Occurrence of ESBL-producing *Klebsiella pneumoniae* in hospital settings and waste

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Abstract: Presence of multiple drug resistant pathogens in hospital waste is a serious public health concern, because it may ultimately be disseminated to the human. Current study was designed with the objective to estimate the occurrence of ESBL-producing *K. pneumoniae* in hospital settings and waste. For this purpose, cross sectional study for a period of one year was designed and non-probability sampling techniques was used to collect total n= 112 samples from various sample sources of hospital waste including ward waste, operation theatre waste, wastewater and hospital sludge. Isolation of the *K. pneumoniae* was done by using selective agar, biochemical identification of the isolates was done through API 20E kit (bioMérieux, France). Molecular identification of the isolates was done by amplifying 16SrDNA with PCR. According to CLSI guidelines disc diffusion assay was performed for antibiotic susceptibility profiling. PCR of MDR isolates was done for the molecular detection of various ESBL genes. Results of the study showed 17 (15%) percentage prevalence of MDR *K. pneumoniae* from all 112 collected samples. Among various sample sources wastewater showed the highest percentage (23%) prevalence of MDR *K. pneumoniae.* In 17 confirmed isolates $bla_{\text{CTX-M}}$ and $bla_{\text{CTX-M1}}$ were found in 13 (76%) and 12 (71%) respectively which showed the highest prevalence as compared to all other investigated genes. While bla_{TEM}, bla_{SHV}, $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ were found with percentage prevalence 9 (53 %), 1 (6%), 9 (53%) and 6 (35 %) respectively. Whereas bla_{KPC}, bla_{IMP}, bla_{VIM} and bla_{GIM} were not detected in any of the isolate. Taking together, strict rules and regulations should be adopted at public as well as hospital level to restrict the dissemination of antibiotic resistance from hospital environment to humans.

Keywords: ESBL-producing *Klebsiella pneumoniae*, hospital waste, public health.

INTRODUCTION

Hospital and other health care settings waste may contain various hazardous materials particularly resistant pathogenic microorganisms (Rowe *et al*., 2017). Inadequate treatment services for hospital waste and deficient sanitation facilities may be the risk factors which ultimately steer the transmission of these super bugs and associated genes responsible for antibiotic resistance into the environment. Occurrence and distribution of resistant bacterial strains and associated resistance genes from various environmental sample source have been reported, these sources include hospital sewage, domestic sewage, underground waters, sewage sludge and river water contaminated with sewage water (Rowe *et al*., 2016). A strong reason to believe the presence of resistant pathogens in hospital waste is the aberrant use of antibiotics to treat infectious diseases in hospital settings (Aslam *et al*., 2018).

According to the World Health Organization (WHO) *Klebsiella pneumoniae*, member of Enterobacteriaceae has emerged a MDR superbug and gained the position of pathogens with critical priority (Ventola, 2015). *K. pneumoniae* is an important pathogen associated with Hospital acquired infections (HAIs), especially pneumonia, UTI (Urinary Tract Infection), septicemia and sepsis. In addition, ESBL-producing *K. pneumoniae* has been reported as one of the most prevalent pathogen with growing frequency rate in various parts of the globe (Chong *et al*., 2011).

Though, in the past there are some reports about the detection of ESBL-producing *K. pneumoniae* in clinical samples and patients from Pakistan (Ejaz *et al*., 2013; Bukhari *et al*., 2016). Since no such study has been designed before which is based on the presence of resistant *K. pneumoniae* in hospital waste particularly at genetic level, so we proposed the current novel study to estimate the burden of MDR *K. pneumoniae* by investigating the various ESBL genes in hospital settings and waste in Pakistan.

MATERIALS AND METHODS

Study design and settings

This cross sectional study is designed for a duration of one year from December 2017 to December 2018 for the estimation of occurrence of ESBL-producing *K. pneumoniae* in hospital settings and waste of various hospital. Ethical approval of the study was granted by the Ethical Review Board (ERB; No. 4162 dated: 23-11-

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2017) of Government College University Faisalabad. Permission for the collection of samples from hospital settings was given by the hospital officials on prescribed consent proforma.

