Antibiotic sensitivity of ESBL-producing gram-negative bacteria among patients of urinary tract infections in Southern Punjab, Pakistan

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Research Article

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Abstract

Urinary tract infections (UTIs) are one of the most commonly presented infections among men and women as they acquire it at least once in their lifetime and disease can recur. In recent era, one of the challenges faced by humans is progressively increasing dissemination of antimicrobial resistance among pathogens causing UTIs and other diseases. A hospital-based investigation was carried out including 200 UTI patients affirmed by clinicians. Samples selected based on initial screening by nitrite test were cultured and gram-stained. 161 isolates of gram-negative bacteria were characterized based on morphological and biochemical analysis. Antibiotic susceptibility testing was done by Kirby-Bauer disc diffusion method against 16 selected antibiotics. Genotyping was done for bla_{TEM} and bla_{CTX-M} by PCR. Out of the 161 isolated gram-negative bacteria, *E.* coli (N=116, 72%) was the most common followed by *Klebsiella oxytoca* (N=22, 13%), *Klebsiella pneumoniae* (N=14, 9%), *Proteus mirabilis* (N=6, 4%) and *Proteus vulgaris* (N=3, 2%). Antibiotic susceptibility testing revealed that isolated gram-negative pathogens were highly sensitive to Amikacin, Fosfomycin, Imipenem and Meropenem where as high level of resistance was observed against Ampicillin, Amoxicillin, Cefotaxime, Nalidixic acid and Norfloxacin. The bla_{TEM} and bla_{CTX-M} genotyping showed that around half of the isolates were positive for either or both of these genes. bla_{CTX-M} (57%) was described as being more common as compared to bla_{TEM} (45%). Pertinent to the rapidly evolving drug resistance patterns amongst pathogenic bacteria, conducting monitoring and surveillance studies to provide updates to physicians regarding latest and emerging trends of drug resistance is crucial globally.

1. Introduction

One of the most commonly arising infection in hospital as well as community is urinary tract infection (UTI) which is especially prevalent among elderly individuals [1]. Uncomplicated UTI is experienced by individuals without any structural or functional abnormality inside the urinary tract while complicated UTI presents in individuals with any structural or functional abnormality that can enhance the risk of developing infection or therapy failure [2]. Females are more inclined towards the acquirement of such infections as it has been estimated that more than half of all women experience at least one episode of UTI in their lifetime and 20 to 30% of them experience recurrent episodes of UTI [3]. Catheterization, without any uncertainty, is the most significant risk factor for UTI while some of the other risk factors include diabetes, kidney transplant, antibiotic use, sexual intercourse and anatomical abnormalities present in urinary tract [4]. Both gram-positive and gram-negative bacteria are frequently reported as being causative agents for UTIs. The most common etiological agent responsible for UTI is *Escherichia coli* although others like *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Proteus mirabilis* and *Enterococcus* spp. are also frequently implicated in the pathogenesis of UTI [5, 6].

Recently, multi-drug resistant (MDR) pathogens are increasingly being implicated in the etiology of UTI which presents an alarming situation where treatment options are becoming limiting with the passage of time [7]. Among MDR gram-negative pathogenic bacteria, extended-spectrum beta lactamase (ESBL) producers have recently been on rise among the isolates obtained from outpatient settings particularly

relevant to UTI [8]. ESBLs are enzymes encoded on plasmids that can make pathogens resistant to third and fourth generation antibiotics including not only cephalosporins but also monobactams which is a grave concern in terms of rapidly increasing antibiotic resistance in the present era [9]. To complicate the matters further, ESBL producing bacteria have been associated with worse clinical outcomes including clinical failure and requirement for parenteral administration of antibiotics as well as worse economical outcomes including cost of antibiotics and total pharmacy expenditures [10]. ESBLs include TEM- SHV- and cefotaxime enzyme (CTX-M) groups which are further divided into several sub-types and these genes are usually present along with other resistance genes on same plasmids contributing towards co-resistance [11]. Risk factors for acquiring infection by an ESBL-producer include recurrent UTI, age older than 64 years, recent antibiotic use and travel to areas with high prevalence of ESBL-producing bacteria [12]. Identification of patients who are at particularly increased risk of developing ESBL-producer infection as well as exploration of prevalence and epidemiology of ESBL-producers is essential not only for the assistance of patients but also for the healthcare system. Hence, the present investigation was aimed isolation and characterization of MDR gram-negative bacteria from UTI patients belonging to local population as well as PCR-based detection of bla_{TEM} and bla_{CTX-M} genes in selected isolates.

