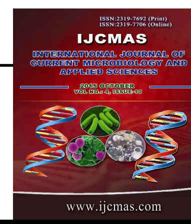

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Original Research Article

Drug Susceptibility and Molecular Mechanism of Resistance in *Klebsiella pneumoniae* Isolated from Cases of Sinusitis

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A B S T R A C T

Twenty five strains of *Klebsiella pneumoniae* isolated from cases of sinusitis were used in this study. Drug susceptible pattern and molecular mechanisms of resistance were evaluated. Drug susceptibility was studied for the following antimicrobial agents - amikacin, gentamicin, ciprofloxacin, ofloxacin, cefuroxime, cefotaxime, ceftriaxone, cefoperazone (sulbactam), ceftazidime, amoxicillin/ clavulanate, ampicillin, chloramphenicol, cotrimoxazole and doxycycline. Sensitivity was maximum to amikacin followed by gentamicin in 84% and 64% of the isolates respectively. Resistance to ciprofloxacin and ofloxacin were 64% and 52% respectively. To the second generation cephalosporin - cefuroxime, 84% strains were resistant. To the third generation cephalosporins viz. cefotaxime, ceftriaxone, cefoperazone (sulbactam) and to cefpodoxime, 18(72%), 23(92%), 17(68%), 23(92%) strains were resistant respectively. To augmentin and ampicillin 23(92%) isolates were resistant and to chloramphenicol 9(36%) strains were resistant. To cotrimoxazole and doxycycline 18(72%) and 23(92%) strains were resistant respectively. Plasmids were isolated in 4(16%) strains only. PCR for TEM-1 gene was positive in 8(32%) strains. Only 2 strains were positive for both TEM and plasmids. This study adds valuable data that can assist the scientific community in the development of a plan for a rational use of antimicrobial agents in the treatment and management of infections occurring due to *K. pneumoniae*.

Keywords

Antibiotic resistance, ESBL, *Klebsiella pneumoniae,* PCR, Plasmids, TEM-1

Introduction

Klebsiella has been reported to be increasingly antibiotic resistant, posing a great challenge to the successful control and treatment of patients with severe underlying diseases or nosocomial infections worldwide (Jurczak *et al.,* 2007; Lederman and Crum, 2005). The advent of *K. pneumoniae* with plasmid-encoded extended-spectrum beta-lactamase activity is resulting in significant morbidity and mortality, due to treatment failure and subsequent septicaemia (Lee *et al.,* 2006).

ESBLs are a group of enzymes encoded by genes described predominantly on plasmid that are common among *Enterobacteriaceae*

(Poole, 2004). Although most ESBLs are mutants of temoneira (TEM) and sulfhydryl variable (SHV) enzymes, the cefotaximase (CTX-M) type-lactamases which have become important, originated from β-lactamases found in environmental species of the genus *Kluyvera*, and this enzyme hydrolyzes cefotaxime and cefriaxone but is weakly active against ceftazidime (Bonnet *et al.,* 2004; Perez, 2007).

At present, there are more than 300 different ESBL variants, and these have been clustered into nine different structural and evolutionary families based on amino acid sequence.

TEM and sulphydryl variable SHV were the major types. However, CTX-M type is more common in some countries (Paterson, 2003). Although complete mechanisms of *K. pneumoniae* virulence are still lacking, the molecular underpinnings of pathogenicity in multiple-resistance for such pathogenic bacteria are generally regarded as plasmid mediated (Hastings, 2004; Shen *et al.,* 2008).

Plasmids have been considered the causative agents because of their ability to acquire antibiotic resistance in different species and can lead to rapid spread of antibiotic resistance (Levy and Marshall, 2004). Determination of ESBL genes by molecular techniques in ESBL producing bacteria and their pattern of antimicrobial resistance can supply useful data about their epidemiology and risk factors associated with these infections (Jain *et al.,* 2008).

The objectives of this study were to determine the antimicrobial resistance profiles of *K. pneumoniae* strains isolated from sinusitis patients. The presence of ESBL encoding gene TEM1 was determined by PCR. Plasmid presence was also noted.

Materials and Methods

Clinical samples

A total of 200 non-repetitive clinical samples collected during the period, June 2007 to January 2010, from the patients attending the Diagnostic Nasal Endoscopy section of the Out Patient (OP), Department of Ear, nose and throat (ENT), General Hospital, Chennai, were included in the study. Endoscopic pus samples were collected from the middle meatus region, in sterile transport medium, after obtaining due permission from the concerned health authorities. Samples collected were immediately transported to the laboratory and processed.

