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Detection of *bla-CTXM* and *bla-TEM* Genes, and Biofilm Forming Ability of *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* Isolated From Salad Sold at a Private University

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ABSTRACT

Food poisoning can be caused by a wide range of substances including but not limited to the presence of pathogenic microorganisms in food e.g. salads. However, there is no report on the occurrence of pathogenic microorganisms in the salads sold in a private University campus and as such the research was performed in order to determine the food safety levels as well as probable sources of contaminants of the salads in the university. The purpose of this research, was to determine the frequency of occurrence of *Escherichia coli*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus* in salads sold in the cafeteria on the campus. A total number of 13 samples of salad were obtained and screened for the possible presence of pathogenic bacteria. Identified bacteria isolates were then tested for virulence traits such as antibiotic resistance, motility, biofilm and possession of *blaTEM* and *blaCTX-M* genes. All the sought for organisms were found in all collected samples. The total heterotrophic count ranged from $0.74 \pm 1.3 \times 10^3$cfu/g to $28.2 \pm 1.75 \times 10^3$cfu/g. The frequency of occurrence of these microorganisms in the salads from the cafeteria stalls ranged from 0(0%) to 5(41%). *S. aureus* and *E. coli* had the highest occurrence at 34%. The result of the antibiotic susceptibility test showed complete resistance of all isolates to amoxicillin clavulanate and cefotaxime and complete sensitivity to gentamycin and ofloxacin. *Pseudomonas aeruginosa* and *S. aureus* isolates showed multi-drug resistance. Multiple antibiotic resistance (MAR) index ranged from 0.16 to 0.83. Out of all the isolates, only one *E. coli* of all the *E. coli* isolates showed the ability to produce biofilm. Amplification of the ESBL genes (*blaTEM* and *blaCTX-M genes*) in *S. aureus, E. coli* and *P. aeruginosa* isolates showed that they possess the gene which encodes for their resistance to cefotaxime, ceftriaxone, penicillin and cephalosporins. The presence of these organisms in ready to eat salads is a cause for concern as these organisms have been associated with serious foodborne infections.

Keywords: *Escherichia coli, Staphylococcus aureus, P.aeruginosa,* Resistant genes, *blaTEM blaCTX-M*

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Introduction

The most common causes of illness and death in underdeveloped nations is food poisoning. The majority of cases of food poisoning are linked to bacterial contaminations, particularly those which are caused by most Gram-negative bacteria which includes *E. coli* and *P. aeruginosa* (Mostafa *et al.,* 2018).

Staphylococcus aureus has been commonly associated with bacterial food poisonings due to its production of enterotoxins (Hennekinne *et al*., 2012). *Staphylococcus aureus* is a pathogenic human bacterium which produces a wide array of protein toxins that are primarily responsible for its high virulence potential. The production of enterotoxins by this bacteria species has been historically linked to a number of illnesses (Fisher *et al.,* 2018).

Poor hygiene throughout food production operations or during food retail and storage can lead to food contamination by pathogenic bacteria like the *S. aureus* which has long been acknowledged as one of the most significant bacteria that cause illnesses. It is the main contributor to cellulitis, abscesses (boils), and other soft tissue infections, according to the Minnesota Department of Health (2010).

Staphylococcus aureus strains which are methicillin resistant, (Methicillin-resistant *Staphylococcus aureus* (MRSA)) are of major concern. The existence of antibiotic-resistant strains in food-derived microbes shows that it may be more essential than previously believed in the transmission of the genes encoding antibiotic resistance (Chajęcka-Wierzchowska and Zadernowska., 2016).

Ready-to-eat (RTE) salads is been consumed globally. and *E. coli* strains has been identified as one of the atielogical agents of gastroenteritis and diarrhoea (Garcia-Fernandez *et al.,* 2009). Food-borne pathotypes, like *E. coli*, which causes diarrheagenic *E. coli* (DEPs) has been on the increase and has been known to be associated with the consumption of RTE-salads (Garcia-Fernandez *et al.,* 2009).

