Klebsiella: An insight into the virulence factors and antimicrobial susceptibility pattern

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Abstract
Introduction: Klebsiella pneumoniae is a frequent pathogen isolated from pneumonia, urinary tract infections, liver abscesses, wound infections, intravascular catheter infections, biliary tract infections, peritonitis and meningitis. The increasing occurrence of serious Klebsiella infections necessitates the need to identify the pathogenic mechanisms that are responsible for high virulence of these strains.

Objectives: Characterization, detection of virulence factors (Hypermucoviscosity, serum resistance, biofilm formation) and determination of antimicrobial susceptibility pattern of Klebsiella isolates from various clinical samples.

Materials and Method: Klebsiella isolates from various clinical samples were analysed in comparison to isolates from stool samples as controls. The isolates were identified by standard microbiological techniques and their antibiotic profile was determined by Kirby-Bauer disc diffusion method. Biofilm production was determined by tissue culture plate method, hypermucoviscosity by string test and serum resistance by serum bactericidal assay.

Results: Amongst the clinical samples, Klebsiella pneumoniae subsp aerogenes (89%) was the predominant species followed by Klebsiella oxytoca (8%) and Klebsiella pneumoniae subsp pneumoniae (3%). The stool samples exhibited a similar pattern with Klebsiella pneumoniae subsp aerogenes (86%) being the predominant species followed by K. oxytoca (14%). The antibiotic susceptibility profile of the isolates showed sensitivity to imipenem, aminoglycosides and fluoroquinolones. Hypermucoviscosity was shown by 12%, serum resistance by 41% and biofilm formation by 36% in clinical isolates. In contrast, hypermucoviscosity, serum resistance and biofilm production was observed in 6%, 7% and 16% of the control isolates respectively.

Conclusion: The rising incidence of Klebsiella infections obligates an insight into the various virulence factors and drug resistance patterns to pave a way for combating the growing menace of the virulent pathogen.

Keywords: Klebsiella pneumoniae, Hypermucoviscosity, Serum resistance, Biofilm formation, Drug resistance

Introduction
Klebsiella pneumoniae is an important pathogen and is a common cause of urinary tract infections, respiratory tract infections and sepsis. In recent years an emerging syndrome of community acquired pyogenic liver abscess caused by highly virulent Klebsiella pneumoniae strains has occurred. A syndrome with metastatic spread to eyes, meninges, brain and other sites has been described which has been strongly associated with K. pneumoniae strains of capsular serotype K1 of a characteristic hypermucoviscous phenotype. The increasing occurrence of severe Klebsiella infections emphasize the need to understand the pathogenic mechanisms of these highly virulent strains. These virulence factors may be responsible for the evasion of host defence mechanisms and damage caused to host cells, tissues and organs in a number of ways. Several mechanisms may contribute towards antimicrobial resistance and virulence in gram negative bacteria which may work in concert to form multi drug resistant profiles. Capsular antigens, fimbriae, serum resistance, siderophore formation constitute some of the important virulence factors of Klebsiella. Hence, the study was undertaken to detect the virulence factors and determine the antimicrobial susceptibility pattern of the Klebsiella isolates in comparison to isolates from stool taken as controls.

Materials and Method
The present study was carried out in the department of Microbiology, at a tertiary care hospital in Bangalore for a period of one and a half years from January 2015 to June 2016. Klebsiella isolates from various clinical samples (urine, pus, blood, peritoneal fluid, sputum, bronchoalveolar lavage and cerebrospinal fluid) were included in the study after correlating with clinical history. Klebsiella isolates from stool samples of healthy individuals were taken as controls. Klebsiella species isolated from non-clinical samples (water analysis) were excluded from the study. Samples obtained were subjected to gram stain and culture. The specimens were streaked on Blood agar, Chocolate agar, MacConkey agar, BHI broth and were incubated overnight at 37°C. They were subjected to speciation by indole production, methyl red test, vogue prostauer test, citrate utilization, urease production, malonate utilization, lysine and ornithine decarboxylation. The Klebsiella isolates obtained from clinical samples were subjected for antibiotic susceptibility testing by Kirby Bauer disc diffusion technique on Mueller Hinton agar (MHA). After 18-24 hrs of incubation the diameter of the clear zone around the disc was measured under transmitted light and results interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.
**Testing for Virulence Factors**

**Hypermucoviscosity:**

Hypermucoviscosity was defined by the formation of viscous strings > 5 mm in length when a loop was used to stretch the colony on agar plate (positive string test). The strains were inoculated on 5% sheep blood agar plates and incubated at 37°C overnight. A standard bacteriological loop was used to stretch a mucoviscous string from the colony.

