coli was 0.57; MAR for *Proteus mirabilis* was 0.71; MAR for *Enterobacter* spp was 0.57; MAR for *Micrococcus* spp was 0.43 and MAR for *Pseudomonas aeruginosa* was 0.64. Bactria having MAR index of ≥0.2 originates from a high risk source of contamination and indicates a high level of multi drug antibiotic resistance.

Plasmid profiling of Isolates: Figure 1 presents the plasmid profile of antibiotic resistance of some of the isolates namely; *Staphylococcus aureus*, *Salmonella typhii*, *Escehrichia coli* and *Shigella dysenteria***.** Plasmid profiling revealed that out of the four isolates analyzed, three strains harbored plasmids and one was without plasmid.The figure 1 below shows that organisms in well 2-4 have plasmid borne resistance of 100bp. This shows that the resistance factor in the organisms are plasmid borne while organism in well 1 has resistance factor that is not plasmid borne but could be chromosomal.

DISCUSSION

The prevalence of antimicrobial resistance among food-borne pathogens has increased in recent time possibly as a result of the use of antimicrobials in food-producing animals (Angulo *et al*., 2000; Bywater, 2004; Teuber, 2001). The co-existence of resistance genes with mobile elements such as plasmids, transposons, and integrons facilitates the rapid spread of antibiotics resistance genes among bacteria (Sunde and Nordstrom, 2006). The bacteria isolated from the salad sampled from three (3) fast food outlets correlates with the work of Tambeker (2006) who reported the presence of *Escherichia coli*, *Enterobacter, Salmonella sp*, *Shigellasp* and *Pseudomonas aeruginosa* in vegetable salad. These contaminants could be as a result of handling by vendors (Eni *et al*, 2010), during harvesting (Alice, 1997) or during processing (Oranusi and Olorunfemi, 2011).

Table 3. Antibiotic resistance profile of test organisms to standard antibiotic

Diameters zone of inhibition in mm																		
Test Ab/ organism	*S.aureus*		*S.typhii*		*S. dysentarea*		*E coli*		*P. mirabilis*		*Enterobacter spp*		*Micrococcus spp*		*Streptococcus spp*		*P. aeruginosa*	
Septrin	-	R	10	S	10	S	10	S	5	R	7	R	10	S	-	R	6	R
Chloramphenicol	8	R	5	R	-	R	10	S	10	S	5	R	10	S	10	S	-	R
Sparfloxacin	-	R	-	R	5	R	5	R	-	R	6	R	-	R	5	R	5	R
Ciprofloxacin	-	R	-	R	10	S	10	S	8	R	10	S	10	S	7	R	10	S
Augtmentrin	-	R	-	R	5	R	-	R	5	R	-	R	10	S	10	S	10	S
Gentamycin	5	R	-	R	10	S	10	S	10	S	9	R	10	S	6	R	5	R
Pefloxacin	12	S	-	R	10	S	10	S	-	R	10	S	5	R	10	S	-	R
Tarivid	-	R	-	R	10	S	-	R	6	R	10	S	-	R	5	R	10	S
Streptomycin	10	S	-	R	15	S	15	S	10	R	-	R	15	S	10	S	6	R
Amoxacilin	-	R	-	R	6	R	5	R	8	R	10	S	10	S	5	R	10	S
Ampiclox	-	R	-	R	-	R	-	R	-	R	5	R	5	R	5	R	-	R
Zinnacef	-	R	-	R	-	R	-	R	8	R	10	S	-	R	7	R	10	S
Rocophin	-	R	5	R	5	R	5	R	10	S	5	R	10	S	6	R	5	R
Erythromycin	-	R	10	S	10	S	8	R	10	S	10	S	-	R	5	R	10	R