2 Patients And Methods

2.1 Sample collection and initial processing

This study was conducted at The Women University, Multan, Pakistan from January to June, 2020 after seeking ethical approval from the Institutional Ethical Review Committee. A total of 200 UTI patients diagnosed and confirmed by clinicians in Multan Institute of Kidney Diseases, Multan were included irrespective of age and gender after obtaining informed written consent. Urine samples of participants were collected in sealed containers so as to avoid contamination from air and transported to the Microbiology Laboratory of Multan Institute of Kidney Diseases. Within three hours of collection, nitrite test was performed on urine samples by dipstick method at 37°C. Samples that were positive for nitrite test were aseptically inoculated onto Cysteine Lactose Electrolyte Deficient (CLED) agar and incubated aerobically under recommended conditions. Glycerol stocks of bacterial isolates were prepared to preserve them at -20°C until further analysis.

2.2 Identification and antibiotic sensitivity testing

Gram staining was performed to distinguish between gram-positive and gram-negative isolates after recording colony characteristics. Biochemical characterization was done only for gram-negative isolates to identify them according to Burgey’s scheme of classification. Biochemical tests performed for this purpose included citrate, indole, urease, triple sugar iron (TSI) tests. For antibiotic sensitivity testing, Kirby-Bauer disc diffusion method was followed involving the use of Mueller-Hinton (MH) agar in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Inoculum of gram-negative bacterial isolates equivalent to 0.5 McFarland standard was prepared for this purpose. After dispensing the antibiotic discs, plates were incubated at 37°C for 24 hours after which zones of inhibition were

measured and recorded. Antibiotics included for sensitivity testing were Amikacin (AK, ≥ 17mm), Amoxicillin/Clavulanate (AMC, ≥ 18mm), Ampicillin (AMP, ≥ 17mm), Cefotaxime (CTX, ≥ 26mm), Ceftazidime (CAZ, ≥ 21mm), Ciprofloxacin (CIP, ≥ 21mm), Co-trimoxazole (SXT, ≥ 18mm), Fosfomycin (FOS, ≥ 16mm), Gentamicin (G, ≥ 15mm), Imipenem (IPM, ≥ 23mm), Meropenem (MEM, ≥ 23mm), Nalidixic acid (NA, ≥ 19mm), Nitrofurantoin (F, ≥ 17mm), Norfloxacin (NOR, ≥ 17mm), Piperacillin/Tazobactam (TZP, ≥ 21mm) and Sulbactam/Cefoperazone (SCF, ≥ 16mm).

2.3 DNA extraction and PCR genotyping

DNA was extracted by boiling method where bacterial colonies were emulsified in nuclease-free water and placed in water bath at 100°C for 15 min. Subsequently, centrifugation was done at 12000 rpm for 4min to separate the supernatant containing DNA which was then stored at -20°C until further use. PCR genotyping for bla_{TEM} was performed using forward primer 5'-TCAACATTTCCGTGTCG-3' and reverse primer 5'-CTGACAGTTACCAATGCTTA-3' and for bla_{CTX-M} genotyping, forward primer 5'-ATGTGCAGYACCAGTAARGT-3' and reverse primer 5'-TGGGTRAARTARGTSACCAGA-3' were used. Composition of the reaction mixture was 5µL DNA, 1µL each of forward and reverse primers, 1µL each of dNTPs and $MgCl_2$, 2.5µL of 10X PCR buffer, 0.3µL of Taq polymerase and 13.2µL of nuclease-free water for a total volume of 25µL. Thermal cycling conditions for both genes were initial denaturation at 94°C at 4 min followed by 35 cycles of denaturation at 93°C for 30 sec, annealing at 51°C for 30 sec and extension at 72°C for 45 sec, and finally extension at 72°C for 4 min. PCR products were resolved on 1.2% agarose gel and visualized using UV transilluminator. Chi-squared test was used to statistically analyze the results for genotyping analysis and p value less than 0.05 was considered significant.

