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Extended-Spectrum-β-Lactamase Producing Multidrug Resistant *Klebsiella pneumoniae* Isolates from Pediatrics

Sumreen Hayat[1,6], Muhammad Hussnain Siddique[2], Bilal Aslam[1], Habibullah Nadeem[2], Asma Ashraf[3], Muhammad Saqalein[1], Mohsin Khurshid[4], Naveed Shahzad[5] and Saima Muzammil[1,*]

[1]*Department of Microbiology, Government College University, Faisalabad*
[2]*Department of Bioinformatics and Biotechnology, Government College University, Faisalabad*
[3]*Department of Zoology, Government College University, Faisalabad*
[4]*College of Allied health Professionals, Directorate of Medical Sciences, Government College University, Faisalabad*
[5]*School of Biological Sciences, University of the Punjab, Lahore*
[6]*Department of Biotechnology, University of Sargodha, University Road, Sargodha*

Sumreen Hayat and M Hussnain Siddique have contributed equally to this article.

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SH and MHS performed the experiments and wrote the manuscript. MS and MK designed the study. AA analysed the data. BA and HN helped in samples collection. SM supervised the work and reviewed the manuscript.

Key words
UTIs, *Klebsiella pneumoniae*, Double disc synergy test, ESBL, Antibiotic resistance.

ABSTRACT

Klebsiella pneumoniae is mainly responsible for nosocomial and urinary tract infections (UTIs) in pediatric and adult population. The ESBL producing *K. pneumoniae* present an alarming situation for clinicians by limiting the treatment options. We aimed to determine the antibiotic resistance pattern and characterization of ESBL producing *K. pneumoniae* isolated from children of different age groups. *K. pneumoniae* isolated from blood and urine samples were identified through API® 20E kit. Antibiogram was determined by disc diffusion method and isolates were screened for ESBL production using double-disc synergy test (DDST). Molecular detection for the presence of *bla*-SHV and *bla*-TEM was carried out by polymerase chain reaction (PCR). Of 392 blood and urine samples, 120 were found positive for microbial growth. Antibiotic susceptibility testing of *K. pneumoniae* isolates illustrated high resistance to ceftazidime (95.6%). Phenotypically, 52.17% of *K. pneumoniae* isolates were found to be ESBL producers as observed by double disc synergy test while the tested ESBL genes were detected in 56.52% of isolates. The study revealed multidrug resistance phenotypes among *K. pneumoniae* isolates with higher prevalence of ESBL producing *Klebsiella pneumoniae among the children with urinary tract infections.* Based on our findings, it is recommended that the ESBL production should be routinely monitored especially among the *K. pneumoniae* strains for the effective management and control of such MDR pathogens.

INTRODUCTION

Urinary tract infections (UTIs) are one of the most common infections among pediatrics, with an annual prevalence of 0.7 % per person and also being responsible for more than $180 million spent per year for treatment purposes (Ranjbar *et al*., 2017). Generally, initial antibiotic treatment is empirically supported until the availability of culture and drug sensitivity results. However, over-reliance on antibiotics to cope with bacteria and their irrational use lead to the emergence of resistance among these pathogens. Indiscriminate use of antibiotics in humans is widely discussed and considered as a primary cause of bacterial resistance development (Hawser *et al*., 2011).

Among *Enterobacteriaceae* family, especially *E. coli* and *Klebsiella* spp. are responsible for Urinary tract infections (UTIs) exhibiting higher degree of resistance to antibiotics used for the treatment and situation is becoming worse in developing countries including Pakistan. The resistance is mainly due to plasmid-encoded enzymes called extended spectrum β-Lactamases (ESBLs) (Livermore, 2008). ESBLs exhibit resistance not only to β-lactam antibiotics, but also sometimes, responsible for cross-resistance towards other antibiotics such as quinolones, trimethoprim-sulfamethoxazole (TMP-SMX), and aminoglycosides (Coque *et al*., 2008; Akhtar *et al*., 2018). The most common types of ESBLs include SHV, TEM, and CTX-M. Other clinically significant types

* Corresponding author: saimamuzammil83@gmail.com
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include BEL-1, PER, VEB, PER, SFO-1, BES-1 and IBC (Livermore, 2008).