**Serum Resistance:** It is implicated as the ability of bacteria to resist the bactericidal effect of serum which plays an important part in the pathogenesis of different infections. Bacteria are grown in nutrient broth and is adjusted to a concentration of 2×10^8 cells/ml of physiological saline. 25 µl of bacterial suspension and 75 µl of pooled normal human serum are put into microtiter trays, mixed and incubated at 37°C. Viability is determined immediately and after 1, 2, and 3 h of incubation. After mixing, samples are taken and serial dilutions are plated on brain heart infusion agar for colony counts. Strains are termed serum sensitive if the viable count drops to <1% of the initial serum value and serum resistant if >90% of organisms survived after 180 mins.

**Biofilm Formation:** Klebsiella strains are sub cultured three times in Luria Bertoni broth for 18 h at 37°C. The (optical density) OD of Klebsiella grown in LB is adjusted to 0.56 to 0.64(2×10^8 CFU/ml). Aliquots of 200µl are transferred to pre-sterilized 96-well polystyrene microtitre plates and incubated for 6 hours at 37°C. After incubation, 25 µl of 1% Crystal Violet is added to each well, shaking the plates three times to help the colorant to get to the bottom of the well. After 15 minutes at room temperature, each well is washed with 200 µl sterile (phosphate buffered saline) PBS to remove the planktonic cells and stain not adhered to the well. The absorbance is determined at 540 nm in an UV spectrophotometer.

**K. pneumoniae** ATCC 700603 strain was used as positive control. Data obtained are used to classify the strains as high producers (OD higher than 0.500), producers (OD between 0.500 and 0.100) or poor producers (OD lower than 0.100). For interpretation of the results, strains were divided into the following categories:

- Non-biofilm producer OD ≤ ODc,
- Weak biofilm producer (+ or 1) = ODc < OD ≤ 2×ODc,
- Moderate biofilm producer (++ or 2) = 2×ODc < OD ≤ 4×ODc,
- Strong biofilm producer (+++or 3), 4 × ODc < OD

**Statistical Methods Used:** Descriptive and inferential statistical analysis has been carried out in the present study. Chi-square/ Fisher Exact test has been used to find the significance of study parameters on categorical scale between two or more groups. Non-parametric setting for Qualitative data analysis.

**Results**

In the present study, out of 100 consecutive non repetitive Klebsiella isolates, 66 were from males and 34 were from females amongst the cases. In the control group, 74 isolates were from males and 26 from females. The maximum number of isolates in both cases and controls were above the age of 40yrs. Speciation of the isolates revealed that *Klebsiella pneumoniae subsp aerogenes* (89%) was the predominant species followed by *Klebsiella oxytoca* (8%) and *Klebsiella pneumoniae subsp pneumoniae* (3%) as depicted in Fig. 1. The isolation of *Klebsiella* species from various clinical specimens is depicted in Table 1. Isolates from sputum constituted the maximum number of samples followed by pus, urine, blood and various body fluids as shown in Table 1.
Virulence Factors: Detection of hypermucoviscosity was performed by string test, of which 12% of the cases and 6% of the controls showed hypermucoviscosity with P-value 0.157 (Table 2). The clinical isolates showed more hypermucoviscosity in comparison to controls but it was not statistically significant. The sputum and bronchoalveolar lavage fluid samples showed maximum hypermucoviscosity.

The clinical isolates and controls were screened for serum resistance which showed that the clinical isolates (41%) were more resistant to the serum bactericidal action than the control isolates (7%) with a significant p value of < 0.001** (Table 2).

Clinical isolates showed more biofilm production (36%) than the control isolates (16%) which was statistically significant with P < 0.001** (Table 2).

Antibiotic susceptibility profile of the isolates: The antibiotic susceptibility profile of the isolates showed sensitivity to imipenem (96.8%), gentamicin (83.6%), amikacin (78.2%) and levofloxacin (81.9%). There was an increasing resistance to third generation cephalosporins cefoperazone (45.2%) and ceftazidime (61.5%). Most of the isolates were resistant to amoxicillin clavulanic acid combination (96.8%) Fig. 2.

### Table 1: Isolation of Klebsiella from various clinical specimens

<table>
<thead>
<tr>
<th>Specimen</th>
<th>K. pneumoniae Subsp Aerogenes (n=89)</th>
<th>Subsp Pneumoniae (n=3)</th>
<th>K. oxytoca (n =8)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum*</td>
<td>33 (37.1%)</td>
<td>3 (100.0%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wet swab</td>
<td>20 (22.5%)</td>
<td>0</td>
<td>3 (37.5%)</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>14 (15.7%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Blood</td>
<td>10 (11.2%)</td>
<td>0</td>
<td>4 (50.0%)</td>
<td></td>
</tr>
<tr>
<td>Ascitic/ Peritoneal fluid</td>
<td>7 (7.9%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BALT fluid</td>
<td>4 (4.5%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CVP tip/ Oral suction tip</td>
<td>1 (1.1%)</td>
<td>0</td>
<td>1 (12.5%)</td>
<td></td>
</tr>
</tbody>
</table>

*On microscopy, sputum samples with pus cells> 25 and epithelial cells< 10/lpf were processed.

