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Original Research Article

Molecular Characterization and Antibiogram of Bacteria Isolated from Irrigated Vegetables in Selected area of Kano Metropolis

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Abstract: Recently, the world's problem has become in the spread of microbes and the extent of their impact on public health, especially the problem of microorganisms causing food spoilage. The study was aimed to characterize and determine antibiogram of bacteria isolated from irrigated vegetables and their sensitivity to commonly used antibiotics was carried out in Kano metropolis which are; Fagge (Kwakwaci wastewater irrigation area), Kumbotso (Shagari quarters wastewater irrigation area) and Ungoggo (Jaba wastewater irrigation area)". In the study, the most common bacteria isolated and identified were *Staphylococcus aureus* (30.43%), *Escherichia coli* (27.54%) followed by *Salmonella* (24.64%) and *Shigella* (17.39%). The results of molecular indicate the genus and species of each isolate. The gel electrophoresis confirmed the presence of PCR products of the expected size. Sequencing and subsequent bioinformatics analysis further validated the results. In Gram-negative bacteria, Septrin, Sparfloxacin, ciprofloxacin, amoxicillin augmentin, gentamycin, perfloxacin streptomycin were sensitive. At the same time, most of the remaining antibiotics (chloramphenicol, tarivid, levofloxacin and nitrofuraticin) are 100% resistant to bacterial isolates. However, in Gram-positive bacteria, ciprofloxacin, norfloxacin, rifampicin, erythromycin, chloramphenicol, levofloxacin, azithromycin and imipenem were sensitive while the remaining antibiotics for Gram-positive bacteria (gentamycin, amoxicillin, streptomycin, ampiclox) were resistance to bacterial isolates.

Keywords: Antibiogram, Bacteria, Irrigation, Kano, Molecular Characterization.

INTRODUCTION

Presently, antimicrobial resistant among microorganism such as bacteria, virus, parasites and other disease-causing organisms is one of the serious threats to the management of infectious disease globally (Umaru *et al*., 2018). The increase in antibiotic resistance has been attributed to a combination of microbial characteristics, the selective pressure of antibiotic use and social and technical changes that enhance the transmission of resistant organisms. The growing threat from resistant organisms calls for concerted action to prevent the emergence of new resistant strains and the spread of existing ones (Thiel and Gutow, 2005). Many procedures use and misuse of antibiotics in man have resulted in antibiotic-resistant bacteria. Antimicrobial resistance is a natural biological phenomenon which often enhanced as a consequence

of infectious agents' adaptation to exposure to antimicrobial agents used in human or Agriculture and the widespread use of disinfectant at the farm and the household levels (Tsou and Mori, 2002). It is now accepted that antimicrobial use is the single most important factor responsible for increased antimicrobial resistance (Cannon *et al*., 2004)

Conventional methods for detecting foodborne bacterial pathogens present in food as based on culturing coliforms on agar plates in lactose fermenting media followed by biochemical characterization, study of cell morphology and colony morphology (Kumar *et al.,* 2013; Law *et al.,* 2015). Conventional methods are usually inexpensive and simple but these methods can be time consuming as they depend on the ability of the microorganisms to grow in different culture media such as pre-enrichment media, selective enrichment media and selective plating media.

Usually conventional methods require 2 to 3 days for preliminary identification and more than a week for confirmation of the species of the pathogens (Zhao *et al.,* 2014). Conventional methods are laborious as they require the preparation of culture media, inoculation of plates and colony counting (Mandal *et al.,* 2011). Furthermore, conventional methods may be limited by their low sensitivity (Lee *et al.,* 2014). False negative results may occur due to viable but non-culturable (VBNC) pathogens and the failure to detect foodborne pathogens rapidly would increase the transmission risk of the pathogens (Law *et al.,* 2015). However, due to the limited specificity and time consuming nature of these techniques, DNA based molecular techniques such as 16S rRNA gene sequencing is highly specific. Molecular methods are necessary for the identification of environmental bacterial isolates and species with an incomplete biochemical/phenotypic description. Isolates well identified at the species level by conventional phenotypic methods served as a control for both conventional and molecular identification procedures (Alvarez-Ordonez *et al.,* 2018).

MATERIALS AND METHODS

Study Area

Samples were collected from three different Local Governments of Kano metropolis which are; Fagge (Kwakwaci wastewater irrigation area), Kumbotso (Shagari quarters wastewater irrigation area) and Ungoggo (Jaba wastewater irrigation area).

Sample Collection

Twenty Seven (27) samples were collected randomly directly from each sampling site a in polythene bags. The samples were immediately taken to the laboratory in an iced box for further analysis (Baker and Silverton, 2007).

