Original Research Article

ISOLATION AND MOLECULAR IDENTIFICATION OF ENTEROBACTER CLOACAE FROM URINARY TRACTINFECTIONAMONG PREGNANT WOMEN IN BIU, NORTH EASTERN NIGERIA, BORNO STATE.

ABSTRACT

Urinary Tract Infections represent a significant, widespread, and expensive public health issue owing to their high frequency and the associated health challenges on pregnant women. This study aimed to isolate, molecularly identify *Enterobacter cloacae* strains from Urinary Tract Infection among pregnant women in General Hospital Biu, Borno State. A total of 250 vaginal swabs from pregnant women who attended Antenatal Care Unit whose age range from 15 to 45 years were recruited using a well-designed questionnaire by random sampling technique. Gram-staining, culture, and wet microscopy were employed to check the presence of bacterial pathogens in the samples. Polymerase Chain Reaction was carried out to further validate the results. The degree of relatedness of the organism was determined using NCBI software. The result revealed that 167 representing 66.8% of the total samples studied showed significant bacterial growth. The ascending order of the percentage of the bacterial agents isolated include: *Enterobactercloacea* 47 (28.1%), *Klebsiella pneumoniae* 36 (21.6%), *Escherichia coli* 23 (13.8%), *Proteus mirabillis* 19 (11.4%), *Staphylococcus epidermis* 15 (9.0%), *Staphylococcus aureus* 14 (8.3%) and *Pseudomonas aeruginosa* 13 (7.8%). The result of the demographic factors

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showed that 17 (36.2%) of pregnant women infected within the age of 15–20 yearshad *Enterobacter cloacae*. Illiterate pregnant women were 19 (40.4%), unemployed pregnant women were 29 (61.7%), those in their third trimester (27 - 40weeks) were 24 (51.1%), women who had 7 and above pregnancies 25 (53.2%), symptomatic and asymptomaticpregnant women within the age of 15–20 years19 (40.4%), and rural dwellers 33 (70.2%). This study also demonstrated culture technique as the best biochemical method for isolation of bacterial agents compared to wet mount and microscopic Gram-staining isolation method. The PCR product was used to generate *Enterobacter cloacae* phylogenetic tree. It can be concluded that there is a 66.8% of prevalence of *Enterobacter cloacae* in Biu LGA of Borno State.

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Comment [SA3]: What about the other stages of pregnancy

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Comment [SA5]: What about the other samples collected from cases above 20 years

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Keywords: Urinary Tract Infection, microscopy, Polymerase Chain Reaction, *Enterobacter cloacae*.

INTRODUCTION

Microorganisms especially bacteria in the family of *Enterobacteriaceae*and *Enterobacter*genus are ubiquitous in nature. These organisms are usually facultative anaerobic Gram-negative strains. Bacteria of the *Enterobacter* genus have been reported to be present in sewage, soil as well as part of the commensal enteric flora of the human GI tract (1). *Enterobacter cloacae* belonging to *Enterobacteriaceae* family has been considered as opportunistic pathogens which cause both national (2) and international (1,3,4).Healthcare-Associated Infections (HAIs). The species of bacteria currently assigned to be *Enterobacter cloacae* complex include *Enterobacter cloacae, E. asburiae, E. carcinogenus, E. hormaechei, E. kobei, E. nimipressuralis, E. ludwigii*and *E. mori*(5).

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Comment [SA10]: healthcare-Associated Infections

Susan and Michael, (2022) reported *Enterobacter cloacae* as nosocomial pathogens that cause several infectious diseases such as endocarditis, septic arthritis, lower respiratory tract infections, osteomyelitis, urinary tract infections (UTIs), intra-abdominal infections, CNS, skin and soft tissue infections, and ophthalmic infections. Multi-drug resistant *Enterobacteriaceae* have been confirmed to be more harmful as a result of their extensive resistance to drugs (6). Common mechanism for antibiotic resistance by *Enterobacteriaceae* involves production of Extended-spectrum β-lactamase (ESBLs) and ESBL-producing *Enterobacteriaceae* (EPE) has been reported as major cause of infection (6-7).

Karanika*et al.*, (2016) established that the distribution of ESBL-producing *Enterobacteriaceae* significantly differs in different locations and its rectal colonization prevalence in healthy human beings was 14% across the world's population (8). The estimate of prevalence of infectious diseases was about 110 million globally in 2016 of which species of *Enterobacter*ranked second after Pseudomonas aeruginosa among other gram-negative organisms (9). High rate of perinatal mortality, low birth weight, pyelonephritis, preterm birth in both the fetus and the mother have been recorded as risks associated with untreated bacteruria during pregnancy especially amongst sexually active women(6). These risk factors of HAIs are predominant in Nigeria yet little or no extensive study has been conducted on the cause of infection (*Enterobacter*species) among pregnant women in Biu, Borno, Nigeria. *Enterobacter cloacae* confirmed as multi-drug resistant bacteria do not only impose difficulty in treatments but also causes death of either the fetus or the mother (6-7).

Bacteria of the *Enterobacteriaceae* family are resistance to wide range of antibioticsand susceptible to very few. The multi-drug resistance potential of these organisms depends their ability to secret an enzyme known as Extended-Spectrum β-lactamase (ESBLs) which inturn

Comment [SA11]: it is highly recommended to focus on the targeted m.o.; *Enterobacter cloacae*and the MDR.
Enterobacteriacae is a huge Family , focus on the targeted bacteria is highly recommended

Comment [SA12]: Refer to incidence of the m.o. in the vagina of pregnant women

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deactivates the potency of several antibiotics when administered (6). Most phenotypic methods used in the microbiological laboratories are not only labour intensive but material consuming. Phenotypic and biochemical methods of identification and characterization of bacterial species often relied on preliminary isolation and culture have over time remained inaccurate and unreliable(9). However, molecular-based techniques have provided unprecedented insights into bacterial identification and typing as the widespread use and availability of molecular tools for bacterial genotyping have resulted in high throughput analysis, more sensitive and discriminatory results, and rapid turn-around-times, which are only likely to get better with automated tools and data analysis pipelines. Most molecular methods for bacterial identification are based on some variation of DNA analysis, either amplification or sequencing based. These methods range from relatively simple DNA amplification-based approaches (PCR, real-time PCR, RAPD-PCR) towards more complex methods based on restriction fragment analysis, targeted gene and whole-genome sequencing, and mass spectrometry (8). This study therefore, aims at biochemically and molecularly identifying *Enterobacter cloacae*among pregnant women in Biu, North-Eastern Nigeria, Borno State.