Bacterial identification

The clinical samples were cultured in appropriate media. *Klebsiella pneumoniae* were isolated and identified by performing standard biochemical tests (Balows *et al.,* 1991). The organisms that were identified as *Klebsiella* species were stored in Brain Heart Infusion agar in air tight vials for further study.

Antimicrobial susceptibility

Antimicrobial susceptibility of all the isolates obtained was performed by the disc diffusion method according to the CLSI guidelines (CLSI, 2010). The following antibiotics were tested by the disk diffusion method (Bauer, *et al.* 1966) - amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ofloxacin (5µg), cefuroxime (30µg), cefotaxime (30µg), ceftriaxone (30µg), cefoperazone- sulbactam (30µg), ceftazidime (30µg), augmentin (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), cotrimoxazole (75µg) and doxycycline (30µg) from Hi-Media Laboratories, BD Diagnostics Pvt Ltd, India.

ESBL detection

ESBL producing isolates were characterized phenotypically for ESBL production using double disc synergy test (DDST) as recommended by the Clinical Laboratory Standards Institute (CLSI) [CLSI 2006]. The test was done by using both cefotaxime (30μg) and ceftazidime (30μg) alone and in combination with clavulanic acid. A > 5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was taken as positive result for ESBL production.

Plasmid DNA extraction

Extraction of plasmid DNA was done for all the 25 strains. Plasmid DNA extraction from bacterial cell pellets was performed by alkali lysis method. Plasmids obtained were electrophoresed through 1% agarose gel with ethidium bromide and bands were visualized under UV light.

PCR detection of ESBL-encoding blaTEM

A total of 25 isolates of *Klebsiella pneumoniae,* exhibiting phenotypic traits compatible with ESBL production as described in a recent consensus paper (Cornaglia *et al.,* 2007) were examined for carriage of blaTEM1 by PCR assays. This was described previously using oligonucleotides blaTEM1A-F 5′ATG AGT ATT CAA CAT TTC CG --3′and blaTEM1B-R5′-CTG ACA GTT ACC AAT GCT TA -3′ which yielded a product of 867 bp (Rasheed *et al.,* 1997). Amplification of the target gene was carried out using bacterial cell lysate as the source of template DNA. *Klebsiella pneumoniae* cells which was grown overnight at 37°C on Luria Bertani agar (LB Agar) were picked up, inoculated into LB broth and kept for overnight incubation in the shaker incubator. The bacterial cells were pelleted by centrifugation at 10000 X g for 5 minutes. The cell pellets obtained were washed with Tris EDTA buffer and were resuspended in 1000µl of Tris EDTA buffer and boiled for 2 minutes in a microwave oven. Cell debris was removed by centrifugation at 10,000 rpm for 10 minutes and the supernatant containing the template DNA was used for PCR assay.

The amplification was performed in a thermocycler (Eppendorf Master Cycler Personal, Germany) Amplifications were carried out in 0.2 ml tubes. Two µl of the primer mix, 12.5 µl of the master nucleotide mix, 3µl of the sample supernatant were added in each reaction of 25µl, made up with nuclease free water. The cycling conditions were: initial denaturation step at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and elongation at 72 °C for 1.5 min. After a final elongation step of 5 min at 72°C, the amplification products were obtained. The PCR products were electrophoresed through 1.5% agarose gel with ethidium bromide, to resolve the amplified products and they were visualized under UV light.

Results and Discussion

ESBLs have become a widespread serious problem. These enzymes are becoming increasingly expressed by many strains of pathogenic bacteria with a potential for dissemination. A total of 25 strains obtained from sinusitis patients were used in the study. Antibiotic resistance profiles of the isolates using disk diffusion tests demonstrated that the isolates were highly resistant to ceftriaxone, ceftazidime, augmentin and doxycycline (23 isolates, 92%) and least resistant to amikacin (3 isolates 12%) followed by chloramphenicol

and gentamicin (9 isolates 36%). Majority (23 of 25, or 92 %) of the strains showed sensitivity to ampicillin. The resistance rates for other antibiotics are listed in table 1.

