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Detection of CTX-M type extended spectrum β-lactamases (ESBLs) in Gram-negative pathogens

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The study of antimicrobial resistance (AMR) has been a challenging field. β-lactamase enzymes have been a subject of concern and interest for researchers all over the world. The aim of this study was to determine the rate of extended spectrum β-lactamases (ESBLs) and the occurrence CTX-M type ESBLs among the Gram-negative pathogens cultured from clinical samples. A total of 95 isolates were cultured from clinical samples. ESBL producers were screened and confirmed by the phenotypic tests. Polymerase Chain Reaction (PCR) was used to detect genes coding for CTX-M type ESBLs. After initial optimization (simplex PCR), multiplex PCR was employed for detection of genes coding for *bla*CTX-M. A total of 28 *Escherichia coli*, 42 *Klebsiella* spp., 16 *Pseudomonas* spp. and 9 *Acinetobacter* spp. samples were investigated. Out of the total 95 isolates, 78.95% (75/95) screened positive for ESBLs by combination disc diffusion test (CDT). Among the isolates, 82.66 % (62/75) were positive for *bla*CTX-M-1 group. This study demonstrates a remarkably high prevalence of *bla*CTX-M-1 group among ESBL-producing isolates. The policies of prescription of antibiotics and infection control in hospitals should be continuously reviewed. Routine antimicrobial susceptibility testing of the isolates is crucial to optimize the treatment and prevent development of antimicrobial resistance.

Keywords: Antimicrobial resistance; ESBLs; Gram-negative pathogens; phenotypic; PCR.

INTRODUCTION

Gram-negative bacterial pathogens are often involved in community acquired and hospital acquired infections, including wound infections, urinary tract infections, gastroenteritis, intra-abdominal infections, septicemia, pneumonia and central nervous system infections (Qu, et al. 2021; Gunasekaran, et al. 2020, Menezes & Menezes, 2013)

These Gram-negative bacteria are also found in the environment (Kibret & Abera, 2011). Antibacterial agents such as fluoroquinolone's and cephalosporins (with or without β-lactamase inhibitors) are often used for treating infections caused by Gram-negative bacterial pathogens (Gajdács, 2019). However, over the counter prescription practices and misuse of antibacterial agents for animal husbandry besides human use leads to emergence of antimicrobial resistance (Pokharel et al. 2020; WHO, 2020).

To specify, Gram-negative bacterial pathogens often carry mechanisms to overcome the critically important β-lactam group of antibacterial agents (such as cephalosporins) with co-resistance to

other conventional antibacterial agents (Dupouy et al. 2019). β-lactamases are termed as extended-spectrum β-lactamases (ESBLs), if they can hydrolyze extended-spectrum β-lactam antibiotics and monobactams, except cephamycins and carbapenems (Bush & Bradford, 2020).

ESBLs belong to Ambler A β-lactamase class and includes *bla*TEM, *bla*SHV and *bla*CTX-M types. ESBL producing strains also tend to co-produce other broad-spectrum β-lactamases extremely limiting the therapeutic options (Gajdács & Albericio, 2019). Among the ESBLs, a CTX-M enzyme has been reported gradually more often being associated with antibacterial resistance. CTX-M enzymes are quickly developing plasmid-mediated cefotaximases. The name "CTX" (cefotaximases) is due to the enzymes extended ability to hydrolyze cefotaxime in comparison to ceftazidime and "M" is due the place of its first isolation (Munich in Germany) (Ur Rahman et al. 2018). The rate of ESBL's in clinical isolates differs within a particular area. Greater prevalence of ESBLs have been reported in Southeast Asia (Sah et al. 2021).

Antibacterial resistance determination requires assistance of molecular methods besides the conventional phenotypic screening (Wright 1999).

Timely detection of ESBL producing strains within a given hospital or region helps in devising policies to restrain the spread of such strains (Raut & Adhikari, 2016). The aim of this study was to determine the rate of extended spectrum β-lactamases (ESBLs), their antimicrobial susceptibility pattern, and the occurrence CTX-M type ESBLs among the Gram-negative pathogens cultured from clinical samples.

