

Molecular detection of *bla*_{NDM} and *bla*_{VIM} in clinically isolated multi-drug resistant *Escherichia coli* in Pakistan

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Abstract: Metallo-β-lactamase (MBL) producing *Escherichia coli* are an emerging and serious threat to public health sector around the globe. MBL are spreading via plasmids to the host pathogens and produce resistance against carbapenems and left limited or no treatment option. Therefore, we designed this study to determine the dissemination of MBL producing *E. coli* in our locality. *E. coli* (n=100) were collected from various clinical samples from different tertiary care hospitals, Faisalabad. Microbes were sub-cultured on MacConkey and UTI Chromo Select agar. Bacteria were identified on the basis of culture characteristics and biochemically confirmed by API 20E. Antimicrobial susceptibility testing, carbapenemase and MBL was performed as per CLSI 2018 guidelines. Molecular identification of MBL genes were performed using specific primers by PCR. Of 100 *E. coli*, majority of them isolated from urine (n=55) followed by pus (n=23) and blood (n=22). Antibioqram displayed that all the *E. coli* were resistant to β-lactam drugs including carbapenems followed by 76% to ciprofloxacin and 60% to amikacin. Among these, 81% were MBL producers. Molecular characterization revealed that 18.4% were *bla*_{NDM} and 15.3% were *bla*_{VIM} producers. This study concluded that there is high prevalence of MBL producing *E. coli* in our clinical settings.

Keywords: *Escherichia coli*, metallo-β-lactamase, New Delhi metallo-β-lactamase, verona imipenemase

INTRODUCTION

Emergence of MBL producing *Escherichia coli* are the serious problem worldwide particularly in developing countries like Pakistan (Qamar *et al.*, 2015). *E. coli* are the notorious pathogens, causing wide range of infections such as urinary tract infections, gastritis, septicemia and meningitis (Batool *et al.*, 2016). Treatment failures due to the infections caused by *E. coli* are becoming very difficult because of the acquisition of mobile genetic elements such as plasmids and transposons that carry various antimicrobial resistomes (Partridge *et al.*, 2018). The prevalence of extended-spectrum-β-lactamase (ESBL) producing bacteria is very high, hence carbapenems are considering the “last resort” to treat such pathogens (Shaikh *et al.*, 2015).

β-lactamases are the enzymes that hydrolyze β-lactam ring. They are broadly classified into two main schemes, Ambler classification and Bush Jacoby classification. Ambler is further divided into four (i.e. A-D) subtypes. MBL enzymes (*bla*_{IMP}, *bla*_{VIM} and *bla*_{NDM}) belong to class B which requires zinc ions for their activity (Qamar *et al.*, 2016). MBL producing bacteria not only hydrolyze the carbapenem drugs (imipenem, meropenem and

ertapenem) but also other classes of antibiotics and left no or limited treatment options (Bush and Jacoby, 2010, Qamar *et al.*, 2019).

Carbapenamase producing *Enterobacteriaceae* notably *Klebsiella pneumoniae* and *E. coli* are responsible for high rate morbidity and mortality (Logan and Weinstein, 2017). A study from Islamabad, Pakistan has firstly reported the spread of New Delhi metallo-β-lactamase-1 (NDM) producing *Enterobacteriaceae* from pediatric patients responsible for 4/9 children’s mortality (Qamar *et al.*, 2016). Recently, another study from Lahore, Pakistan also revealed that NDM-4 producing *E. coli* ST405 were isolated from a child’s urine sample and displayed resistant to most β-lactam drugs (Qamar *et al.*, 2018b). Similarly, another study from Karachi, has also documented that clinically isolated NDM-1 producing Gram-negative bacteria mainly *K. pneumoniae* (66%) and *E. coli* (31%) were recovered and 57% patients expired due to bacteremia and septicemia caused by such pathogens (Khan *et al.*, 2016).

As per our knowledge, there is limited or no data available on the prevalence of carbapenem resistant *E. coli* from clinical settings from Faisalabad metropolis which is the 3rd populated city of Pakistan. Therefore, we

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have planned this study to determine the MBL producing *E. coli* from clinical setting.

MATERIALS AND METHODS

Collection of clinical isolates

A total of 100 *Escherichia coli* was collected using convenience sampling technique from clinical samples of blood, urine and pus from different tertiary care hospitals, Faisalabad during September 2017 to February 2018. All isolates were collected and transported using aseptic techniques.

