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Molecular Characterization and Antimicrobial Susceptibility Profile of Bacteria found in Urine of Students in Owerri

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Abstract

The study was carried out to investigate the molecular characterization and antimicrobial susceptibility profile of bacteria found in urine of female university students. 200 mid-stream urine samples were collected from the female students who were aged 16-30 years, using sterile containers. Standard microbiological methods of sterilization and media preparation were observed. Nutrient, MacConkey and Muller Hinton media were prepared, inoculated and incubated at 37^0C for 24 hours. Bacteria were further identified using 16s rRNA gene sequencing method Subcultured colonies were identified using morphological characteristics and biochemical test. The total heterotrophic plate bacterial count ranged from 1.2-5.2 x 10^6 cfu/ml. The total coliform bacteria plate count ranged from 1.0-4.5 x 10^6 cfu/ml and the total staphylococcal plate count ranged from 1.2-4.6 x 10^6 cfu/. Bacterial Isolates characterized and identified from female students were *Escherichia coli, Salmonella typhi, Shigella dysenteriae, Pseudomonas aeruginosa* and *Staphylococcus aureus*. Female students showed significance to urinary tract infections. Age group of 25-30 years showed the most bacterial significant while age group 16-18 years showed the least bacterial significant. Antibiogram showed that most of the isolates were sensitive to Augmentin, Ceftazidime, Cefuroxime, Cefixime, Ciprofloxacin, Ofloxacin and while all bacterial isolates were resistant to Erythromycin, Gentamicin and Nitrofurantoin. There is need to examine the urine of female students to create awareness of the presence of urinary tract infection.

Keywords: Antibiogram, Bacteriuria, Media, Urinary Tract Infection

Introduction

Urine is a liquid by-product of metabolism excreted in humans and as well excreted in many animals. Urine flows from the kidneys through the ureters to the urinary bladder. Urination therefore results in urine being excreted from the body through the urethra. (Arthur and John, 2006) [3]. The urine contains about 91-96% of water. Urine also contains an assortment of inorganic salts and organic compounds, including proteins, hormones, and a wide range of metabolites, this varies based on the food intake of a person. The total solids in urine are on average 59 g per person per day (Rose *et al.,* 2015) [18]. The presence of bacteria in urine is known as 'bacteriuria'. Bacteria may results from contamination during or after collection of urine, contamination of urine can arise from the sample container or it may indicate the presence of bacteria in the bladder. Kass, (2005) [9] distinguished between these possibilities by referring to the term "significant bacteriuria" which has been defined as the presence of 10^5 or more bacteria per ml of clean catch midstream urine aseptically collected, Bacteriuria can be categorized into symptomatic and asymptomatic. Asymptomatic bacteriuria therefore refers to the presence of bacteria pathogen in the bladder, also urine that is greater than 105 bacterial/ml in the absence of symptoms of urinary tract infection (UTI) while symptoms are present in symptomatic cases (Kunin, 2005) [11]. Urinary tract infections (UTIs) are caused by the presence and growth of microorganisms anywhere in the urinary tract and are perhaps the single commonest bacterial infection of mankind. Urinary tract infection occurs when bacteria is introduced into the urinary system usually through the urethra, when it gets into the urinary system, it multiples and travels up the urinary tract causing inflammation and irritation along the way (Kolawole *et al.,* 2009) [10]. When more than105 cells of bacterial colonies are found in urine, infection of the urinary tract bacteriuria is considered to exist (Williams, 1996; Kass, 2005) [19, 9] Common organisms that are commonly implicated in asymptomatic bacteriuria and infection of the urinary tract includes species of *Enterobacteriaceae* especially *Escherichia coli* which is

predominant followed by, *Staphylococcus aureus, Enterococcus faecalis, Klebsiella, pneumonia, Proteus specie* and *Pseudomonas aeruginosa* (Nicolle *et al*., 2005) [13]. Poor hygiene and inadequate sanitary conditions are predisposing factors to urinary tract infection (Ehinmidu, 2003) [7]. The bacterial infections encountered by clinicians in developing countries are the cause of significant morbidity and mortality. Annually, about more than 150 million cases are infected with Urinary tract infection (UTI) worldwide (Mazzariol, *et al*., 2017) [12].

