Prevalence and molecular characterization of extended spectrum beta-lactamase producing uropathogenic *Escherichia coli* in a tertiary health care facility in Anambra State, Nigeria

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Abstract

**Background:** The prevalence of ESBLs-producing *Escherichia coli* has recently increased worldwide due to the expression of ESBL genes which had led to high rate of multidrug resistance antibiotics.

**Aim:** To determine the antimicrobial susceptibility patterns of *E. coli* isolates and evaluating the ESBL carriage of these isolates at phenotypic and genotypic levels.

**Methods:** One hundred and two clinical *Escherichia coli* isolates were recovered from UTI suspects and analyzed for ESBL production at phenotypic and genotypic levels using Modified Double Disc Synergy Test and Polymerase Chain Reaction respectively.

**Results:** Of the 102 isolates, 100(98.04%) were associated with MDR phenotypes. The isolates showed variable resistance to all the antibiotics used in the study. The resistance rates were 99.0%, 97.1%, 88.2%, 82.4%, 81.4%, 65.7%, 54.9%, 46.1%, 46.1%, 23.5% for ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, ceftriaxone, ceftazidime, amoxicillin-clavulanate, gentamycin, cefoxitin, nitrofurantoin and imipenem, and respectively. The prevalence of phenotypic ESBL production was 74.5%. Based on the PCR results, the randomly selected 20 ESBL-positive isolates possessed one or more ESBLs genes. CTX-M type was the predominant ESBLs type (100%), while those for TEM and SHV-types were 85% and 80% respectively. Four genotype patterns were detected (CTX-M, TEM+CTX-M, SHV+CTX-M and SHV+TEM+CTX-M). The genotype SHV+TEM+CTX-M, was the predominant (70%), followed by the genotype TEM+CTX-M combination (15%). The occurrences of the genotypes, CTX-M and SHV+CTX-M were 5% and 10% respectively.

**Conclusion:** This study found a high rate of Phenotypic ESBL production (74.5%) among the isolates with multidrug resistance, CTX-M as the predominant ESBLs type (100%) and combination of SHV+TEM+CTX-M as the predominant genotype (70%).

**Keywords:** *Escherichia coli*; Extended spectrum beta-lactamases; Modified double disc synergy test; Anambra State

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1. Introduction

A urinary tract infection (UTI) is a situation in which one or more sites of the urinary system (kidneys, ureters, bladder, and urethra) become infected. Urinary tract infections are a universal health issue in both outpatient and inpatient locations, and urine cultures bear most of the workload in practically all clinical Microbiology laboratories. Approximately 95% of cases of UTIs are produced by bacteria that consistently proliferate at the orifice of the urethra and navigate up to the bladder. Occasionally, bacteria disseminate to the kidney from the bloodstream. Urinary tract infections are severe public health problems and are caused by a diverse collection of organisms, but frequently by *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus saprophyticus* [1]. Extended-spectrum β-lactamase-producing Enterobacteriaceae is an emerging public health concern. Complications in UTIs have increased because of the prevalence of extended spectrum beta-lactamas (ESBL) producing bacterial pathogens which are also causing many management and epidemiological issues [2]. These complications came out from the increasing resistance among UTI pathogens to conventional drugs. Although a wide range of pathogens can cause UTI, *E. coli* continues to be the most common cause due its ubiquitous presence in the perianal area.

Production of beta-lactamase is one of the most critical mechanisms in Gram-negative bacteria against beta-lactam antibiotics. Due to the treatment of bacterial infections with the broad-spectrum antibiotics, it leads to the production of broad-spectrum enzymes called beta lactamas [3]. These hydrolyse penicillins, broad-spectrum cephalosporins, and monobactams. Beta-lactamases species are ordinarily derived from TEM and SHV-type enzymes [4]. Extended spectrum beta lactamas (ESBLs) -producing *Enterobacteriaceae* have been responsible for various outbreaks of infection around the globe and pose challenging infection prevention problems [5]. In the previous 20 years, Gram-negative bacteria have elaborated their resistance to broad spectrum beta-lactam antibiotics [6]. Scientists have discovered more than 400 types of ESBLs, and majority of them reside in the *Enterobacteriaceae* family [7].

