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Antibiotic Resistant and Plasmid Borne Bacteria Associated with Locally Produced Honey in Enugu State, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study is to isolate and identify bacteria contaminants present in the honey samples sold in Ogbete market and detect the presence of imipenem resistance genes in the isolates.
Place and Duration of Study: A total of four samples were collected from Ogbete market at Zenith bank, Peace mass, Wheelbarrow and Peace park stations between the early hours of the morning (8-9am) within the month of April, 2023.

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Methodology: Microbiological analysis was carried out on the samples, including antimicrobial susceptibility test using disc diffusion method., after which Molecular analysis involving DNA extraction, PCR and Plasmid Profiling, were carried out.
Results: The total mean heterotrophic bacterial count ranged from $0.03\pm2.5\times10^3$cfu/ml to $22.1\pm25.5\times10^3$cfu/ml. The highest total mean heterotrophic bacterial count was recorded in Zenith bank while the lowest was in Peace mass. The bacteria isolated and characterized through morphological and biochemical tests include *Escherichia coli* (45%) *Klebsiella spp.* (41%), *Staphylococcus spp.* (9%) and *Streptococcus spp.* (5%). *Escherichia coli* had the highest frequency of occurrence at 45% while *Streptococcus spp*. had the lowest at 5%. The sensitivity test showed that the isolates with the highest frequency of occurrence, *Escherichia coli* and *Klebsiella spp*. posed a high resistance to Imipenem and Nalidixic at 80% while the least resistance was in Levofloxacin at 10% from these two isolates. The gene of interest, Bla_{IMP} was found after PCR assay. Plasmid DNA was further carried out using the alkaline lysis method using the Zymo research kit. All isolates possessed a single plasmid with molecular weight of 23.1kbp.
Conclusion: These isolates may undergo horizontal gene transfer and confer resistance to other microorganisms. Thus, there is need for the development of proper measures to counter bacterial contamination of locally produced honey as this poses a threat to public health.

Keywords: Honey; bacteria contaminants; imipenem; resistance; Escherichia coli; Klebsiella spp.; horizontal gene transfer.

1. INTRODUCTION

Honey is a natural sweetener made from flower nectars and other chemical secretions that bees collect from flowers. They bring these materials (nectar and other materials) to their hive and transform them into the thick, golden, and sweet liquid we call honey. In addition to the fact that honey is utilized as normal sugar for food varieties, it likewise contains restorative characteristics which can be utilized for dealing with medical problems like hack, therapy of wounds, and a few other bacterial circumstances and diseases [1]. As earlier stated, honey has been an ancient treatment remedy for infected wounds, etc. It has been greatly implemented into the medical sector and its use in this section increases day by day because of its numerous importance [2].

Soffer, [3], stated that “honey, however, despite its exclusive health qualities and benefits as well as its great components and aftermath results, it still serves as reservoir for certain microbes. Although, the presence of these microbes do not diminish the good qualities of which honey entails”. It possesses microbes such as bacteria and yeast. These microbes found in honey may have come from different sources and means such as the flower nectar, from which the bees feed on their juices, pollen from the bees bodies, digestive secretions from the honeybees, also from external sources which include human purification process for the honey product, packaging of the honey, etc [4].
Since bacteria are everywhere, honey may contain them. Bacterial contaminants and other microorganisms tend thrive in the setting where the honey is sold. That might result in food poisoning. Due to the presence of microbes (especially bacteria) that are able to survive and adapt to the antimicrobial conditions of honey, the honey may or may not be suitable for human consumption and may result in health issues for the consumer.

Antibiotics are organic compounds of biological origin gotten from certain bacteria to counter the effect of other bacterial strains. They can either be bacteriostatic or bactericidal. They were devised to work against corresponding bacteria. However, due to certain factors, most bacteria have showcased resistant mechanisms to these antibiotics, hence; the effectiveness of the antibiotics on the bacteria is reduced greatly.

As was mentioned earlier, microorganisms can use honey as a reservoir or habitat because they are everywhere and can withstand its antimicrobial properties. According to observations, the honey that is sold at Ogbete is typically kept in a basin, while other samples are already kept in unsanitized bottles (e.g. eva bottles), which may not have been properly cleaned or sanitized. Also, it could be that these honey sold at Ogbete might be adulterated. As a result, the honey becomes more susceptible to bacterial contamination as a result of this

exposure. The honey that is sold at Ogbete (or main) market—which is the most common and largest market in Enugu metropolis—is the sole focus of this study because honey there is purchased at higher prices. As the most recent antibiotics developed for the treatment of bacterial infections, particularly abdominal infections, antibiotics in the class of carbapenems known as "Imipenem" are utilized due to their broad spectrum of activity. Despite the unique qualities and properties of these antibiotics, the majority of bacterial strains have developed resistance to them because they have become accustomed to them. As a result, the antibiotic resistance genes of these bacterial contaminants to carbapenems, specifically Imipenem, are the focus of this study, which expands on secondary bacterial contamination of honey from the external environment, which includes contaminants from the air, products/cans used for storing the honey during the purification process, and even humans.

