Extremely High Mortality Rates in Patients with Carbapenem-resistant, Hypermucoviscous *Klebsiella pneumoniae* Blood Stream Infections

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**Abstract**

**Background:** Infections caused by carbapenem resistant *K. pneumoniae* are increasing and associated with high mortality rates. There are increasing reports of hypermucoviscous/hypervirulent *K. pneumoniae* isolated from various sources. However, there is limited data on the prevalence of hypermucoviscous strains among carbapenem-resistant *K. pneumoniae* from invasive infections in India and its association with mortality. *rmpA*, *rmpA2* and *magA* genes are associated with these hypervirulent strains. In this study, we investigate the prevalence of hypermucoviscous strains amongst carbapenem resistant *K. pneumoniae* isolated from blood culture. Association of mortality rate with meropenem minimum inhibitory concentration and hypermucoviscous strains are determined.

**Methods:** 86 non-repetitive carbapenem resistant *K. pneumoniae* isolated from bacteremia underwent E-test for meropenem minimum inhibitory concentration (MIC) determination and PCR for detection of carbapenamase genes. String test, PCR for *rmpA*, *rmpA2* and *magA* were performed for characterisation of hypervirulent strains.

**Results:** 31.3% of the 86 isolates displayed hypermucoviscous phenotype as indicated by a positive string test. Among the two genotypic markers, 7% were positive for *rmpA2* and all were negative for *rmpA* and *magA*. 74.1% and 67.9% mortality were seen among string test positives and isolates meropenem MIC of ≥16µg/ml respectively (p 0.036 and 0.008 respectively). Isolates with both string positivity and meropenem MIC of ≥16µg/ml had a very high mortality rate of 84.2%.

**Conclusion:** String test, aids prediction of disease severity, and is independently associated with increased mortality in invasive carbapenem resistant *K. pneumoniae* health care-acquired infections. High meropenem MIC is a significant risk factor for mortality. Combination of string positive carbapenem resistant hypermucoviscous *K. pneumoniae* resulted in mortality rate of 84.2%. It is important to monitor prevalence of carbapenem resistant hypermucoviscous/hypervirulent *K. pneumoniae* among invasive isolates especially in a setting with high resistance rates as combination of increased virulence and decreased susceptibility to antimicrobials results in worse outcomes.

**Background**

*Klebsiella pneumoniae* is a common pathogen and causes a wide range of infections including pneumonia, urinary tract infection, intra-abdominal infection and wound infection. Infections with *K. pneumoniae* are usually hospital-acquired and occur primarily in patients with impaired host defences including solid tumours, haematological malignancies, liver cirrhosis and diabetes mellitus.

Increased and poorly regulated antibiotic use has resulted in a rise in incidence of carbapenem-resistant *K. pneumoniae* (CRKp). These infections have a very high mortality rate of 30 to 44%. Recent years have also brought a rise in incidence of a hypermucoviscous/hypervirulent variant of *K. pneumoniae* (hvKp) associated with liver abscess. The string test is a phenotypic marker used to screen for these strains. The genes *rmpA* and *rmpA2* (both regulators of mucoid phenotype) and *magA* (mucoviscosity associated gene) are associated with string test positivity and are sometimes used as genomic markers of hypervirulence. Other virulence genes that are expressed more frequently in hypervirulent *K. pneumoniae* than the classical *K. pneumoniae* include siderophores such as *iutA* (aerobactin), *ybtS* (yersiniabactin), *cntB* (enterobactin), *kfu* (mediating ferric iron uptake); *allS* (allantoin metabolism) and *mrkD* (type3 fimbriae mediating attachment to extracellular matrix).

Unfortunately, there is no internationally agreed definition for hvKp strain and this hinders surveillance and literature review. Some authors define hypervirulence by clinical syndrome alone including patients with liver abscess, metastatic infection and high mortality rate caused by *K. pneumoniae*. Others define hypervirulence phenotypically by string test positivity or genotypically by *rmpA* or *rmpA2* positivity. Further authors use mouse lethality studies.

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Received: 07.06.2017; Accepted: 12.09.2018
There have been no reports on the prevalence of hypermucoviscous variants among CRKp causing bacteremia in India. This study aims to characterize carbapenem-resistant hypermucoviscous strains isolated from bacteremia. We investigate the phenotypic and genotypic prevalence of the hypermucoviscous together with meropenem MIC and carbapenemase genes. The association of string test and meropenem MIC with patient mortality was determined.

