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PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF EXTENDED–SPECTRUM β-LACTAMASE PRODUCING CLINICAL ISOLATES OF *Klebsiella Pneumoniae*

Microbiology

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ABSTRACT

Introduction: *K.pneumoniae*, often associated with nosocomial infections, has acquired resistance against β-lactam antibiotics. Production of β-lactamase is considered as one of the most common mechanism that accounts for the resistance. It is known to produce significant percentage of ESBLs. Thus, the present study was aimed to characterize ESBL producing isolates of *K.pneumoniae*.

Material & methods: Sixty strains of *K.pneumoniae* were collected from patients visiting VPCI. Isolates were subjected to antibiotic susceptibility, phenotypic and genotypic confirmation of ESBL genes.

Results: Isolates exhibited (31-100%) resistance to various antibiotics. 53.33% tested positive using phenotypic confirmatory test. 91.7% isolates were positive for various ESBL genes. The most prevalent ESBL genotypes were bla_{TEM-1}, bla_{SHV-1} and $bla_{CTX-M-1}$.

Conclusion: Multiplex PCR is sensitive and rapid method for identifying the ESBL genes. The prevalence of MDR ESBL producing *K.pneumoniae*appears to be high and majority of them tested positive for TEM-1, SHV-1 and CTX-M-1.

KEYWORDS

Klebsiella pneumoniae ESBL (Extended-spectrum β-Lactamase), PCR (Polymerase chain Reaction)

Introduction

Klebsiella pneumoniae is known to cause both community and hospital-acquired infections [1]. Carriage rate of *K.pneumoniae* are generally low in healthy humans, but increase exorbitantly in hospitalized patients [2]. β-lactam antimicrobial agents are often employed to counteract the infections associated with *K.pneumoniae*. However, *K.pneumoniae* has acquired resistance against β-lactams by producing β-lactamases, enzymes which destroy these antibiotics. Persistent exposure of bacterial strains to β-lactam antibiotics leads to mutations in the β lactamase genes producing extended spectrum β lactamases (ESBLs) leading to resistance to a wider range of antibiotics. [3, 4].

ESBLs have the potential to hydrolyze extended-spectrum Cephalosporins and Monobactams but are inhibited by clavulanic acid. These enzymes are encoded by genes that can either be present on chromosome or on plasmids. Till date several ESBL genes have been identified [4]. At present, the transferable nature of plasmid bearing ESBLs probably accounts for increased basal level of multidrug resistance among nosocomial pathogens [5]. Chromosomally located genes disperse by means of vertical gene transfer while plasmid located genes undergo efficient dissemination both by vertical and horizontal gene transfer [6].

The occurrence of ESBL producing *K. pneumoniae* is highest in Latin America (44%) compared with Asia/Pacific (22.4%), Europe (13.3%) and North America (7.5%). However, higher ESBL rates occur in India (72%) and Mexico (71.4%) followed by Latin American countries (37.8% to 55.3%), Greece (43.1%) and Poland (37.5%) [7]. The most common hospital infection caused by ESBL strains of *K. pneumoniae* are Urinary tract infections (36.9%), followed by Respiratory tract infections (30.5%), Gastrointestinal systems (14.2%), surgical sites (8.5%), Bloodstream infections (7.1%) and other infection (2.8%) including skin, Central Nervous System, gynecological and eye infection [8].

Thus, the aim of the present study was to identify and characterize multidrug resistant clinical isolates of *K. pneumoniae* with reference to their antibiotic susceptibility, presence and types of ESBLs.

Materials And Methods

Clinical Isolates: A total of sixty non-repetitive clinical isolates of *K. pneumoniae* (Sputum – 34, ET Aspirate – 12, Blood - 7, Bronchial Asp. - 3 Urine – 3, Tip – 1), collected during May, 2013 to February, 2015 at Vallabhbhai Patel Chest Institute, Delhi, India, were included in the study.