Identification of Bacteria Isolates

The bacterial isolates were characterized on the basis of their colonial morphology, cellular morphology through Gram staining and biochemical characteristics such as catalase, coagulase, citrate utilization, methyl red, VP, indole, and triple sugar iron tests as demonstrated by (Cheesbrough, 2010).

Antibiotics Sensitivity Test

The method of Ali *et al.,* (2024) was used. Mueller Hinton agar was prepared according to the manufacturer's instructions. The inoculum of each test organism was prepared by picking discrete colonies of organisms in the nutrient agar and transferred into sterile distilled water in a bijou bottle to obtain a turbidity equivalent to 0.5 McFarland turbidity standards which gives an approximate density of the test organism (Cheesbrough, 2010). The suspension was seeded unto the surface of Mueller Hinton agar plates in duplicates with a sterile wire loop. These of different antibiotics (Amoxicillin, Ampicillin, Ampiclox, Ciproxin and Ciprofloxacin) were placed at the surface on the media by means of sterile forceps at 22mm apart. The plates were incubated at 37^0C for 24hrs after overnight the plates were examined for zone of inhibition and the value was recorded in mm.

Molecular Identification

DNA Extraction

DNA was extracted using SDS-CTAB method as described by Sambrook *et al.,* (1989). The DNA extraction buffer was comprise of: 100mM sodium EDTA (pH 8.0), 100mM Tris-HCL (pH 8.0), 1% CTAB (Cetylmethyl Ammonium Bromide), 100mM sodium phosphate (pH 8.0), 1.5M NaCl. About 13.5mL DNA extraction buffer and 50µL of proteinase K (20mg/mL) was mixed with approximately 1.ml of each bacterial isolate into a 50mL centrifuge tube. The mixture was incubated at 37^0C for 30mins with horizontal shaking at 200 rpm in shaking incubator. Then, 1.5mL of 20% SDS was added into the mixture, and cell lysis was carried out with incubation at 65^0C for 2 h in a water bath. The samples was then be centrifuged at 6000g for 10mins at room temperature, and the supernatant was collected into a new centrifuge tube. The remaining cell pellet was subjected into two additional rounds of cell lysis by adding 4.5mL of DNA extraction buffer, and 0.5mL of 20% SDS, which was vortexes for 10sec and incubated at 65^0C for 30mins, with horizontal shaking at 200 rpm. The supernatants obtained from the three cycles of extractions was pooled together, and mixed with an equal volume of phenol: chloroform: isoamyl alcohol in a ratio of 25:24:1,

1phase. The mixture was mixed gently by end-over-end inversions of the microcentrifuge tube, and centrifuge at 6000g for 10mins at room temperature. The aqueous phase was collected using a pipette and then transferred into a new 1.5mL microcentrifuge tube (750µL), and mixed with 0.6 volumes (450µL) of isopropanol. DNA precipitation was carried out at room temperature for 24 h. The samples was then be centrifuged at 16000g for 30mins at room temperature to obtain DNA pellets. The DNA pellets were washed with cold 70% ethanol by centrifuging at 16000g for 30mins at room temperature. Lastly, the pellets was allowed to dry, and re-suspended in 100µL of sterile ultrapure water, and the DNA was stored at a temperature of -20^{0}C.

Agarose Gel Electrophoresis

The presence of DNA in the extracts of the isolate was confirmed using 1% agarose gels. For the preparation of 1% agarose gel, 0.5g of SeaKem® LE Agarose powder (LONZA, USA) was added to 50mL of 1x TAE buffer (40mM tris-acetate and 1mM ethylenediaminetetraacetic acid, EDTA) (Bro-Rad, USA). The agarose solution was heated in a microwave using medium power, until the solution becomes clear and the agarose powder dissolved completely. RedSafe™ Nucleic Acid staining solutions (2.5µL) (20000x) (iNtRON, Korea) was added into warm agarose solution before gel casting. After the agarose gel had solidified, appropriate amounts of sample and DNA ladder (1 kb) was loaded into the wells of the agarose gel. Electrophoresis was carried out using the Mupid-ex U system (TaKaRa, Japan) filled with 0.5 x TAE buffer at 100V for 35mins. Finally, the gel was visualized, and the photograph was taken using the Gel Doc™ EZ system (Bio-Rad, USA).

DNA Quantification

DNA concentration and purity was measured using NanoDrop® ND-1000 (Thermo Fisher Scientific, USA) or Biospectrometer® (Eppendorf, Germany). About 1.5 – 2.0µl of DNA sample was needed for each measurement. Deionized water was used as blank during the measurements.