As a result, this study tends to identify and list the prevalent bacteria contaminants in the honey, as well as their concentration per gram (CFU) and resistance pattern, if any.

2. METHODS

2.1 Study Area

The study was carried out in Ogbete Market, along market road, Enugu East LGA with a geographic latitude 7487'23'' E and longitude 6438'66'' N.

2.2 Sample Collection

A total of four (4) samples of honey were collected from different vendors at four different locations which include; Zenith bank, Peace Mass, Wheelbarrow and Peace park stations; one from each at ogbete market during the early hours of the morning (between 8am-9am) and were taken to the laboratory for microbiological assays and further molecular assay

2.3 Microbiological Assays

2.3.1 Preparation of media

All the media to be used (MacConkey agar, Nutrient agar and Eosin Methylene Blue agar) were prepared according to the manufacturer's standards and specifications and autoclaved to achieve sterility.

2.3.2 Serial dilution

Peptone water was used to carry out dilution instead of distilled water because peptone water contains nutrients that can enable the microorganisms in the sample to survive. Two gram of peptone water was measured and diluted in 100ml of water. Ten-fold serial dilution was carried out, that is, 9ml of the diluted peptone water was pipetted into 5 test tubes for each of the samples, that is, 5 test tubes to 1 sample. The test tubes were then autoclaved to achieve sterility. After autoclaving at 121ºC for 30 minutes and cooled, one ml of honey was added to the first test tube to give a 10^{-1} dilution. Using another sterile syringe, one ml was taken from that test tube and transferred into the next tube, giving a 10^{-2} dilution factor and this procedure continued till the last tube (10^{-5}) [5,6].

2.3.3 Culture preparation

After serial dilution of the honey samples, using pour plate technique, 1ml of the diluted samples were put in the petri dish and at the same time, the prepared medium (Nutrient agar, MacConkey agar and Eosin Methylene Blue agar) were put in each petri dish, one after the other. The plates were immediately covered to avoid contamination and swirled for the contents to mix well and thereafter, allowed to gel and put in the incubator for 24-48 hours to observe bacterial growth.

2.3.4 Determination of colony forming units (bacteria load)

After microbial growth on the incubated plates were observed, the colonies formed were counted using plate count method, recorded and later calculated to get the CFU/ml (total colony count).

2.3.5 Preparation of McFarland's standard

To prepare McFarland's standard for the standardization of test organisms; One gram pf Barium chloride (Bacl2) was weighed out and dissolved in 99ml of water. One ml of Conc. H2SO4 was measured and dissolved in 99ml of water. 0.05ml of the 1% Bacl2 was poured in a bijoux bottle and 9.95ml of dilute 1% H2SO4 was added into the same bottle. The mixture was kept in a dark place for 24-48 hours before use.

2.3.6 Standardization of test organisms

The test organisms to be used for antimicrobial susceptibility test were standardized using

McFarland's standard prepared as a control. This was done by 1ml of normal saline was measured into the ten test tubes and autoclaved. After autoclaving and cooling, using a sterile wire loop, a small quantity of each isolate was inoculated into each test tube, using the McFarland's standard as a control, till the test organisms were standardized with McFarland's solution.

2.3.7 Antimicrobial susceptibility test

Nutrient agar was prepared and poured into sterile petridish. A sterile wireloop was used to collect samples and put on the plates. After isolation of bacteria, antimicrobial susceptibility test was carried out in order to determine which isolate(s) were susceptible to imipenem or not. This was done by carefully inserting 10 antimicrobial discs into 10 pure culture plates (that had been prepared with nutrient agar) and put back into the incubator for 24 hours to check for susceptibility and zones of inhibition. After twenty four hours, the plates were taken out and the zones of inhibition were measured with a ruler.

2.3.8 Stock culture preparation

Stock cultures (agar slants) were prepared in order to store the isolates that were cultured for future use. 1.4g of nutrient agar was measured and diluted in 50ml of water. The mixture was stirred well and heated a bit with low heat. 5ml of the media was put into 10 bijou bottles and sterilized in the autoclave for 45 minutes. After autoclaving, the bottles were slanted and allowed to gel before the isolates were inoculated. The stock was carefully wrapped in a foil paper and stored in the fridge for future use.