The isolates were identified as *K. pneumoniae* on the basis of phenotypic characteristics exhibited on culture and biochemical reactions. Pink-coloured, large dome-shaped mucoid, sticky, lactose fermenting colonies were identified using the standard microbiological methods [9]. They were confirmed by Vitek (Biomerieux). They were maintained in the laboratory as stab culture in Nutrient Agar slants, kept at 4°C and suspension in Brain Heart Infusion (BHI) broth with 16% glycerol, stored at -80°C for further testing.

Antimicrobial Drug Susceptibility Testing: Susceptibility of isolates to various classes of antibiotics was tested by Kirby-Bauer's disk diffusion method [10]. piperacillin (100 µg) , piperacillin/ tazobactam (100/10 µg), cefepime (30 µg), ceftriaxone (30 µg), cefoxitin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefpodoxime ((30 µg), aztreonam (30 µg), meropenem (10 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (30 µg), levofloxacin (5 µg), tigecycline (30 µg), and colistin (10 µg) were used. Briefly, the test organism was grown in Mueller Hinton broth and incubated at 37°C for 2-3 hours. The turbidity was adjusted to match 0.5 McFarland standards. A lawn culture of the organism was made in Mueller Hinton Agar (MHA) plates. Antibiotic impregnated disks (HIMEDIA) were placed on the surface of the plate and were incubated overnight at 37°C. The diameter of the zone of inhibition was recorded and interpreted as sensitive, intermediate or resistant, as per the CLSI guidelines 2015. Escherichia coli ATCC 25922 and Pseudomonas. aeruginosa ATCC 27853 were used as control strains.

Screening of Extended Spectrum β–lactamases (ESBLs):. Isolates that exhibited the zone of inhibition as indicated to at least one of the following antimicrobials: cefpodoxime (10 µg) ≤17 mm, ceftazidime (30 µg) ≤22 mm, aztreonam (30 µg) ≤27 mm, cefotaxime (30 µg) ≤27 mm, and ceftriaxone (30 µg) ≤25 mm indicated the production of ESBLs, CLSI, 2015 [11].

Phenotypic Confirmation of Extended Spectrum β–lactamases (ESBLs): The CLSI (2015) advocates the use of cefotaxime (30 µg) or ceftazidime (30 µg) disks with or without clavulanate (10 µg) for confirmation of the presence of ESBLs in K. *pneumoniae.* Briefly, a 0.5 McFarland's suspension of each isolate was spread on a MHA plate and the relevant disks were placed aseptically at a distance of about 24 mm between the two disks and the cultures were incubated at 37°C overnight. A difference of a ≥5 mm between the zone diameters of either of Cephalosporin disks and their respective zephalosporin/ Clavulanate disk is confirmatory of ESBL production.

ESBL PCR: Isolates were further identified and confirmed for the presence of the genes that encodes for ESBLs. DNA extraction was carried out using Bacterial Genomic DNA purification Kit (HIMEDIA, catalogue no. MB505-250PR HiPurATM) by following the manufacturer's instructions. DNA thus extracted was stored at -20°C and used for various PCR reactions. The DNA was subjected to multiplex PCR using specific primers for different types of ESBLs [12].

Amplified products were then separated by electrophoresis in 1.3% agarose gels containing SYBR safe. A 100 bp DNA ladder was used as a molecular marker (New England BioLabs Inc. Catalog No. N3231S).

Results

Sixty non-repetitive isolates of *Klebsiella pneumoniae* were included in the study, 24, 10, 26 isolates were from the Ward, OPD and ICU respectively.

Antibiotic Susceptibility: Isolates showed 100% resistance to various members of Cephalosporins, Monobactams and Carbapenems, 95-98% resistance to Penicillins. 79-85% resistance to Aminoglycosides and 90% resistance against Fluoroquinolones. All the clinical isolates showed sensitivity towards colistin. 10% isolates showed resistance while 31.66% were intermediate sensitive against Tigecycline (Table 1).