Polymerase Chain Reaction

The presence of bacterial DNA was confirmed by amplifying 16S rRNA gene sequences with bacteria specific primers. For PCR, 10ng of extracted DNA was used in 20 µL reactions consisting of 1 µL of each forwarded and reverse primer, 2µL of $MgCl_2$ buffer, 2 µL dNTP mix, and 0.5 units Ex *Taq* DNA polymerase. PCR was carried out in a Bio-Rad Thermal Cycler with parameters set as 94^{0}C for 1 min, 55^{0}C for 1 min, 72^{0}C for 4 mins (35cycles), followed by 10mins extension at 72^{0}C. PCR products were confirmed using 1% agarose gel electrophoresis. The PCR products was separated by electrophoresis on a 1% agarose gel, and stained with RedSafe™ Nucleic Acid Staining Solution. The presence of bacteria was confirmed by amplifying 23S rRNA gene sequencing using universal plastid primers (Prestige, 2006). The PCR mixture amplification was carried out at 94^{0}C for 1 min, 50^{0}C for 1 min, 72^{0}C for 4 mins (35cycles), followed by extension of 72^{0}C for 10 min. The PCR was separated electrophoretically with 2% agarose gel. The PCR product sequences were used in MegaBlast search of the NCBI database to get maximum species similarity.

DNA Sequencing

PCR products were sent for sequencing to a service provider. Sanger DNA sequencing was carried out by MyTACG Bioscience Enterprise (Malaysia) using an ABI DNA Analyzer (Life Technologies, USA)

Phylogenetic Analysis

The phylogenetic analysis was conducted using sets of sequences data (16S rRNA and 16S-23S ITS) separately for each of the selected strains. The first set included all 16S rRNA gene sequences of the selected strains which were deposited in GenBank. Homologous sequences was identified using a MegaBlast search of the NCBI database in which only closely related sequences was selected to build the phylogenetic tree. The sequences were aligned using the CLUSTAL W program in Mega 6 version 6 (Tamura *et al.,* 2013). The second set included the 16S-23S ITS sequences of all the isolates, which were identified through MegaBlast search and compared directly without tree-building analysis. The presence and absence of tRNAs sequence lengths, and spacers was identified using a tRNA scan- SE Search Server (Lowe and Chan, 2016).

RESULTS

Occurrence of Bacteria Isolates from the Vegetables Samples

The occurrence of bacteria isolated from vegetables samples is presented in table 1. The results showed that 4 different isolates were identified as follows; *Shigella, Salmonella, Escherichia coli* and *Staphylococcus aureus.* Among the bacterial isolated, *Staphylococcus aureus* had the highest percentage of occurrence of 30.43% while Shigella had the least percentage of occurrences of 17.39%.

Table 1: Occurrence of bacteria Isolates from the Vegetables samples

Bacteria Isolates	No of occurrence (n)	Percentage occurrence (%)
Escherichia coli	21	30.43
Staphylococcus aureus	19	27.54
Salmonella	17	24.64
Shigella	12	17.39
Total	**69**	**100**

Susceptibility Profile of Gram Negative Isolates

The zone of inhibition produced by the Gram negative isolates against the commonly used antibiotics is presented in Table 2 while the interpretation of the susceptibility (sensitive or resistant) profile of the isolates is presented in Table 3 below. The result showed that *Shigella* is susceptible to Gentamycin, Perfloxacin, Levofloxacin, Streptomycin, Septrin, Ciprofloxacin, Augmentin, Amoxicillin and Sparfloxacin. therefore; *Salmonella* Gentamycin, Perfloxacin, Tarivid, Streptomycin, Septrin, Ciprofloxacin, Augmentin, Chloramphenicol and Sparfloxacin while *Escherichia Coli* is sensitive in Gentamycin, Perfloxacin, Streptomycin, Ciprofloxacin, Augmentin, Amoxicillin, Chloramphenicol, and Sparfloxacin.

Table 2: The zone of Inhibition of antibiotic against *Shigella, Salmonella* and *E. coli*

Isolates/Zone of inhibition (mm)			
Antibiotics (µg)	***Shigella***	***Salmonella***	***E. Coli***
Septrin (30)	19	22	14
Chloramphenicol (30)	15	21	17
Sparfloxacin (10)	21	18	24
Ciprofloxacin (10)	21	18	23
Amoxicillin (10)	18	11	16
Augmentin (25)	23	19	20
Gentamycin (10)	22	20	16
Perfloxacin (10)	20	17	20
Tarivid (30)	12	18	13
Streptomycin (10)	23	20	18
Levofloxacin (10)	19	15	15
Nitrofuraticin (10)	14	13	15

Key: mm= millimeter.