Uropathogenic *Escherichia coli* (UPEC) are one bacterium that can induce ESBL enzymes and can distinguish between phenotypic or genotypic methods [8]. The genotypic methods are more specific at identifying such resistant strains. ESBLs comprise many plasmid-mediated derivatives such as TEM, OXA, and SHV. Since 2000, a new group of ESBLs, called CTX-M (i.e., “active on CefoTaXime, first isolated in Munich”), has emerged [9]. Since then, CTX-M β-lactamas have been the predominant ESBL type worldwide [10]. This group of ESBLs has been associated with an extensive pattern of antimicrobial resistance to many antibiotics, including β-lactam agents such as penicillins, cephalosporins, monobactams, and carbapenems [11]. In addition, CTX-M-producing *E. coli* strains are often associated with co resistance to other large antimicrobial families such as aminoglycosides and fluoroquinolones. This increasing level of antimicrobial resistance of UPEC is of great concern since it can limit the therapeutic choices used for treating common bacterial infections such as UTIs and highlights the growing threat of the emergence of pan drug resistance in UPEC.

Molecular genotyping has been used along with phenotyping techniques to screen and confirm expression of antimicrobial drug resistance within a population [12]. Genotyping may help in controlling the spreading of pathogens, by tracing the origin of outbreaks. This area is often referred to as molecular epidemiology. The prevalence of ESBLs is increasingly being reported worldwide, and it varies according to geographic location and is directly linked to the use and misuse of antibiotics [13]. Incidentally the laboratory detection of ESBLs can be more complex and is not routinely performed in most laboratories. In appreciation of the above-outlined issues, this study was aimed at determining the antimicrobial susceptibility patterns of a collection of clinical *E. coli* urine isolates, to phenotypically assess the ESBL carriage of these isolates and to characterize the ESBL genotypes (TEM, SHV, and CTX-M genes) of the isolates.

2. Material and methods

2.1 Ethical approval

This study was approved by the Ethics Committee of Nnamdi Azikiwe University Teaching Hospital, (NAUTH), Nnewi (Approval No. NAUTH/CS/66/VOL.11/149/2018/083).

2.2 Sources and identification of Bacterial Isolates

A total of 102 nonconsecutive, non-duplicate clinical *Escherichia coli* urine isolates were recovered from urinary tract infections suspects within the period of May to December, 2020 in Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State, Nigeria. Urine specimens were inoculated on CLED, EMB, and MacConkey using standard techniques. Plates were incubated overnight at 37 °C and inspected for growth [14]. Isolates were identified based on
morphological and biochemical characteristics. Morphological characters include colony diameter, shape, texture, height, edge, color and reaction on the standard media used. Gram staining for all isolates was done. Routine biochemical tests i.e., lactose fermentation, indole production, Methyl red test, citrate utilization, urease and motility were tested according to Bergey’s manual [15].

2.3. Antibiotic susceptibility testing

Antimicrobial susceptibility was determined by using Kirby-Bauer disk diffusion technique recommended by Clinical and Laboratory Standards Institute CLSI guidelines [16]. Antimicrobial agents used in this study were: Ampicillin (AMP-10µg), Trimethoprim/Sulphamethoxazole(SXT-1.25/23.75µg), Ceftazidime(CAZ-30µg), Ceftriaxone(CRO-30µg), Cefoxitin(FOX-30µg), Gentamicin(CN-10µg), Nitrofurantoin(F-300µg), Ciprofloxacin(CIP-5µg), Amoxicillin-clavulate(AMC-20/10µg) and Imipenem (IPM-10µg). Discs were purchased from Oxoid Ltd, Basingstoke, Hampshire U.K.

2.4. Phenotypic detection of ESBL production

All the isolates which showed a diameter of less than 22mm for ceftazidime(30µg) and less than 25mm for ceftriaxone were identified as potential ESBL producers, then selected for ESBL production [17,18]. The ESBL production was tested by the Modified Double Disc Synergy Test (MDDST) by using a disc of amoxicillin-clavulanate (20/10 µg) along with four cephalosporins; 3GC-cefotaxime, ceftriaxone, ceftazidime and 4GC-cefepime [18]. A lawn culture of the organisms was made on a Mueller-Hinton agar plate, as was recommended by CLSI guidelines [16]. A disc which contained amoxicillin-clavulanate (20/10 µg) was placed at the centre of the plate. The discs of 3GC and 4GC were placed 15mm and 20mm apart respectively, centre to centre to that of the amoxicillin-clavulanic acid disc. Any distortion or increase in the zone towards the disc of amoxicillin-clavulanate was considered as positive for the ESBL production.