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MATERIALS AND METHODS

Study Area

Study was carried out in Biu General Hospital on a latitude of 10° 36' 42.12"N of the Equator and Longitude of 12° 11' 30.84"E of the Greenwich Meridian in Biu local government area (LGA) in Borno State Nigeria. The Biu Plateau is a highland area in North-eastern Nigeria containing many recently extinct volcanoes. It covers about 5,200 km^2 and has an average elevation of 700 m above the mean sea level. The plateau lies between the Upper Benue Basin to the south and the Chad Basin to the north has road connections to Damaturu, Mubi and Shani.

Study Period and Design

This study was carried out from August, 2021 to February, 2021. An analytical cross-sectional investigation was conducted.

Subjects

The study population consisted of pregnant women who attended antenatal care units at General Hospital Biu, Borno state during their first antenatal visit. Individual consent was approved and patient confidentiality was ensured.

Ethical Consideration

Ethical approval was obtained from the ethical review committee of the Borno State Ministry of Health before the study was carried out.

Comment [SA16]: Ethical committee approval shall be clearly defined

Clinical Sample Collection

Method described by (10) was used to obtain the vaginal swab from each participating woman. A sample size of 250 pregnant women was collected aseptically with the use of a sterile swab labeled with laboratory identification numbers during sample collection of duplicate High Vaginal swabs from the posterior fornix of the vaginal tract with the aid of plastic disposable speculum and gently rotated about 2-3 times in order to pick the vaginal exudate with the help of a Gynecologist of each subject. It was immediately replaced in its casing and transported to the laboratory for visual and microscopic examination.

Bio-Data collection

Information such as age, occupation, educational qualification, trimester of pregnancy, patience religion status, vaginal hygiene practice, toilet hygiene, gravida, history of premature labor,

Comment [SA17]: Results shall define the isolation relevant to the trimester and age for all tested positive cases

number of pregnancies and vaginal signs and symptoms was obtained from the subjects. An interview was conducted and previous clinical history was obtained from the hospital record cards in the antenatal department.

Determination of Sample Size

The sample size will be calculated based on the report prevalence rate of 20.5% reported by Hamafyelto and Ikeh, (2017).

Using the formula: $N = Z^2 X P (1-P)/ D^2$

Where N – Sample size; Z – Statistic corresponding to level of confidence of 95% (Cl=1.96); P – Expected prevalence-based study; D – Precision corresponding to effect size 5% at 95% Cl

$N= 1.96^2 X 0.205(1-0.205) / 0.05^2 = 250$

Thus, the cross-sectional researcher has to take at least 250 subjects.

Inclusion Criteria

All apparently healthy pregnant women between the age range of 15–45 years who attended antenatal care unit of the hospital whose verbal consent was included in the study.

Exclusion Criteria

All pregnant women who are not within the age of 15–45 years, pregnant women who declined, pregnant women who had renal or on any antibiotic therapy within 72 hours to encounter were excluded in the study.

Visual Examination

The posterior vaginal fornix of the pregnant women was observed while swab samples were collected for the presence or absence of discharge.

Comment [SA18]: results are not correlated with the clinical findings

Isolation and Characterization of Target Bacteria

According to the indicated conventional microbiological techniques, bacterial isolates were detected as described by (10), It involves inspecting samples in order to find, separate out, and identify infections or their byproducts using microscopy, culture techniques, and biochemical traits.

Comment [SA19]: outlining the methodology in use shall be stated

Isolation of Target Bacteria from Swab samples

The swab samples were innoculated unto McConkey's agar. The plates were incubated for 18 to 24 hours at 37°C. All suspected isolates with *Enterobacter cloacae*-like growth characteristics underwent routine bacteriological and biochemical identification procedures.

Comment [SA20]: what about the results of S.aureus, and other Gram +ve bacteria which should have been inhibited on McConkey agar?????

Microscopic Examination (Wet preparation)

This was accomplished using the techniques of (10). A wet mount of each swab sample was created by rolling the first swab on a slide, to which a drop of ordinary saline was added to look for actively motile, spear-shaped flagellates. This method was used to diagnose *Enterobacter cloacae* under the microscope using X10 and X40 objective lenses, the homogenate was covered with a slide and checked for motile flagellates.

Gram stain microscopy

- The dry vaginal swab collected from each woman was examined under the microscope using the Gram stain.

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- Each vaginal swab was carefully rolled onto a glass slide, and the samples were then fixed by momentarily holding the rear of the slides (the side without the cells) in a flame.
- A crystal violet dye was applied on slides for 1 minute, then they were submerged in an iodine solution for 1 minute, and they were exposed to alcohol for 20 seconds before being exposed to safranin for 1 minute.
- Slides were rinsed with water for two seconds after each immersion step.
- The gram-negative rods were observed on the slides under a microscope using an oil immersion objective lens at a magnification of X100.

Comment [SA22]: Gram staining methodology shall not be given in details

Bacterial Vaginosis and Candida cells were also seen in the gram-stained vaginal swab. It was scored using the Nugent scoring system for laboratory-based BV diagnosis while being observed under a microscope at 40X/100X magnification. All slides were rated by two people in a single-blind fashion for quality assurance. Slides were reevaluated and inspected in the event of a categorization difference (normal vaginal microbiota, intermediate microflora, and BV according to Nugent scoring).

Biochemical Characterization of Target Bacteria

The following biochemical tests were performed on each isolate: Citrate utilization, urease, and indole (10).

DNA Extraction

For DNA extraction, bacteria isolate from pure colonies were put into correctly labeled Eppendorf tubes. Following the manufacturer's instructions, commercially available kits

(Accuprep Genomic DNA extraction kit from (Bioneer) were used to extract the DNA of the bacterium isolates. Birefly, 20µl of Proteinase K was added to a clean 1.5 ml tube, 20µl of cultured cells were added to the tube containing Proteinase K, 20µl of binding buffer (GC) was added to the sample and mixed immediately by the vortex mixer to suspend the bacterium cells. After that, the sample mixes underwent 10-minute incubation at 60°C. The samples were homogenized by adding tissue lysis buffer, 20µl of proteinase K, and 10µl of RNAs, and then they were heated at 60°C for an hour. 100µl of isopropanol was then added and well mixed using a pipette.