Materials and methods

The study area is Imo State. Imo state is located in the south eastern part of Nigeria and is bounded to the North by Anambra State, to the East by Abia State, to the South by Rivers State and to the West by Delta State. Imo State is made up of 3 Senatorial zones, namely: Orlu zone, Owerri Zone and Okigwe zone. The Imo State lies between latitude 5° 30' and 6° 15' North. Longitude 6° 38' and 7° 18' East. (Federal Republic of Nigeria official gazatte, 2007) [8]. The site of study is Imo State University, Owerri.

The subjects are female students of Imo State University Owerri from faculty of biological sciences, comprising of different age groups ranging from 16 years through 30 years old.

Collection of Samples: 200 urine samples were aseptically collected from female students and taken to the laboratory for analysis.

Sterilization of glass wares

All glass wares were washed with detergent and rinsed with water, dried in hot air oven, they were then sterilized. Media were also sterilized by autoclaving at 121^0 C, 15Psi. All wire loops were flamed to red hot using Bunsen burner. Laboratory benches were cleaned before and after work with 75% alcohol (Ohazuruike *et al.*, 2017) [15].

Preparation of media

The powder components of the following media namely, Nutrient agar, MacConkey agar and Muller Hinton agar were dissolved in a conical flask, the manufacturer's instruction was followed to disperse appropriate concentration of agar into the conical flask then mixed with equal volume of water. The conical flask was closed with cotton plug and covered with Aluminium foil, placed into an autoclave and sterilized at 121^0C for 15mins. The media was cooled to 45^0C after sterilization, the cotton plug was then removed and the mouth of the flask flamed over a Bunsen burner in other to ensure sterility, and the medium was poured into sterile, empty petri dishes (15-20ml into each petri dish).The petri dishes were kept horizontally until the media were completely solidified, then they were turned upside down and stacked for storage.

The plates were labeled according to the media and also a sterility test was performed on them by incubating some plates at 37^0C for 24hrs and after which they were examined to check for growth. (Ohazuruike *et al.*, 2017) [15].

Isolation and enumeration of microorganisms

Ten (10ml) of each urine sample was homogenized and aseptically added to 9ml sterile normal saline and serially diluted up to 10^{-3}. 0.1ml of the various dilutions was inoculated into sterile Nutrient agar, MacConkey agar and Muller Hinton agar using streak plate method. The plates were incubated at 37ºC for 24hours and observed for bacterial growth. Isolates were sub cultured to obtain pure cultures (Cheesbrough, 2011) [5].

Sub-culturing of the cultures

After the Culturing and incubation period, the plates were observed and colonies were counted. The discrete colonies that were seen were sub-cultured into a freshly prepared Nutrient agar and other media plate to get a pure culture. The sub-cultured plates were incubated for 24hrs, and examined for pure culture. The pure culture growth was used for gram staining, motility test and biochemical characterization of the organisms. biochemical tests like Catalase, Oxidase tests, Coagulase, Citrate utilization test, Indole test, Methyl-red test, Voges proskaeur test and Sugar fermentation were carried out. A stock culture was prepared using a bijou bottle: This stock culture was used in storing the organisms for further biochemical characterization, Nutrient broth isolates were used for molecular characterization (Ohazuruike *et al.*, 2017) [15].

Identification of bacterial isolates

The bacterial isolates were identified using colonial, cellular characteristics; Colony's shape, colour, consistency, surface appearances and size of the colony (diameter in mm), Gram Staining, Motility test and biochemical properties like Catalase, Oxidase tests, Coagulase, Citrate utilization test, Indole test, Methyl-red test, Voges proskaeur test and Sugar fermentation (Ohazuruike *et al.*, 2017) [15].