Multidrug resistant (MDR) *Klebsiella* species especially *K. pneumoniae* infection is spreading worldwide (Coque *et al*., 2008). *K. pneumoniae* is an opportunistic pathogen associated with various infections in humans such as urinary tract infections (UTIs), septicemia, pneumonia, wound infections and infections associated with intensive care units (I.C.U) (Dhillon and Clarck, 2012). Moreover, there are multiple reports describing association of *Klebsiella pneumoniae* with hospital acquired infections, especially sepsis (Afridi *et al*., 2011). *K. pneumoniae* is detected in 40% UTI patients, thus making it the second most prevalent pathogen followed by *E. coli* (Mythri *et al*., 2014).

Although, there are a few studies from Pakistan indicating the incidence of ESBL producing *Klebsiella pneumoniae* responsible for UTIs (Batool *et al*., 2016; Kausar *et al*., 2014) but information regarding the occurrence among children particularly in Southern areas of Punjab, is limited. Therefore, the current study was designed to elucidate the status of ESBLs producing MDR *Klebsiella pneumoniae* and their antibiotic susceptibility pattern among children of different ages. Blood and urine samples from UTI patients belonging to District Multan, Punjab, Pakistan were collected due to the lack of availability of data from this region.

MATERIALS AND METHODS

Isolation and identification of bacteria

A total number of 392 samples (Urine = 197 and Blood = 195 while two of the patients refused to give blood samples) were aseptically collected during March 2018 to June 2018, from 197 patients with urinary tract infections (UTIs) in Nephrology and Urology wards of Children Hospital Complex and Nishtar Hospital from Multan, Punjab, Pakistan. Only the admitted patients were included in the study and sampling was done by non-probability convenient sampling method, and all the subjects were grouped based on demographic features such as gender and age (< 5 years, between 5-10 years and > 10 years). Blood specimens (1-3 mL) were primarily inoculated in BD BACTEC™ Peds Plus™ medium culture vials (BD™, USA) and monitored for bacterial growth for a period of 5-7 days. The culture positive bottles were streaked onto non-selective media *i.e.,* nutrient agar (Oxoid™, UK) followed by Gram staining (Batool *et al*., 2016). Later, selected colonies were streaked onto Blood agar (Oxoid™, UK) and MacConkey agar (Oxoid™, UK) media as well. Whereas urine specimens were cultured on CLED (BD™, USA) agar and incubated for 24-48 h at 37°C. The isolates were identified by various biochemical tests and finally confirmed by multi-test identification systems *i.e.* API® (Biomerieux™, France).

Antibiotic susceptibility testing

Antibiotic susceptibility testing of *K. pneumoniae* isolates was carried out by using disc diffusion method according to the guidelines of Clinical and Laboratory Standards (CLSI, 2015). Following antibiotic discs were used for susceptibility profiling: ceftazidime (30µg), c iprofloxacin (5µg), Amikacin (30µg), piperacillin-tazobactam (100/10µg), meropenem (10µg) and chloramphenicol (30µg). The interpretation of susceptibility results was done according to the guidelines of Clinical Laboratory Standards Institute (2015) (Kausar et at., 2014). Organisms exhibiting intermediate or resistant in at least 3 of above classes of antibiotics were multidrug resistance (MDR).

Screening of ESBL producers

K. pneumoniae isolates were screened for their ESBLs production by double-disc diffusion/synergy test (DDST); a phenotypic identification method for ESBL positive *K. pneumoniae*. Ceftazidime (30µg) with and without clavulanic acid (10 µg) (Oxoid™, UK) were used for phenotypic detection and interpretation of ESBL production according to the methodology published in Clinical and Laboratory Standard Institute guidelines (CLSI, 2015).

Etraction of plasmid DNA and PCR amplification

Plasmid DNA was extracted from each isolate (detected phenotypically as ESBLs producers) using QIAGEN® Plasmid Mini Kit (QIAGEN®, USA). PCR amplification was carried out for beta-lactamase genes of the family SHV and TEM using following pairs of primers: 5’ ATGCGTTATATTCGCCTGTG 3’ and 5’ AGATAAATCACCACAATGCGC 3’ for *bla*SHV and 5’ AAAATTCTTGAAGACG 3’ and 5’ TTACCAATGCTTAATCA 3’ for *bla*TEM (Macrogen™, Korea). The PCR conditions used for *bla*SHV were initial denaturation at 95°C for 5 min followed by 35 cycles at denaturation: 95°C for 30 sec, annealing: 56°C for 30 sec, amplification: 72°C for 2 min and final extension at 72°C for 10 min (Du *et al*., 2014). For the amplification of *bla*TEM, PCR conditions comprised initial denaturation: 94°C for 3 min followed by 35 cycles at denaturation: 94°C for 30 sec, annealing: 50°C for 30 sec, amplification: 72°C for 2 min and final extension at 72°C for 10 min (Sharma *et al*., 2010). Following gel electrophoresis, PCR product was visualized under UV transilluminator after staining with ethidium bromide. The product size was 896bp and 980bp for *bla*SHV and for *bla*TEM, respectively.