Table 1: Antibiotic susceptibility pattern of *Klebsiella pneumoniae* using disk diffusion (n = 60)

Antibiotic	Resistant (%)	Inter-mediate (%)	Sensitive (%)
Piperacillin	59 (98.33)	0 (0)	1 (1.67)
Piperacillin-Tazobactam	57 (95)	1 (1.67)	2 (3.33)
Ceftazidime	60 (100)	0 (0)	0 (0)
Cefoxitin	60 (100)	0 (0)	0 (0)
Cefpodoxime	60 (100)	0 (0)	0 (0)
Cefotaxime	60 (100)	0 (0)	0 (0)
Cefepime	30 (100)	0 (0)	0 (0)
Ceftriaxone	60 (100)	0 (0)	0 (0)
Aztreonam	60 (100)	0 (0)	0 (0)
Meropenam	60 (100)	0 (0)	0 (0)
Ciprofloxacin	33 (89.19)	0 (0)	4 (10.81)
Levofloxacin	18 (90)	0 (0)	2(10)
Tigycycline	6 (10)	19 (31.66)	32 (53.33)
Amikacin	26 (78.79)	2 (6.06)	5(15.15)
Gentamicin	22 (84.62)	1 (3.85)	3 (11.53)
Colistin	0 (0)	0 (0)	60 (100)

Detection of β-lactamases by Screening and Confirmatory Tests: All isolates tested positive for ESBLs in the screening test. 53.33% screen positive isolates were confirmed phenotypically to produce ESBL (Table 2)

Table 2: Isolates positive for various β-lactamases by Phenotypic tests and PCR

Number of isolates tested	Number of ESBL positive isolates = n(%)		
	Screening test	Confirmatory test	PCR
60	60 (100)	32 (53.33)	55 (91.7)

PCR for ESBL genes: Clinical isolates were subjected to several multiplex as well as uniplex PCR's using specific primers [11] for the identification of various ESBL genes. Clinical isolates were tested for the presence of bla_{TEM-1}, bla_{SHV-1}, bla_{OXA-1}, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, bla_{VEB}, bla_{PER}, bla_{GES}, $bla_{CTX-M\ 8/25}$ genes that encode for ESBLs. 55 (91.7%) isolates were positive for various ESBL genes. 71.67%, 66.67%, 26.67%, 61.66%, 5%, 5%, 15%, tested positive for bla_{TEM-1}, bla_{SHV-1}, bla_{OXA-1}, $bla_{CTX-M-1}$, $bla_{CTX-M-9}$, bla_{VEB}, bla_{PER}, gene respectively. None were positive for bla_{CTXM-2}, bla_{Ges} and $bla_{CTX\ M8/25}$. One clinical isolate tested positive for five genes, namely bla_{TEM-1}, bla_{SHV-1}, bla_{OXA-1}, $bla_{CTX-M-1}$ and bla_{PER} while six samples showed the presence of bla_{TEM-1}, bla_{SHV-1}, bla_{OXA-1} and $bla_{CTX-M-1}$ genes. None of the isolates tested positive for the presence of all the genes tested, indicating selective expression of genes (Fig. 1).

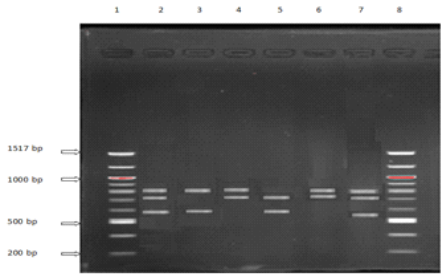

Fig. 1: Band pattern obtained for (bla_{TEM-1}, bla_{SHV-1}, bla_{OXA-1}) PCR
Lane 1,8 represents 100 bp Ladder marker. Other lanes depicts banding pattern of some included isolates. Lane 2, 3, 4, 6 and 7 shows the presence of bla_{TEM-1} gene (800 bp). Lane 2, 4, 5, 6 and 7 shows the presence of bla_{SHV-1} gene (713 bp). Lane 2, 3, 5 and 7 shows the presence of bla_{OXA-1} gene (564 bp).