Identification of the *Escherichia coli*

Preliminary isolates were sub-cultured on MacConkey agar (Oxoid, UK) and further selected on UTI Chromo Select agar (Sigma, Aldrich, UK) and plates were incubated at 37°C aerobically. Isolates were identified on the basis of colony morphology and culture characteristics and biochemical confirmation of the isolates were carried out using API 20E (bioMerieux, France).

Antimicrobial susceptibility testing

Antibiogram profile of all the isolates was determined by Kirby-Bauer disk diffusion method as per CLSI 2018 guidelines. Briefly, 0.5 McFarland standard suspensions of each isolates was prepared and lowned on Mueller-Hinton agar (MHA) plate. Plates were incubated at 37°C overnight for 18-20 hours and zone of inhibition was interpreted as per CLSI 2018. The implanted antibiotics were ampicillin (10µg), amoxicillin/clavulanate (20/10 µg), cefepime (30µg), ceftriaxone (30µg), ceftazidime (30µg), imipenem (10µg), meropenem (10µg), ertapenem (10µg), amikacine (30µg), ciprofloxacin (5µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg).

Phenotypic detection of carbapenemases

Modified Hodge's test (MHT) was carried out for carbapenemase activity. Briefly, 0.5 McFar and dilution of the *E. coli* (ATCC 25922) was prepared and streaked MHA. Meropenem (10µg) disc was placed centrally and isolates were streaked from the edge of the disc to the edge of the plate. After incubation at 35°C±2°C, cloverleaf like indentation showed positive results (Qamar *et al.*, 2018a).

Phenotypic determination of Metallo-β-lactamase (MBL)

MBL was detected using double disc synergy tests as described previously (Qamar *et al.*, 2018a). In short, tested organisms were leaned on MHA plate and two imipenem (10µg) and two meropenem (10µg) discs were planted at 25 mm. 10µg of 5M EDTA solution was added in each imipenem and meropenem discs. After incubation, the MBL was considered positive with an enlarged zone of inhibition of EDTA containing discs compared to non EDTA discs.

Multiplex PCR for carbapenemases genes

DNA extraction was carried out using commercially available kit (Tiagen, China). Carbapenemase genes were amplified using specific primers (table 1) with the following conditions; initial denaturation at 94°C for 10 minutes, denaturation at 94°C for 30 sec, annealing at 52°C for 40 sec, extension at 72°C for 50 sec and final extension at 72°C for 5 sec. Amplicons were separated on 2% agarose gel containing ethidium bromide under UV in Gel Documentation (Bio-Rad, UK).

Ethical approval

Before starting the research work, ethical approval was obtained from the ethical review committee, Government College, University, Faisalabad and informed consent was obtained from patients.

RESULTS

Clinical isolates identification

Of 100 clinical isolates, 55 *E. coli* were identified from urine culture followed by 23 from pus cultures and 22 from blood cultures. Majority of the *E. coli* were recovered from female as compared to male with 2:1 ratio.

Antimicrobial susceptibility testing

Antimicrobial susceptibility pattern revealed that 100% of the *E. coli* showed resistance against commonly used β-lactam antibiotics and β-lactam inhibitors such as ceftriaxone, ceftazidime, cefepime, imipenem, meropenem while 76% to ciprofloxacin, 60% to both amikacina and trimethoprim. However, most sensitive drugs were colistine and tigecycline (fig. 1).

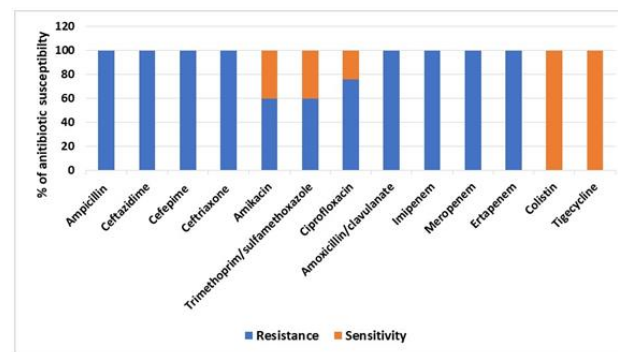


Fig 1: Antimicrobial susceptibility pattern of *E. coli*. The isolates display resistance against commonly used antibiotics.