Most nosocomial infections and other fatal diseases in immunocompromised individuals is caused by the bacterium, *Pseudomonas aeruginosa* (Gale *et al.,* 2015; Wu *et al*., 2015; Gomila *et al*., 2018). It is an opportunistic pathogen that preys on gaps in the hosts' defences to develop an infection.

Due to of *P. aeruginosa'*s adaptability and inherent drug resistance, common antibiotics will have a low efficacy and thereby, increasing mortality. Also, biofilms formed by *P. aeruginosa*, protects them from environmental stressors and prevent phagocytosis. This attribute allows them to colonise and survive for long periods of time which even further complicates treatment of these infections.

In natural environments, antibiotic resistance genes (ARGs) are extensively dispersed. In the context of food production, ARGs and antibiotic-resistant bacteria are thought to be stored in the phyllosphere of plants and vegetables as well as in harvested and processed ready-to-eat foods. By horizontal gene transfer (HGT), it has been demonstrated that ARGs can later spread to human pathogens and commensal bacteria, potentially resulting in severe illness (Zhou *et al*., 2020). The aim of this study was to determine the presence of ARGs in pathogenic bacteria isolated from salad samples.

Through mobile genetic components like transposons and plasmids, genes giving resistance to these medications can be effectively passed from one harmful bacterium to another (Partridge *et al.,* 2018).

Materials and Methods

Sample collection

Thirteen samples of salad were obtained from 5 cafeteria stalls as 3 samples per food stall from a private University campus in Nigeria. Each sample was aseptically collected in a sterile plastic bags and transferred to the microbiology laboratory for analysis within 24 hrs. of collection.

Bacteriological analysis

Sample Preparation

A gram of homogenized sample was then placed in a test tube which contained 9ml of sterile peptone water, after which the homogenate was used for isolation. Serial dilutions were made up to five-fold for plating. All the dilutions were used in plating, 1m of the dilutions were plated on Mannitol salt agar, Eosine Methelyne Blu media and Centrimide media using pour plate method and were then incubated at 37^0C for 24h. After 24hrs the isolates obtained where sub cultured onto Nutrient agar plates to attain pure cultures. The isolates obtained were identified based on their cultural and biochemical characteristics using a microbiology laboratory manual by Naveena and Joy., 2014.

Standardization of the inoculum and multi-sensitivity disk test

Isolates gotten from a 24hr. pure were inoculated into test tubes which contained 5ml of sterile peptone water broth. The media was thereafter, incubated at 37°C for 24 hrs. After which the broth was diluted using peptone water to obtain an opacity of 0.05 - 0.1 absorbance units at a wavelength of 625nm, using a spectrophotometer. After the standardization, the multisensitivity disk test was carried out. This test is done to detect possible drug resistance and susceptibility. The Disk diffusion method was carried out in order to test the extract which will diffuse through the agar medium seeded with the test microorganism. Muller Hinton agar was used for the culture. Sterile swab sticks were used to streak the inoculum on the Muller Hinton agar plates and allowed to dry. The disk was then placed on top of the plate containing the inoculated media with the aid of a sterile forcep and was incubated for 24hrs at 37°C. The zones of inhibition were measured in millimetres (mm) after incubation. The Clinical and Laboratory Standard Institute (CLSI) protocol was used to explain the readings of the susceptibility test.

Biofilm formation of isolates

This test is based on the characteristic cultural morphology of biofilm-forming bacteria on congo red agar medium. The medium was consist of brain heart infusion broth 37g/l, sucrose 36g/l and 0.8g/l of congo red. The mixture was sterilized at 121℃ for 15 mins. Agar plates ere made and inoculated with isolates and incubated for 24hrs at 37℃. The production of blackish colonies indicates biofilm production and non-biofilm producing isolates developed pinkish colonies.