Methods

Study population

The study was performed at the department of Clinical Microbiology, Christian Medical College, Vellore, India. Cases of bacteremia from 2014 and 2015 were considered and 86 non-repetitive, first positive blood culture isolated from patients that grew carbapenem resistant K. pneumoniae were included in the study.

Phenotypic characterisation of bacterial isolates:

Isolation and identification of the K. pneumoniae isolates were performed using standard biochemical reactions. The antimicrobial susceptibility testing for the first and second line antimicrobials was performed using Kirby Bauer disc diffusion as recommended by CLSI (Clinical and Laboratory Standards Institute) and the results were interpreted according to CLSI guidelines. 86 carbapenem resistant isolates were chosen. Further, these were tested for meropenem by E-test (Biomerieux) to determine the minimum inhibitory concentration (MIC). E. coli ATCC 25922 was used as control strain for antimicrobial susceptibility testing.

String test was performed for the detection of hypermucoviscous K. pneumoniae and was considered positive when a viscous string of >5mm was produced when the colonies were stretched with an inoculation loop.15

Molecular characterization of bacterial isolates

Bacterial genomic DNA was isolated by boiling the bacterial suspension at 100°C for 15 minutes and the supernatant was collected after centrifugation and used for molecular assays. Molecular characterization of bacterial isolates was carried out using conventional multiplex PCR for the detection of carbapenemase genes which included bla IMP, bla VIM, bla NDM, bla OXA-48, bla KPC, and bla GES, as described by Poirel et al., 2011, Dallene et al., 2010, Yigit et al., 2001 and Ellington et al., 2007.16,17 For the detection of hypervirulent K. pneumoniae, rmpA, rmpA2 and magA were detected. PCR for rmpA2 and magA were performed as described by Brisse et al., 2009.18 magA also corresponds to the capsular type1. The primers used are as mentioned in Table 1.

Multi-locus Sequence Typing

Ten representative isolates were sequenced and typed using the protocol mentioned by Diancourt et al., 2005.18 Four string test positive isolates and six string test negative isolates were typed. The sequence type was determined using the database maintained by Pasteur Institute at http://bigd.db.pasteur.fr/klebsiella/klebsiella.html

Clinical details

Clinical details of the patients, including age, sex, co-morbidities, source of infection and clinical outcome, were collected retrospectively from electronic medical records at Christian Medical College, Vellore.

Statistical analysis

Results were analysed for association between 30-day mortality with meropenem MIC, string test positivity and carbapenemase genes using Chi square test and logistic regression with 95% CI. The variables associated with risk factors for mortality were considered significant if the p value was <0.05. For analysis of meropenem MIC, isolates were stratified into four groups based on meropenem MIC. The first group comprised of susceptible isolates with MIC ≤1µg/ml (n=11), the second of intermediate susceptible isolates with MIC 2µg/ml to ≤16 µg/ml (n=11), and the third of resistant isolates with MIC between ≥16 µg/ml to ≤64 µg/ml (n=53), a fourth group considered these isolates that were highly resistant to meropenem.

Results

CRKp isolates from 86 patients obtained during 2015 and 2016 were included in this study. 65% of the patients included in the study were male with a median age of 37.5 years. 52% were immunocompromised. 92% of infections were associated with healthcare. Further clinical details can be found in Table 2.

Twenty-seven (31.3%) isolates were string test positive and 59 (68.6%) were negative. Only three string positive and three string negative isolates contained the rmpA2 gene. rmpA was absent in all the isolates. None of the isolates belonged to K1 capsular type since