2.4 Molecular Analysis

2.4.1 DNA extraction, Polymerase Chain Reaction (PCR) and plasmid profiling

The isolates were further taken to the biotechnology lab to determine the genes for antimicrobial resistance.

2.4.2 DNA extraction

DNA was extracted using Zymo research kit by following the extraction protocol which are stated as follows; 50-100 mg (wet weight) of bacteria cell that has been re-suspended in up to 20µl of water or isotonic buffer (PSB), was added to a ZR (Zymo Research) bashing bead lysis tube (0.1 mm and 0.5 mm) with 750 µl bashing bead buffer. Bead buffer fitted with a 2ml tube holder assembly was used and processed at maximum speed for ≥ 5minutes. The bashing bead tubes were centrifuged at ten thousand xg for 1minute. After centrifuging, 400µl of supernatant were transferred to a Zymo spin III-filter collection tube and centrifuged for 1 minute at 800xg. 1200µl of genomic lysis buffer was added to the filtrate collection tube after centrifuging and 800µl of mixture from the filtered collection tube was added to a zymo spin IIC column collection tube and centrifuged at10000xg for 1 minute. The flow-through were discarded and the last step was repeated once again. 20µl of DNA pre-wash buffer was added to a new zymo spin IIC column collection tube and centrifuged at ten thousand xg for 1 minute. 500µl of gDNA buffer was added and centrifuged at 10,000xg for 1 minute. The zymo spin IIC column was then transferred to a clean 1.5ml micro centrifuge tube with 100µl DNA elusion buffer containing the matrix and centrifuged at 10,000xg for 30seconds to elute DNA.

2.4.3 1% Agarose gel electrophoresis

This was carried out to confirm if DNA extraction was successful. A 1% agarose gel was prepared and allowed to gel.

2.4.4 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction was used to detect antibiotic resistant genes in the *Escherichia coli* and *Klebsiella* spp*.* using specific primers. This technique was performed in 45µl of reaction buffer containing 0.05 unit/ml of Taq polymerase (as recommended by the manufacturer's manual) and 5µl of DNA template. The protocol for the thermal cycler (BIORAD) was as follows; bla_{IMP}genes- initial denaturation at 94°C for 5minutes followed by 30 cycles of denaturation at 96°C for 30 seconds. Annealing occurring at 55°C for 30 seconds and elongation at 72°C for 1 minute and a final extension at 72°C for 5 minutes.

2.4.5 2% Agarose gel electrophoresis

7µl of PCR Products (that is, 5µl of PCR product and 2µl of loading dye were analyzed by gel electrophoresis in a 2% agarose.

2.4.6 Plasmid profiling

Plasmid profiling was done to confirm the results after PCR with the following protocol; 600µl of bacterial growth of the four samples was added to a 1.5ml centrifuge tube. 100µl of 7X lysis buffer (blue) was added to it and mixed by inverting the tube 4-6 times. After 2 minutes, 350µl of cold Neutralization buffer (yellow) was added and mixed thoroughly. The sample turned yellow when neutralization was complete, with a yellowish precipitate as the result. The samples where then inverted 2-3 times to ensure complete neutralization. The samples were then centrifuged at 11,000- 16,000xg for 2-4 minutes. Approximately 900µl of the supernatant was transferred into the Zymo-spin IIN column carefully. The column was then placed in a collection tube and centrifuged for 15 seconds. The flow-through was discarded and the column was placed back into the collection tube. 200µl of Endo-wash buffer was added to the column. It was then centrifuged for 30 seconds. 400µl of Zyppy wash buffer was added to the column and centrifuged for 1 minute. The column was transferred into a clean 1.5ml microcentrifuge tube. 30µl of Zyppy elution buffer was added directly to the column matrix and let to stand for 1 minute at room temperature. It was then centrifuged for 30 seconds to elute the plasmid DNA.

2.4.7 1% Agarose gel electrophoresis

A 1% gel was run in order to check for plasmid bands from the isolates.