Gram's staining

This Gram's staining technique is used to differentiate microorganisms into Gram-positive and Gram-negative as a result of their gram reaction (Cheesbrough, 2011) [5]. The gram's staining method described by Cheesbrough (2011) [5] was adopted. With the aid of a sterile inoculating wire loop; smears of the isolates was made on clean, grease-free microscope glass slides, air-dried and heat-fixed by passing the slides 2-3 times over a Bunsen burner flame. Afterwards, each smear was covered with a Crystal violet (primary stain) for 60 seconds, after which it was quickly washed off with clean water. The smear was flooded with lugol's iodine (Mordant) for 30 seconds. After, they were decolorized with 75% alcohol for 30seconds, which was washed off quickly with clean water and counter stained with a secondary stain safranin for 30 seconds. The safranin stain was washed off quickly with clean water. The back of the slides were then wiped and placed in a draining rack to air-dry. The smear was then examined microscopically using the oil immersion objective (X100). Gram positive cells showed purple while gram negative cells showed red colour. (Cheesbrough, 2011) [5].

Motility test

The method also described by Cheesbrough (2011) [5] was adopted. It is used to differentiate between motile and non-motile organisms due to the presence of locomotory structures like flagella and cilia. This test was carried out using the stab culture method. Test tubes of semi-solid motility medium were inoculated by stabbing a sterile straight wire loop charged with inoculums from the isolated pure culture vertically into the media and it was incubated at 37^0C for 24hours. Non-motile bacteria produced growths that were un-diffused from the line of stab while motile

bacteria produced diffused growth away from the line of stab into the medium and rendered it opaque (Cheesbrough 2011) [5].

Biochemical test

Catalase test

A drop of 3% hydrogen peroxide was placed on each end of a microscope slide, with the aid of a sterile wire loop, colonies of the test organisms were transferred on to one end of the microscope slide, and the other end was not inoculated but served as a control. The presence of gas bubbles indicates a positive catalase test, while absence of bubbles indicates a negative catalase test (Ohazuruike *et al.*, 2017) [15].

Citrate utilization test

The test is carried out to-demonstrate the use of citrate as a sole source of carbon by alkalinisation of the medium and ammonia as the only source of nitrogen by the bacteria, *Enterobacteriaceae* family. The test was carried out by inoculating sterilized Simmon's citrate agar with the test organisms using a sterile wire loop and incubating at 37^0C for 48 hours and observing for changes in colour. Positive result shows a change of the medium colour from green colour to royal blue colour, indicating the presence of citrate utilizing bacteria (Ohazuruike *et al.*, 2017) [15].

Coagulase test

A drop of distilled water was placed on each end of the microscope slide. A colony of test organism was emulsified in each of the drops of distilled water that was placed on the ends of the microscopic slide, to make thick suspensions. A 100cfu/l of plasma was added to one of the suspension and mixed gently. No plasma was added to the same suspension serving as control. Clumping of the mixture within 10 seconds will indicate positive coagulase test, while absence of clumps within 10seconds indicates a negative result (Cheesbrough, 2011) [5].

Indole test

The test organism is cultured in a medium which contains tryptophan. Indole production is detected by Kovac's reagent which, contain 4-p-dimethylaminobenzaldehyde; it reacts with the indole to produce a red coloured compound. The test organisms were inoculated in a bijou bottle containing 3ml of sterile tryptone water, which was incubated at 37^0C for 48hrs, after incubation, 0.5ml of kovac's reagent was added, the tubes were gently shaken, and the appearance of a red colour surface layer within 10mins indicates a positive indole test (Cheesbrough, 2011) [5].

Oxidase test

A piece of filter paper was soaked with a few drops of oxidase reagent (tetra-ethyl-p-phenylendeamine dihydrochloride). A colony of the test organism was picked with a sterile glass rod and smeared on the filter paper. A blue purple colour develops within a few seconds if the organism is an oxidase producer as a result of the oxidation of the phenylendeamine, while the absence of a blue purple colour indicates a negative result (Cheesbrough 2011) [5].