MATERIALS AND METHODS

It is a cross-sectional and retrospective study. The Gram-negative bacterial isolates from various patients (including all infections) isolated in January 2019 to March 2019, at a tertiary care hospital in Ras Al Khaimah, UAE were included. Institutional Ethical Clearance and regional ethical clearance approval has been obtained for the study.

Bacterial isolation, identification and antibiotic susceptibility testing: Bacterial identification and antibiotic susceptibility testing (Minimum Inhibitory Concentration- MIC based) were performed using Vitek 2 (bioMerieux Inc., Durham, NC). The Vitek panel preparation had included QC strains as recommended by Clinical & Laboratory Standards Institute (CLSI) guidelines.

Phenotypic Testing for ESBLs:

The isolates considered screen positive for ESBL production (resistance to the penicillin's; first-, second- and third-generation cephalosporins; and aztreonam (but not the cephamycins or carbapenems) (Rawat & Nair, 2010) were tested for ESBL via the combination disc diffusion test (CDT). CDT uses cefotaxime (30 µg) and ceftazidime (30 µg) and a disc of cefotaxime-plus-clavulanate (30 µg plus 10 µg) and ceftazidime-plus-clavulanate (30 µg plus 10 µg). A ≥5 mm increase in diameter of the inhibition zone of the cefotaxime-plus-clavulanate and ceftazidime-plus-clavulanate disc, when compared to the cefotaxime and ceftazidime disc alone, will be interpreted as phenotypic evidence of ESBL production (Watt et al. 2000).

Molecular-biological Studies

Preparation of Template DNA:

A single bacterial colony from an overnight grown culture will be suspended in 100 µl of sterile MilliQ water and boiled for 5 minutes. The suspension will be centrifuged at 8,000 rpm for 10 min. The supernatant containing bacterial DNA will be used as template for PCR (Khan et al., 2012).

Detection of *bla*CTX-M genes

*bla*CTX-M: After initial optimization (simplex PCR), template DNA will be amplified by multiplex PCR, with use of oligonucleotide primers (Woodford et al., 2006).

Group 1: 5'-AAAAATCACTGCGCCAGTTC-3' & 5′-AGCTTATTCATCGCCACGTT-3';

Group 2: 5'-CGACGCTACCCCTGCTATT-3' & 5'-CCA GCGTCAGATTTTTCAGG-3';

Group 9: 5'-CAAAGAGAGTGCAACGGATG-3' & 5′-ATTGGAAAGCGTTCATCACC-3'.

Fragments of alleles encoding enzymes of groups 8 and 25 will be amplified with two specific forward primers and a shared reverse primer:

5′-TCGCGT TAAGCGGATGATGC-3' (group 8 forward);

5′-GCACGATGACATTCGGG-3' (group 25 forward); and

5′-AACCCACGATGTGGGTAGC-3' (groups 8/25 reverse).

The PCR was performed in a final reaction volume of 50 µl containing 25 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 20 pmol of each primers, 2.5 U Taq polymerase and 5 µL template DNA. An Eppendorf thermocycler was

used for amplification. The program for amplification included a step of initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 2 min, 60°C for 1 min and 72°C for 2 min and a final extension step at 72°C for 10 min. The PCR products were loaded in 1% wt/vol agarose gel prepared in Tris-borate-EDTA buffer and detected by ethidium bromide staining after electrophoresis (BioRad, USA)

RESULTS AND DISCUSSION

A total of 95 isolates were collected over a period of 6 months. The studied samples included urine, blood, sputum, wound and respiratory secretions. During the study, 28 *Escherichia coli* (29.47%), 42 *Klebsiella* spp. (44.2%), 16 *Pseudomonas* spp. (16.84%) and 9 *Acinetobacter* spp. (9.47%) samples were investigated. Out of the total 95 isolates, 78.95% (75/95) screened positive for ESBLs via CDT (Figure 1, 2 and 3).