Discussion

A high degree of resistance was observed among isolates of *K. pneumoniae*. Multi drug resistant (MDR) isolates have increased during the period of study (Our isolates were five times more in-patients than the outpatients. According to community surveillance study report on pathogenic organisms, resistance is encountered more among hospitalized patients in comparison to OPD [13]. This could be due to the extensive use of broad spectrum antibiotics in hospitalized patients. In the present study tigecycline and colistin showed promising results. Tigecycline has already been reported for lowering the all-cause mortality [14]. Although, isolates showed 100% sensitivity, but colistin resistance has already been reported [15, 16]. Tigecycline and colistin being reserve drugs, must be strictly monitored to prevent the development and dissemination of resistance in them.

In *K. pneumoniae*, production and spread of β-lactamase contributes towards attaining β-lactam resistance. Their continued spread can be attributed to overuse and misuse of inhibitory drugs, emergence of new class of β-lactamases like ESBLs (CTX-Ms) [17] and delay in administering appropriate antimicrobial therapy. Agriculture and food products further help in its dissemination [18].

In the study, multidrug resistance is correlated with ESBL production. Genotypic identification of ESBLs aids in treatment, prevention and control. It also helps in tracking these organisms in surveillance systems [3].

Till date, hundreds of new ESBL enzymes have been identified. The variants arise as a result of point mutations. The bla_{TEM} genes that codes for the TEM-1 enzyme is usually found on plasmids [4] and confer resistance towards Ampicillin and other Penicillins. SHV enzymes are chromosomally encoded, but can also be plasmid-mediated [19, 20]. Likewise, CTX-M enzymes are plasmid-mediated [21]. ESBLs showed variable degree of resistance to all class of antibiotics [22]. The coexistence of different ESBLs can render them multidrug resistant. TEM-1, SHV-1 and CTX-M-1 were the most widespread of all the ESBL enzymes observed in this study. Some ESBL groups, namely CTX-M-2, CTX-M-8/25 and GES were conspicuous by their absence.. There exists a possibility that the untypable bla_{CTX-M} genes were from another group and were not detected by $bla_{CTX-M-1}$, $bla_{CTX-M-9}$ and $bla_{CTX-M-8/25}$ group specific PCR.. Likewise bla_{SHV}-gene was not detected in isolates. Similar results have already been reported [23, 24]. The above results indicates that resistance can be conferred by the expression of a single gene or synergistic effect of multiple genes.

As evident from results above, PCR detected higher percentage (91.7%) of ESBLs in comparison to the phenotypic confirmatory test (51.33%). This could be due to the fact that PCR (for ESBL) detects genes while phenotypic confirmatory test is based on the presence of the gene products. There exist a possibility that gene might be present but does not undergo transcription or if at all it is transcribed, it does not undergo translation or there may occur post-translational modifications that might curb the expression or activity of enzyme, thereby resulting in negative phenotypic test. The sensitivity of PCR

being only 91.7 % explains that there may be other mechanisms of resistance other than ESBLs viz; efflux pumps.

Conclusions

Multidrug resistance in *K. pneumoniae* is increasing worldwide that invokes the need for judicious use of antibiotics This warrants continuous surveillance programs to monitor the emergence and spread of ESBLs. Multiplex PCR is a rapid and sensitive method that can be adopted in laboratories for rapid detection of *K. pneumoniae* from clinical isolates. To conclude, our study showed high prevalence of TEM-1, SHV-1 and CTX-M-1 type ESBLs which could have been the cause of MDR in our isolates.

Conflict of interest: The authors declare that they have no conflict of interest.

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