Methyl red/ voges-proskauer {mr/vp}

The bacteria isolates were inoculated into 2mls of glucose phosphate (peptone water) and was incubated at 37^0C for 48hours. After the period of incubation, 4 drops of methyl red indicator was added to the tube. The solution was homogenized and observed immediately for colour change. The appearance of a red colour indicates a positive result while the appearance of a yellow colour indicates a negative result (Ohazuruike *et al.*, 2017) [15]. For Voges proskauer test, the method described by Ohazuruike *et al.*, (2017) [15] was adopted, the bacteria isolates were added to 2ml of glucose phosphate (peptone water) and it was incubated at 37^0C for 48hrs, after incubation, 40% KOH and 3ml of 5% alcoholic alpha-naphthol were added, the appearance of a pink colour after 2-5 minutes indicates a positive result.

Sugar fermentation test

This test was employed to check for the ability of an organism to ferment sugar. The agar used in this test is called Triple Sugar Iron (TSI). This test engines the ability of the organism to produce gas, Hydrogen sulphide, to ferment Glucose, maltose and fructose to also ascertain if its Slant and Base are acidic or basic. The agar was sterilized at 121^0C at 15mins. The test organism was inoculated at a slanted test tube. A colour change from purple to yellow indicates the utilization of several sugars. A black duct at the slanted area indicates the presences of H_2S. Also a gaseous bubble at the bottom or slant of the test tube indicates the presence of gases while displacement in the durham's tube indicates gas production (Ohazuruike *et al.*, 2017) [15].

Molecular identification of bacterial isolates.

The deoxyribonucleic acid (DNA) was extracted using Zymo research bacterial DNA mini prep extraction kit. The qualitative estimation of genomic DNA was done using agarose gel electrophoresis. The extracted DNA was amplified using Polymerase chain reaction amplification protocol. 16S rRNA sequencing protocol was used to characterize bacterial isolate. PCR products were cleaned using ExoSAP Protocol. Fragments were sequenced using the Nimagen brilliant dye terminator cycle sequencing kit according to manufacturer's instructions (Platt *et al*., 2007) [17]. The sequenced data were subjected to Basic Local Alignment Search Tool Nucleotide (BLASTn) to identify corresponding organisms from National center for bioinformatics information (NCBI) as described by (Altschul *et al*., 1990).

Antibiotic susceptibility testing

Antimicrobial Susceptibility of the isolates was tested using modified Kirby-Bauer disc diffusion method on Muller Hinton agar (Oxoid) for testing bacterial isolates. The results of the antimicrobial susceptibility was interpreted based on the guidelines of the Clinical Laboratory and Standards Institute (2006) [6].

Antimicrobial susceptibility test was carried out using the Kirby-Bauer disk diffusion susceptibility test on Muller Hinton agar medium. The following antibiotics was employed for sensitivity analysis; Augmentin (30µg), Ceftazidime (30 µg), Cefuroxime (30 µg), Nitrofurantoin (30 µg), Gentamicin (10 µg), Cefixime (5 µg),

Ciprofloxacin (5 µg) Erythromycin (5 µg) and Ofloxacin (2 µg). The growth was standardized by diluting the culture with normal Saline to match the turbidity of 1.0×10^6 cfu/ml (0.5 McFarland standards).Then 0.1ml was collected and spread on the surface of Muller Hinton agar using sterile glass rod. The antibiotic disc was placed carefully to make good contact with the agar surface using sterile forceps and sufficiently separated from each other in order to prevent overlapping of the zones of inhibition. The agar plates was left on the bench for 30mins to allow for diffusion of the antibiotics and was incubated at 37°C for 24hrs and results was interpreted as sensitive and resistant. The inhibition zone diameters were measured using meter rule after 24 hours incubation and recorded in millimeter (mm). It was further interpreted according to Clinical Laboratory Standards Institute (CLSI, 2006) [6].

Statistical analysis

The data obtained from this work were statistical analyzed using frequency distribution tables sample percentages were graphically represented with a pie chart.

