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ANTIBIOTIC RESISTANCE PATTERNS OF *Campylobacter jejuni* and *Salmonella* Typhi ISOLATED FROM READY-TO-EAT VEGETABLE SALADS HAWKED IN KANO METROPOLIS

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ABSTRACT
A total of 200 samples of Ready – to – Eat (RTE) vegetable salads were aseptically purchased randomly from hawkers in eight Local Governments of Kano State, Nigeria. The Aerobic mesophilic bacterial count was conducted according to standard techniques. Samples were further screened for S. Typhi and C. jejuni using standard procedures. Isolates of the two bacterial species were subjected to antibiotic sensitivity testing using Kirby Buer disk diffusion technique. The total aerobic bacteria count ranged from 1.200 ×10^5 to 1.70×10^5 cfu/g. A total of 36 bacterial isolates from the RTE vegetables were identified as C. jejuni (18%) and 97 (48.5%) as S. Typhi. Ninety percent (90%) of the bacterial isolates were found to be resistant to the assayed antibiotics. C. jejuni was highly sensitive (98.4%) to gentamicin. TEM genes were detected in 40% of the C. jejuni isolates while 60% were detected in S. Typhi isolates. RTE vegetable salads hawked in the study areas are contaminated with C. jejuni and S. Typhi and the isolates were resistant to most of the antibiotics tested. It is recommended that hazard analysis and critical control point of ready to eat food should be observed.
Keywords: Vegetable salads, Aerobic mesophilic bacterial counts, C. jejuni and S. Typhi, Kirby-Baeur disc diffusion technique

INTRODUCTION

Salad is a mixture of fresh vegetables (tomatoes, cucumber, carrots, onions, cabbage, and lettuce etc.) that provides a rich source of vitamins, minerals and dietary fiber of low fat and calories to the consumer (Abdul-Raouf and Ammar, 2011; Adeshina *et al.,* 2012; Udo *et al.,* 2008; Itohan *et al.,* 2011). In recent years, salad has become a very popular component of menu served at birthday and wedding parties; they are also sold in fast food centers in most major cities in Nigeria. The consumption rates of vegetables and vegetable salads have also greatly increased based on their proven medical and nutritional benefits (Udo *et al.,* 2009; Adeshina *et al.,* 2012; Puspanadan *et al.,* 2012). Recently, vegetables are sliced and beautifully arranged in layers in transparent plastic containers and hawked in almost every market, motor parks and other public places. Media reports of unverified rampant cases of gastroenteritis following consumption of meals served with fresh vegetable salads have become serious public health concern (Udo *et al.,* 2008).

Salad has high water content because of its dressing but it is low in calories and hence, it is used by people who are aiming at weight loss, help in disorders and strokes.

Moreover, the availability of potable water for proper washing of these vegetables is also lacking in different areas. As a result of which dirty or contaminated water is used for washing which could lead to further increasing the microbial load on these vegetables which some people buy and eat without further washing and also it can become contaminated with pathogenic microorganisms during harvesting, through human handling, harvesting equipments and transport containers (Hassan *et al.,* 2006; Elexson *et al.,* 2017; New *et al.,* 2017).

Campylobacter jejuni and *Salmonella* Typhi have been implicated in many food borne disease outbreaks related to fresh produce (Beuchat, 2002; Maffei *et al.,* 2013) making them potential threat to consumers. Several outbreaks of human gastro-enteritis have been linked to the consumption of contaminated fresh vegetable salad (Udo *et al.,* 2008). Among the well-related food borne pathogens are; *Esherichia coli* 0157 (Shiga toxin *E. coli*), *Listeria monocytogenes, Salmonella* sp., *Vibrio* sp., *Yersinia* sp., and *Campylobacter* sp. (Jong, 2010).

Antimicrobial resistance is a growing public health threat and has been designated by the World Health Organization as an emerging public health problem (Komolafe, 2003). The problem arises when bacteria causing disease withstand therapy. Thus, the issue on biosafety with regard to antibiotic resistance must be addressed at a global level (Chai *et al.,* 2008). The prevalence of antimicrobial resistance among pathogens from vegetables has increased during the recent decade in developed countries (Holvoet *et al*., 2013). However, reports of the antibiotic sensitivity of these bacteria are only presently emerging in developing countries. Transmission of antimicrobial resistant bacteria is a potential concern with unhygienic handling of vegetables (Adesetan *et al.,* 2013).

In view of the growing concerns on the bacteriological safety of the salads sold in Kano, growing resistance of bacteria to antibiotics and increase in consumption of the salads by the teeming populace, there was need for the study on the antibiotic resistance pattern of C. jejuni and S. Typhi isolated from ready to eat vegetable salads hawked in Kano Metropolis..