3. RESULTS

The following results were obtained from the experiment carried out;

Table 1. Colony count of isolates from honey

S/N	Isolates	Colony forming unit/ml (CFU/ml) (10^3)	Mean bacterial counts ($\times10^3$ CFU/ml) ± S.D
1	NZ^{-1}	1.82	1.82 ± 1.5
2	MZ^{-1}	1.20	1.20 ± 5.0
3	EZ^{-1}	1.73	1.73 ± 12.5
4	NZ^{-2}	22.1	22.1 ± 25.5
5	NPM^{-1}	0.13	0.13 ± 0.5
6	MPM^{-1}	0.03	0.03 ± 2.5
7	EPM^{-1}	0.13	0.13 ± 12.5
8	NPM^{-2}	1.6	1.6 ± 12.0
9	MPM^{-2}	0.2	0.2 ± 1.5
10	EPM^{-2}	0.1	0.1 ± 1.0
11	NWB^{-1}	1.81	1.81 ± 19.0
12	MWB^{-1}	0.96	0.96 ± 16.0
13	EWB^{-1}	1.86	1.86 ± 20.6
14	NWB^{-2}	1.01	1.01 ± 5.0
15	MWB^{-2}	5.3	5.3 ± 7.5
16	EWB^{-2}	4.3	4.3 ± 5.0
17	NPP^{-1}	1.61	1.61 ± 2.5
18	MPP^{-1}	0.39	0.39 ± 6.5
19	EPP^{-1}	1.66	1.66 ± 6.0
20	NPP^{-2}	1.34	1.34 ± 6.0
21	MPP^{-2}	5.0	5.0 ± 2.0
22	EPP^{-2}	6.5	6.5 ± 2.0

KEYS: N= Nutrient Agar, M= MacConkey Agar, E= EMB, Z= Zenith bank, PM= Peace Mass, WB= Wheelbarrow, PP= Peace Park

Table 2. Morphological and biochemical characterization of isolates from honey samples

Sample Isolates	Colour	Texture/Appearance	Shape	Elevation	Catalase	Oxidase	Lactose Fermentation	Gram Stain	Probable Organism
NZ^{-1}	Golden yellow	Wet/dry	Circular	Flat	**+**ve	-ve	A.	+ve cocci in chains	*Staphylococcus spp.*
MZ^{-1}	Pink	Dry/rough	Irregular	Flat	+ve	**-**ve	A.G	-ve short rods in clusters	*Escherichia coli*
EZ^{-1}	Green metallic sheen	Wet/shiny	Circular	Raised	+ve	**--**ve	A.G	**-**ve short rods in clusters	*Escherichia coli*
NZ^{-2}	Yellow	Wet/smooth	Circular	Flat	**+**ve	**-**ve	A	**+**ve cocci in chains	*Staphylococcus spp.*
NPM^{-1}	Pale yellow	Wet/mucoid	Irregular	Slightly raised	+ve	-ve	A.G	-ve long rods in pairs	*Klebsiella spp.*
MPM^{-1}	Pink	Wet/mucoid	Irregular	Flat	**+**ve	-ve	A.G	-ve long rods in pairs	*Klebsiella spp.*
EPM^{-1}	Purple-black	Wet/mucoid	Circular	Flat	**+**ve	-ve	A.G	-ve long rods in pairs	*Klebsiella spp.*
NPM^{-2}	Creamy white	Wet/mucoid	Circular	Flat	+ve	-ve	A.G	**-**ve long rods in chains	*Klebsiella spp.*
MPM^{-2}	Pink	Wet/mucoid	Irregular	Flat	+ve	-ve	A.G	**-**ve long rods in chains	*Klebsiella spp.*
EPM^{-2}	Purple-black	Wet/mucoid	Circular	Flat	**+**ve	-ve	A.G	**-**ve long rods in chains	*Klebsiella spp.*
NWB^{-1}	White	Dry/smooth	Irregular	Flat	**+**ve	**-**ve	A.G	**-**ve short rods in clusters	*Escherichia coli*
MWB^{-1}	Pink	Dry/smooth	Irregular	Flat	**+**ve	-ve	A.G	-ve short rods in clusters	*Escherichia coli*
EWB^{-1}	Green metallic sheen	Wet/shiny	Circular	Raised	+ve	-ve	A.G	**-**ve short rods in clusters	*Escherichia coli*
NWB^{-2}	White	Wet/mucoid	Irregular	Flat	**+**ve	-ve	A.G	**-**ve long rods in pairs	*Klebsiella spp.*
MWB^{-2}	Pink	Dry/mucoid	Irregular	Flat	+ve	-ve	A.G	**-**ve long rods in pairs	*Klebsiella spp.*
EWB^{-2}	Pink	Wet/mucoid	Circular	Raised	+ve	-ve	A.G	**-**ve long rods in pairs	*Klebsiella spp.*
NPP^{-1}	Creamy white	Wet/smooth	Circular	Flat	**+**ve	-ve	A.G	**-**ve short rods in chains	*Escherichia coli*