Comment [SA23]: Need correction

To collect the droplets that were still clinging to the lid, pulse centrifugation was used. The binding column tube was then sealed and centrifuged at 8,000 rpm for one minute or until the binding column tube was empty, after the lysate had been carefully transferred into the top reservoir (fit in a 2ml tube) without soaking the rim. After that, the binding columns were moved to a fresh 2 ml tube for filtering. After adding 500µl of washing buffer 1 (W1), the mixture was centrifuged for one minute. The fluid spilled out after the tube was opened. After carefully adding 500µl more of washing buffer 2 (W2) to the columns, they were centrifuged at 8,000 rpm for one minute.

To guarantee that all ethanol and buffer W2, which may cause issues in later applications, were completely removed, the final centrifugation phase was repeated at about 12,000 rpm for 1 minute. The binding column tube was transferred to a new 1.5 ml tube for elution, 200 l of elution buffer was added directly onto the binding column tube, and it was left at room temperature (15–25 °C) for at least 1 minute until all of the elution buffer had been absorbed into the glass fiber of the binding column tube. After this time, the binding column tube was

centrifuged for 1 minute at 8000 rpm to elute the DNA. For further analysis, the eluted genomic DNA was kept in a freezer at 4°C.

Amplification of 16S rRNA Gene using PCR (AccupowerHotstart PCR premix, Bioneer)

On a thermal cycler (Thermal cycler PTC 100, MJ Research), PCR amplification for the confirmation of *Enterobacter cloacae* was carried out using a total of 5µl of DNA solution and a master mix Accupower hot start PCR premix by Bioneer. The premix contains 1.5 mM MgCl2, 250 M of each dNTP, 2µl of each of the species' forward and reverse primers, 1µl of taq DNA polymerase, and 1x PCR buffer. After that, 20µl of deionized water (water devoid of nucleases) was added. After separation on a 1.5% agarose gel, the thermocycler was run under the following conditions: pre-denaturation at 94°C for 5 minutes; denaturation at 94°C for 1 minute; annealing at 51°C for 1 minute; extension at 72°C for 1 minute for 25 cycles; and a final extension at 72°C for 5 minutes.

Primers

List 1 :Primers used for Molecular Analysis

S/N	Target Gene	Primer Sequence (5' – 3')	Amplicon Size (Bp)	Cycles	Reference
1	16S rRNA	Forward: TCT GGA ATG GCT GAA GAA GAC G Reverse: CAG GGT ACA TCG TAT	300	25	(11).

TGG TC

Reconstituting primer pellets in 100µl of distilled water was used to make the primers. The suspension was briefly vortexed followed by a brief 2-minute centrifugation. 100µl of distilled water was used to make the working solution after 10µl of suspension has been pipetted into a new tube.

Gel Extraction

The gel extraction buffer kit will be used for the procedure. A 1.5% agarose in TAE (Tris-acetate-EDTA-buffer) solution containing 5µl of ethidium bromide was made into an agarose gel. A solution containing 3g of agarose was heated in a water bath until the agarose was entirely dissolved, then the temperature was allowed to cool to between 50°C and 55°C. The cooled gel was then mixed with 5µl of ethidium bromide and placed into a gel tray. After then, it was allowed to cool at ambient temperature for 15–30 minutes. The combs were taken out, the gel put in the electrophoresis chamber, and TAE buffer was added on top of the gel. Next, 100bp of DNA ladder was added separately to each gel well. After carefully pipetting the PCR products into the comb's wells, they were electrophoresed for at least an hour. With the use of an ultraviolet (UV) light box, DNA bands may be seen (Bio-Rad).

Dye Terminator Cycle Sequencing with Quick Start Kit.

In a 2.0ml tube, a sequencing reaction was prepared. While preparing the sequencing reactions, all reagents were kept on ice and introduced in the following order: Pipette 0-9.5µl of deionized distilled water, 10.0µl of DNA template, 2.0µl of primers, and 8.0µl of DTCS into the tube. In the PCR machine, a quick start master mix and sequencing reaction will be set up with an initial

hold cycle of 95°C for 10 minutes, 45 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 30 seconds, and a final extension consisting of one cycle of 72°C for 10 minutes.

Ethanol Precipitation

For each sample, sterile 0.5ml tubes will be made and labeled. In accordance with the following sequencing reaction, a fresh stop solution/glycogen mixture will be created: Pipette 2µl of 3M Sodium acetate, 2µl of 100mM Na2-EDTA, and 1µl of 20mg/ml of glycogen into the tubes (as provided in the DTCS kit). The stop solution/glycogen combination will be put in 5µl to each of the tubes once they have been labeled. The sequencing reaction will then be moved to and thoroughly mixed in each of the tubes with labels. A chilly 60µl. The tube will be filled with 95% (v/v) ethanol from a -200°C freezer, properly mixed, and immediately centrifuged at 14,000 rpm for 15 minutes at 40°C. A visible particle will be obtained when the supernatant has been delicately removed with a micropipette. The pellet will be centrifuged at a speed of 14,000 rpm for two minutes at 40°C after being rinsed with 200µl of 70% (v/v) ethanol and kept in a freezer at -200C. A micropipette was used to extract the supernatant, and it was then vacuum-dried for 12 minutes until dry. Next, 40µl of the sample loading solution was added to the specimen to re-suspension it.

Sample Preparation for Loading into the Instrument

The samples were re-suspended and added to the appropriate wells on the sample plate (PN 609801). Each re-suspended sample in the wells received a drop of mineral oil from the kit. The apparatus was loaded with the sample plate to begin the sequence analysis.

Comment [SA24]: Shall be summarized and refernces shall be added with the clear definition of the kits in use

Statistical Analysis

The Statistical Package for Social Science (SPSS), version 23.0, was used to analyze the data. Calculated p-values were regarded as statistically significant with $p > 0.05$.

RESULTS

Occurrence and distribution of bacterial pathogens in UTIs among pregnant women

Figure 1 shows the percentage occurrence of bacterial pathogens in Urinary tract infections among pregnant women. In this study, cultures that showed 105 bacterial colonies per ml of vaginal swab were said to have significant growth. The pathogens were identified as *Enterobacter cloacae, Klebsiella Pneumoniae, E. coli, Proteus mirabilis, Staphylococcus aureus, Pseudomonas aeruginosa, and Staphylococcus epidermis*. *E. cloacae* 47 (28.1%) was the highest occurring bacteria pathogen followed by *K. Pneumoniae*, 36 (21.6%), *P. mirabilis* 19 (11.4%), *E. coli* 23 (13.8), *S. aureus* 14 (8.3%), *S. epidermidis* 15(9.0%) and *P. aeruginosa* 13 (7.8%) which had the least percentage occurrence.