Polymerase chain reaction (PCR) based detection of *bla*CTX-M

Among the isolates, 65.3% of the isolates were positive for *bla*CTX-M-1 group. Genus wise results showed 6.25% *Pseudomonas* spp., 83.3% *Klebsiella* spp., 44.44% *Acinetobacter* spp., and 78.57% *E. coli* isolates positive for *bla*CTX-M-1 group, corresponding to 415bp's (Figure 1, 2 and 3). We did not carry out sequencing of the amplification products to classify the specific gene type under the *bla*CTX-M-1 group.

Due to several resistant genotypes, Gram-negative bacterial infections create major challenge. *E. coli* and *K. pneumoniae* happen to be the main causative agents associated with severe infections including wound infections, urinary tract infections, gastroenteritis, intra-abdominal infections, septicemia, pneumonia and central nervous system infections (Qu, et al. 2021; Gunasekaran, et al. 2020, Menezes & Menezes, 2013).

In many parts of the world, CTX-M type of ESBL's have been often reported (Ur Rahman et al. 2018). Alfaresi et al. in 2011 and 2018 have reported CTX-M type of ESBLs in majority of the isolates in UAE. Intermittent reports of CTX-M types exist from various countries, like Brazil, India and China (Alfaresi et al. 2018). ESBLs among the pathogens is a matter of concern worldwide as it poses difficulty in treating infections and leads to prolonged hospital stay. The rate of ESBLs varies between countries and also between Institutions. Additionally, particular ESBL types have been demonstrated to be exclusive to a certain region (Hawkey et al. 2018).

ESBL producing organisms have been a matter of major concern in treating infections worldwide. The prevalence of ESBLs among clinical isolates varies from country to country and from institution to institution. Furthermore, specific ESBLs appear to be unique to a certain country or region.

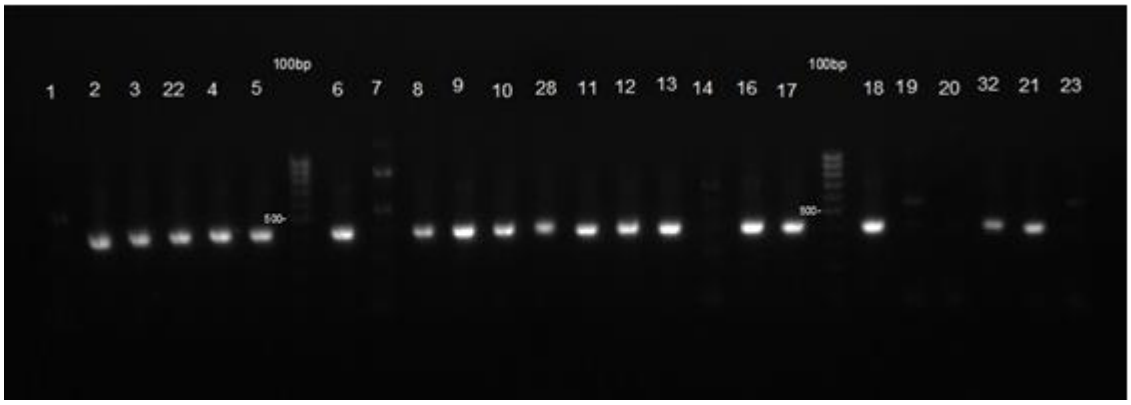

Figure 1: A representative Polymerase Chain Reaction (PCR) gel image demonstrating *bla*CTX-M. The image shows L2: positive control for *bla*CTX-M-1 group; (100bp, marker DNA (100–1000 bp), and L20: negative control. PCR results showed PCR products of same lengths. Lane's 3-18 represent test strains positive for *bla*CTX-M-1 group (corresponding to 415bp's). Lane 2 is positive control and lane 20 is a negative control. Lanes 1, 14, 19 and 23 are test strains negative for *bla*CTX-M.