Antibiotic Resistant genes detection

The genes coding for cefotaxime resistance (CTX-M) and ampicillin resistance (TEM), were targeted in the *P. aeruginosa, S. aureus* and *E. coli* isolates using the polymerase chain reaction (PCR). Amplification of the targeted genes was carried out with the following sets of primers; *blaTEM* F 5’ AAACGCTGGTGAAAGTA 3’ , *blaTEM* R 5’ AGCGATCTGTCTAT 3’ , *blaCTX-M* F 5’CGCTTTGCGATGTGCAG 3’ , *blaCTX-M* R 5’ ACCGCGATATCGTTGGT 3’.

The reaction mixture contained inqaba PCR Premix (Inqaba, South Africa), which is premixed ready-to-use solution containing Tag DNA polymerase, dNTP, and $MgCl_2$. The reaction mixture was prepared in 0.2 ml PCR tubes with 25 µl reaction volumes (12.5 µl Premix, 8.5 µl nuclease free water, 0.5 µl

forward primer, 0.5 µl reverse primer and 3.0 µl DNA template). The amplification reaction was executed in a thermocycler. Gene Amp PCR system (Gene Amp Singapore) using the following cycling conditions 94 °C for 4 min followed by 31 cycles, each at 94 °C for 45 secs. Annealing temperatures of 55 °C for 1 min and 68 °C for 1 min were respectively set for the *CTX-M and TEM* genes. The final extension was performed at 68 °C for 8 mins. Amplicons were confirmed with 1.5% agarose gel electrophoresis and viewed with a UV trans illuminator (Geneix biotech, Asia).

Results and Discussion

At the University campus, all foods are purchased from various vendors available as the rules of the school do not permit cooking by students. Hence, it is important in ensuring the safety of the students by ensuring that the foods sold by these vendors have acceptable microbiological standards. Thirty-eight (38) bacterial isolates, characteristic of *P. aeruginosa*, *S.aureus* and *E. coli* were obtained from all the samples, and screened, based on their cultural, morphological and biochemical characteristics.

The total heterotrophic count from the results, ranged from 0.74 ± 1.3 $\times 10^3$cfu/g to 28.2 ± 1.75 $\times 10^3$cfu/g which is relatively low compared to the work of Uzeh *et al.,* 2009 in which the count ranged from 26.0 $\times 10^3$cfu/g to 59.0 $\times 10^3$cfu/g. Table 1 shows the total heterotrophic count recorded for salad samples obtained.

The frequency of occurrence of the *E. coli* isolates ranged from 1(8%) to 3(23%), *S. aureus* isolates ranged from 1(8%) to 4(30%) and *P. aeruginosa* isolates ranged from 0 (0%) to 5(41%). *S. aureus* and *E. coli* had the highest and equal percentage frequency of occurrence (34%) in the salad samples as shown in Table 2.

Table 1: Mean heterotrophic count for salad sampled from the cafeteria stalls

Salads Samples	Mean bacterial heterotrophic counts (NA) ($\times 10^3$ cfu/g) ± S.D
1	6.2 ± 1.2
2	10.3 ± 0.4
3	4.0 ± 0.4
4	17.1 ± 0.65
5	5.5 ± 0.55
6	0.74 ± 1.3
7	9.15 ± 0.75
8	6.4 ± 0.25
9	15.5 ± 0.45
10	3.6 ± 0.35
11	17.6 ± 0.65
12	27.9 ± 1.0
13	28.2 ± 1.75

KEYS: (N.A) Nutrient Agar, (S.D) Standard Deviation

Table 2: Frequency of occurrence of isolates

Isolates	Shop 1	Shop2	Shop3	Shop4	Shop 5	Total
Escherichia coli	3 (23%)	3(23%)	3(23%)	3(23%)	1(8%)	13 (100%)
Staphylococcus aureus	4 (30%)	2 (15%)	3 (23%)	3 (23%)	1 (8%)	13 (100%)
Pseudomonas aeruginosa	2 (17%)	5 (41%)	3 (25%)	0 (0%)	2 (17%)	12 (100%)