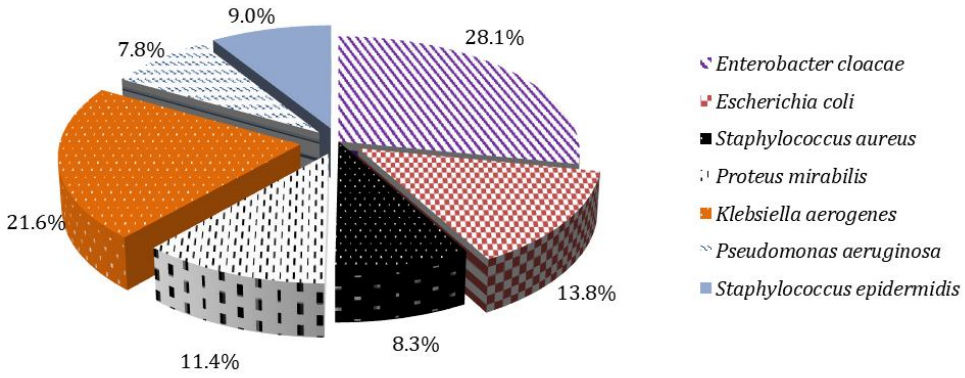

Figure 1: percentage occurrence of bacterial pathogens in Urinary tract infections among pregnant women.

Prevalence of UTIs in Relation to Age Distribution of Pregnant Women

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Comment [SA28]: *K. Pneumoniae*, 36 (21.6%), not isolated from cases of UTIs???? it is associated with pneumonia, bloodstream infections, wound or surgical site infections, or meningitis

Comment [SA29]: *S. aureus* 14 (8.3%), *S. epidermidis* 15(9.0%) never grow onto McConkey agar??

Table 1 shows the number of vaginal swab samples that tested positive or negative in urinary tract infections in relation to the age distribution of pregnant women in General Hospital Biu, North-Eastern Nigeria, Borno State. A total of 250 vaginal samples were collected from the pregnant women and cultured for the presence of bacterial pathogens, 167 (66.8%) showed significant bacterial growth while 83 (33.2%) showed no significant bacterial growth. The result revealed that the incidence of UTIs in relation to age distribution were prevalent among pregnant women within the of 15–20 years 84 (33.6%) out of which, 57 (67.9%) tested positive followed by 21–25 years 58 (23.2%), 26–30 years 45 (18.0%), 31–35 years 31 (12.4%), 36–40 years 20 (8.0%) and those within 41 years and above 12 (4.8%).

Table 1: Prevalence of UTIs in relation to age distribution of pregnant women

Age (Years)	No. Tested (%)	No. Positive (%)	No. Negative (%)
15 – 20	84 (33.6%)	57 (67.9%)	27 (32.1%)
21 – 25	58 (23.2%)	43 (74.1%)	15 (25.9%)
26 – 30	45 (18.0%)	36 (80.0%)	09 (20.0%)
31 – 35	31 (12.4%)	15 (48.4%)	16 (51.6%)
36 – 40	20 (8.0%)	11 (55.0%)	09 (45.0%)
41 – Above	12 (4.8%)	05 (41.7%)	07 (58.3%)
TOTAL	**250 (100%)**	**167 (66.8%)**	**83 (33.2%)**

Comment [SA30]: Prevalence of bacterial infection in urinary tracts...

Comment [SA31]: No of positive isolates shall be distributed among each group

Age Distribution of *Enterobacter cloacae* Infection among Pregnant Women

Table 2 shows the age distribution of *Enterobacter cloacae* infection among pregnant women determined using gram stain, wet techniques and culture techniques. Age distribution of *Enterobacter cloacae* infection among pregnant women determined using gram stain, wet

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techniques and culture techniques. The result showed that the infection rate among the pregnant women studied differ significantly ($p > 0.05$) by their age differences as well as the techniques used. 47 (28.1 %) *E. cloacae* were isolated in 250 specimens examined in this study as shown in Table 2. A higher percentage of pregnant women 17 (36.2%) infected with *E. cloacae* were found within the age brackets of 15-20 years, followed by age groups 21–25 years 11 (23.4%), 26-30 years 10 (21.3%), while the least infection rate were recorded within the age bracket of 31–35, 36–40, and 41 and Above having a total of 5 (10.6%), 3 (6.4%) and 1 (2.1%) *E. cloacae* isolate respectively. However, culture method of isolation showed the highest number of isolates compared to wet isolation and microscopy in all the age groups considered.

Table 2: Age Distribution of *Enterobacter cloacae* Infection among Pregnant Women

Age (Years)	**PW/Rate**	**GS/Inf. Rate**	**Wet/Inf. Rate**	**Culture/Inf. Rate**
15 – 20	17 (36.2%)	3 (17.6%)	5 (29.4%)	9 (52.9%)
21 – 25	11 (23.4%)	2 (18.2%)	3 (27.3%)	6 (54.5%)
26 – 30	10 (21.3%)	3 (30.0%)	2 (20.0%)	5 (50.0%)
31 – 35	5 (10.6%)	1 (20.0%)	2 (40.0%)	2 (40.0%)
36 – 40	3 (6.4%)	1 (33.3%)	1 (33.3%)	1 (33.3%)
41 – Above	1 (2.1%)	0 (0%)	0 (0%)	1 (100.0%)
TOTAL	**47 (100%)**			

Key: PW/Rate = Number of pregnant women examined; GS/Inf. Rate = Isolation by Gram-Staining; Wet/Inf. Rate = Isolation by wet preparation; Culture/Inf. Rate = Isolation by culture.

Distribution of *Enterobacter cloacae* Cases based on the Literacy Level of the Pregnant Women.

Table 3 showed the result of distribution of *Enterobacter cloacae* cases based on literacy level of the pregnant women studied. The result established that the rate of the bacteria infection differs significantly ($p > 0.05$) based on their literacy level. Highest rate 19 (40.4%) of infection was recorded among women with no formal education (illiterate) background followed by those that attended primary school 14 (29.9%) secondary school 9 (19.1%) while 5 (10.6%) of the pregnant women that attended tertiary institution had the least infection rate. Table 4 also revealed that there was significant difference in isolation of the bacteria using different techniques at $p > 0.05$. Isolation by culture preparation had the highest number of positive isolates 9 (47.4%) among illiterate pregnant women followed by isolation by wet preparation 7 (36.8%) and then, Gram-Staining technique 3 (15.8%). Similarly, isolation by culture techniques showed 6 (42.9%), 5 (55.6%) and 3 (60.0%) pregnant women who attended primary, secondary and tertiary institution respectively. Wet techniques can be considered after culture techniques as 5 (35.7%), 3 (33.3%) and 1 (20.0%) were respectively recorded for pregnant women who attended primary school, secondary school and tertiary institution while microscopic (Gram-staining) had the least result across the literacy status of the pregnant women examined in this study.