Sample collection

A Total (n=112) samples suspected for bacterial contamination were collected from various hospitals of Faisalabad. Non probability sampling technique was employed to collect samples from hospital settings and waste of Allied Hospital, DHQ Hospital, General Hospital, National hospital, Aziz Fatima Hospital, Independent Medical College Teaching Hospital. Various sample sources include ward waste, operation theatre waste and wastewater of the hospitals (table 1). Samples were collected in sterile containers. Ward waste and operation theatre waste were collected with the help of sterile cotton swabs whereas the wastewater samples were collected in sterile water containers.

Isolation and characterization of K. pneumoniae

Sterile cotton swabs were dipped into the 1 ml of PBS and then inoculated on the cultural media whereas in case of wastewater samples inoculum was directly streaked on the petri plates containing culture media. Culture media used in the study are Nutrient agar which served as general purpose media, Mackonkey, s agar and HiChrom Klebsiella Selective agar (M1573 Himedia®) were used as selective agar for the isolation of *K. pneumoniae*. Further Biochemical identification of the isolates was done by using API 20E Kit (bioMérieux, France) (Alvi *et al*., 2018). Molecular Identification of the isolates was carried out through Polymerase Chain Reaction (PCR) by using universal primers against 16SrDNA with the sequence (table 2). Briefly, downstream to the initial melting at 95°C for 3, total 35 PCR cycles were carried out with following scheme: each cycle consists of denaturation at 95°C for 30 sec, annealing at 50°C for 25 sec, extension at 72°C for 65 sec and then at 72°C for 5 min the final extension was done. Subsequently, agarose gel electrophoresis was performed and results were interpreted in gel documentation system (Bio-Rad, USA).

Antibiotic sensitivity profiling

According to the guidelines of CLSI (Wayne, 2015) disc diffusion assay was employed for antibiotic sensitivity testing (AST) of the isolates. Various cephalosporin antibiotics used in the study include Cefepime, Ceftriaxone, Cefixime and Cefuroxime. Additionally, AST profiling of *K. pneumoniae was* also estimated against various other antibiotics like ciprofloxacin, ampicillin, meropenem, tetracycline, amikacin, tazobactum, Tigecycline and chloramphenicol.

Phenotype based ESBL confirmation of the isolates

For phenotype based ESBL confirmation of *K. pneumoniae* the isolates were subjected to DDST (Double Disc Synergy Test) as described by (Wayne, 2015). In brief, a 30µg containing cefotaxime disc alone and a disc of cefotaxime in combination with clavulanic acid having the conc. of 30:10µg respectively were placed at a distance of 20 mm, and difference between disc in terms of zone of inhibition was observed, difference of ≥5 mm was considered positive for ESBL production.

Modified Hodge Test (MHT) for phenotypic characterization of carbapenem resistance

Isolates were subjected to MHT for phenotypic characterization to detect the carbapenem resistance (Sultan *et al*., 2013). Briefly, growth lawn of *E. coli* (ATCC 25922) on Mueller Hinton agar (Oxoid, UK) was prepared and disc of IMP (10 µg) was placed in the center of that petri dish. Subsequently, streaking of the sample isolate was done along the disc edge to edge of the petri dish, and kept at 37°C overnight. The MHT positive results were recorded on the basis of cloverleaf indentation at intersecting point of sample isolate within inhibition zone of IMP disc.

Molecular detection of ESBL genes

Overnight grown cultures of the isolates were subjected to phenol-chlorophorm method for the extraction for the genomic DNA (Sambrook *et al*., 1989). Quantity of the DNA was estimated with the help of Nanodrop (Thermo Fisher Scientific, MA). DNA concentration of 60ng/µl and above was used as template for PCR. Molecular detection of various β-lactamase genes listed in table 2 was performed through PCR by using specific primers. A Final volume of 25µl PCR reaction mixture was made to perform the PCR, 5µl template DNA, 100pM both forward and reverse primers 10µl of PCR Master Mix (Thermo-Scientific™), remaining volume was made by UltraPure (Thermo-Scientific™) water. All the reaction was carried out in the presence of negative control. Annealing temperatures according to the gene amplification is listed in table 2. After the PCR amplification all the PCR products were analyzed through 1.2% agarose gel electrophoresis and examined under the gel documentation system (Bio-Rad, USA).