MATERIALS AND METHODS

Study area

The Kano urban area covers 137sq.km and comprises eight Local Government Areas; Kano Municipal Council (KMC), Dala (DAL), Gwale (GWL), Fagge (FGE), Ungoggo (UGG), Kumbotso (KBT), Nassarawa (NSR) and Tarauni (TRN).

Sampling Sites

The samplings were market places, motor parks and roadsides in all the eight (8) local government areas.

Sample size

Sample size was calculated using the formula;

$n = Z^2 PQ/ d^2$

Where n- number of samples = ?

Z- Normal distribution =1.960

P- Prevalence obtained from previous research = 5.56% and 11%.

Q- 1-P =

d- 0.05

n = 200

Sample collection and Processing

Two hundred vegetable salads (25 from each local government) were purchased randomly from different hawkers at markets, motor parks, schools as well as road sides in a sterile aluminium foil paper. The samples were immediately taken to Postgraduate Laboratory at the Department of Biological Sciences, Bayero University Kano in an ice box for analyses.

Fresh and apparently healthy vegetables (cabbage, lettuce, cucumber, onions and tomatoes) were purchased from Rimi Market in Kano Municipal Council Local Government and washed with clean water. Using sterile knife and chopping board, the vegetables were cut and mixed together and analysed in the laboratory for bacteriological contamination (Partially Treated Control).

Fresh and apparently healthy vegetables (cabbage, lettuce, cucumber, onions and tomatoes) were purchased from Rimi Market and washed with clean water. Water and distilled white vinegar were poured in a clean bowl at ratio 3:1, the vegetables were soaked inside the bowl for 3 minutes and rinsed under clean running water according to the Manufacturer's instructions. Using sterile knife and chopping board, the vegetables were cut and mixed together. This was used for bacteriological analyses (Treated Control).

Enumeration Bacteria

This was carried out by serial dilution and pour plating (Egboh and Emeshili, 2007). Twenty five gram (25g) of the homogenized sample was transferred into a conical flask containing 225ml of buffered peptone water (BPW) using sterile pipette syringe and labeled 10^{-1} (stock solution). One millilitre from tube 10^{-1} was transferred after agitation into another test tube containing 9ml of BPW (using a separate syringe) and labeled 10^{-2}, this was repeated to obtain 10^{-3}, 10^{-4} and 10^{-5}. Using another fresh syringe, 1ml of sample from each dilution was transferred into two sterile petridishes and labeled accordingly. This was followed by pouring nutrient agar in each petridish, swirled clockwise and anti-clockwise and allowed to solidify. Finally the plates were incubated at 37^0C for 24hours. Colonies that developed were thereafter counted and expressed as colony forming unit per gramme (CFU/g).

Detection of *Campylobacter jejuni*

The selective media used to isolate *Campylobacter jejuni* was charcaol cefoperazone desoxycholate agar (CCDA; Oxoid, Basingstoke, UK). Then, 22.75g of the media was suspended in 500ml of distilled water and brought to boil to dissolve. The media was sterilized by autoclaving at 121^0C for 15minutes and cooled to 50^0C. One vial of CCDA selective supplement SR0155 was aseptically added and reconstituted as directed. It was mixed well and poured into sterile petridishes containing 1ml of homogenised vegetable sample. Plates were anaerobically incubated at 37^0C for 48 hours. Plates were observed for colonial and morphological appearance.

Detection of *Salmonella* Typhi

Salmonella-Shigella agar was used to isolate *Salmonella* Typhi*.* The media was prepared by suspending 31.5g of SS agar into 500ml of distilled water. The media was heated to boiling with frequent agitation to dissolves completely not autoclaved or overheated because overheating may destroy the selectivity of medium. The media was cooled to about 50^{o}C. The media were mixed well and poured into sterile Petri dishes containing 1ml of homogenised vegetable sample. Plates were incubated at 37^{0}C for 24 hours. This was further confirmed with gram staining and biochemical test as described by Cheesbrough (2006).

Gram Staining

All the isolates were subjected Gram staining according to the standard method as described by Cheesbrough (2006).

Biochemical test for characterization of bacteria

Bacterial isolates were characterized using biochemical tests (catalase, oxidase, indole, urease, motility, hydrogen sulphide production etc.) as demonstrated by Cheesbrough (2006).

Antimicrobial Susceptibility testing of the Isolates

Preparation of Inoculum

A single isolated colony was picked using sterile wire loop and carefully streaked on the surface of sterile nutrient agar plate to give well distinct isolated colonies after incubation at 37℃ for 18h (Cheesbrough, 2002).