Table 3: Distribution of *Enterobacter cloacae* Cases based on their Literacy Level

Literacy status	PW/Rate	GS/Inf. Rate	Wet/Inf. Rate	Culture/Inf. Rate
Illiterate	19 (40.4%)	3 (15.8%)	7 (36.8%)	9 (47.4%)
Primary	14 (29.8%)	3 (21.4%)	5 (35.7%)	6 (42.9%)
Secondary	9 (19.1%)	1 (11.1%)	3 (33.3%)	5 (55.6%)
Tertiary	5 (10.6%)	1 (20.0%)	1 (20.0%)	3 (60.0%)
TOTAL	**47 (100%)**			

Distribution of *Enterobacter cloacae* based on Socio–Economic Status

The distribution of *Enterobacter cloacae* based on socio–economic status of the pregnant women studied was presented in Table 4. The result differs significantly ($p > 0.05$) in their socio–economic status. Out of the 167(66.8%) specimens that tested positive to different UTIs, 47(28.1%) of them were infected with *Enterobacter cloacae*. Only 18 (38.3%) of the pregnant women infected with *E. cloacae* were employed while 29 (61.7%) of them were unemployed. The results of the biochemical test showed that culture technique had 8 (44.4%) for employed samples and 11(37.9%). Meanwhile, isolation by wet techniques showed 7 (38.9%) and 10 (34.5%) respectively whereas Gram-staining method had 3 (16.7%) for employed group and 7 (24.1%) for unemployed pregnant women examined.

Table 4: Distribution of *Enterobacter cloacae* based on Socio–Economic Status

Economic Status	PW/Rate	GS/Inf. Rate	Wet/Inf. Rate	Culture/Inf. Rate
Employed	18 (38.3%)	3 (16.7%)	7 (38.9%)	8 (44.4%)
Unemployed	29 (61.7%)	7 (24.1%)	10 (34.5%)	11 (37.9%)
TOTAL	**47 (100%)**			

Key: PW/Rate = Number of pregnant women examined; GS/Inf. Rate = Isolation by Gram-Staining; Wet/Inf. Rate = Isolation by wet preparation; Culture/Inf. Rate = Isolation by culture.

***Enterobacter cloacae* Cases among Pregnant Women Based on their Trimester of Pregnancy**

Table 5 showed the number of *Enterobacter cloacae* cases among pregnant women based on their trimester of pregnancy. The result revealed that trimester of pregnancy brought about

significant difference ($p > 0.05$) in the infection rate among the pregnant women. Out of the total number of women studied, 7 (14.9%) of them were in their first trimester (1-13 weeks) while 16 (34.0%) were in their second trimester (14-26 weeks) and 24 (51.1%) were recorded in third trimester (27–40 weeks). Among the isolation methods used, culture method showed the highest infection rate 3 (47.9%), 8 (50.0%) and 13 (54.2%) respectively across the three trimesters (1st, 2nd and 3rd) followed wet isolation 2 (28.6%), 5 (31.3%) and 7 (29.2%) respectively while Gram-staining method 2 (28.6%), 3 (18.8%) and 4 (16.7%) respectively showed the least number of isolate.

Table 5: Number of *Enterobacter cloacae* cases among pregnant women based on their trimester of pregnancy

Trimester	PW/Rate	GS/Inf. Rate	Wet/Inf. Rate	Culture/Inf. Rate
1st (≤ 13 weeks)	7 (14.9%)	2(28.6%)	2(28.6%)	3 (47.9%)
2nd (14 - 26weeks)	16 (34.0%)	3(18.8%)	5(31.3%)	8(50.0%)
3rd (27 - 40weeks)	24(51.1%)	4(16.7%)	7(29.2%)	13(54.2%)
TOTAL	**47 (100.0%)**			

Key: PW/Rate = Number of pregnant women examined; GS/Inf. Rate = Isolation by Gram-Staining; Wet/Inf. Rate = Isolation by wet preparation; Culture/Inf. Rate = Isolation by culture.

Enterobacter cloacae Case among Pregnant Women based on their Frequency of Pregnancy

Table 6 shows the number of *Enterobacter cloacae* case among pregnant women based on their frequency of pregnancy. The result revealed the number of pregnancies increases *Enterobacter cloacae* infection rate at $p > 0.05$. They showed that 9 (19.1%) of pregnant women infected with *E. cloacae* have had 1–3 pregnancies, 13 respondents representing 27.7% specimens infected

with *E. cloacae* have had 4 – 5 pregnancies whereas 25 respondents representing 53.2% have had from 7 pregnancies and above. Therefore, the observation showed that most of the pregnant women infected with *E. cloacae* have had from 7 pregnancies and above. To identify most effective isolation technique, the study revealed 4 respondents that have had 1 to 3 pregnancies representing 44.4% were positive by culture isolation technique, 3 (33.3%) for wet isolation technique and 2 (22.2%) for isolation by Gram-Staining. Among groups that have had 4 to 5 pregnancies, 6 samples representing 46.2% were positive by culture isolation technique, 4 (30.8%) for wet isolation technique and 3 (23.1%) for microscopic technique (Gram-Staining). Similarly, respondents who have had 7 pregnancies and above showed that 10 representing 40.0% were recorded from culture isolation technique, 9 (36.0%) from wet isolation technique and 6 (24.0%) for Gram-Staining techniques. Therefore, Culture method showed the highest number of *Enterobacter cloacae* infection in each group of pregnant women followed by wet isolation method while Gram-staining method showed the least result as shown in Table 6.