Table 3: Antibiotics susceptibility profile of the isolates

Antibiotics (µg)	***Shigella***	***Salmonella***	***E. Coli***
Septrin (30)	S	S	S
Chloramphenicol (30)	R	R	R
Sparfloxacin (10)	S	S	S
Ciprofloxacin (10)	S	S	S
Amoxicillin (10)	S	S	S
Augmentin (25)	S	S	S
Gentamycin (10)	S	S	S
Perfloxacin (10)	S	S	S
Tarivid (30)	R	R	R
Streptomycin (10)	S	S	S
Levofloxacin (10)	R	R	R
Nitrofuraticin (10)	R	R	R

Key: *E. coli* = *Escherichia coli,* S= sensitive and R= resistance, Note: Antibiotic susceptibility according to CLSI (2010)

Susceptibility Profile of *Staphylococcus Aureus*

The zone of inhibition produced by *Staphylococcus aureus* from different antibiotics used is presented in Table 4 while the interpretation of the susceptibility (sensitive or resistant) profile of the isolates is presented in Table 5. The results showed that *S. aureus* is sensitive in Ciprofloxacin, Norfloxacin, Rifampicin, Erythromycin, Chloramphenicol, Levofloxacin, Azithromycin and Imipenem.

Table 4: The mean zone of Inhibition in millimeter of antibiotic against *S. aureus*

Antibiotics	Concentration (µg)	Zone of inhibition (mm)
Ciprofloxacin	10	19
Norfloxacin	10	20
Gentamycin	20	14
Amoxicillin	10	10
Streptomycin	30	13
Rifampicin	30	17
Erythromycin	10	20
Chloramphenicol	30	18
Ampiclox	10	12
Levofloxacin	10	18
Azithromycin	10	20
Imipenem	10	16

Key: mm= millimeter.

Table 5: Antibiotics susceptibility profile of *Staphylococcus aureus*

Antibiotics	Concentration (µg)	Susceptibility Profile
Ciprofloxacin	10	S
Norfloxacin	10	S
Gentamycin	20	R
Amoxicillin	10	R
Streptomycin	30	R
Rifampicin	30	S
Erythromycin	10	S
Chloramphenicol	30	S
Ampiclox	10	R
Levofloxacin	10	S
Azithromycin	10	S
Imipenem	10	S

Key: S= sensitive and R= resistance

16S RNA Sequencing Results of the Isolates

The 16S rRNA sequencing has successfully identified the bacterial isolates. The results indicate the genus and species of each isolate. The gel electrophoresis confirmed the presence of PCR products of the expected size as shown in Table 6.

Table 6: Summary of 16S RNA sequencing results of the isolates to identify the closet homologs

Sample ID	Sequence bp	Identity of the closet homologs of the sequence	% identity	Accession Nº of the closet homolog
S1	265	*Staphylococcus strain Ng016*	97.87%	MH517387.1
S2	662	*Escherichia coli isolate C3*	98.78%	LK985368.1
S3	815	*Shigella flexneri strain IKA3*	97.67%	MW448733.1
S4	796	*Salmonella enteric subsp. Enteric serovar Hadar strain cvm*	98.73%	CP158230.1

Key: S = Sample

The gel electrophoresis results for the PCR products obtained from the 16 and 18S rRNA gene amplification is presented in Figure 1. The lanes 1, 6, 7 and 8 (Fig 1 by left) represent the amplified product of the bacterial isolate (1-8). Lane 1 in figure 1 represents the ladder. The presence of DNA band in the lanes confirms the success of the PCR amplification.