Standardization of the inoculum

Well isolated colonies from each overnight culture of the isolates on nutrient agar were aseptically transferred into a 5ml sterile physiological saline, shake vigorously and its turbidity compared to 0.5 McFarland Standard (approximately 1.5×10^{8} cfu/ml). This was done for each of the test bacterial isolates. The standardized inocula were used for the antibacterial susceptibility testing (Cheesbrough, 2002).

Antibiotic susceptibility test

The antibiotics susceptibility pattern was determined using the Kirby-Bauer disc diffusion technique as described by CLSI (2008).

Detection of drug resistance gene by polymerase chain reaction (PCR)

DNA Extraction

Genomic DNA of the bacterial isolates was extracted using alkaline lyses method as described by Sambrook *et al.* (1989). The extracted DNA was amplified using polymerase chain reaction and the products were analyzed by ethidium bromide stained 2% agarose gel electrophoresis. Following electrophoresis, the PCR products were viewed and the picture of the bands was taken.

Statistical Analyses

The data obtained was subjected to two way analysis of variance (ANOVA)

RESULTS

The mean aerobic mesophilic bacterial counts of all the samples and the negative control were far above the acceptable limit, while the positive control (vinegar treated) was below the acceptable limit set by International Commission on Microbiological Specifications for Foods (ICMSF) (Table 1).

Campylobacter jejuni was detected more in samples from Ungogo, Kumbotso and Fagge, while least frequency of occurrence occurred in Tarauni. *Salmonella Typhi* was detected in high frequencies in samples collected from Fagge, Dala, Kumbotso and Ungogo, while the positive control has l zero frequency of occurrence (Table 2).

Campylobacter jejuni was sensitive to only gentamicin, *Salmonella* Typhi was only sensitive to ciprofloxacin, while the two bacteria were resistant to other antibiotics tested against them (table 3 and 4). Bla-TEM gene was detected in 60% of *S.* Typhi and 40% in *Campylobacter jejuni* (Table 6).

Table 1: Mean Aerobic Mesophilic Bacterial Counts of ready to eat vegetable Salads hawked in Kano Metropolis

S/N	Sampling sites	AMBC (cfu/g×10^5)	ICMSF FAO	
1	FAG	1.37 ± 0.99^{aj}	10^3	10^5
2	GWL	1.40 ± 1.63^{bj}		
3	TRN	1.36 ± 1.36^{cj}		
4	KMC	1.20 ± 1.06^{dj}		
5	DAL	1.70 ± 1.27^{ej}		
6	KBT	1.50 ± 1.49^{fj}		
7	NSR	1.84 ± 0.89^{gj}		
8	UGG	1.23 ± 2.11^{hj}		
9	Control (Partially Treated)	0.84 ± 0.20^{i}		
10	Control (Treated)	0.00016 ± 0.00^{ji}		

Footnote: Values are mean±SD of triplicate data, Values with the same alphabet along the column are considered significant.
Key: AMBC – Aerobic Mesophilic Bacterial Count, cfu/g – Coliform Forming Unit/ Gram, FAG – Fagge, GWL – Gwale, TRN – Tarauni, KMC – Kano Munincipal, DAL – Dala, KBT – Kumbotso, NSR – Nassarawa, UGG – Ungogo

Table 2. Frequency of occurrence of isolates sourced from ready-to-eat vegetable salad hawked in Kano metropolis

S/N	Sampling sites	Samples collected	*C. jejuni*	Percentage occurrence (%)	*S.* Typhi	Percentage occurrence (%)
1	FAG	25	5	25	15	75
2	GWL	25	4	20	10	50
3	TRN	25	2	10	9	45
4	KMC	25	4	20	11	55
5	DAL	25	3	15	14	70
6	KBT	25	6	30	12	60
7	NSR	25	5	25	10	50
8	UGG	25	7	35	12	60
9	Control (Part. Treated)	25	0	0	3	15
10	Control (Treated)	25	0	0	0	0
	Total	250	36		97	

Table 3.Mean zone of inhibition of isolates sourced from ready-to-eat vegetable salad

S/N	Antibiotics	Disk potency(µg)	Zone of inhibition (mm)	
			C. jejuni n=36	*S.* Typhi n=97
1	AUG	30	6.0	6.0
2	CXM	05	6.0	6.0
3	GEN	10	20	8.0
4	CRX	30	6.0	6.0
5	CAZ	30	6.0	6.0
6	CPR	05	10	20
7	OFL	05	14	15
8	NIT	300	15	9.0

Key: AUG- Augmentin, CAZ- Ceftazidime, CXM- Cefixime, CPR- Ciprofloxacin, GEN- Gentamicin, OFL- Ofloxacin, CRX- Cefuroxime, NIT- Nitrofurantoin, 6mm-disk diameter which indicate no activity.