RESULTS

Out of 392 blood and urine specimens of children (different age groups) collected from Children Hospital Complex and Nishtar Hospital Multan, Punjab, Pakistan. 120 samples were positive for microbial growth, whereas remaining samples did not depict any visible growth. It was observed that frequency of positive cultures (blood and urine specimens) was 30.61%. Of 120 positive cultures, 107 were Gram-negative rods (GNRs), 9 were Gram-positive cocci and 4 were fungal cultures as shown (Table I).

Table I.- Frequency distribution of different microorganisms from UTIs patients (n= 120).

Organisms	Frequency	Percentage
Gram negative rods (GNRs)	107	89.16%
Gram positive cocci (GPC)	9	7.5%
Fungi	4	3.33%

Table II.- Frequency distribution of various isolates from UTIs patients.

Organisms	Frequency	Percentage
Gram negative rods (n= 107)		
K. pneumoniae	46	43%
E. coli	52	48.6%
Pseudomonas spp.	6	5.6%
Proteus spp.	3	2.8%
Gram positive cocci (n= 9)		
Staphylococcus spp.	5	55.55%
Streptococcus spp.	4	44.44%

It was also noticed that 29.44% urine (n=197) and 25.13% blood (n=195) samples were positive for GNRs. Thus, relatively high frequency of GNRs was found in urine (58/197) as compared to blood samples (49/195). Among the Gram-negative isolates, *E. coli* was the predominant one (48.6%) followed by *K. pneumoniae* (43%) as indicated in Table II the frequency of various isolates from urine and blood specimens of patients.

Our data had indicated that prevalence of GNRs isolated from urine and blood specimens was relatively high among females (52.33%) as compared to males (47.67%). While group wise distribution had indicated that the highest frequency of GNRs was found among children of age group <5 years (41.12%) followed by age group of >10 years (33.65%) as shown in Table III.

Antibiotic susceptibility pattern of *K. pneumoniae* isolates against routinely used antibiotics had indicated that 71.7% isolates were resistant to amikacin, 67.4% against ciprofloxacin, and 95.6% to ceftazidime. Majority of *Klebsiella* isolates exhibited multidrug resistance against routinely used antibiotics, while only 10.8 % and 6.5% of isolates exhibited resistance to piperacillin-tazobactam and meropenem, respectively. In the present study, phenotypic detection of ESBL production by double disc synergy test indicated that a high frequency (52.17%) of clinical isolates of *K. pneumoniae* was ESBL producers whereas 47.83% of *K. pneumoniae* were non-ESBL producers (Table IV).

Table III.- Gender and age wise distribution of GNRs isolated from urine and blood samples of UTIs patients.

Targeted population	Frequency	Percentage
Males	51	47.67%
Females	56	52.33%
<5 years	44	41.12%
5-10 years	27	25.23%
>10 years	36	33.65%

Table IV.- Molecular detection of percentage occurrence of beta lactamase genes (*bla*-SHV and *bla*-TEM) in *K. pneumoniae* isolates.

***K. pneumoniae* isolates (n=46)**	**Beta lactamase genes detection**		
	SHV (%)	**TEM (%)**	**SHV/TEM (%)**
Phenotypic ESBL producers (n=24) (52.17%)	21 (87.5)	19 (79.16)	17 (70.83)
Phenotypic non ESBL producers (n=22) (47.83%)	5 (22.72)	4 (18.18)	4 (18.18)
Total ESBL producers	56.52%		

Molecular detection of ESBL genes was also carried out for *bla*-SHV and *bla*-TEM genes using plasmid DNA extracted from *K. pneumoniae* isolates. The 1080 bp PCR products of TEM and 896 bp PCR products of SHV genes were amplified as shown in Figure 1. The results indicated that 56.52% of ESBL producers among *K. pneumoniae* isolates carried either SHV or TEM genes. 21 out of 24 isolates were positive for SHV gene while 19 isolates carried TEM gene. Interestingly, 5 out of 22 isolates (confirmed as non-ESBL producers phenotypically) were also positive for having SHV gene and 4 out of these were also positive for TEM as shown in Table IV.