Legend;
R—Resistant
S—Susceptible
- --- No activity

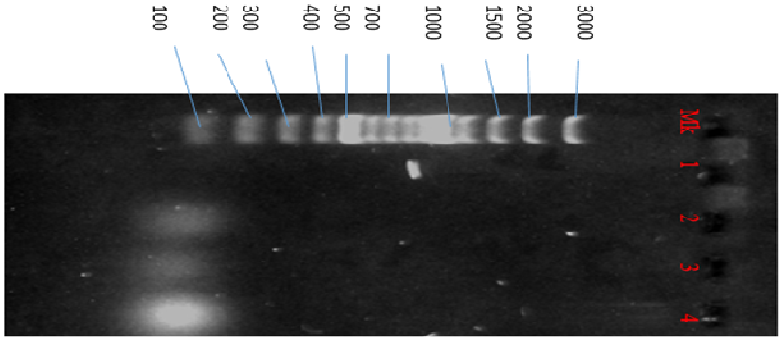

Fig 1. Plasmid profile of resistant bacteria

The problem of contamination can also be from preparation environments which may have been contaminated with *Micrococcus spp* and *Staphylococcus spp* (Mensah *et al*., 2005). Contamination with *S. aureus,* has been linked to carriage in nasal passages of food handlers or by infected workers (Tambeker, 2006, Itohan *et al.*, 2011). Food handlers could be a source of food contamination in *Staphylococcus aureus* food poisoning, but equipment and environmental surfaces are also implicated sources of contamination with *S. aureus* (Meldrum *et al.,* 2009).The presence of *S. aureus* in ready-to-eat salad vegetables sampled from specific outlets is an indication of poor hygiene practices **(**Harris *et al.*, 2003**)**. According to the WHO (2002), effect of microbiological hazards such as *Salmonella* on food safety is now a major public health concern worldwide (Itohan *et al.,* 2011). Presence of *E. coli* indicates recent contamination by faecal matter and possible presence of other enteric pathogens known to be causative agents of food borne gastroenteritis and bacterial diarrhea disease. In a study from UK it was reported that *E. coli* was present in 1.5% of ready-to-eat organic vegetable samples (Sagoo *et al.,* 2001). Of the total nine (9) isolates tested for antibiotic susceptibility using standard antibiotic discs. The MAR of resistant bacteria ranged from 0.43-0.93. Research has shown that MAR greater than 0.2 shows a high risk level of resistance to antibiotics. Such organism can be referred to as multi-drug resistant bacteria (MDRB). This result indicates high risk of contamination where antibiotics are often used (Olayinka *et al.*, 2009).

Hence, antibiotic resistance of bacteria is a major threat to public health and can be significant reservoirs of genes encoding antibiotic resistance determinants. An increase in the emergence of multi-drug resistant bacteria in recent years is worrisome and the presence of antibiotic resistance genes on plasmids has further helped in the transmission and spread of drug resistance among pathogenic bacteria. It has become increasingly apparent that a variety of important properties of microorganisms are plasmid mediated. Figure 1 which shows the plasmid profiling of the isolates confirms this as three out of the four isolates analyzed have their resistant factor to be plasmid-borne with high molecular weight base pairs. The best-known example of the plasmid pool of bacteria is the plasmid mediated antibiotic resistance determinants, so called R- plasmids which can transfer themselves from one cell to another and thus capable of spreading rapidly through a bacterial population (Smillie *et al.*, 2010). The plasmid antibiotic resistance genes prove useful in identifying the bacterial cells that have taken up the recombinant DNA molecule in a high background of untransformed cells (Brown, 2010). It is well known that plasmid is one of the most important mediators facilitating the fast spreading of antibiotic resistance among bacteria (Dale and Park, 2004). Since plasmids are easily transferable from bacterium to bacterium, the environmental strains can undergo sudden changes in their plasmid carriage causing diversity in plasmid profile and the resulting antibiotic resistance pattern.

Conclusion

The prevalence of multi-drug resistance is quite high in bacteria and most bacteria acquire antibiotic resistance by means of plasmids and they are capable of transferring the resistance from one bacterium to another. The presence of plasmids may pose a potential health hazard, since plasmids may be transferred to humans from bacteria either directly or indirectly. Therefore, Promotion of good hygiene practices among all food services providers should be encouraged, Safety regulators should also be implemented and mentored, Vegetables agricultural practices should also be reviewed and strict regulators should be implemented especially the use of safe irrigation water and fertilizers, Local food standards and guidelines should also be set based on local data. A study to determine the role of food borne bacteria in transferring antibiotic resistance is highly recommended, Frequent assessment of bacterial resistance and their plasmid profiles should be considered and the threat posed by overuse of antibiotics which has led to the buildup of resistance in bacteria should be recognize and dealt with.

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