3. Results

Initial screening and bacterial prevalence

Initial screening of samples was done by nitrite test as the presence of nitrite in urine serves as an indicator for the presence of pathogenic bacteria. Samples that produced positive nitrite test result were proceeded for further analysis. Gram staining was performed for all isolates obtained on CLED agar plates so as to distinguish gram negative bacteria from gram positive bacteria. Biochemical characterization was done only for gram negative cultures which were 161 in total. Out of these 161 isolates, 116 (72%) were *E. coli*, 22 (13%) were *K. oxytoca*, 14 (9%) were *K. pneumoniae*, 6 (4%) were *Proteus mirabilis* and 3 (2%) were *Proteus vulgaris*. Gender distribution of isolates was such that 68 (42%) were from females and 93 (58%) were from males.

Antimicrobial sensitivity profile

A total of 16 different antibiotics were used for determining the antibiotic sensitivity profile of selected pathogens. A vast majority of the isolates were resistant to AMC, AMP, CAZ, CIP, CTX, NA and NOR whereas most of the gram-negative pathogens were susceptible to AK, FOS, IPM and MEM as shown in Fig. 1. Intermediate results were produced by the remaining five antibiotics. *E. coli* was the predominant

isolate accounting for around three quarter of the total isolates (Fig. 1E). The most effective (> 90% sensitive) antibiotics against *E. coli* were AK, F, FOS, IPM and MEM while AMC, AMP, CIP, CTX, NA and NOR were the least effective (> 90% resistant). Against CAZ as well, more than 85% of the *E. coli* isolates were resistant while intermediate observations were recorded for the remaining four antibiotics.

Genotyping for bla $_{TEM}$ and bla$_{CTX-M}$

Results for PCR genotyping of *bla*$_{TEM}$ and *bla*$_{CTX-M}$ indicated that *bla*$_{CTX-M}$ (57%) was more common among the bacterial isolates from UTI patients as compared to *bla*$_{TEM}$ (45%). In the remaining samples, *bla*$_{TEM}$ and *bla*$_{CTX-M}$ could not be genotyped indicating that they were not ESBLs expressing these genes. Distribution of *bla*$_{TEM}$ and *bla*$_{CTX-M}$ positive as well as negative genotypes among different bacterial isolates is represented in Fig. 2. *bla*$_{CTX-M}$ gene was the most prevalent (67%) among *Proteus mirabilis* and the least prevalent (33%) among *Proteus vulgaris*. *bla*$_{TEM}$ gene was observed as being the most common (67%) among *Proteus vulgaris* and the least common (33%) among *Proteus mirabilis*. A greater number of *E. coli* isolates were detected as being *bla*$_{CTX-M}$ positive as compared to those that were *bla*$_{TEM}$ positive.

Differential distribution of *bla*$_{TEM}$ and *bla*$_{CTX-M}$ based on gender was also evaluated and chi-squared p value was used to document statistical difference between the two groups as shown in Fig. 3. The *bla*$_{CTX-M}$ was present among *Proteus mirabilis* and *Proteus vulgaris* isolates obtained from males but not among those from females and it was equally distributed among *K. oxytoca, K. pneumoniae* and *E. coli* isolates obtained from both genders. However, none of the differences achieved statistical significance. As far as *bla*$_{TEM}$ is concerned, its prevalence was greater among *K. pneumoniae* and *E. coli* isolated from males but lower among *K. oxytoca* and *Proteus mirabilis* isolated from males in comparison with females. *bla*$_{TEM}$ was absent among *Proteus vulgaris* isolates from males but present in the only isolate from females. Again, none of the differences was statistically significant.

4. Discussion

UTIs are a group of inflammatory diseases that involve urinary tract and are found to affect all individuals irrespective of age and gender. Symptoms of UTI include pain during urination, flanking pain, hematuria, fever as well as nausea although the disease may be asymptomatic as well [13]. Although the present study included more males, generally UTIs are much more prevalent among females due, in part, to the anatomical differences between two genders [14]. Among gram-negative bacteria, *E. coli* was the predominant pathogen responsible for UTI in present exploration followed by *Klebsiella* spp. and *Proteus* spp. It is already well established that *E. coli* is the most frequently isolated pathogen from UTI cases followed by *Klebsiella* spp. [5, 15].