RESULTS

Confirmed ESBL producing K. pneumoniae

Out of total 112 samples 17 (15%) were confirmed as ESBL producing *K. pneumoniae*. A detail of the percentage prevalence of *K. pneumoniae* is given in table 1. Out of 17 confirmed isolates highest percentage prevalence (23%) of isolates was found in wastewater while among various sample sources the least percentage (3%) of the isolates was found in operation theatre waste (table 1).

Antibiotic susceptibility profiling

All the positive samples were found MDR *K. pneumoniae,* all 17 (100%) isolates exhibited resistance

Table 1: Details of all the collected samples and percentage isolation of *K. pneumoniae* from various sample sources

Sample Source	Number of Samples	Isolation % of *K. pneumoniae*
Ward Waste	19	3 (16 %)
Operation Theater Waste	31	1 (3 %)
Wastewater	39	9 (23 %)
Hospital Sludge	23	4 (17 %)
Total Samples	112	17 (15 %)

Table 2: Details of sequences of all the primers and prevalence of AMR genes

Name of the gene	Primers sequence	Ann. temp (°C)	size (bp)	Prevalence %	Reference
*16*S rDNA	AGAGTTTGATCTGGCTCAG AAGGAGGTGWTCCACC	51	˜1500	For Mol. Identi.	(Srinivasan *et al*., 2015)
bla_{TEM}	TCAACATTTCCGTGTCG CTGACAGTTACCAATGCTTA	56	860	9 (53 %)	(Schlesinger *et al*., 2005)
bla_{SHV}	ATGCGTTATATTCGCCTGTG AGATAAATCACCACAATGCGC	56	896	1 (6 %)	(Schlesinger *et al*., 2005)
bla_{CTX-M}	GGATATCGTTGGTGGTGCCATA TTTGCGATGTGCAGTACCAGTAA	57	544	13 (76 %)	(Rhodes *et al*., 2014)
bla_{CTX-M1}	CCGTTTCCGCTATTACAAACCGTTG GGCCCATGGTTAAAAAATCACTGC	55	944	12 (71 %)	(Krishnamurthy *et al*., 2013)
$bla_{CTX-M-2}$	TGGAAGCCCTGGAGAAAAGT CTTATCGCTCTCGCTCTGTT	55	833	8 (47 %)	(Kawakami *et al*., 2000)
$bla_{CTX-M-9}$	ATGGTGACAAAGAGAGTGCA CCCTTCGGCGATGATTCTC	52	865	7 (41 %)	(Messai *et al*., 2008)
$bla_{CTX-M-14}$	GAGAGTGCAACGGATGATG TGCGGCTGGGTAAAATAG	54	941	7 (41 %)	(Espedido *et al*., 2013)
bla_{KPC}	TGCAGAGCCCAGTGTCAGTTT CGCTCTATCGGCGATACCA	52	880	Not detected	(Gootz *et al*., 2009)
bla_{IMP}	GGAATAGAGTGGCTTAATTCTC CCAAACCACTACGTTATC	55	624	Not detected	(Kaczmarek *et al*., 2006)
bla_{VIM}	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	52	390	Not detected	(Ellington *et al*., 2006)
bla_{GIM}	TCGACACACCTTGGTCTGAA AACTTCCAACTTTGCCATGC	52	477	Not detected	(Poirel, *et al*., 2011)
bla_{NDM-1}	TGCCCAATATTATGCACCCGG CGAAACCCGGCATGTCGAGA	60	292	9 (53 %)	(Huang *et al*., 2013)
bla_{OXA-48}	TTGGTGGCATCGATTATCGG GAGCACTTCTTTTGTGATGGC	56	743	6 (35 %)	(Poirel *et al*., 2004)

against all the cephalosporin antibiotics used in the study. Furthermore, the highest resistance of *K. pneumoniae* showed was found against amikacin (55%). Tigecycline was found the most effective antibiotic against the isolates of the study, as only 8% of *K. pneumoniae* isolates were found resistant to Tigecycline (Graph 1).