Table 4. Antibiotic Resistance profile of isolates sourced from ready-to-eat vegetable salad hawked in Kano

S/N	Antibiotics	Resistance profile	
		C. jejuni n=36	*S.* Typhi n=97
1	AUG	R	R
2	CXM	R	R
3	GEN	S	R
4	CRX	R	R
5	CAZ	R	R
6	CPR	R	I
7	OFL	I	I
8	NIT	I	R

Key:

AUG	≤13mm (R)	14mm (I)	≥18mm (S)
CXM	≤15mm (R)	16mm (I)	≥19mm (S)
GEN	≤12mm (R)	13mm (I)	≥15mm (S)
CRX	≤14mm (R)	15mm (I)	≥18mm (S)
CAZ	≤14mm (R)	15mm (I)	≥18mm (S)
CPR	≤15mm (R)	16mm (I)	≥21mm (S)
OFL	≤12mm (R)	13mm (I)	≥16mm (S)
NIT	≤14mm (R)	15mm (I)	≥17mm (S)

Classification of sensitive and resistant *S.* Typhi and *C. jejuni* to antibiotics adopted from Cheesbrough (2002).

Table 5: Percentage Resistance profile of isolates sourced from ready to eat vegetables isolates

S/N	Antibiotics	Resistant (%)	Susceptible (%)	Resistant (%)	Susceptible (%)
		C. jejuni		*S.Typhi*	
1	AUG	100	0	100	0
2	CXM	100	0	100	0
3	GEN	98.4	1.6	99.3	0.6
4	CRX	100	0	100	0
5	CAZ	100	0	100	0
6	CPR	99.2	0.8	98.4	1.6
7	OFL	98.8	1.2	98.8	1.2
8	NIT	98.8	1.2	99.2	0.8

Table 6: percentage of occurrence TEM resistance gene on *Campylobacter jejuni* and *Salmonella* Typhi isolated from ready to eat vegetable salads

S/N	Name of Orgnism	No of sample collected	No of positive samples	Percentage of occurrence (%)
1	*Campylobarterr jejuni*	5	2	40
2	*Salmonella* Typhi	5	3	60

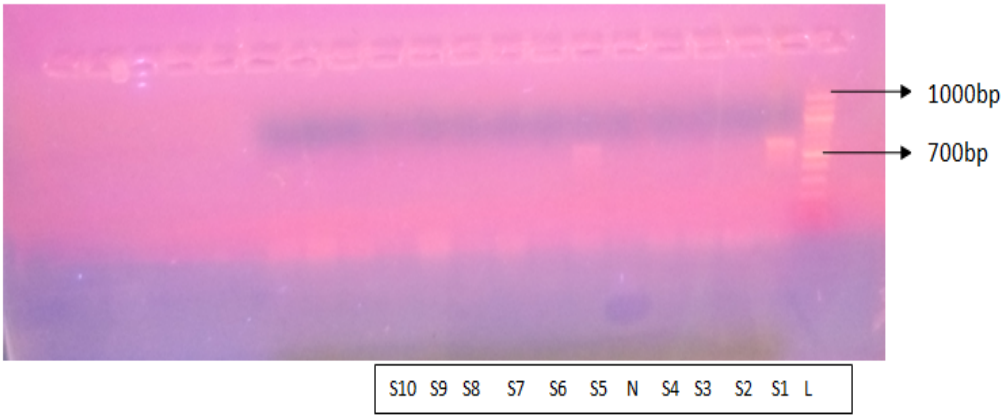

Plate I: Gel picture of TEM gene detection on *Campylobacter jejuni* and *Salmonella* Typhi

KEY:
L is 100 bp DNA ladder
S1 and S6 are positive for TEM, Product size is 700 bp
S2-S4 and N are Negative for TEM
N is Negative control
S stands for sample
S1 = Ca.1 (from client and so on)
Some positive bands are faint.