The antibiotics used for the susceptibility profile of the isolates consist of 15 commonly used antibiotics; nitrofurantonin (300 µg), erythromycin (5 µg), ciprofloxacin (5 µg), cefuroxime (30 µg), azithromycin (5 µg), ceftriaxone sulbactam (45 µg), nalidixic acid (30 µg), ampiclox (10 µg), gentamycin (10 µg), cefixime (5 µg), ofloxacin (5 µg), levofloxacin (5µg, amoxicillin clavulanate (30 µg), cefotaxime (25 µg)

A majority of the isolates 58% (14) were resistant to amoxicillin clavulanate and cefotaxime, resistance through Extended spectrum β lactmases (ESBL) genes in accordance with Iseppi *et al,* 2018. The antibiotic to which most isolates found sensitive was ofloxacin and gentamycin 4% (1). Seventy five percent (75%)of isolates were multidrug resistant.

This result is similar to the work by Nuesch-Inderbinen *et al.,* 2015, which confirms that minor ESBL's can be detected in the vegetables. Since salads are eaten raw, ingestion of relatively harmless ESBL-producing environmental bacteria could cause these bacteria to be potential reserviours in the intestine having the potentials to carry out horizontal transfer of these ESBL genes to pathogens (Huddleston, 2014). The MAR index ranged from 0 (which was mostly with the *Escherichia coli* and *Staphylococcus aureus isolates*). Table 3 shows the MAR for the isolates. The MAR index for *Pseudomonas aeruginosa* isolates is in line with the study of Jolapamo *et al.,* 2019.

A majority of the samples were higher than the standard 0.2 MAR index. Values of high-risk sources of contamination are usually greater than 0.2, indicating the frequent use of antibiotics.

blaCTX-M and *blaTEM* were found to be possessed by the isolates in fig 1 and 2 from the salad. The isolates used in this study showed an inability to produce biofilm. Only one *E. coli* isolate demonstrated the ability to form biofilm. Studies have shown that *E. coli* can attach to and form biofilms on a variety of surfaces, including those that come into contact with food, like stainless steel, PVC, polystyrene, polypropylene, glass, etc.

Table 3: Antibiotic susceptibility profile of *P. aeruginosa* isolates

Isolates	Sensitive (%)	Resistant (%)	Intermediate (%)
LBC	8(100)	0 (0)	0 (0)
CXM	1 (12.5)	7 (87.5)	0 (0)
CRO	6(75)	1 (12.5)	1 (12.5)
CTX	0 (0)	8 (100)	0 (0)
ZEM	1 (12.5)	7 (87.5)	0 (0)
OFX	8(100)	0 (0)	0 (0)
GN	8(100)	0 (0)	0 (0)
NA	2 (25)	2 (25)	4 (50)
NF	2 (25)	5 (62.5)	1 (12.5)
IMP	3 (37.5)	3 (37.5)	2 (25)
AUG	0 (0)	6(75)	2 (25)
ACX	0 (0)	8 (100)	0 (0)

Table 4: Antibiotic susceptibility profile of *S. aureus* isolates

Isolates	Sensitive (%)	Resistant (%)	Intermediate (%)
AZN	8 (100)	0 (0)	0 (0)
CXM	8 (100)	0 (0)	0 (0)
OFX	8 (100)	0 (0)	0 (0)
CTX	2 (25)	5 (62.5)	1 (12.5)
CRO	4 (50)	3 (37.5)	1 (12.5)
ZEM	3 (37.5)	4 (50)	1 (12.5)
LBC	8 (100)	0 (0)	0 (0)
CIP	8 (100)	0 (0)	0 (0)
IMP	6 (75)	2 (25)	0 (0)
AUG	3 (37.5)	5 (62.5)	0 (0)
ERY	4 (50)	2 (25)	2 (25)
GN	8 (100)	0 (0)	0 (0)

Table 5: Antibiotic susceptibility profile of *E. coli* isolates.