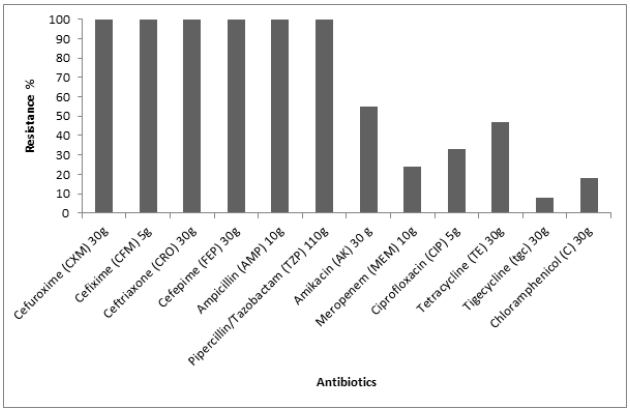
Distribution of ESBL genes

Among confirmed (17) isolates most prevalent CTX-m genes were bla_{CTX-M} and bla_{CTX-M1} which showed 13 (76 %) and 12 (71%) prevalence respectively as compared to various other genes. Subsequently, bla_{TEM} and bla_{NDM-1} were found with same percentage prevalence of 9 (53%) while bla_{OXA-48} showed 6 (35%) prevalence and the least prevalence 1 (6%) was estimated in case of bla_{SHV}. While bla_{KPC}, bla_{IMP}, bla_{VIM} and bla_{GIM} were not present in any of the confirmed MDR *K. pneumoniae*.

DISCUSSION

The antibiotics of β-lactam and carbapenems group are the common antibiotics which are used to treat the infections caused by Gram-negative group of bacterial pathogens, but infection control become demanding due to the emergence of ESBL producing pathogens across the global. Although ESBL producing Enterobacteriaceae is disseminated worldwide and have been studied extensively in the developed countries but developing countries like Pakistan still lacking in such complete investigations regarding ESBL producing gram negative

bacteria especially *K. pneumoniae.* Distribution of ESBL harboring *K. pneumonia* has been reported from various parts of the world, From USA it has been reported with the rate of 44%, from Asia region 22.4% and from Europe it was reported with the rate of 13% (Falagas *et al*., 2009).



Graph 1: Percentage resistance of *K. pneumoniae* against various antibiotics (along conc.) used in the study

In the present study, we have estimated the distribution of ESBL-producing *K. pneumoniae* and various ESBL genes from the hospital waste. There are some studies from Pakistan which have demonstrated the occurrence of ESBL harboring *K. pneumoniae* associated with hospital infection (Habeeb *et al*., 2013). In the near past a study has been conducted and they recorded about 25% incidence of ESBL producing *K. pneumoniae* (Ahmed *et al*., 2015). In our results we found the highest percentage (23%) of ESBL producing *K. pneumoniae* in hospital wastewater as compared to all other sample sources, strong reason behind the highest percentage from wastewater is the washing of all the hospital premises and settings so it should contain the highest percentage of microbes and associated resistance genes. Our study showed the same frequency of *K. pneumoniae* as the previous reports of (Bukhari *et al*., 2016), they found 15.8% *K. pneumoniae* in various samples of hospital settings and hospitalized patients, but they have included a small sampling fraction of hospital environment whereas our whole study is based on various sources of contamination in hospital environment. Both the studies showed the presence of various ESBL genes which include blaCTX-M, blaTEM and blaSHV. Moreover, diversity of the results in the present study is significantly different to the findings of the studies which have been conducted in the past (Jabeen *et al*., 2005). Because these investigations were on the basis of phenotypic characterization of the isolates and no molecular characterization was used in these studies.