2.5. Molecular (Genotypic) Characterization of ESBLs genes

Out of the 76 confirmed ESBL phenotypes detected in this study, twenty (20) isolates were randomly selected for ESBL molecular characterization to detect blaCTX-M, blaTEM and blaSHV genes by PCR technique.

2.5.1. Extraction of the E. coli DNA for PCR

The extraction of the DNA was done using Quick-DNATM Miniprep Plus Kit (Zymo Research) according to manufacturer’s instructions. The primers used and their amplicon sizes were as shown in Table 1.

| Target Gene | Primer name | Primer sequences(5’-3’) | Product size (bp) | Reference |
|-------------|-------------|-------------------------|-------------------|-----------|
| blaCTX-M    | CTX-M-F     | CGCTTTGCGATGTGCAG        | 500/512           | 19        |
|             | CTX-M-R     | ACCGCGGATATCGTTGGT       |                   |           |
| blaTEM      | TEM-F       | TCAACATTTCCGTGTGGC       | 800               | 19        |
|             | TEM-R       | CTGACAGTTACCAATGCTTA     |                   |           |
| blaSHV      | SHV-F       | CGCCTGTGTATTATCTCCCT     | 300               | 19        |
|             | SHV-R       | CGAGTAGTCCACCAGATCCT     |                   |           |

2.5.2. Amplification of DNA

The PCR amplification was performed in 12.5µl of One Taq Quick-Load 2X Master Mix with Standard Buffer (New England Biolabs Inc.); 0.5µl each of forward and reverse primers; 8.5µl of Nuclease free water and 3µl of DNA template was used to prepare 25µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to an Eppendorf nexus gradient Mastercycler (Germany), for thermal cycling. The cycling parameters were at initial denaturation of 94°C for 5 minutes, denaturation of 94°C for 30secs, annealing at 55°C for 1 min, followed by extension step of 72°C for 2 minutes and final extension of 72°C for 5 minutes for both CTX-M and TEM genes, then initial denaturation at 94°C for 5 minutes,denaturation of 94°C for 30secs, Annealing at 54°C for 30 secs, followed by extension of 72°C for 1 minute and final extension at 72°C for 10 minutes for the SHV gene.
2.5.3. Preparation of agarose gel Electrophoresis

After amplification the products were separated on a 2% agarose gel electrophoresis and amplified bands were visualized alongside a 100bp DNA ladder using gel documentation system with the help of ultraviolet light.

2.5.4. Statistical Analysis

Results were entered into an Excel spread sheet for Windows 9 and analysed using the Stata corp 16. Chi square test was used to determine the association or relationship between ESBL production and multi-drug resistance among the isolates. Results were presented in tables, bar chart and percentages. P value was considered at 95% confidence level and P values less than 0.05(p<0.05) were considered statistically significant.

3. Results

3.1. Demographic Characteristics of study population

In this study, a total of 102 Uropathogenic Escherichia coli (UPEC) isolates were obtained from urine samples of UTIs suspects that attended the Nnamdi Azikiwe University Teaching Hospital, Nnewi within the period of the study. Among these isolates, 63(61.76%) were from female participants, while 39 (38.24%) were from male participants. Majority of the study participants are within the age range of 60-69 years (22.55%) with a mean± SD of 50.6 ± 19.364 years.

Demographic characteristics of patients are shown in Table 2.