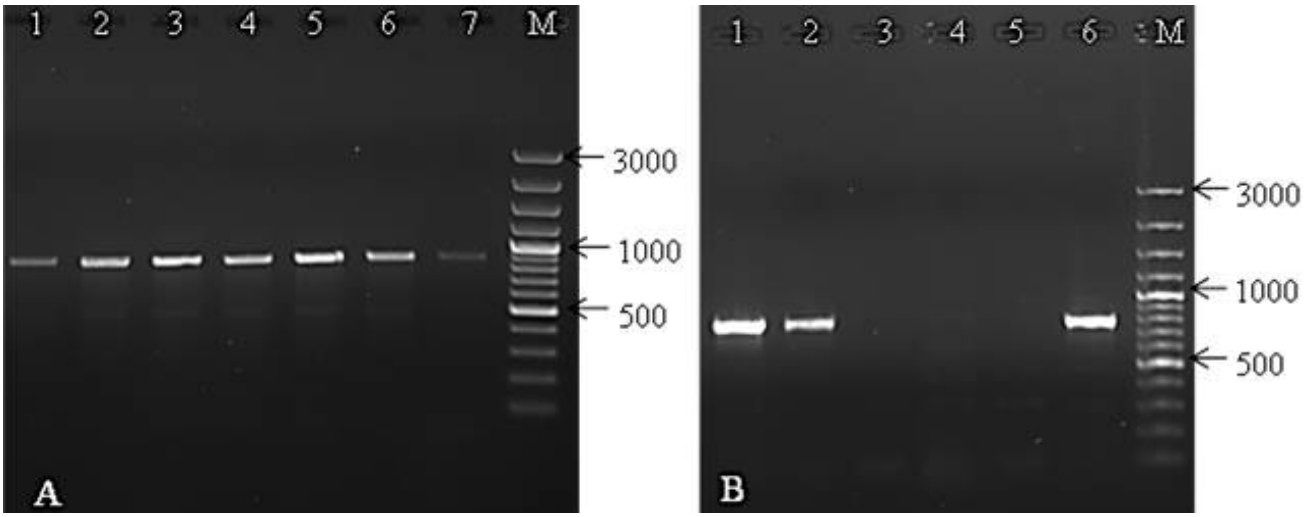

Fig. 1. A, PCR products amplified using the specific primers for *K. pneumoniae* TEM gene (Lane M, gene ruler 100-bp DNA ladder (Thermo Fisher Scientific); Lanes 1 to 7, PCR products of the positive samples); B, PCR products amplified using the specific primers for *K. pneumoniae* SHV gene (Lanes 1, 2 and 6, positive samples; Lanes 3 to 5, negative samples).

DISCUSSION

Urinary tract infections (UTIs) are the most common among all nosocomial infections especially in children and multi drug resistant ESBLs producing *K. pneumoniae* plays significant role. In the current study, overall 30.61% samples (urine and blood, n=392) yielded microbial growth and this figure correlates with the earlier reports (Ahmad, 2013; Jamil *et al*., 2014) Furthermore 29.44% urine (n=197) and 25.13% blood (n=195) samples were positive for GNRs. But according to available data 9 to 34% positivity of urine (Jahanzeb *et al*., 2008; Oh *et al*., 2013) and about 20 to 50% positivity of blood specimens had been reported in preceding studies (Lee *et al*., 2012; Livermore, 2008). The disparity in results could be due to the differences in sample size and sample processing technique (Jamil *et al*., 2014). In present investigation, GNRs were recorded 89.16%, 7.5% GPC and 3.33% fungi. The number of GNRs was comparable with the previous data from Pakistan (91%), Turkey (89%), Iran (90.3%) and Australia (96%) (Ayazi *et al*., 2010; McMullan *et al*., 2007; Tumbarello *et al*., 2006). The frequency of GNRs isolated from urine specimens was relatively high (29.44%) in contrast to blood (25.13%), it is coherent with a previous report suggesting that the most common clinical sample to yield the GNRs was urine (McMullan *et al*., 2007). However, bacteremic infections caused by Enterobacteriaceae members has witnessed increasing trend around the globe (Tumbarello *et al*., 2008).