Isolates	Sensitive (%)	Resistant (%)	Intermediate (%)
LBC	7(87.5)	1 (12.5)	0 (0)
CXM	5 (62.5)	1 (12.5)	2 (25)
CRO	8 (100)	0(0)	0 (0)
CTX	4 (50)	1 (12.5)	3 (37.5)
ZEM	8 (100)	0(0)	0 (0)
OFX	7 (87.5)	1 (12.5)	0 (0)
GN	7 (87.5)	1 (12.5)	0 (0)
NA	7 (87.5)	1 (12.5)	0 (0)
NF	4 (50)	1 (12.5)	3 (37.5)
IMP	3 (37.5)	5 (62.5)	0 (0)
AUG	3 (37.5)	3 (37.5)	2 (25)
ACX	5 (62.5)	1 (12.5)	2 (25)

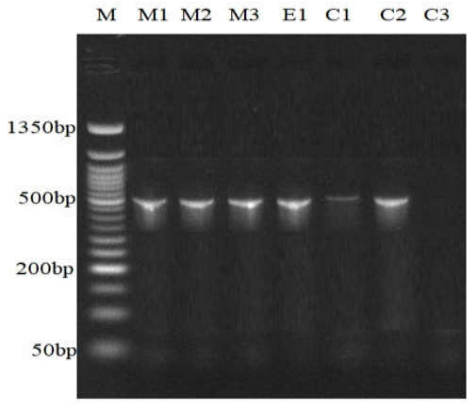

Fig 1: Gel image showing amplification of *CTX-M* gene at about 550bp. M is a 50bp ladder.

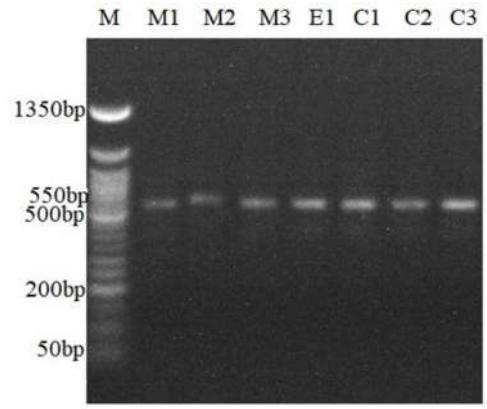

Fig 2: Gel image of *blaTEM* gene amplification at about 500bp. M is a 50bp ladder

Table 6: MAR index of salad isolates

Isolates		MAR Index
1SAM	3/12	0.25
4SAM	2/12	0.16
6SAM	2/12	0.16
7SAM	2/12	0.16
9AM	5/12	0.42
10SAM	0/12	0
11SAM	3/12	0.25
12SAM	4/12	0.33
1SAE	0/12	0
3SAE	1/12	0.08
5SAE	0/12	0
6SAE	10/12	0.83
9SAE	2/12	0.16
10SAE	1/12	0.08
12SAE	2/12	0.16
13SAE	0/12	0
1SAC	6/12	0.5
2SAC	6/12	0.5
4SAC	6/12	0.5
8SAC	6/12	0.5
9SAC	6/12	0.5
10SAC	5/12	0.42
12SAC	7/12	0.58
13SAC	5/12	0.42

Conclusion

The examined salads from the campus were found to be contaminated with diverse numbers of known potentially pathogenic bacteria some of which exhibited varying degrees of multi-drug resistance to all antibiotics which were used for this study. Amongst, the numerous isolates, one was found to possess biofilm-forming abilities which could subsequently lead to food-borne illnesses. This shows little practice of kitchen hygiene during salad preparation and as such may present severe health threats to the students consuming such foods. The salad procured is of poor microbiological quality for consumption. To minimize the microbial burden as much as possible, the food handlers should be advised on the importance of excellent hygiene and thorough washing of vegetables.

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