Table 2 The socio-demographics of the study participants

| Socio-demographics | Frequency | Percentage (%) |
|--------------------|-----------|----------------|
| Age group          |           |                |
| <20 years          | 5         | 4.90           |
| 20-29 years        | 10        | 9.80           |
| 30-39 years        | 19        | 18.63          |
| 40-49 years        | 12        | 11.76          |
| 50-59 years        | 14        | 13.73          |
| 60-69 years        | 23        | 22.55          |
| 70-79 years        | 12        | 11.76          |
| 80-89 years        | 7         | 6.86           |
| Mean ± SD          | 50.6±19.364 |                |
| Gender             |           |                |
| Females            | 63        | 61.76          |
| Males              | 39        | 38.24          |
| Ward               |           |                |
| A/E                | 18        | 17.65          |
| ANC                | 1         | 0.98           |
| CHER               | 1         | 0.98           |
| CHOP               | 1         | 0.98           |
| ENDO               | 2         | 1.96           |
| FMW                | 8         | 7.84           |
| FSW                | 1         | 0.98           |
| GOPD               | 10        | 9.80           |
3.2. Antimicrobial Susceptibility Profiles

The antibiotic susceptibility testing results are shown in Table 3. Among the ten different antibiotics tested, imipenem was the most active agent with 48.04% and 28.4% susceptible and intermediately susceptible respectively. Out of the 102 isolates, 101 (99.02%), 67 (65.7%), and 56 (54.9%) were resistant to ampicillin, amoxicillin-clavulanic acid, and gentamycin respectively. The resistance rates for ceftazidime, ceftriaxone, cefoxitin, trimethoprim-sulfamethoxazole and imipenem were 81.4%, 82.4%, 46.1%, 97.1% and 23.5% respectively. Furthermore, 88.2% and 46.1% of the isolates were resistant to ciprofloxacin and nitrofurantoin respectively.

Table 3 The antibiotic susceptibility pattern of the different *E. coli* isolates tested in this study

| Antibiotic | Susceptible (%) | Intermediate (%) | Resistant (%) |
|------------|-----------------|------------------|--------------|
| AMP        | 1 (0.98)        | Nil              | 101 (99.02)  |
| AMC        | 14 (13.73)      | 21 (20.59)       | 67 (65.69)   |
| CN         | 46 (45.10)      | Nil              | 56 (54.90)   |
| CAZ        | 11 (10.78)      | 8 (7.84)         | 83 (81.37)   |
| CRO        | 6 (5.88)        | 12 (11.76)       | 84 (82.35)   |
| FOX        | 38 (37.25)      | 17 (16.67)       | 47 (46.08)   |
| SXT        | Nil             | 3 (2.94)         | 99 (97.06)   |
| IPM        | 49 (48.04)      | 29 (28.43)       | 24 (23.53)   |
| CIP        | Nil             | 12 (11.76)       | 90 (88.24)   |
| NIT        | 38 (37.25)      | 17 (16.67)       | 47 (46.08)   |

3.3. Multi-drug resistance

Hundred (98.04%) of the isolates were found to be multidrug resistant, while two (1.96%) isolates were non-Multidrug resistance (Table 4). Of the 102 UPEC isolates used in this study, 100 isolates were resistant to at least one agent in three or more antibiotic class; thus, indicating that most of the isolates were ESBLs-producers.
Table 4: The drug resistance pattern of the different \textit{E. coli} isolates used in the study

| Drug resistance pattern | Frequency | Percentage |
|-------------------------|-----------|------------|
| MDR                     | 100       | 98.04      |
| Non-MDR                 | 2         | 1.96       |
| Total                   | 102       | 100        |

MDR: Multi drug resistance

3.4. ESBL Prevalence

With regard to ESBL production, the phenotypic detection method using the Modified Double Disc Synergy Test (MDDST) revealed that 76 (74.5\%) of all the isolates were ESBL producers (positive), while 26 (25.5\%) were non-ESBL producers (negative).

3.5. Molecular characterization of ESBL genes

Twenty (20) ESBL-producing \textit{E. coli} isolates were randomly chosen and tested for the production of the ESBL genes (\texttt{bla} \texttt{CTX-M}, \texttt{bla} \texttt{TEM}, and \texttt{bla} \texttt{SHV}) by PCR. Information on the PCR primers used for gene detection is shown in Table 1. Amplified PCR-products for \texttt{bla} CTX-M, TEM and SHV genes gave molecular weights (band sizes) of 500/512,860 and 300 base pairs respectively as shown in Figure 1.

![Figure 1A](image1.png)

A.