The resistance rates for amikacin and gentamicin were 3 (12%) and 9 (36%) respectively which were however, lower than those in the earlier studies on *K. pneumoniae* strains from Malaysia in 1999 (Parasakthi *et al.,* 2000) and 2000–2004 (Loh *et al.,* 2007). Researchers also reported the resistance rates from another public Malaysian hospital but over a 4-year period. They showed that the resistance rate for combined amoxicillin–clavulanic acid and ampicillin– sulbactam was 58.4% whereas the resistance rate for aminoglycosides (combined gentamicin and amikacin) was 42.5 %. These figures are in sharp contrast to our study.

The resistance rates of *K. pneumoniae* to the third generation cephalosporins- ceftazidime and ceftriaxone were lower than those in the study carried out in 1999 (Parasakthi *et al.,* 2000) but were higher than those in the study carried out for the years 2000–2004 (Loh *et al.,* 2007). Fluctuating resistance rates of 18–21% to third-generation cephalosporins (combined ceftazidime, ceftriaxone and cefotaxime were reported. In contrast our study showed resistance rates for cefotaxime, ceftriaxone, cefoperazone-sulbactam and cefpodoxime of 18(72%), 23(92%), 17(68%), 23(92%) resistant respectively by Loh *et al.,* (2007).The resistance rates of the Malaysian *K. pneumoniae* strains to amikacin, chloramphenicol and trimethoprim–sulfamethoxazole were also lower than those of strains isolated from Thailand (resistance rates of 58 %, 45% and 74 %, respectively (Chaikittisuk Munsrichoom, 2007).

Among the 25 *K. pneumoniae* strains, 16(64%) were MDR (i.e. resistant to three or more classes of antimicrobial agents). In the study conducted by King Ting Lim *et al.* (2009) reported ESBL production was 32%. On the contrary in various other studies, ESBL production rate varies from 17% to 70% (Meeta Sharma *et al.,* 2013).

In present study endoscopic pus samples were the major source of ESBL. In a study conducted by Meeta Sharma, (2011) respiratory tract samples (63.83%) were the major source of ESBL producing strains followed by stool samples, urine, body fluid, pus and blood. However, in other studies urine was the major source of ESBL producers (Saba *et al.,* 2012). The rate of MDR *K. pneumoniae* strains in Malaysia is relatively low (53 %) when compared to the 94% MDR rate reported in India (Manchanda *et al.,* 2005).

Nuesch & Hachler reported that although molecular methods appear sensitive, but are expensive, time consuming and require specialized equipment and expertise (Nuesch and Hachler, 1996). However, definitive identification is possible only by molecular detection methods. Keeping in view this scenario, the current study was investigated on *K. pneumoniae* to look for the presence of TEM gene. Two studies from Chennai in 2006 show different rates of ESBLs - 21.2% & 42.85% (Menon T *et al.* 2006, Padma M *et al.* 2006). Similarly two studies in 2012 from Davangere showed different rates of ESBLs- 2 6.53% & 37.23%. It has been already reported that incidence of ESBLs differs not only across the country but from institute to institute (Eshwar singh *et al.,* 2012). The incidence of ESBLs in Manipal has actually decreased over the years from 41% in 2007 to 27.39% in 2009 (Shobha *et al.,* 2009). Prevalence of ESBL producing *Klebsiella* around the world varies between 3%–8% to 100% (Cristina, 2011).

Table.1 Antimicrobial susceptibility profile of the 25 *K. pneumoniae* isolates used in the study

Antibiotics	Resistant	Intermediate	Sensitive
Amikacin	3(12%)	1 (4%)	21(84%)
Gentamicin	9(36%)	0 (0%)	16(64%)
Ciprofloxacin	16(64%)	3(12%)	6(24%)
Ofloxacin	13(52%)	2(8%)	10(40%)
Cefuroxime	21(84%)	2(8%)	2(8%)
Cefotaxime	18(72%)	6(24%)	1(4%)
Ceftriaxone	23(92%)	0 (0%)	2(8%)
Cefoperazone -sulbactam	17(68%)	5(20%)	3(12%)
Ceftazidime	23(92%)	1(4%)	1(4%)
Augmentin	23(92%)	1(4%)	1(4%)
Ampicillin	23(92%)	1(4%)	1(4%)
Chloramphenicol	9(36%)	4(16%)	12(48%)
Cotrimoxazole	18(72%)	2(8%)	5(20%)
Doxycycline	23(92%)	2(8%)	0(0%)