Gram-negative pathogens demonstrated complete resistance against AMP and AMC in present investigation both of which are β-lactam antibiotics. A high degree of resistance was measured against

CAZ, CIP, CTX, NA and NOR, two of which are β-lactams. Previously, a substantial amount of resistance has been reported against β-lactam antibiotics by some of the gram-negative pathogens in other populations which is in accordance with our findings, although some differences also exist possibly due to differential geographical distribution of pathogens and varying antibiotic susceptibility profiles across the globe [16, 17]. *E. coli* was established as being resistant completely against AMP, AMC, CIP, CTX, NA and NOR, three of which are β-lactam antibiotics whereas considerable degree of resistance was documented against CAZ, SCF and SXT, two of which are β-lactams. Earlier, a similar pattern of resistance of *E. coli* strains was reported against AMP, AMC, CIP and CTX in Sudanese population [17]. Furthermore, comparable degree of resistance of *E. coli* was reported against CAZ, CIP and NA in a study from Uganda [18]. This signifies that majority of the β-lactams are becoming ineffective for administration to UTI patients over time and rationally devised and planned strategies are required to minimize further spread of resistance among pathogenic bacteria.

The most effective drugs against gram-negative bacteria isolated here were AK, FOS, IPM and MEM while F and TZP were also documented as being effective, though to a lesser extent. Similar observations regarding efficacy of these antibiotics have been reported earlier though the exact percentage of sensitivity of gram-negative pathogens varied somewhat [19, 20]. Against *E. coli* strains isolated in the present study, AK, F, FOS, IPM and MEM were very much effective and TZP was also effective, but to a lesser degree. Similar to our observations, *E. coli* was reported as being particularly sensitive to all of these antibiotics in a Russian study conducted in recent times [21]. Analogous observations were also reported formerly in an Iraqi population with a difference that TZP was not very effective in their study subjects [22]. Together, these observations imply that FOS is one of the best drugs of choice for treatment of UTIs particularly when *E. coli* is the etiological agent. Carbapenems are also efficacious for treatment of UTIs and hence, must be recommended over other less operative antibiotics.

Molecular analysis was done due to its superiority over phenotypic methods so as to detect ESBL producing bacteria and it was revealed that approximately half of the isolates were possessing bla_{TEM} and $bla_{\text{CTX-M}}$ genes making them ESBL-producers. An elevated prevalence rate has previously been reported for $bla_{\text{CTX-M}}$ genes in another Pakistani study [23]. A UK-based investigation also stated similarly higher prevalence of ESBL genes amongst children [24]. In a different population, ESBL genes have been reported as being less prevalent among pathogens in comparison with our observations [25]. This highlights the fact that ESBL prevalence is dissimilar from region to region and country to country pointing towards global differences. We documented comparatively higher positivity rate for $bla_{\text{CTX-M}}$ than for bla_{TEM} amongst all pathogenic isolates except for *Proteus vulgaris* which can be explained as chance observation based on the low number of *Proteus vulgaris* isolates which were only three. Gender-based differences in the prevalence rate for $bla_{\text{CTX-M}}$ and bla_{TEM} were not statistically significant and could not be ascertained with meticulousness and certainty due to small sample size. The higher prevalence of $bla_{\text{CTX-M}}$ is in line with the observations in an Irani as well as an Egyptian cohort [26, 27]. Several anthropogenic factors serve as determinants of ESBL prevalence rate among different populations accounting for the similarities as well as disparities observed in different studies.

Additionally, inclusion of a larger dataset may provide more valuable and reliable information as the sample size was limited for the present study.

Conclusions

ESBL producing pathogenic bacteria are gradually on rise among the etiological agents responsible for development of UTIs in our population as evidenced by high prevalence of bla_{TEM} and bla_{CTX-M} genes in our study sample. Owing to this, resistance against β-lactam antibiotics is increasing progressively rendering these drugs ineffective for prescription to UTI patients in this population which highlights the need to increasingly use other classes of antibiotics for the treatment of these patients. Encouragement of antimicrobial stewardship programs so as to avoid misuse and overuse of antibiotics is the need of the hour.

Declarations

FUNDING

No funding was received to conduct this study.

CONFLICT OF INTEREST STATEMENT

There is no conflict of interest to declare.

DATA AVAILABILITY

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

ETHICAL APPROVAL

All the procedures used in this study were in compliance with the declaration of Helsinki and it was approved by the Ethical Review Committee of The Women University, Multan, Pakistan.

CONSENT TO PARTICIPATE & PUBLISH

All the study participants provided written informed consent or assent with consent provided by a family member.