![Figure 1B](image2.png)

B.
Figure 1 Amplified products of CTX-M, TEM and SHV genes. 2.0% Agarose gel analysis of PCR amplified fragment of (A) bla CTX-M (510/512bp) using CTX-M-F and CTX-M-R primers, (B) blaTEM (860bp) using TEM-F and TEM-R primers, (C) bla SHV (300bp) using the SHV-F and SHV-R primers. The 20 different lanes which were represented by the isolates project identification numbers are the PCR products of the ESBL genes from *E. coli* isolates. Lane M is the molecular weight ladder (DNA markers) spanning from 100-1000bp; NC and PC are PCR products of *E.coli* negative and positive controls respectively.

3.6. The prevalence of the three different ESBL genes

This study showed that CTX-M gene was the predominant type (20/20; 100%) while TEM and SHV genes were less predominant (17/20; 85% and 16/20; 80%, respectively). Figure 2 below showed the distribution of the ESBL genes in different wards of the hospital.

Figure 2 A column bar chart showing the distribution of the ESBL genes in the different wards in NAUTH, Nnewi.

3.7. Genotypic patterns of ESBLs

Analysis of the amplified products of the 20 *E. coli* ESBLs producers using the specific primers revealed that four genotype patterns were obtained (Table 5). These results showed that the most prevalent genotype was SHV+TEM+CTX-M (70.0%) combination, followed by TEM+CTX-M (15.0%) combination.
Table 5 ESBLs genotype patterns among the E.coli isolates

| ESBLs genotype          | Number of isolates | Percentage |
|-------------------------|--------------------|------------|
| blaCTX-M only           | 1                  | 5.0        |
| blaSHV only             | 0                  | 0          |
| blaTEM only             | 0                  | 0          |
| blaTEM and blaSHV       | 0                  | 0          |
| blaTEM and blaCTX-M     | 3                  | 15.0       |
| blaSHV and blaCTX-M     | 2                  | 10.0       |
| blaSHV + blaTEM + blaCTX-M | 14              | 70.0       |
| Total                   | 20                 | 100.0      |

4. Discussion

The emergence of Multidrug resistant (MDR) E. coli strains and the progressive rise in antimicrobial resistance threatens the effective treatment of UTIs leading to increased morbidity, prolonged hospital stay, increase in the cost of treatment and disease related mortality [20]. Extended spectrum beta-lactamases (ESBLs) production is an important mechanism used by these pathogens to jeopardize UTI treatment options; thus, early detection of the characteristics of antimicrobial resistance of these pathogens in a particular region will help to devise strategies that will reduce the extensive misuse of antimicrobial agents and prevent the emergence and subsequent spread of such MDR isolates.

The results of the antibiotic susceptibility showed that the isolates were highly resistant to ampicillin (99.0%), trimethoprim-sulfamethoxazole (97.1%), ciprofloxacin (88.2%), ceftriaxone(82.4%), ceftazidime(81.4%), amoxicillin-clavunate(65.7%). This finding is in agreement with other studies [21-23]. Alqasim and co discovered in their study that UPEC isolates have shown an increased level of antimicrobial resistance to frontline antibiotics such as trimethoprim-sulfamethoxazole and ciprofloxacin [17]. The high level of antimicrobial resistance seen could be attributed to the inappropriate and excessive use of these agents in the empirical treatment of UTIs [24]. Also imipenem in this study showed reduced susceptibility of 48.04%, intermediately susceptible (28.43%), and resistance rate of 23.5%. This is in contrast to other findings such as [23, 15, 18, 14, 24] with high susceptibility (for imipenem) rates of 100, 98, 100, 100 and 97.1% respectively. However, the finding is in conformity with studies of [25 - 26] with a resistance of 17.32 % and 22% respectively. The reduced susceptibility of imipenem seen in this study might be due to the production of carbapenemase enzyme by some of these isolates. This enzyme is capable of hydrolyzing carbapenems antibiotic group. The carbapenems (imipenem, meropenem, doripenem, e.t.c), which are the last resort in the effective treatment of severe ESBL-producing E. coli infections, have recently witnessed rise in resistance by E. coli strains that produced carbapenem-hydrolyzing enzymes [27].