Sample Isolates	Colour	Texture/Appearance	Shape	Elevation	Catalase	Oxidase	Lactose Fermentation	Gram Stain	Probable Organism
MPP^{-1}	Pink	Wet/rough	Irregular	Slightly raised	+ve	**-**ve	A.G	**-**ve short rods in chains	*Escherichia coli*
EPP^{-1}	Green metallic sheen	Wet/shiny	Circular	Raised	**+**ve	**-**ve	A.G	-ve short rods in chains	*Escherichia coli*
NPP^{-2}	Creamy white	Wet/smooth	Circular	Flat	**-**ve	**-**ve	-ve	**+**ve cocci in chains	*Streptococcus spp.*
MPP^{-2}	Pink	Wet/rough	Irregular	Slightly raised	+ve	-ve	A.G	-ve short rods in clusters	*Escherichia coli*
EPP^{-2}	Green metallic sheen	Wet/shiny	Circular	Slightly raised	**+**ve	**-**ve	A.G	**-**ve short rods in clusters	*Escherichia coli*

KEYS: A=Acid A.G=Acid and Gas -ve= Negative for either Acid or Gas or both +ve= Positive

One millimeter of all four honey samples collected from different sites at Ogbete (Main market) were inoculated in culture plates containing Nutrient agar, MacConkey agar and Eosin methylene blue agar using pour plate method. These plates were incubated for 24 hours to observe microbial growth. Table 1 shows the colony count of the isolates on each plate. The highest colony forming unit/ml was from sample NZ^{-2} having a colony forming unit of 22.1×10^3 while sample MPM^{-1} with 0.03×10^3 had the lowest colony forming unit/ml.

Table 2 shows the morphological and biochemical characteristics of the colonies from each culture plate ranging from its colour, texture, elevation, biochemical reactions, Gram reaction and probable identity. The organisms identified via these characterizations were *Staphylococcus spp., Escherichia coli, Streptococcus spp.* and *Klebsiella spp.*

Table 3 shows the frequency of isolates that were cultured from each honey sample, ranging from the most reoccurring to the least. The most reoccurring organism is seen to be *Escherichia coli* with a total of 45.4% while the least organism shown to be present is *Streptococcus spp.* with a total of 4.54%. The range for other isolates was; *Staphylococcus spp.* with 9.1% and *Klebsiella* with 40.8%. *Staphylococcus* was only present in Zenith bank samples, *Escherichia coli* was present in Zenith bank, Wheelbarrow and Peace park samples, *Klebsiella spp.* was found in only Peace mass and Wheelbarrow samples and *Streptococcus spp.* was found only in the Peace park samples. From the frequency of occurrence of isolates in Table 3, *Klebsiella spp.* and *Escherichia coli* were the most reoccurring isolates. Thus, five each, of the highest colonies formed/ml of these isolates were selected for antimicrobial susceptibility test.

Ten antimicrobial discs were used to test for the susceptibility and resistance of the bacterial isolates to the antibiotics, including imipenem. Table 4 shows the Multiple Antibiotic Resistance of the selected isolates (*Escherichia coli* and *Klebsiella spp.*) to various antibiotics. Numbers 1-5 are for *Escherichia coli* isolates whereas 6-10 are *Klebsiella spp.* isolates. The highest resistance occurred in EPP^{-1} (*Escherichia coli*) and NPM^{-1} (*Klebsiella spp.*) both with a resistance of 0.58 and the lowest resistance rate in NPP^{-1} (*Escherichia coli*) with a resistance rate of 0.083.

Table 3. Frequency of occurrence of Isolates in honey samples

Isolates	Zenith Bank	Peace Mass	Wheelbarrow	Peace Park	Total
Staphylococcus spp.	2(9.1%)	0(0%)	0(0%)	0(0%)	2
Escherichia coli	2(9.1%)	0(0%)	3(13.6%)	5(22.7%)	10
Klebsiellaspp.	0(0%)	6(27.2%)	3(13.6%)	0(0%)	9
Streptococcus spp.	0(0%)	0(0%)	0(0%)	1(4.54%)	1
Sum total of Isolates					22

Table 4. Multiple Antibiotic Resistance (MAR) Index of the Isolates

S/N	Isolates		MAR Index
1.	NPP^{-1}	1/12	0.083
2.	MPP^{-2}	3/12	0.25
3.	EPP^{-2}	2/12	0.16
4.	EPP^{-1}	7/12	0.58
5.	EWB^{-1}	4/12	0.33
6.	NPM^{-1}	7/12	0.58
7.	EPM^{-1}	6/12	0.5
8.	NPM^{-2}	5/12	0.41
9.	MWB^{-2}	6/12	0.5
10.	EWB^{-2}	5/12	0.41