DISCUSSION

The results of the aerobic mesophilic bacterial counts of the ready-to-eat vegetable samples sourced from the eight metropolitant Local Government areas in Kano revealed that the mean counts of all samples were above 1.00×10^5 cfu/g. These findings were similar to that of Bukar *et al.* (2010), who recorded aerobic mesophilic count above the maximum acceptable limit set by Food and Agricultural Organization in lettuce, cabbage and tomato ($1.40x10^6$ to 1.60×10^7CFU/g) sourced from Kwakwaci irrigation site, in Fagge LGA of Kano State. Similarly, the findings were similar to that of Gbonjubola *et al.* (2012) and Chikodili *et al,* (2015) who recorded high bacterial load ranging from 6.0×10^4 cfu/g to 2.0×10^6 cfu/g on vegetable salads sourced from restaurants in Zaria, Kaduna State, Nigeria. The high bacterial counts of the samples investigated in this research could be attributed to the usage of animal dungs as fertilizers, cultivation of vegetables with sewage polluted water (domestic sewage), contact with soil and dust, poor handling and processing, use of contaminated water during processing, contaminated utensils and usage of bare hands during servicing of the product to the end users.

The aerobic mesophilic bacterial counts of all the samples were found to be above the maximum acceptable counts of 10^3 cfu/g as reported by International Commission on Microbiological Specification for Foods. In view of this, the ready-to-eat vegetable salads sold in the sampling sites can be reported as unsafe for human consumption.

The mean aerobic mesophilic count of control (partially treated) was found to be 8.41×10^4 cfu/g, while control (treated), the AMBC dropped to 1.6×10^2 cfu/g which was within the acceptable limit and were safe for consumption.

The presence of these bacteria (*Campylobacter jejuni* and *Salmonella* Typhi) in the ready-to-eat vegetable salads could be attributed to poor cultivation practices, bad handling and transportation practices, poor personal and environmental hygiene during processing and selling of the vegetable salads.

Campylobacter jejuni shows high resistance to most of the assayed antibiotics, while very low resistance to gentamicin was observed. Similarly, *Salmonella* Typhi demonstrated very high resistance to most antibiotics used in the study. This observed resistance to the antibiotics is an indication of earlier exposure of the isolates to these drugs or the acquisition of the genes from other bacteria through processes such as conjugation, transduction or transformation.

Resistance of food borne pathogens including *Campylobacter jejuni* and *Salmonella* Typhi to multiple antibiotics is becoming an emerging public health issue worldwide. In addition, the use of antibiotics in agricultural practices may have contributed immensely to the development resistant food borne pathogens. This can lead to some bacteria developing resistance against the antibiotics being used to control them. Subsequently, humans and animals share these pathogens which find their way into water bodies some of which are used to irrigate vegetables. The result of this is that vegetables get contaminated with these resistant pathogens which can also be easily transferred to other food sources. Sources including application of manures to the farm from slaughter houses, in vitro propagation of crops (tissue cultured plants), antibiotics spray on the crops in the orchard, soil and water contamination with faecal material and effluent from farm animals at the field, and genetic engineering causing increased antibiotic resistance have been noted as sources by which antibiotic resistance are incorporated into fruits and vegetables.

The antimicrobial resistance profile of the isolated *Campylobacter jejuni* revealed a high percentage of the organisms showing multiple drug resistance to commonly used antibiotics. Infections with antibiotic resistant bacteria make the therapeutic options for infections treatment extremely difficult or virtually impossible in some instances.

This study further shows that TEM resistance gene was detected in both *Campylobacter jejuni* and *Salmonella* Typhi isolates. The antimicrobial resistance gene in microflora, food spoilage or opportunistic pathogenic strains contaminating ready to eat vegetable salads form an indirect risk to public health as they increase the gene pool from which pathogenic bacteria can pick up traits. The observation of resistance to multiple antibiotics by the organisms isolated from ready to eat vegetable salads in this study suggest a substantial chance for transfer of antimicrobial resistance to humans because the eventually resistant bacteria are not killed as they are often consumed without cooking or pre-heating, as a consequence, transfer of antimicrobial resistance genes between bacteria after ingestion may occur.

CONCLUSION AND RECOMMENDATION

In conclusion, the results of this study on ready to eat vegetable salads collected from eight Local Governments of Kano State clearly revealed high bacterial contamination above the acceptable limit. The ready to eat vegetable salads were contaminated with *Campylobacter jejuni* having 36(18%) and *Salmonella* Typhi having 97(48.5%) rates of occurrence. Over 90% of the isolates were resistant to tested antibiotics and as such pose substantial risk for transfer of antimicrobial resistance to humans as they are consumed without having undergone prior preservation or additional processing.TEM resistance gene was detected in *Campylobacter jejuni* with 2(40%) and *Salmonella* Typhi with 3(60%). It is recommended that vegetable salad hawkers should be enlighted on hygienic vegetable salad processing and handling methods as well as the public health importance of campylobacteriosis and salmonellosis thereby ensuring food safety. Washing of vegetable salads with just water is inadequate to remove all contaminating pathogens. Therefore, the use of distilled white vinegar should be employed in order to reduce the bacterial load.

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