Results and Discussion

The total heterotrophic bacteria count ranges from 2.7-4.2 x 10^5 cfu/g; Total coliform count ranges from 2.1-3.3 x 10^5 cfu/g; and Total Staphylococcal count count ranges from 2.5-3.8 x 10^5 cfu/g as shown in Table 1. The Table 2 shows the colonial and morphological characteristics of bacterial isolates. Table 3 shows the physiological properties of bacterial isolates, including their biochemical tests. Table 4 is the molecular sequence 16S rRNA identity of various bacteria. Bacteria isolated and characterized were *Escherichia coli*, *Salmonella typhi*, *Shigella dysenteriae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Figure 1 shows the Percentage occurrence of bacterial isolates in a pie chart. The percentage occurrence of significance bacteriuria of the urine of female students according to their ages is shown in Table 5. Table 6 is the Antibiogram, the zone of inhibition for isolated organisms in mm.

Table 1: Total viable counts obtained from urine of female students

Sample range	Total viable count $x10^6$ Cfu/ml		
Samples range	THBC	TCC	TSC
1-50	2.7×10^5 - 3.1×10^5	2.1×10^5 - 2.7×10^5	2.5×10^5 - 2.5×10^5
51-100	3.2×10^5 - 3.6×10^5	2.8×10^5 - 3.0×10^5	2.6×10^5 - 3.7×10^5
101-150	2.8×10^5 - 4.0×10^5	2.7×10^5 - 3.2×10^5	2.4×10^5 - 3.8×10^5
151-200	3.3×10^5 - 4.2×10^5	3.0×10^5 - 3.3×10^5	2.9×10^5 - 3.8×10^5

Keys: THBC = Total Heterotrophic Bacteria count
TCC = Total Coliform count
TSC = Total Staphylococcal count.

Table 2: Colonial and morphological characteristics of bacterial isolates

Sample	Colour	Shape	Surface	Arrangement	Probable organism
1	Pink colonies	Raised growth	Smooth and dry	Rods in singles	*Escherichia coli*
2	Dark colonies	Elevated	Round, small opaque and entire	Rods in cluster	*Salmonella* spp
3	Pale colonies	Circular and entire	Flat, pin size and transparent	Rods	*Shigella* spp
4	Blue or yellow green	Irregular margins	large, opaque, flat	Rods	*Pseudomonas* spp
5	Yellow colonies	Round	Glassy	Cocci in clusters	*Staphylococcus aureus*

Table 3: Physiological properties of bacterial isolates

Isolates	**Bacteriological tests**			**Biochemical tests**										**Probable organism**
	Gram reaction test	**Cellular arrangement**	**Motility test**	**Catalase test**	**Citrate test**	**Indole test**	**Oxidase test**	**Coagulase test**	**Voges Proskuer test**	**Methyl red test**	**Glucose test**	**Lactose test**	**Sucrose test**	
1	-	Rod	+	+	-	+	-	-	-	+	**A**	**A**	**A**	*Escherichia coli*
2	-	Rod	+	+	-	-	-	-	-	+	+	-	-	*Salmonella spp*
3	-	Rod	-	+	-	-	-	-	-	-	+	-	-	*Shigella spp*
4	-	Rod	+	+	-	-	-	-	-	-	-	-	-	*Pseudomonas spp*
5	+	Cocci	-	+	+	-	-	+	+	+	**A**	**A**	**A**	*Staphylococcus aureus*

Key
- = Negative
+ = Positive
A = Acid
A/G = Acid and Gas production

Table 4: Molecular sequence 16S rrna identity of various bacteria

S. No.	Biochemical isolates	Percentage (%)	NCBI match
1	*Escherichia coli*	99	*Escherichia coli* M75029
2	*Salmonella* spp	99	*Salmonella typhi* L21912
3	*Shigella* spp	100	*Shigella dysenteriae* LR739008
4	*Pseudomonas* spp	96	*Pseudomonas aeruginosa* WE 41437
5	*Staphylococcus aureus*	97	*Staphylococcus aureus* MH401415