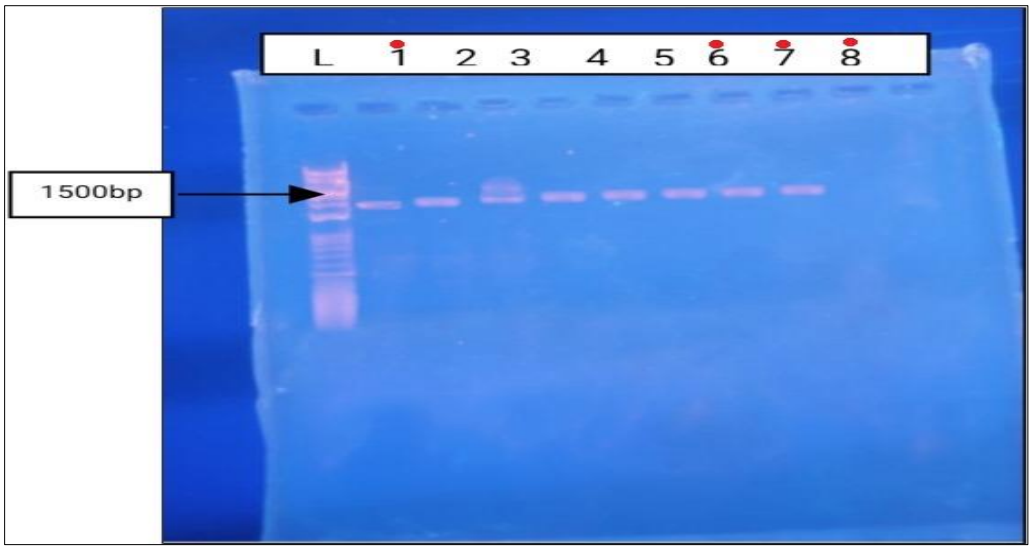

Figure 1: Gel image showing the PCR products from the 16 and 18S rRNA gene amplification

DISCUSSION

According to the study, bacteria associated with vegetables samples include *Shigella, Salmonella, Escherichia coli* and *Staphylococcus aureus.* The prevalence of *S. aureus* in the current study in all vegetables was lower than what is mentioned in the study of Halablab *et al*., (2011) who reported higher prevalence of *S. aureus* (51.5%) from Lebanon. The 16S rRNA sequencing has successfully identified the bacterial isolates. The results indicate the genus and species of each isolate as identified phenotypically. The gel electrophoresis confirmed the presence of PCR products of the expected size. The antibiotic resistance patterns of *S. aureus* isolates in the current study showed low percentage of resistance to erythromycin, azithromycin, norfloxacin (20mm), ciprofloxacin (19), levofloxacin (18mm), rifampicin (17mm) and imipenem (16mm). This is partly similar to previous report by Donokor *et al*., (2008) from Ghana. In the current study, all *S. aureus* isolates were resistant to penicillin G, ampicillin, and cefuroxime sodium (100.0% each). This finding is partly in agreement with Sina *et al*., (2011).

In the present study, high number of *Salmonella* spp. was susceptible to *Shigella* is susceptible to gentamycin, perfloxacin, levofloxacin, streptomycin, septrin, ciprofloxacin, augmentin, amoxicillin and sparfloxacin. therefore; *Salmonella* gentamycin, perfloxacin, tarivid, streptomycin, septrin, ciprofloxacin, augmentin, chloramphenicol and sparfloxacin while *Escherichia Coli* is sensitive in gentamycin, perfloxacin, streptomycin, ciprofloxacin, augmentin, amoxicillin, chloramphenicol, and sparfloxacin. This result is fairly in line with Akbarmehr (2012) who reported that *Salmonella* spp. were highly susceptible to chloramphenicol (100%) followed by gentamycin (91.89%). However, isolates of *Salmonella* spp. exhibited resistance to tetracycline, erythromycin, penicillin G and cefuroxime sodium (100% each) followed by ampicillin (90.3%). Cardoso *et al*., (2006) have also reported 100% resistance of *Salmonella enteritidis* to both tetracycline and erythromycin from Brazil. The marked resistance of strains of *Salmonella* spp. to ampicillin as shown in the present study agrees with the findings of Ash *et al*., (2002) and Ikpeme *et al*., (2011), working on rivers in the United States and Nigeria, respectively.

CONCLUSION

Finding of the present study showed that 4 different isolates were identified as follows; *Shigella, Salmonella, Escherichia coli* and *Staphylococcus aureus.* Among the bacterial isolated, *Staphylococcus aureus* had the highest percentage of occurrence of followed by *E. coli* while *Shigella* had the least percentage of occurrences. The 16S rRNA sequencing has successfully identified the bacterial isolates. The results indicate the genus and species of each isolate. The result showed that *Shigella* is susceptible to gentamycin, perfloxacin, levofloxacin, streptomycin, septrin, ciprofloxacin, augmentin, amoxicillin and sparfloxacin. On the other hand, *Salmonella* is sensitive to gentamycin, perfloxacin, tarivid, streptomycin, septrin, ciprofloxacin, augmentin, chloramphenicol and sparfloxacin while *Escherichia Coli* is sensitive in gentamycin, perfloxacin, streptomycin, ciprofloxacin, augmentin, amoxicillin, chloramphenicol, and sparfloxacin.

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