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Figures

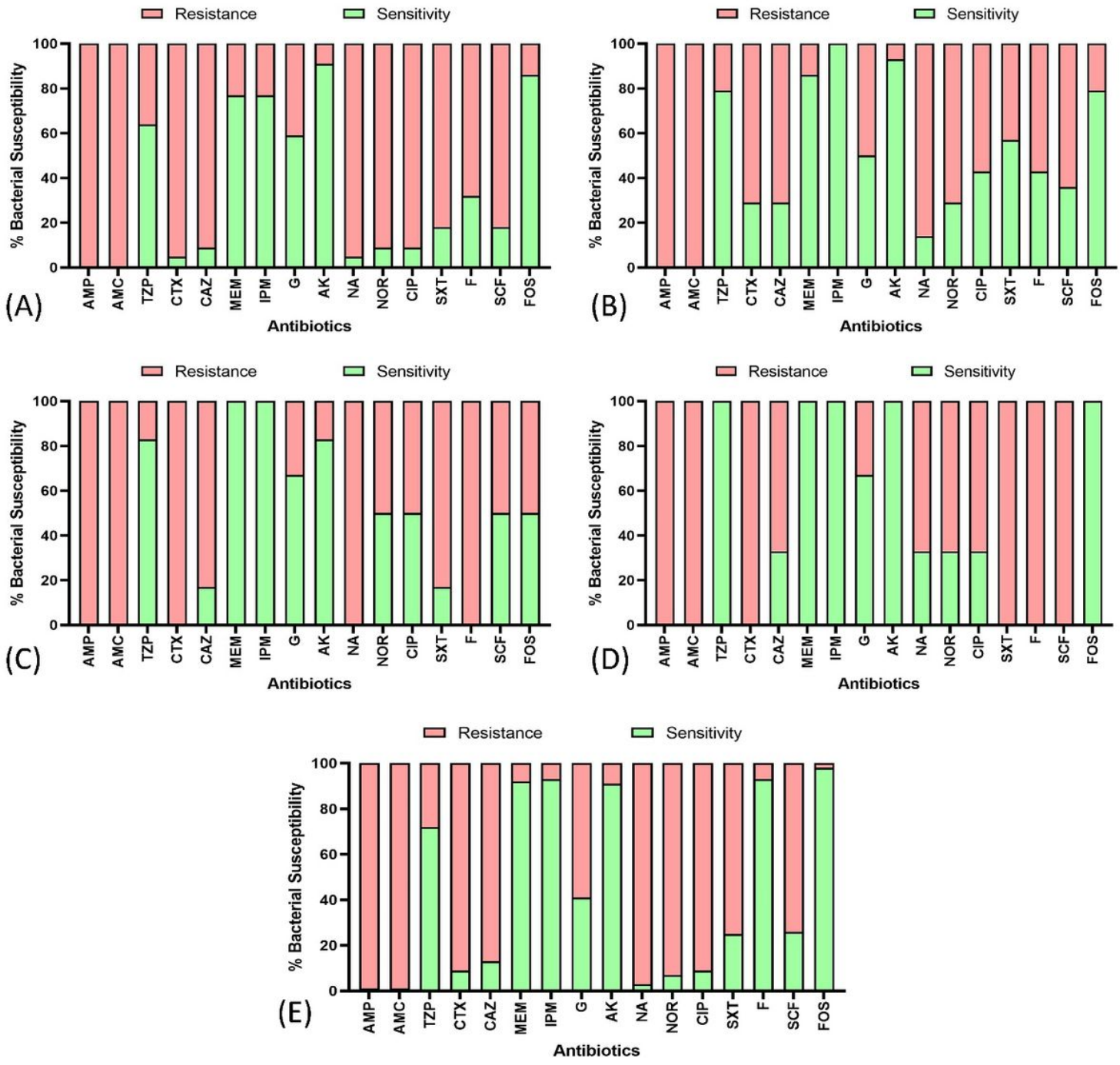

Figure 1

Antibiotic susceptibility pattern of Gram-negative ESBL-producing bacterial isolates. (A). *Klebsiella oxytoca.* **(B).** *Klebsiella pneumoniae.* **(C).** *Proteus mirabilis.* **(D).** *Proteus vulgaris*. **(E).** *E. coli.* AMP (Ampicillin), AMC (Amoxicillin/Clavulanate), TZP (Piperacillin/Tazobactam), CTX (Cefotaxime), CAZ (Ceftazidime), MEM (Meropenem), IPM (Imipenem), G (Gentamicin), AK (Amikacin), NA (Nalidixic acid), NOR (Norfloxacin), CIP (Ciprofloxacin), SXT (Co-trimoxazole), F (Nitrofurantoin), SCF (Sulbactam/Cefoperazone), FOS (Fosfomycin).