### Table 2: Virulence factors in klebsiella species from cases and controls

<table>
<thead>
<tr>
<th>Virulence factors</th>
<th>Cases (n=100)</th>
<th>Controls (n=100)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
<td>No</td>
</tr>
<tr>
<td>Hypermucoviscosity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Positive</td>
<td>12</td>
<td>12.0</td>
<td>6</td>
</tr>
<tr>
<td>• Negative</td>
<td>88</td>
<td>88.0</td>
<td>94</td>
</tr>
<tr>
<td>Serum resistance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Resistant</td>
<td>41</td>
<td>41.0</td>
<td>7</td>
</tr>
<tr>
<td>• Intermediate</td>
<td>24</td>
<td>24.0</td>
<td>13</td>
</tr>
<tr>
<td>• Sensitive</td>
<td>35</td>
<td>35.0</td>
<td>80</td>
</tr>
<tr>
<td>Biofilm production</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Producers</td>
<td>36</td>
<td>36.0</td>
<td>16</td>
</tr>
<tr>
<td>• Non-biofilm producers</td>
<td>64</td>
<td>64.0</td>
<td>84</td>
</tr>
</tbody>
</table>

Chi-Square test/fisher Exact test


**Discussion**

*Klebsiella* has emerged as a potential virulent pathogen over the years. Previously, a common cause of community acquired pneumonia, urinary tract infections, bacteremia and neonatal septicemia, hypervirulent forms with a potential for metastatic spread has occurred. These hypervirulent forms are primarily the cause of amoebic liver abscess, meningitis and metastatic endophthalmitis. An insight of different virulence factors and their mechanism of interference with the host immune mechanism responsible for disease causation would enable to curb their harmful effects.

The male to female ratio in cases is 1.94:1 and in controls is 2.84:1 in this study. The higher preponderance of males as compared to females correlates well to a study conducted by Namratha K G et al\textsuperscript{15} where a higher incidence of *Klebsiella* infections were reported in males as compared to females. The maximum number of cases in our study were in the age group 41-50 yrs followed by the age group of 51-60 yrs. A study done by Namratha K G et al revealed that individuals aged above 60 years were more susceptible to *Klebsiella* infections which was closely followed by age group between 45-60 yrs.

Species identification is critical in the diagnosis and treatment of persons infected with *Klebsiella*. It is also required in disease prevention, patient management and surveillance of infection. This practice is usually ignored in most of our hospitals mainly due to limited resources, time and labour.\textsuperscript{16} This study was performed to characterize, identify virulence factors and to understand the antimicrobial susceptibility of clinical isolates of *Klebsiella* species in comparison to *Klebsiella* isolates from stool sample as controls.

Majority of the isolates were *Klebsiella pneumoniae subsp aerogenes* (89%), followed by *Klebsiella Oxytoca* (8%) and *Klebsiella pneumoniae subsp pneumoniae* (3%). This corresponds well to study conducted by Namratha K.G. et al\textsuperscript{15} where *Klebsiella pneumoniae* was the major isolate followed by *Klebsiella oxytoca*. In a study conducted by D. O Acheampong, L. K Boamponsem et al\textsuperscript{16} in 201, *Klebsiella pneumoniae* was the most common isolate followed by *Klebsiella oxytoca*, 2 isolates of *Klebsiella rhinoscleromatis* and one isolate of *Klebsiella ozaenae*.

Bacterial virulence factors are structural components or products produced by bacteria that allow the organism to harm the host in some manner. Knowledge of a microorganisms capacity to cause specific types of infections plays a major role in the development of diagnostic microbiology procedures used for isolating and identifying microorganisms.\textsuperscript{4}

**Hypermucoviscosity:** 

Hypermucoviscous (hypervirulent) strain of *K pneumoniae* poses a great threat. A defining virulence feature in these organisms is the ability to resist the bactericidal activity of antimicrobial peptides complement and phagocytes in the absence of antibody. It has shown to be significantly more resistant to the complement mediated and neutrophil mediated bactericidal activity than the classical *K pneumoniae* isolates.\textsuperscript{17}