Table 6: Number of *Enterobacter cloacae* case among pregnant women based on their frequency of pregnancy

Pregnancy No	**PW/Rate**	**GS/Inf. Rate**	**Wet/Inf. Rate**	**Culture/Inf. Rate**
1 -3	9 (19.1%)	2 (22.2%)	3 (33.3%)	4 (44.4%)
4 -5	13 (27.7%)	3 (23.1%)	4 (30.8%)	6 (46.2%)
7 & Above	25 (53.2%)	6 (24.0%)	9 (36.0%)	10 (40.0%)
TOTAL	**47 (100.0%)**			

Key: PW/Rate = Number of pregnant women examined; GS/Inf. Rate = Isolation by Gram-Staining; Wet/Inf. Rate = Isolation by wet preparation; Culture/Inf. Rate = Isolation by culture.

Age Distribution of Symptomatic case of *Enterobacter cloacae* Infected Women Examined.

Table 7 shows the age distribution of symptomatic cases of *Enterobacter cloacae* infected women examined. Among other pathogens isolated in this study, the frequency of *Enterobacter cloacae* was 47 representing 28.1% of the total isolates. Symptomatic pregnant women within the age bracket 15 – 20 years were 19 representing 38.3%. However, 7 samples representing 36.8% within the age bracket 15 – 20 years tested positive out of which 1 (14.3%) was from Gram-staining method, 2 (28.6%) from wet preparation and 4 (57.1%) from culture preparation. In 21 – 25 years age group were 4 (36.4%) tested positive representing1 (25.0%), 1 (25.0%) and 2 (50.0%) for Gram-staining, wet preparation and culture preparation respectively. Age group of 26 – 30 years had 3 (42.9%) positive results which includes 1(33.3%) in all the techniques. Within 31 – 35 years age bracket, 2 (40.0%) tested positive identified by on culture preparation and wet preparation methods with 1 (50.0%) each. Age 36 – 40 and 41-45 years had 1 (33.3%) and 1 (50.0%) respectively, each were identified using culture preparation method. Among the isolation techniques used, Culture method showed the highest number of *Enterobacter cloacae* infection in each age group of pregnant women followed by wet isolation method while Gram-staining method showed the least result.

Table 7: Age distribution of symptomatic case of *Enterobacter cloacae* infected women examined.

Age (Year)	**PW/Rate**	**No of Positive (%)**	**GS/Inf. Rate**	**Wet/Inf. Rate**	**Culture/Inf. Rate**
15 – 20	19 (40.4%)	7 (36.8%)	1 (14.3%)	2 (28.6%)	4 (57.1%)
21 – 25	11 (23.4%)	4 (36.4%)	1 (25.0%)	1 (25.0%)	2 (50.0%)

26 – 30	7 (14.9%)	3 (42.9%)	1(33.3%)	1 (33.3%)	1 (33.3%)
31 – 35	5 (10.6%)	2 (40.0%)	0 (0%)	1 (50.0%)	1 (50.0%)
36 – 40	3 (6.4%)	1 (33.3%)	0 (0.0%)	0 (0.0%)	1 (100.0%)
41-45	2 (4.3 %)	1 (50.0%)	0 (0%)	0 (0%)	1 (100.0%)
TOTAL	**47(100.0%)**	**18**			

Key: PW/Rate = Number of pregnant women examined; GS/Inf. Rate = Isolation by Gram-Staining; Wet/Inf. Rate = Isolation by wet preparation; Culture/Inf. Rate = Isolation by culture.

Age Distribution of Asymptomatic Case of *Enterobacter cloacae* Infected Women Examined.

Table 8 shows the age distribution of asymptomatic cases of *Enterobactercloacae* infected women examined. Among other pathogens isolated in this study, the frequency of *Enterobactercloacae* was 47 representing 28.1% of the total isolates. Asymptomatic pregnant women within the age bracket 15 – 20 years were 29 representing 61.7%. However, 12 samples representing 63.2% within the age bracket 15 – 20 years tested positive of which 2 (16.7%) was obtained by Gram-staining method, 4(33.3%) from wet preparation and 6 (50.0%) from culture preparation. In 21 – 25 years age group 7 (63.6%) tested positive representing 2 (28.6%), in both Gram-staining and wet preparation method and 3 (42.9%) for culture isolation method. Age group of 26 – 30 years had 4 (57.1%). Within 31 – 35 years age bracket, 3 (60.0%) tested positive which includes 1(33.3%) in all the techniques. Age 36 – 40 years had 2 (66.7%) of positive result identified by culture preparation. Among the isolation techniques used, Culture method showed the highest number of *Enterobactercloacae* infection in each age group of

pregnant women followed by wet isolation method while Gram-staining method showed the least result.

Table 8: Age Distribution of Asymptomatic Case of *Enterobactercloacae* Infected Women Examined.

Age (Year)	PW/Rate	No of Positive (%)	GS/Inf. Rate	Wet/Inf. Rate	Culture/Inf. Rate
15 – 20	19 (40.4%)	12 (63.2%)	2 (16.7%)	4(33.3%)	6 (50.0%)
21 – 25	11 (23.4%)	7 (63.6%)	2 (28.6%)	2 (28.6%)	3 (42.9%)
26 – 30	7 (14.9%)	4 (57.1%)	1(25.0%)	1 (25.0%)	2 (50.0%)
31 – 35	5 (10.6%)	3 (60.0%)	1 (33.3%)	1 (33.3%)	1 (33.3%)
36 – 40	3 (6.4%)	2 (66.7%)	0 (0.0%)	1 (50.0%)	1 (50.0%)
41-45	2 (4.3 %)	1 (50.0%)	0 (0%)	0 (0%)	1 (100.0%)
TOTAL	**47(100.0%)**	**29**			

Key: PW/Rate = Number of pregnant women examined; GS/Inf. Rate = Isolation by Gram-Staining; Wet/Inf. Rate = Isolation by wet preparation; Culture/Inf. Rate = Isolation by culture.

Distribution of Pregnant Women Infected with *Enterobactercloacae*based on their Resident

Table 9 presented the distribution of the respondents infected with *E. cloacae* based on their residents. The result showed that the residents of the pregnant women brought about significant difference in the infection rate at $p > 0.05$. Among the pregnant women attending General Hospital Biu, Borno State, 33 respondents representing 70.2% are rural dwellers while 14 representing 29.8% were from urban area. Therefore, the observation showed that most of the pregnant women infected with *E. cloacae* were from different rural areas. On the techniques

used, isolation by culture had 16 (48.5%) for rural dwellers and 9 (64.3%) for urban dwellers followed by Isolation by wet preparation which had 11 (33.3%) for rural dwellers and 2 (14.3%) for urban dwellers while the least result was observed in Gram-Staining techniques that had 6 (18.2%) for rural settlers and 3 (21.4%) for urban dwellers. This implies that isolation by culture techniques was the best for technique for examining UTIs among the pregnant women studied.