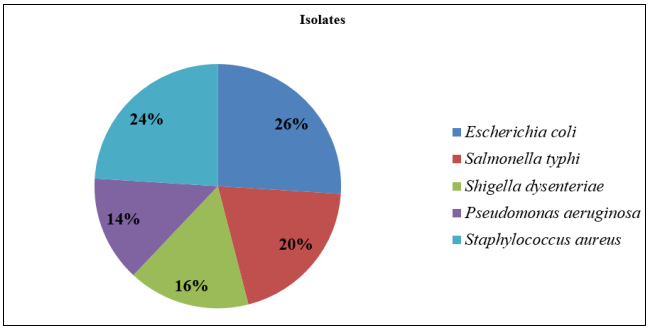

Fig 1: Percentage occurrence of bacterial isolates

Table 5: Significance bacteriuria of the urine according to the age of the students

Age of Students	Number of samples	Significant bacteriuria (%)	Suspected bacteriuria (%)	Non-significant Bacteriuria (%)
16-18	50	19 (38)	13 (26)	18 (36)
19-21	50	22 (44)	13 (26)	15 (30)
22-24	50	28 (56)	10 (20)	12 (24)
25-30	50	33 (66)	8 (16)	9 (18)

Table 6: Antibiogram zone of inhibition for isolated organisms in mm

Antibiotics	*Escherichia coli* (mm)	*Salmonella typhi* (mm)	*Shigella dysenteriae* (mm)	*Pseudomonas aeruginosa* (mm)	*Staphylococcus aureus* (mm)
Augmentin (30µg)	26 ± 1.000	24 ± 0.577	27 ± 1.154	26 ± 1.000	25 ± 1.000
Ceftazidime (30µg)	27 ± 1.154	25 ± 1.527	26 ± 1.000	25 ± 1.527	26 ± 1.000
Cefuroxime (30 µg)	24 ± 0.577	23 ± 1.000	24 ± 1.000	25 ± 1.527	25 ± 1.527
Nitrofurantoin (30µg)	10 ± 0.577	8 ± 1.000	12 ± 1.154	9 ± 0.577	8 ± 1.000
Gentamicin (10 µg)	12 ± 1.154	9 ± 0.577	8 ± 1.000	5 ± 1.154	7 ± 0.577
Cefixime (5 µg),	23 ± 1.000	18 ± 1.000	16 ± 1.000	23 ± 1.000	25 ± 1.527
Ciprofloxacin (5 µg)	26 ± 1.000	23 ± 1.000	26 ± 1.527	25 ± 1.000	26 ± 1.000
Erythromycin(5 µg)	6 ± 1.000	3 ± 0.577	5 ± 1.154	7 ± 0.577	9 ± 1.000
Ofloxacin (2 µg),	25 ± 1.000	23 ± 1.000	24 ± 0.577	23 ± 1.000	25 ± 1.527

Key: Breakpoint table for *Staphylococcus aureus* Susceptible (S) ≥ 25 mm, Intermediate (I) 18-24 mm, Resistance (R) ≤ 19mm. Enterobacteriaceae Susceptible (S) ≥ 23mm, Intermediate (I) 15-22 mm, Resistance (R) ≤ 14mm (CLSI, 2006).

Urinary tract infections (UTIs) are fast growing infections in the world due to poor hygiene, inadequate sanitary conditions and resistance among antimicrobials. This findings show that *Escherichia coli* was the predominant organism. Other organisms like *Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa,* were also identified. This concurred with the research of (Ojo and Anibijuwon, 2010) [16]. According to (Angus *et al*., 2017) [2] *Escherichia coli* and *Staphylococcus aureus* were the predominant bacteria isolated from urine samples and this concurred with this study. Significant bacteriuria that was observed in this study and agrees with the report of (Obirikwurang *et al*., 2012) [14] that observed significant bacteriuria in females among age groups 16-30 years. The result of the susceptibility tests in Table 3 shows that most of the isolates were sensitive to Augmentin, Ceftazidime, Ciprofloxacin and Cefuroxime and resistant to Erythromycin which concurred with the report of (Chaudhary *et al*., 2014) [4].

Conclusion

This study showed that the urine samples collected from female students of Imo State University Owerri showed significant urinary tract infections. Most of the bacteria isolated and characterized were sensitive to most of the antibiotics used. There is need to examine the urine of female students to create awareness of the presence of urinary tract infection as well as to educate students on preventive measures.

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