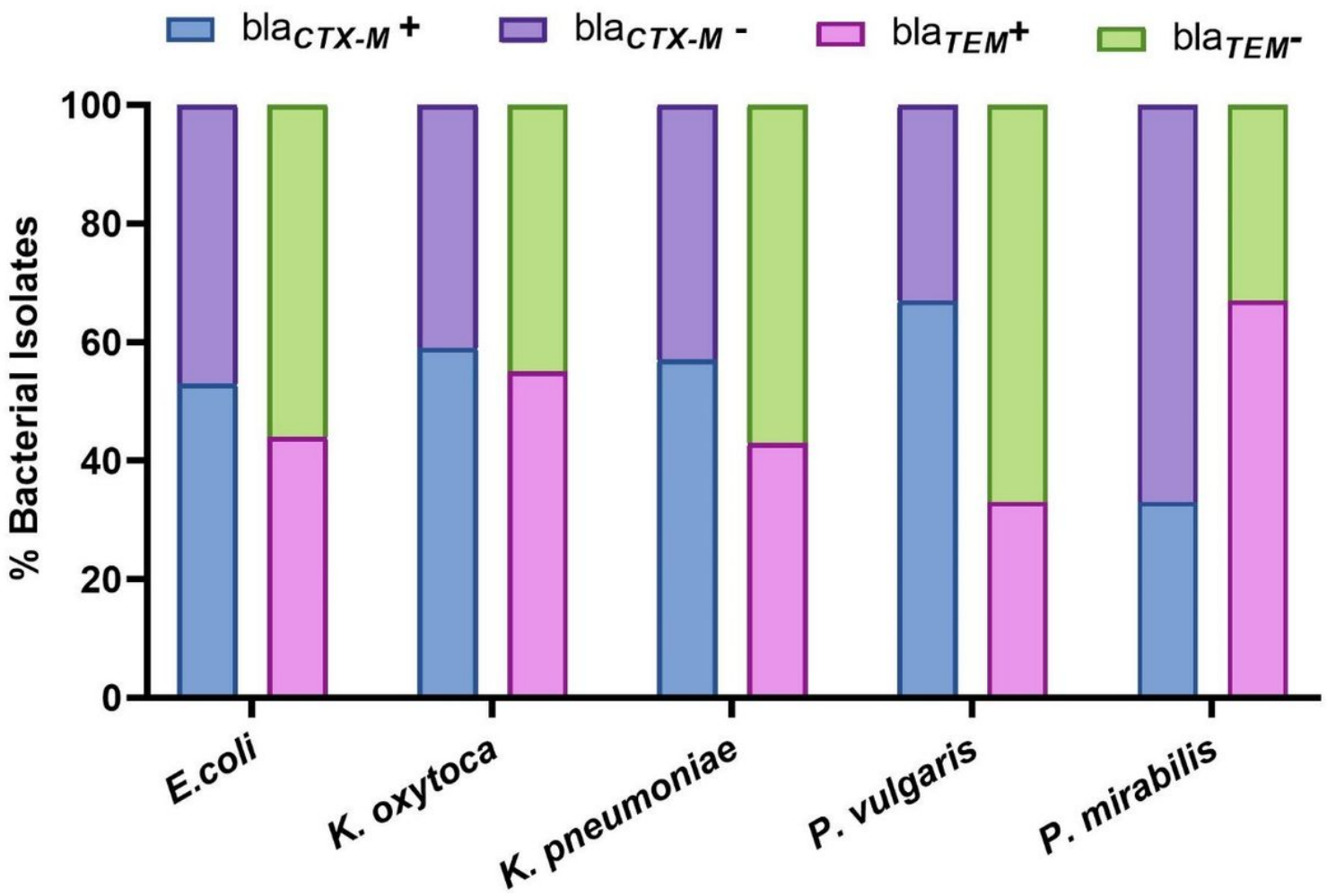

Figure 2

Distribution of β-lactamase genes *bla*$_{TEM}$ and *bla*$_{CTX-M}$ among ESBL-positive Gram-negative bacterial isolates.