Fig.1 Amplification with blaTEM lane 1: 100 bp ladder, lane 4-6 samples, lane 3 positive control and lane 2 negative control

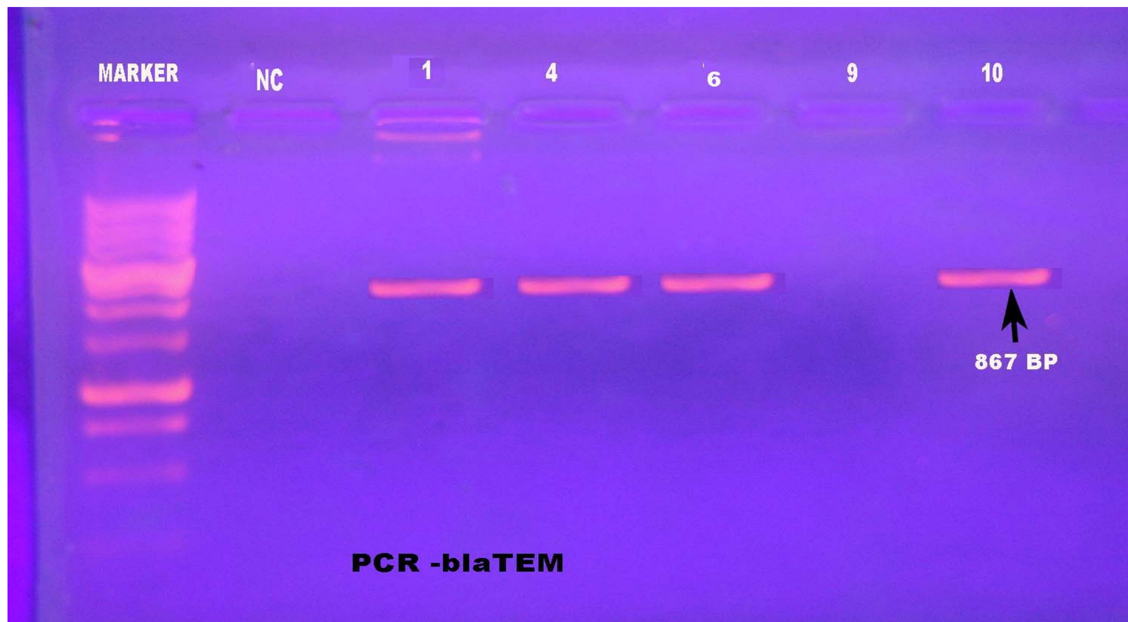

Fig.2 Agarose gel demonstrating the presence of plasmids ranging from 90 to100 Kb

PCR detection of ESBL-encoding gene

Using specific primers, PCR was carried out on the genomic DNA of the 25 *K. pneumonia* strains for the ESBL-encoding genes: blaTEM-1. One third of the strains (8 of 25) showed positive amplification for blaTEM-1 (Figure 1). According to King – Ting Lim *et al.* (2009) majority of the ESBL-positive isolates from Malaysia harbored TEM-1 (88%), which is quite high as compared to this study

In another study from Turkey the most frequent β-lactamase type was CTX-M (92%), followed by TEM (39%), SHV (5%) and Vietnamese extended-spectrum beta-lactamase (VEB, 1.6%) (Nazik, 2011). Thus, blaSHV appeared to be more common among Malaysian *K. pneumoniae* strains even though blaCTX-M is now reported to be the most prevalent ESBL gene worldwide, replacing TEM and SHV b-lactamases in many European countries (Livermore *et al.,* 2007).

Of the 25 ESBL positive *K. pneumoniae* isolates 15 harboured TEM-1 gene (Figure 1). A study by Grover *et al* (2006) on phenotypic and genotypic methods of ESBL detection concluded PCR to be a reliable method of ESBL detection. Preliminary plasmid analysis results indicated that 21 of the *K. pneumoniae* strains had not harboured any plasmids. Plasmids of sizes ranging from 90 to 100 kb were, however, detected in the other 4 *K. pneumoniae* strains and efforts are on-going to further characterize these plasmids (Figure 2). Molecular detection and identification of beta lactamases would be essential for a reliable epidemiological investigation of antimicrobial resistance. Reporting of ESBL producing isolates from clinical samples is

useful for the clinicians to select appropriate antibiotics for the treatment of ESBL producing strains and to take proper precaution to prevent the spread of these resistant organisms to other patients.

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