According to our results *E. coli* was the predominant one (48.6%) followed by *K. pneumoniae* (43%), *Pseudomonas* spp. (5.6%) and *Proteus* spp. (2.80%), the findings with respect to the range of Gram negative urinary tract pathogens are in line with the previously published data (Manisha *et al*., 2012; Sohail *et al*., 2015). Urinary tract infections mostly result from GNRs (80 to 85%) and *E. coli* plays major role (75 to 95%) followed by *Klebsiella pneumoniae* and a few cases develop due to GPC as well (Manisha *et al*., 2012). It is an established fact that members of Enterobacteriaceae family are the leading culprits for UTIs. As these microbes reside in human gut as normal flora, so can easily infect the persons suffering from other diseases and with poor hygiene during their stay at hospital (Sohail *et al*., 2015). *E. coli* is still by far the most frequent pathogen among all uropathogens (Ghafourian *et al*., 2012; Shaikh *et al*., 2015). But in contrast to our results a few reports from the recent past had pointed out that *K. pneumoniae* was the most prevalent UTI pathogen (Ahmad, 2013; Garg *et al*., 2007).

In the present study, total of 46 *K. pneumoniae* isolates were confirmed, out of which 52.17% (24/46) were declared positive for ESBLs production and 47.83% (22/46) as non-ESBLs producers. In current study, urinary tract was the hub of ESBL producing *K. pneumoniae* infections 58.33% (14/24) followed by bacteremia 41.67% (10/24), which resembles with other investigations suggesting *K. pneumoniae* to be culpable for 6-17% of nosocomial UTIs and 4 to 15% of bacteremic infections (Ghafourian *et al*., 2012). Concerning the resistance patterns of *K. pneumoniae* the study showed that resistance percentage of ceftazidime (95.6%) was highest. Antibiotic resistance was reported for *K. pneumoniae* from blood and UTI isolates in Lahore

(Mythri *et al*., 2014). Resistance to certain antibiotics is well known phenomenon for *K. pneumoniae*, which are acquired by resistance genes like ESBLs. *K. pneumoniae* isolates were divided into two groups: (i) ESBLs producing and (II) non-EBSLs producing. The frequency of ESBLs producing *K. pneumoniae* in present study was comparable to a study from India and Iran (Sharma *et al*., 2008; Ayazi *et al*., 2010; Mohajeri *et al*., 2018). The frequency of EBSLs producing *K. pneumoniae* is high as compare to other reported studies in Pakistan. ESBLs prevalence in the clinical isolates varies worldwide and in different geographical areas and rapidly change over time. This high frequency may be due to poor hygienic conditions in Multan as compared to other cities in Pakistan such as Lahore, or may be because of lack of facilities and knowledge about health a mong peoples. While we found the difference of values between phenotypic screening by double disc synergy test and molecular detection of ESBLs producing genes as in the case with Sharma *et al*. (2008). Phenotypic tests for detection of ESBL could not be hundred percent reliable as may be ESBLs production could not reach to detectable level by disc diffusion test. This situation can create problem for clinical researcher. These phenotypic tests need to be evaluated regularly as new enzymes or mutations in the genes may change the performance of these tests (Ghafourian *et al*., 2012). In our study, 3 out of 24 ESBLs producing *K. pneumoniae* isolates could not be detected at molecular level for *bla*SHV and *bla*TEM. It might be due to the involvement of some other genes in ESBL production such as CTX-M or other subtypes of SHV or TEM. Although molecular detection is more sensitive, reliable and definitive for ESBL subtype detection but it is expensive and requires specialized expertise (Burgess *et al*., 2003). Furthermore, it is not possible in developing countries due to facilities constraint.

Treatment of ESBL-producing *K. pneumoniae* is difficult because of its resistance to multiple antibiotics. Carbapenems has been experienced to cure the infection caused by ESBL-producing microorganisms (Shiri *et al*., 2017). A set of antibiotics used in clinical infections were selected for antibiotic susceptibility. Our results reported the most effective and least effective antibiotic against *K. pneumoniae*. Current study provides an insight about the frequency distribution of ESBLs producing *K. pneumoniae* that will help clinicians in selection of antibiotics for the treatment of *K. pneumoniae* infections.

CONCLUSIONS

In our study, it was observed that the most effective drug against clinical isolates of *K. pneumoniae* was meropenem along with piperacillin-tazobactam exhibiting resistance of 6.5% and 10.8%, respectively. The present study warrants the empirical use of meropenem for the therapeutic management of infections caused by ESBL producing bacterial pathogens, however, a large multicenter study involving molecular tools should be performed to assess the exact magnitude of the problem due to ESBL producing pathogens among children.

Statement of conflict of interest

The authors declare no conflict of interest.

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