Table 9: Distribution of Pregnant Women Infected with *Enterobacter cloacae* based on their Resident

Resident	PW/Rate	GS/Inf. Rate	Wet/Inf. Rate	Culture/Inf. Rate
Rural	33 (70.2%)	6 (18.2%)	11 (33.3%)	16 (48.5%)
Urban	14 (29.8%)	3 (21.4%)	2 (14.3%)	9 (64.3%)
TOTAL	**47 (I00.0%)**			

Key: PW/Rate = Number of pregnant women examined; GS/Inf. Rate = Isolation by Gram-Staining; Wet/Inf. Rate = Isolation by wet preparation; Culture/Inf. Rate = Isolation by culture.

Electropherogram of DNA Ladder and PCR Product on Agarose Gel

Figure 2 showed the PCR amplification for the confirmation of *Enterobacter cloacae* was carried out for the confirmation of *Enterobacter cloacae* was carried out using a total of 5µl of DNA solution and a master mix Accupower hot start PCR premix by bioneer. Figure 2 represents the electropherogram of 100bp DNA ladder and the PCR product of *Enterobacter cloacae* separated on 1% agarose gel. Gel-well labelled "L" contains 100Bb DNA ladder, "GS" represents *E. cloacae* isolated using Gram-staining method. "W" represents *E. cloacae* by wet isolation method while "C" stands for isolate obtained from culture isolation method.

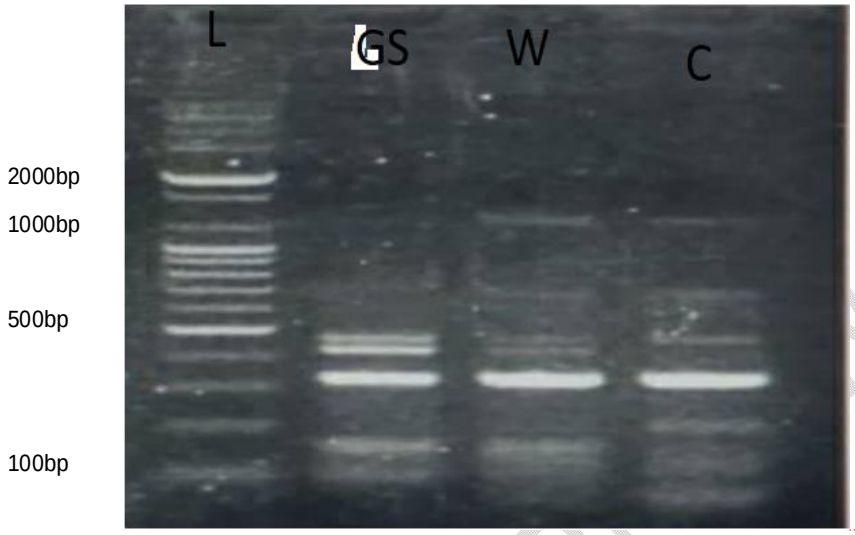

Figure 2: Electropherogram of DNA Ladder and *Enterobacter cloacae* on 1% Agarose Gel.

Well labelled L contains 100Bb DNA ladder, GS represents *E. cloacae* isolated using Gram-staining method. W represents *E. cloacae* by wet isolation method while C stands for isolate obtained from culture isolation method.

Phylogenetic Tree of *Enterobactercloacae*

Figure 3 showed the degree of relatedness among species of *Enterobactercloacae*. The sequence of amplified DNA of *Enterobacter cloacae* isolated from the virginal swabs confirmed that the isolate was truly the suspected pathogen.

Comment [SA35]: The results of tested samples ar not clear at all , several bands are found,
Did the author select only one sample for all cases ??

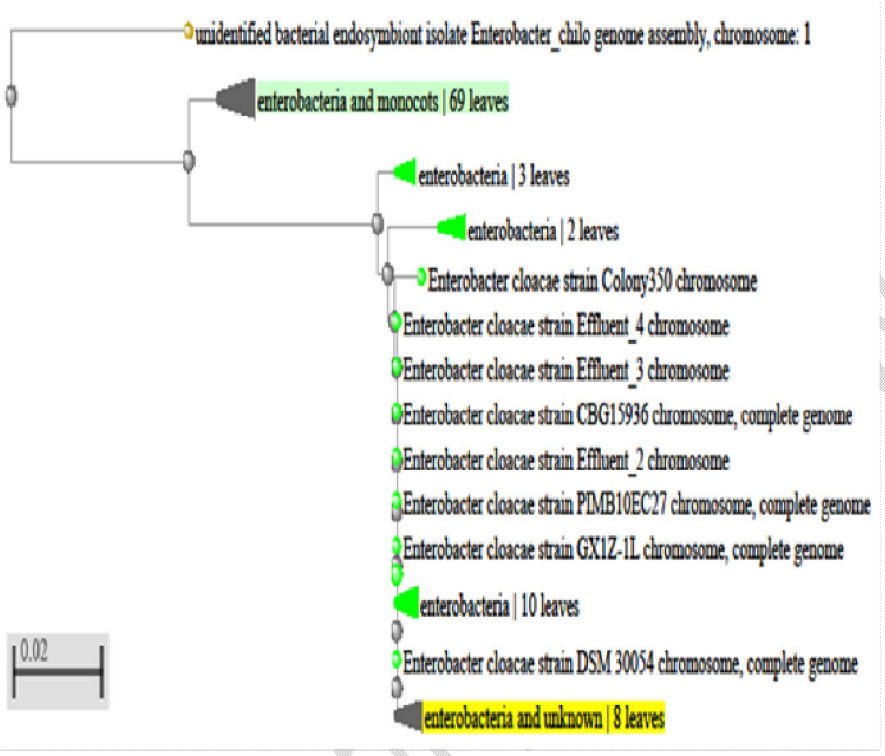

Figure 3 Phylogenetic tree of *Enterobactercloacae*

Discussion

Comment [SA36]: Discussion could be revised after correcting the material and method and the results of the manuscript.

The high prevalence rate of UTIs (66.8%) in this study is higher than the prevalence of UTIs among pregnant women studied from other regions of the nation. Onoh*et al*., (2013) reported 46.5% of UTIs in Ebonyi, Eastern Nigeria, 35.5% in Ilorin, North Central Nigeria (12), 31.6% in Kano, Northern Nigeria (12), and 32.7% in Benin, Southern Nigeria (13). This is in agreement to a study reported in Makurdi, North central Nigeria, which accounted for 66.7% of the bacteria isolated (14).