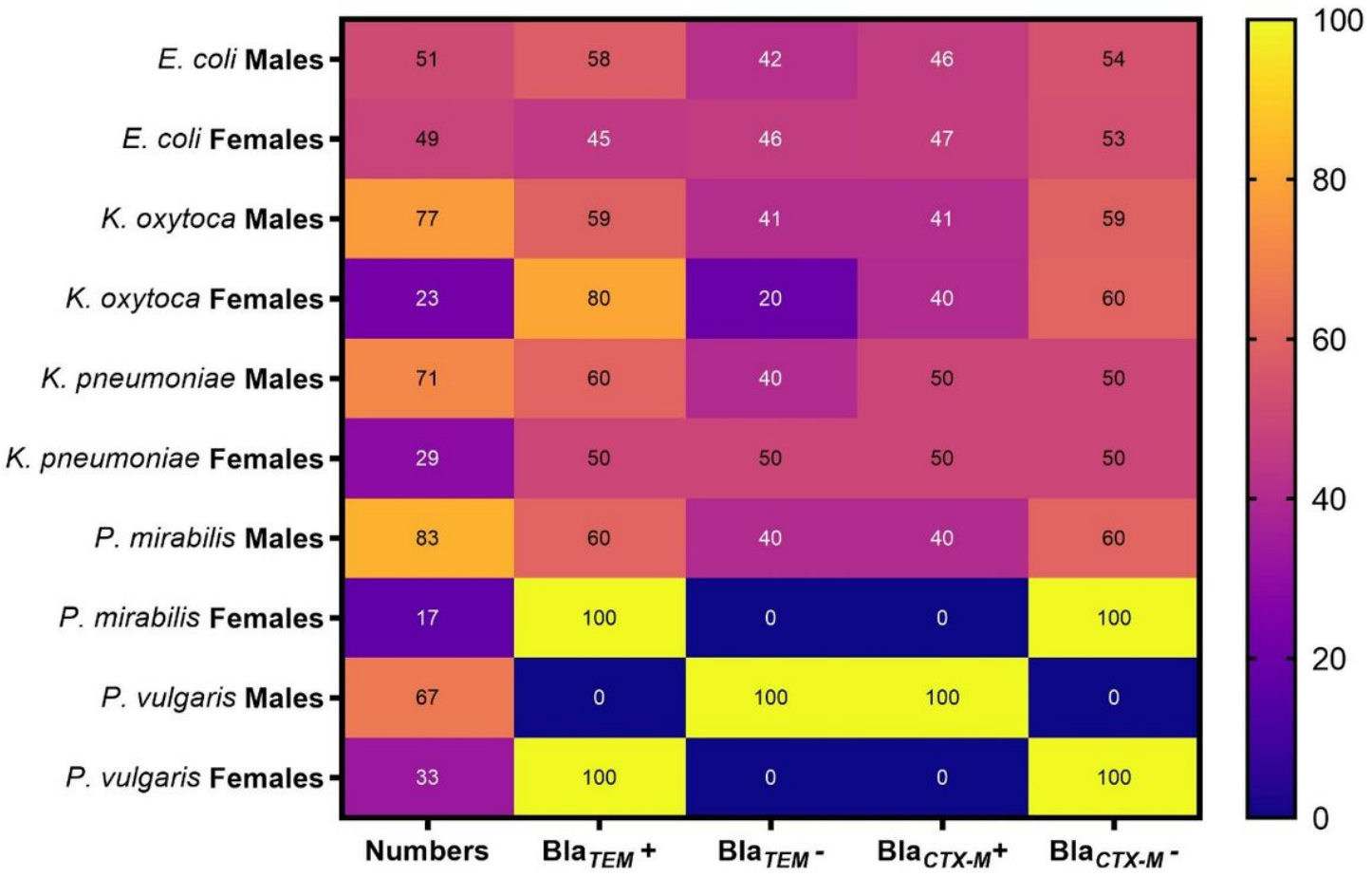

Figure 3

Heatmap of gender-based percentage prevalence of ESBL genotypes *bla*$_{tem}$ and *bla*$_{ctx-m}$ among gram-negative bacterial isolates. *E. coli* (n=116), *K. oxytoca* (n=22), *K. pneumoniae* (n=14), *P. mirabilis* (n=6), *P. vulgaris* (n=3). Data represents Chi-square test with degree of freedom (df) of 699.5, 36 and *p value* of <0.0001.