During the past two decades CTX-M emerged as the most common ESBL type in clinical settings and its association with MDR pathogens has been reported from various regions of the globe from Asia to Africa and Europe to America (Pitout *et al*., 2008). In Pakistan scanty data is available regarding the clinical significance of CTX-M, but the present investigation revealed significant prevalence (76%) of CTX-M in *K. pneumoniae* in hospital premises and environment. Results of the present study are in agreement with various studies conducted nationally and internationally. In Pakistan some studies conducted in the past showed blaCTX-M as one of the most prevalent ESBL type as compared to blaTEM and blaSHV. Earlier results showed 93.84% prevalence of ESBL harboring *K. pneumoniae* from a hospital of Karachi, Pakistan (Khan *et al*., 2010). Results of the present stud are in accordance of the findings of these studies as the association of CTX-M and MDR has been established in the studies, because a very high percentage (76 %) of the MDR *K. pneumoniae* exhibited the presence of CTX-M.

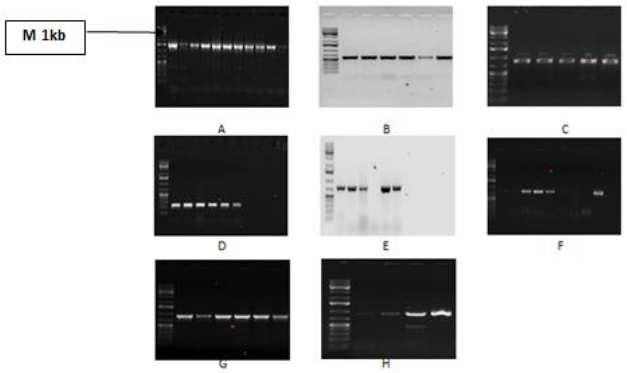


Fig. Agarose gel (1%) electrophoresis image of various samples showing the results of respective gene amplification with their PCR product size A)1500bp PCR product for 16SrDNA amplification, M lane is the Marker (1kb plus Gene Ruler-Themo Fisher™), whereas L1 is showing positive control and all the subsequent lanes up to Lane 9 showing positive samples followed by a negative control B) Amplification of $bla_{\text{CTX-M}}$ showing the PCR product of 544bp C) Amplification of bla_{TEM} showing the PCR products of 860bp D) Amplification of $bla_{\text{NDM-1}}$ showing the PCR product of 292bp E) Amplification of $bla_{\text{OXA-48}}$ showing the PCR product of 743bp F) Amplification of $bla_{\text{CTXM-14}}$ showing the PCR product of 941bp G) Amplification of $bla_{\text{CTXM-1}}$ showing the PCR product of 944bp H) Amplification of $bla_{\text{CTXM-9}}$ showing the PCR product of 865bp

In Pakistan at present physicians are prescribing the carbapenems frequently due to better results, sensitivity and availability. Very few reports have been published on resistance against carbapenems in Pakistan. The reason behind this may be researcher have not studied the carbapenem resistance encoding genes. Studies showing the prevalence of *bla*NDM-1 in *Enterobacteriaceae* have been reported sporadically. While in the present study we have investigated carbapenem resistance at phenotypic as well as at genetic level by studying various carbapenem resistance encoding genes. Various genes which were

found associated with carbapenem resistance in the isolates of *K. pneumoniae* in present study these include bla_{NDM-1} and bla_{OXA-48}. These findings are similar to the results of limited data available in Pakistan which have also reported the prevalence of bla_{NDM-1} in *K. pneumoniae* (Qamar *et al*., 2018), as same is the case which $bla_{KPC,}$ bla_{VIM} and bla_{GIM} which has not been found in those studies.

In conclusion, findings of the current study reveal a significant percentage prevalence and dissemination of MDR *K. pneumoniae* harboring various ESBL genes in hospital environment of Pakistan. Suitable and adequate control measures from awareness to prescription should be adopted which may ultimately play a significant role in restraining the spread and transmission of antibiotic resistance from hospital environment to community.

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