Table 5. Antibiogram of the isolates

Antibiotics	µg	Susceptible (%)	Resistant (%)	Intermediate (%)
AUG	30	1(10)	7(70)	2(20)
CTX	25	2(20)	7(70)	1(10)
IMP	10/10	0(0)	8(80)	2(20)
OFX	5	6(60)	2(20)	2(20)
GN	10	4(40)	4(40)	2(20)
NA	30	0(0)	8(80)	2(20)
NF	300	5(50)	2(20)	3(30)
CXM	30	0(0)	7(70)	3(30)
CRO	45	5(50)	4(40)	1(10)
ACX	10	1(10)	7(70)	2(20)
ZEM	5l	2(20)	2(20)	6(60)
LBC	5	6(60)	1(10)	3(30)

KEYS: AUG= Amoxicilin Clavulanate CTX= Cefotaxime IMP= Imipenem OFX= Ofloxacin GN= Gentamicin NA= Nalidixic NF= Nitrofurantoin CXM= Cefuroxime CRO= Ceftriaxone Sulbactam ACX= Ampiclox ZEM= Cefexime LBC= Levofloxacin

A record of susceptible, resistant and intermediate strains to various antibiotics, including imipenem. Table 5 shows the interpretation of the zones of inhibition at which the isolates were either susceptible, resistance or nearly susceptible (intermediate) in response to the antibiotics. The highest susceptibility was levofloxacin and ofloxacin at 60% while the lowest was imipenem and cefuroxime at 0%. The highest resistance was imipenem and nalidixic at 80% while the lowest was levofloxacin at 10%. The highest intermediacy was cefexime at 60% while the lowest was cefoxatime and ceftriaxone at 10%.

(OBSERVATION - WHICH ISOLATES WERE SUSCEPTIBLE OR RESISTANCE)

3.1 DNA Extraction

Four of the selected isolates which were resistant to Imipenem (EPP^{-1}, NPM^{-1}, EWB^{-1} and MWB^{-2}), two each for *Escherichia coli* and *Klebsiellaspp.* at a high rate were used for DNA extraction and PCR assay in order to evaluate the resistant genes in the isolates to imipenem. Gel electrophoresis was first carried out to ascertain if DNA extraction was carried out accurately.

3.2 PCR Assay

The DNA extracted which were stored at for PCR assay to check for the amplification of the resistant genes. Polymerase Chain Reaction was carried out using the *BLa$_{IMP}$*gene specific primer in order to target this gene.

3.3 Plasmid Profiling

Since the resistant genes were not seen genotypically, it was suspected that the imipenem resistant gene was plasmid borne. Thus, plasmid profiling was carried out to determine the presence of plasmid in the isolates.

4. DISCUSSION

Honey is known for its delicious and therapeutic ingredient since the ancient times, containing fructose, glucose, proteins, amino acids, organic acids, enzyme values, phenolic acids, flavonoids, antioxidants, and high osmotic pressure [7]. The present study studied commercially sold honey samples from various central market regions, determining the antimicrobial susceptibility of the isolates to imipenem and detecting the resistant gene, *Bla$_{IMP}$* for imipenem, using PCR-assay. Honey is not sterile and is expected to contain a limited number of microorganisms [8]. Numerous researches have reported isolated microorganisms from honey samples produced in different topographical locations ranging from 0 - 1000 colony forming units (CFUs) per gram [9,10]. The present study revealed low bacterial load in honey samples commercially sold in various market regions of Enugu, however showed the highest colony forming unit/ml of 22.1×10^3 in just a sample. Comparable results were reported in previous studies conducted by Pajor et al*.,* [11] in Poland, Fernández et al*.*, [12], in Argentina and Sinacori et al*.,* [9] from different cities of Southern Italy. Honey is a rich source of beneficial bacteria that might be potential bio-control agents against bee pathogens [13],

however, pathogenic bacteria that develop colonies is an indication of hygienic safety of honey.