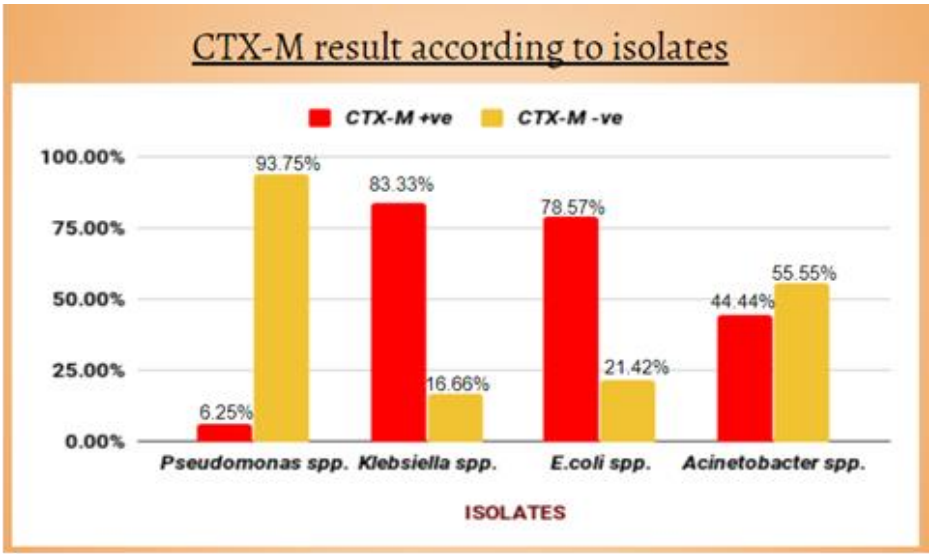

Figure 2: Representation of genus wise positivity of isolates for *bla*CTX-M by Polymerase Chain Reaction (PCR)

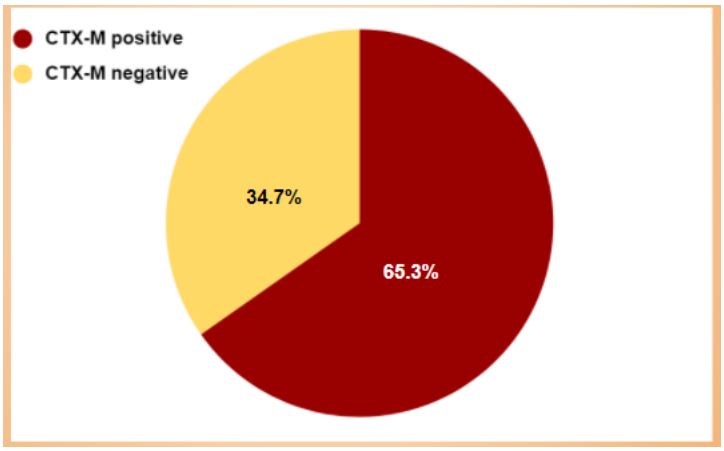

Figure 3: Representation of percentage of isolates positive or negative for *bla*CTX-M by Polymerase Chain Reaction (PCR).

CONCLUSION

In our study majority of the isolates tested were *Klebsiella* spp. Our phenotypic test (CDT) demonstrated a prevalence of 78.9% ESBL producing organisms among the total isolates. Whereas PCR detected 65.3% to be *bla*CTX-M group 1 producers. The remaining 13.6% of ESBL production among our isolates could be due to either TEM or SHV type of ESBLs. Among the isolates, *Klebsiella* spp. and *E. coli* were dominant in ESBL production. The findings are similar to as observed in Alfaresi et al., 2018. Our study showed results that were comparable with many other studies that determined the prevalence of CTX-M type ESBLs globally. The high rates of MDR isolates is an alarming finding and needs to be taken in consideration. The current trend as described in our study pertaining to this geographic area will require more studies and amendments in pattern of antibiotic usage.

Containment of antimicrobial resistance (AMR) requires executing basic policies for proper use of medications; institute AMR surveillance system; understand emergence & spread of AMR; reinforce infection prevention and control measures; and reassure the innovation of newer effective antimicrobials. Our study was of short duration and involved isolates from a single center. Multicentric studies needs to be practiced to determine the changing trend of resistance mechanisms of the

isolates to antimicrobial agents.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

SFM, FM, SK, MAFA and GAM designed the study and experiments. MAFA arranged for clinical samples. SFM, FM and SK performed the experiments and wrote the draft manuscript. GAM supervised the study and reviewed the manuscript. All authors read and approved the final version.

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