The prevalence of multiple drug resistance (MDR) among the studied isolates was 98.04%. This is considered to be very high and in accordance with the reported findings of 85-100% in various parts of Nigeria and other African countries[28- 32 ]. Hassan et al and Elsayed et al [33, 14] also reported 100% and 95% MDR phenotypes respectively. These findings indicate that most of these isolates are ESBL producers capable of causing multidrug resistance to drugs; thereby jeopardizing UTIs treatment.

The genotypic (molecular) detection of the resistant ESBL genes among the randomly selected twenty ESBL positive isolates in this study revealed that the bla CTX-M gene was the predominant type (100%), while TEM and SHV-types were 85% and 80% respectively. This has been reported in other studies done in Nigeria [34,35,36,23] and studies done across the globe [22, 37, 38, 17]. Studies in Iraq and neighboring countries have declared that CTX-M type was the predominant gene type in both E. coli and Klebsiella pneumonia [39] and currently, CTX-M enzymes have replaced the traditional ESBL types such as SHV and TEM enzymes as the predominant ESBL type in E. coli[6]. However, studies from Turkey and India revealed that TEM was the predominant type [40].

The analysis of the PCR amplified products of the 20 isolates revealed that four genotype patterns of ESBLs existed among the isolates. Combination of SHV+TEM+CTX-M was the most prevalent genotype (70%), followed by the genotype combination TEM+CTX-M (15%). Further illustrations are seen in the study. The multiple genes (SHV, TEM and CTX-M) harboured by some of these isolates in their genome suggested the carriage of resistance plasmids [37]. A
This study by Polse et al., revealed six genotype patterns [22]. This might be because larger sample size of 55 isolates was used, but only 20 isolates was used in this study. The presence of more than one resistance ESBL genes in some of the isolates means that the ESBL-producing strains may be related to a complex antimicrobial resistance [22]; this has resulted to co-resistance to other antibiotics group apart from the β-lactams antibiotic groups. The TEM gene is a broad spectrum ESBLs that is always combined with CTX-M on the same plasmid [22]. The occurrence of SHV+TEM+CTX-M combination can cause resistance to carbapenems [41], as seen in the study. This is worrisome and more serious for the community, because carbapenems which is regarded as the antibiotic of last choice and mainstay of treatment used against infections caused by ESBLs are equally experiencing setback gradually due to emergence of carbapenamase-producing strains.

5. Conclusion

This study found a high rate of ESBL production (74.5%) in Escherichia coli isolates with high multi drug resistance pattern. Clinical Microbiology laboratories should routinely incorporate ESBL detection methods for continuous surveillance of multidrug resistant isolates and antibiograms to guide empirical therapy. Effective hospital-based infection prevention and control and antibiotic stewardship programs aimed at limiting the spread and emergence of resistant isolates should be instituted in our health-care facilities.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors declare that they have no conflict of interest to disclose.

Statement of ethical approval

The study protocol was reviewed and approved by the Research Ethics committee at Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State.

Authors’ Contributions

Conceptualization: Nneka R. Agbakoba; Investigation: Ifeoma G. Nwafuluaku; Methodology: Ifeoma G. Nwafuluaku, Simon N. Ushie; Supervision: Nneka R. Agbakoba; Writing-original draft: Ifeoma G. Nwafuluaku; Writing-review and editing: Eucharia A. Dilihe, Ifeoma G. Nwafuluaku, Simon N. Ushie, Nneka R. Agbakoba.

Abbreviations

MDR: Multidrug-resistance
UPEC: Uropathogenic Escherichia coli
E. coli: Escherichia coli
PCR: Polymerase Chain Reaction
MDDST: Modified Double Disc Synergy Test
ESBLs: Extended-Spectrum beta-lactamases
TEM: Temoneira (an ESBL enzyme named after a Greek patient)
SHV: Sulfhydryl variable
CTX-M: An extended-spectrum beta-lactamase with greater activity against cefotaxime: CTX for cefotaximase, and M for Munich.
NAUTH: Nnamdi Azikiwe University Teaching Hospital, Nnewi.
UTIs: Urinary Tract infections
CLSI: Clinical and Laboratory Standards Institute
Bla: Beta lactamase
CLED: Cysteine lactose Electrolyte Deficient
EMB: Eosine Methylene Blue
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