Phenotypic detection of carbapenemase and MBLs

MDR *E. coli* were confirmed for carbapenemase and MBL production. of 100 *E. coli* 86 and 81 were positive for carbapenemase and MBL activity, respectively (fig. 2 and 3).

Molecular detection of bla_{VIM} and bla_{NDM} producing E. coli

Among 86 carbapenemase producing *E. coli*, 18 (20.9%) were bla_{NDM} producers and 13 (15.1%) were positive for bla_{VIM} (fig. 4 and 5). Of bla_{NDM} producing isolates, 8 (44%) were recovered from urine samples, 7 (38%) from blood and 3 (16.6%) from pus samples. However, 7 (53.8%) bla_{VIM} positive *E. coli* were identified from blood samples followed by 4 (30.7%) from urine and 2 (15.3%) from pus samples.

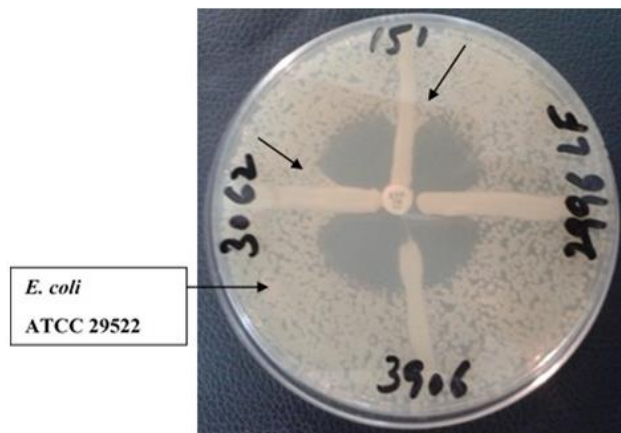


Fig. 2: Modified Hodge's test for the detection of MBL. Black arrows show the indentation of the isolates towards ertapenem disc.

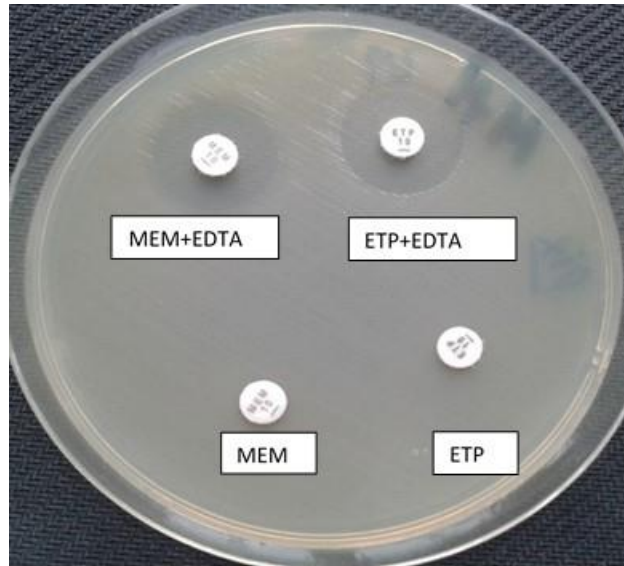


Fig. 3: Combined disk synergy test for MBL producing *E. coli*. Both the MEM+EDTA and ETP+EDTA show large zones of inhibition compared to MEM and ETP alone disc. MEM: Merpenem; EDTA: Ethylene diamine tetra aminoacetic acid; ETP: Ertapenem.

DISCUSSION

Carbapenemase producing *E. coli* are the serious public health concerns due the acquisition of various

antimicrobial resistomes (Qamar et al., 2018a). *E. coli* are causing a variety of hospital and community acquired infections such as UTI, bacteremia, septicemia, catheter associate infections and wound infections (Lee et al., 2018). In present study, most of the *E. coli* were recovered from urine (n=55) and pus samples (n=23). Similarly, study conducted in Ethiopia also reported that 45% and 19% *E. coli* were recovered from urine and pus culture respectively (Kibret and Abera, 2011). However, Pakistani study has also documented that 73% *E. coli* were responsible for UTI in hospitalized patients (Tanvir et al., 2012). Likewise, another study conducted in Peshawar, Pakistan they have also found that 21% *E. coli* were recovered from urine culture and 12% from pus while none of the *E. coli* were identified from blood samples (Khokhar et al., 2016). This difference could be due to the variation in sample size, demographic and research methodology.