The high prevalence of *Enterobacter cloacae* (28.1%) and Klebsiella pneumoniae (21.6%) as seen among pregnant women within 15- 20 years age bracket could be attributed to differences in study areas as the principal microorganisms causing Early Onset Neonatal Sepsis (EONS) responsible for premature rupture of membrane, low birth weight, prematurity, low Apgar score at birth and meconium stained amniotic fluid. This result is in line with the report of Mulinganya*et al*., (2021)(15) who noted that the two main microbes responsible for EONS in Bukavu (DRC) are *Enterobacter cloacae* and *Klebsiella pneumoniae*. Several studies also opined that Gram-negative bacteria are responsible for more than 85% cases of UTI (16-18). This is in contrast to the studies carried out in Minna (Northern Nigeria), North India, Kano (Northern Nigeria) reporting Escherichia coli and Staphylococcus aureus as the prevalent causes of UTIs ((19). Reports on the incidence of bacteria causing UTIs established that E. coli, S. aureus, Klebsiella, Pseudomonas, and Proteus are the most prevalent species (20-21) which is in disagreement with the present study.

Age distribution of *Enterobactercloacae* infections among pregnant women in this study established that pregnant women within the age of 15–20 years old were mostly affected by UTIs caused mainly by *E. cloacae*. This high prevalence among these categories of women could be as a result of consumption of contaminated food, socio-economic status, attitude to personal hygiene and educational exposure (18). This study is also in line with study carried out by Yunusa and Kolade, (2015) which suggested that UTIs caused by bacteria are common among elderly women but most common among pregnant women especially in sexually active women. Pregnant women are immuno-compromised hosts for bacterial infections (22). In terms of technique for isolation of bacterial agents, this study indicated that culture method of isolation showed the highest number of isolates compared to wet isolation and gram staining.

The prevalence of *E. cloacae* in terms of literacy level in this study was high among illiterates (40.4%) who had never attended any formal education. This established the fact that the level of education/literacy plays a significant role in the management or control of bacterial infection among pregnant women. This may be due to lack of proper knowledge on how to maintain good personal hygiene. Findings of this study are consistent with that made in Kano, who proposed that people without a formal education had a greater prevalence of UTI (19).

This present study established that the prevalence of *E. cloacae* based on the socio-economic status of pregnant women infected with UTI was high among the unemployed (61.7%). This favors the report made by Ali and Abdallah, (2015) who noted that infectious diseases are encouraged by poverty and a low standard of living, which are both correlated with unemployment.

The present study proved that the number of *Enterobacter cloacae* cases among pregnant women based on their trimester of pregnancy differs significantly ($p > 0.05$). The infection rate increased from 23.2 to 51.1%. The prevalence (51.1%) was recorded among pregnant women in their third trimester (27–40 weeks). This study is in accordance with the reports on gestational age of the patients which stated that the prevalence of UTI in pregnant women has higher incidence among those in the third trimester (19, 23-26). This is because, in the third trimester, a larger uterus puts pressure on the ureter, and pregnancy hormones' growing ability to relax smooth muscles as well as pressure from the descending region of the bladder can cause urine to become stagnant, which can promote the growth of bacteria (27)(23)(26). However, this finding contrasts with that of Akpan*et al*., (2019) who found that the highest prevalence (66.7%) of severe bacteriuria was higher in the second trimester (14 – 26 weeks) (13).

The high prevalence of UTI among pregnant women with high parity or number of pregnancies (7 & above) in this study was (53.2%) is in consonant with the report made by Okonko*et al*., (2009) (28) who demonstrated that parity is one of the potential variables influencing the prevalence and incidence of UTIs in women. However, this study disagrees with the findings of Onuh*et al*., (2006) who claimed that there was no relationship to parity, this conclusion also connects to the research of (29). These variations could be due to the different study areas in which these studies were conducted.

In this study, pregnant women without symptoms (asymptomatic) had a frequency of substantial bacteriuria (63.2%) within the age bracket of 15 – 20 years, compared to pregnant women with symptoms (symptomatic) who had a frequency of 36.8%. This may be attributed to the shortness of their urethra and certain social circumstances, such as personal hygiene and sexual activity (30)(31). However, several studies disagreed with this opinion, noting differences in the frequencies between study areas might be due to different cultural practices, living standards, and category of the study population (2)(32)(17).

The majority of UTI patients among the study subjects were rural residents of which rural resident had the highest rate (70.2%) of *E. cloacae* compared to urban residents. This may be a result of the poor environmental sanitation and living circumstances in rural areas, as well as the fact that most rural residents had poor personal hygiene habits and that could increase their risk of developing *E. cloacea.* This result supported the findings of studies carried out in Abakaliki by Nworie and Eze, (2010) (14) and in Kano by Ali and Abdallah (2015) (19), which discovered that among the reasons contributing a high prevalence of UTI among rural residents were inadequate personal cleanliness and poor housing conditions (4).

This study also demonstrated culture technique as the best biochemical method for isolation of bacterial agents compared to wet mount and microscopic Gram-staining isolation method.

The result of gel electrophoresis showed that the bands formed by the DNA sequences of *Enterobacter cloacae* were comparable to the molecular weight of the 100bp DNA ladder used. The PCR product was used to generate *Enterobacter cloacae* phylogenetic tree.

Conclusion

The high occurrence rate of UTIs found in this study should be of great concern as the UTI during pregnancy do not only pose threats to health of the mother and the fetus but also lead to economic and social burden due to the stigma associated with these infections. The results of this study necessitate improve on personal hygiene as one of the strategies for combatting UTIs among pregnant women and other vulnerable hosts.

More than a single test procedure may be required to adequately diagnose the presence of *E. cloacae* in infected individuals. The use of Polymerase Chain Reaction (PCR) technique has proven to be the most sensitive technique for the diagnosis of *E. cloacae* infection. However, this technique is quite expensive and requires a high level of technical skill compared to Gram staining, wet mount and culture techniques. Meanwhile, culture technique can also be considered as the gold standard for the diagnosis of *E. cloacae* infection while Gram-stain and wet mount technique may also be used for the diagnosis of *E. cloacae* infection in resource limited settings. Age and literacy level are considered as a risk factor of the bacterial pathogen because in terms of Age, pregnant women within the age range of 12–17 are been given out for marriage and they are not lack educational exposure.

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