Although the intrinsic properties of honey affects the development and survival of several microorganisms in honey, yet some microbial species mostly bacteria have been isolated from different honey types and samples. Microbiological analysis revealed the presence of four (4) species of bacteria from the honey samples, including Gram-positive and Gram-positive bacteria in the present study. The highest occurring bacteria was observed in 10 samples representing 45.4.5% were identified as *Escherichia coli*, followed by *Klebsiella* spp. (40.8%) in 9 samples, while the least bacteria were *Staphylococcus* spp. (9.1%) in 2 samples and *Streptococcus* spp. with a total of 4.54% in just a sample. This is in line with Sarbojoy S et al*.,* [14] that identified *Escherichia coli* (8*%), Klebsiella pneumonia* (8%), *Micrococcus luteus* (75%), *Streptococci* spp., *Staphylococci* spp*.,* and lactobacilli from honey contaminants in Bangladesh. This could be that these bacteria thrive as contaminants from adulteration of the

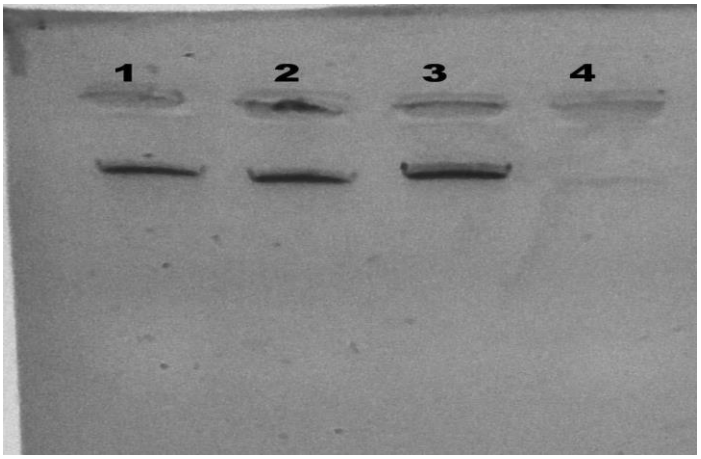

Fig. 1. Agarose Gel showing visible bands from extracted DNA for Pre-Gel electrophoresis
Wells 1, 2 and 3 showed thick bands for DNA while well 4 has a thin band. Well 1 and 2 is for the *Escherichia coli* isolates whereas Well 3 and 4 is for *Klebsiella* spp.

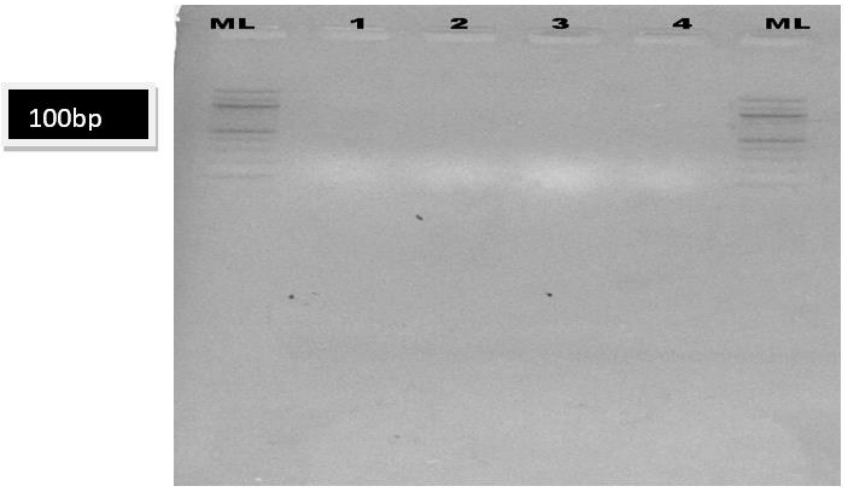

Fig. 2. Agarose Gel image showing the absence of the amplification of *Bla$_{IMP}$* gene in selected isolates
Wells 1 and 2 is for Escherichia coli isolates while wells 3 and 4 are for Klebsiella isolates

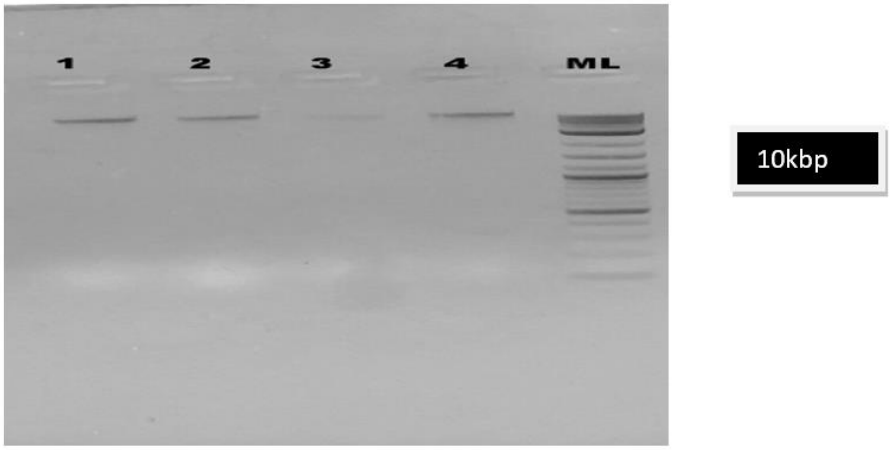

Fig. 3. Agarose Gel image showing bands for plasmids at 10,000 base pair, confirming the presence of plasmids in the isolates