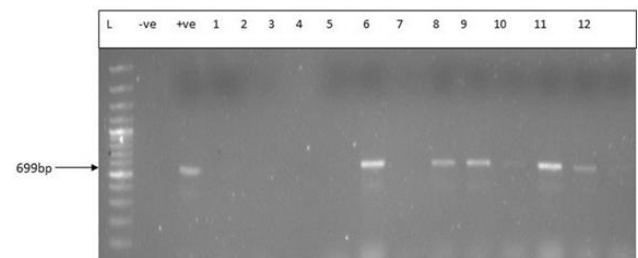


Fig. 4: Gel electrophoresis of PCR products of bla_{NDM}. L: ladder; -ve: negative control; +ve: positive control; isolates (1-12).



Fig. 5: Gel electrophoresis of PCR products of bla_{VIM}. L: ladder; isolates (1-4).

Treatment failure due to MDR *E. coli* is becoming great threat which can lead to the morbidity and mortality in our clinical settings (Qamar et al., 2015). In this study, all the isolates showed high resistance (100%) against commonly used antibiotics while moderate resistance to ciprofloxacin (76%) and amikacin (60%), while most effective drugs were colistin and tigecycline. These findings are in accordance with previously published studies from Pakistan that documented 100% of the isolates were resistant to β-lactams drugs and low to moderate resistance was observed against aminoglycosides and quinolones (Hannan et al., 2013, Qamar et al., 2015). Recently, another case report study from Pakistan also highlighted that NDM-4 producing *E. coli* ST405 showed 100% resistance to commonly used antibiotics including carbapenems and most effective drug was colistin (Qamar et al., 2018b). Moreover, a study

Table 1: Primers used for gene amplification

Carbapenemase genes	Nucleotides sequence (5' - 3')	Amplicon size (bp)
NDM-F	GGTTTGGCGATCTGGTTTTC	699
NDM-R	CGGAATGGCTCATCA CGATC	
VIM-F	AGTGGTGA GTATCCGACAG	261
VIM-R	ATGAAAGTGCCTGGA GAC	

conducted in Islamabad, also revealed high prevalence of NDM and other carbapenem resistant bacteria with resistance to various classes of antibiotics (Qamar *et al.*, 2015).

In present study, 86% and 81% *E. coli* were positive for carbapenemase and MBL and among these, 18 (20.9%) were *bla*_{NDM} and 13 (15.1%) were *bla*_{VIM} producers. Most of the NDM and VIM producing *E. coli* (n=8; 44%) were recovered from urine and blood samples (n=7; 53%) respectively. These findings have also been reported from Luanda, Angola that stated 50/57 were *bla*_{OXA} and 7/57 were NDM producing Enterobacteriaceae (Kieffer *et al.*, 2016). Nordman *et al.* (2011) also described the global spread of carbapenemase (notably NDM, VIM and IMP) producing bacteria particularly in Indian subcontinent. Likewise, there are various local studies also reported the spread of carbapenem resistant Enterobacteriaceae from different parts of Pakistan (Hasan *et al.*, 2014, Kumarasamy *et al.*, 2010, Qamar *et al.*, 2017, Braun *et al.*, 2018).

In our local clinical settings, high antimicrobial resistant burden is mainly due to the irrational use of antibiotics, use of broad spectrum empirical therapy, sub-standard hospital practices, lack of effective infectious control committee, lack of health care workers in the hospitals, lack of microbiology facilities or proper diagnosis, improper infectious waste disposal and sharing of beds which can lead to the cross contaminations (Hannan *et al.*, 2013, Qamar *et al.*, 2014). Moreover, according to the national antimicrobial survey, physicians prescribe antibiotics to 70% patients with 2 to 3 different classes of antibiotics without diagnosis (Khan *et al.*, 2017). According to Leekha *et al.* (2011), the empirical regime should be revised after every three months based on the hospital antimicrobial susceptibility testing reports.

CONCLUSION

This study has found the spread of carbapenem resistant *E. coli* in our local hospitals. This can lead to morbidity and mortality due to the presence of highly resistant bugs. Therefore, there should be performed a comprehensive surveillance studies and to give awareness to the physicians to combat this emerging threat.

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