In this study, hypermucoviscosity was shown by 12% of the clinical isolates and 6% of the control isolates with P value 0.157. In a study of community acquired bacteremia conducted by Lee H-C, Chuang Y-Cet al, the hypermucoviscous phenotype of *K pneumoniae* were associated with development of a distinct invasive syndrome.\textsuperscript{18}

In a seroepidemiological study of stool samples of *K pneumoniae* from healthy Chinese adults, hypermucoviscosity was demonstrated in K1 and K2 serotypes of *K pneumoniae* pointing to the fact that certain Asians are colonized with hypervirulent *K pneumoniae* strains.\textsuperscript{17}
Serum resistance: Serum bactericidal activity is supposed to prevent micro-organisms from establishing infection in the blood. The isolates which show resistance to serum bactericidal activity are associated with the onset of infection with severe symptoms. In our study, 41% of clinical isolates showed resistance to serum bactericidal activity, 24% were of intermediate susceptibility and 35% were susceptible to serum bactericidal activity. In contrast, 7% of isolates from controls were resistant to serum bactericidal action, 13% showed intermediate susceptibility 80% isolates were susceptible to serum bactericidal action. The clinical isolates were significantly resistant to serum bactericidal action in contrast to faecal isolates with P value < 0.001.

Studies conducted by Benge et al[9] and Podschun et al[10] revealed that 40% of *Klebsiella* isolates from different infections were more resistant to the serum bactericidal action than the faecal isolates. The findings of our study are consistent with the studies done by Benge and Podschun whereby *Klebsiella* isolated from clinical specimens were more resistant to the bactericidal effect of serum than the faecal isolates. Virulence of the strains resistant to the bactericidal effect of serum is greatly increased by its ability to grow in the presence of bactericidal serum components thereby causing more severe infections.

Biofilm formation: Microorganisms growing in a biofilm are associated with chronic and recurrent human infections that are resistant to antimicrobial agents. The gel-like state, predominantly consisting of polysaccharides, prevents the access of antibacterial agents, such as antibiotics, white blood cells and antibiotics. 36% of clinical isolates in our study were biofilm producers in contrast to 16% of the faecal isolates showing biofilm production. The present study correlates with the study conducted by Hemchandran[11] et al showing a biofilm formation in 31% of the *Klebsiella* isolates in their study. This study does not show a very high biofilm production as it may depend on the clinical condition and the antimicrobial susceptibility pattern of the isolates.

Antimicrobial susceptibility of *Klebsiella* Isolates: The multidrug resistant strains of *Klebsiella* species are constantly increasing.[12] Knowledge about the resistance pattern of these bacterial strains will help in the judicial use of antibiotics, formulation of antibiotic policies apt for the hospitals and implementation of infection control programmes.

The present study reveals the incidence of infections due to *Klebsiella* and their tendency towards antibiotic resistance. Antibiotic susceptibility testing revealed that *Klebsiella* isolates were sensitive to imipenem (96.8%), gentamycin (83.6%), amikacin (78.2%), levofloxacin (81.9%), meropenem (77.42%) piperacillin/tazobactam (70.37%) and cefepime (64.3%), followed by third generation cephalosporins ceftoperazone (54.8%) and cefazidime (38.47%). They were least susceptible to amoxicillin and clavulanic acid combination (3.2%).

The sensitivity pattern of the isolates to imipenem were similar to a study conducted by Bhaumik et al[13] and Namratha et al.[14] The decreased susceptibility to meropenem might be due to the loss of porin and the presence of plasmid mediated beta lactamases. The increasing resistance to third generation cephalosporins could be due to the production of extended spectrum beta-lactamases and Amp C beta lactamases. Similar observation was recorded by Kothari et al[15] where the isolates showed an increasing resistance to third generation cephalosporins.

Apart from the inherent resistance to ampicillin, other group of antibiotics are also being rendered ineffective against this virulent pathogen. The escalating multidrug resistant antibiotic profile of the organism would leave us with limited therapeutic options to combat the infections caused by it. Thus, we need to choose the antibiotics with precision against the infections caused by *Klebsiella*.

Limitations of the Study

The study was a phenotypic study of the virulence factors and speciation was done by the conventional biochemical methods. Detection of biofilm forming genes, hypermucoviscosity genotypes by PCR and confirmation of antimicrobial susceptibility procedures by the various automated methods would give a better insight into the virulence factors, drug resistance pattern and enable management of *Klebsiella* infections.

Conclusion

*Klebsiella* infections are a significant cause of mortality and morbidity amongst the patients. Therefore, the study of infections caused due to *Klebsiella* and their virulence factors coupled with the antimicrobial susceptibility pattern would be of utmost importance in combating the growing menace. Regular surveillance of antibiotic susceptibility pattern would help in formulation of proper antibiotic policies and prevent indiscriminate usage of drugs which lead to drug resistance.

References


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