Wells 1 and 2 are for Escherichia coli isolates while wells 3 and 4 are for Klebsiella spp

honey by the producers/sellers. However, the bacteria isolated are contrary to the study carried by Seraglio et al*.,* [15], reported that 25% of the isolates were from the genus *Lactobacillus*, including *L. plantarum* (24%), *L. Kazie* (28%), and *L. acidophilus* (48%), other isolates belong to *Bacillus, Enterococcus, Lactococcus,* micrococcus. Mustar and Ibrahim [16] isolated *Enterococcus* (23.8%), *Micrococcus* (18.8%), *Str eptococcus* (13.8%), *Pediococcus* (13.8%), *Lactococcus* (10.0%) and *Lactobacillus* (10%). The findings of studies mentioned above disagreed with the present study regarding the bacteria obtained from the honey samples. This could be that bacteria in honey might be derived from primary and secondary sources during production [17]; moreover, bacteria contaminants can be inoculated to honey during its extraction and post-harvest processing often from hygiene and storage conditions [18].

An antibiogram of bacterial isolates used in this study assayed against a total of 12 antibiotics which include; Amoxicilin (30µg), Cefotaxime (25µg), Imipenem (10/10µg), Ofloxacin (5µg), Gentamicin (10µg), Nalidixic (30µg), Nitrofurantoin (300µg), Cefuroxime (30µg), Ceftriaxone Sulbactam (45µg), Ampiclox (10µg), Cefexime (5µg), Levofloxacin (5µg), indicates that *Escherichia coli* and *Klebsiella* spp were resistant to imipenem, but susceptible to other antibiotics. Similarly, a study in Turkey by Dolunay G. et al*.,* [19] reported that imipenem-resistant E. *coli* and *Klebsiella* spp., had lost major outer membrane proteins, produced OXA-48-like carbapenemases and also had group 1 CTX-M-type ESBLs. Additionally, Elizabeth B. and Vincent H. (2010) stated that Klebsiella pneumoniae carbapenemases (KPCs) enzymes are capable of hydrolysing a broad spectrum of beta-lactams of cephalosporins, carbapenems and monobactam. More researches are required for well-controlled clinical trials to establish the optimum treatment of infections caused by these World Health Organization (WHO) priority organisms especially *Escherichia coli* and *Klebsiella* spp.

The emergence of carbapenem-resistance bacteria has gradually become a major global health problem [20]; because of its frequent use in hospitals in treating bacterial infections due to its broad spectrum activity [21]. In this study, the resistant isolates *Escherichia coli* and *Klebsiella* spp., was analyzed for the resistant gene Bla_{IMP}. *IMP*F and *IMP*R primers were used to detect the presence of the gene sequence for Bla_{IMP}at. Conversely, there was no amplification of the gene of interest at the genome level of these isolates. Plasmid profiling was successful as visible bands were shown on the agarose gel, indicating that the imipenem resistance by these isolates is plasmid-borne. This is in agreement with Shams et al*.,* [22] which described that antibiotics resistance may have been as a result of an outer membrane/extra chromosomal DNA (Plasmid). This resistance is as a result of horizontal gene transfer through transduction [23-26].

5. CONCLUSION

According to the findings of this study, the honey samples that were sold at Ogbete Market, contained bacteria that are potentially harmful.

Some of these strains are *Staphylococci spp.*, *Streptococcus spp., Escherichia coli* and *Klebsiella spp.* The presence of their colony-forming unit and frequency of occurrence confirmed the prevalent isolates. The most common isolates, *Escherichia coli* and *Klebsiella spp*., were highly resistant due to the virulent factors these isolates possessed during the susceptibility test to Imipenem. This resistance may not be at the genome level but rather be caused by an additional chromosomal factor, as demonstrated by the absence of amplification of the Bla_{IMP} gene during PCR. Plasmid profiling affirmed that these disengages opposition is plasmid-borne because of flat quality exchange by means of transduction and this prompts the exchange of protection from different microorganisms. Subsequently, inferable from the presence of this component of obstruction, there is need for the improvement of legitimate measures to counter bacterial contaminants in locally produced honey examples as it represents a danger to general wellbeing.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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