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Table 1: List of primers used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaIMP</td>
<td>GGAAATAGATTGCTTTAAAYTCTC</td>
<td>TTTATGTGCAATAAGGATGTT</td>
<td>10</td>
</tr>
<tr>
<td>blaVIM</td>
<td>GATGCTTTTGGTGCTCGGATA</td>
<td>CCTCCTGGAGAGTAAGCATT</td>
<td>16</td>
</tr>
<tr>
<td>blaNDM</td>
<td>CACCTTCTTGACATGCTGCAA</td>
<td>CTGTCATGGTGAATTCGCC</td>
<td>16</td>
</tr>
<tr>
<td>blaOXA48</td>
<td>TATATCGTTAGACAGGAAACG</td>
<td>TATATCGTTAGACAGGAAACG</td>
<td>16</td>
</tr>
<tr>
<td>blaKPC</td>
<td>AGAATGCGGTCAGAAGAACC</td>
<td>CACACAAATGGCCTAACC</td>
<td>16</td>
</tr>
<tr>
<td>rmpA1</td>
<td>TCTGATCGTCACCTCAGAAGACC</td>
<td>CTCATCCCTGGCAGCACC</td>
<td>17</td>
</tr>
<tr>
<td>rmpA2</td>
<td>ATTCAGACCTGCTTGG</td>
<td>ATTCAGACCTGCTTGG</td>
<td>16</td>
</tr>
<tr>
<td>magA</td>
<td>GGTGCCTTACATCCATTGC</td>
<td>CTTCTGGAATGCGAATGTT</td>
<td>10</td>
</tr>
</tbody>
</table>

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Table 2: Demographic details of patients with carbapenem resistant K. pneumoniae (n=86)

<table>
<thead>
<tr>
<th>Numbers (%):</th>
<th>Sex:</th>
<th>Male: 56 (65)</th>
<th>Female: 30 (35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age:</td>
<td>Range: 0.002 to 80 yrs</td>
<td>79 (92)</td>
<td>5 (6)</td>
</tr>
<tr>
<td>Immune status:</td>
<td>Immunocompromised: 45 (52)</td>
<td>Other co-morbidities: 28 (33)</td>
<td>No comorbidities: 13 (15)</td>
</tr>
</tbody>
</table>
they lacked magA gene. The results of the multiplex PCR for carbapenemase genes are tabulated in Table 3. blaNDM was the most common gene expressed by the isolates followed by blaOXA48 blaKPC, but there was no difference in the distribution among string test positives and negatives.

30 day mortality was 55.9% (n=49). There was no association between mortality and age, sex, co-morbidity, immunosuppression or carbapenemase gene. However, there was a positive association between mortality rate and meropenem MIC (Figure 1). Meropenem MIC ≥16 µg/ml was a significant and independent risk factor for mortality (p 0.008, OR 9.5). String test positivity was also independently associated with mortality (p 0.036, OR 2.23).

Among the ten isolates that were typed, six were negative by string test and four were positive. Among the isolates that were string test negative, three belonged to ST14 and ST231 each. Among the four isolates positive for string test, two isolates belonged to ST231 and one each to ST11 and ST43. However, both the string test positive isolates belonging to ST231 lacked rmpA and rmpA2 genes.

### Discussion

**CRKP are a common cause of nosocomial infections and have previously been associated with a high 30 day mortality rate of 42%,**19,20 Ben-David et al., found that carbapenem resistance is an independent risk factor for mortality in blood stream infections caused by *K. pneumoniae*.21 In this study 92% of the CRKP infections were health-care associated and the overall mortality was higher than previously described at 55.9%. In this study, we describe increasing mortality rate with increasing meropenem MICs. As mentioned in Figure 1, the mortality in patients with meropenem MIC ≤12 µg/ml is 20% but is double this for patients with MIC of 24 µg/ml. Mortality rates in patients with MIC ≥16 µg/ml and ≥16 µg/ml are 54.5% and 67.9% respectively.

In India, carbapenem resistance is mostly due to the NDM and OXA enzymes22,23 as seen in this study while in Europe KPC is the major carbapenemase encoded by the bacteria. However, the type of carbapenemase gene did not correlate with meropenem MIC or outcome (p >0.05) as shown in Table 3. Sixty two percent of the isolates had an MIC of ≥32 µg/ml for meropenem and this was found to be an independent risk factor for mortality (p 0.008).

Immunosuppression (p 0.049) and high meropenem MICs (p 0.03) have found to be risk factors for mortality in CRKP infections in other studies.24 Although we found that meropenem MIC ≥16 µg/ml (p 0.008) was a significant risk factor, immunosuppression (p 0.533) was not. Nature and source of infection as mentioned in Table 2 were not risk factors mortality.

hvKP usually causes community acquired sepsis with a high mortality (55%)25 and is almost always susceptible to beta-lactam antibiotics. There is limited data on hospital acquired CR-hvKP infections which we describe in this study. Very few cases of CR-hvKP has been described;25 one such being from China where a single isolate was obtained from tracheal secretion and was pan drug resistant.26 The other report by Zhang et al., 2015, describe five CR-hvKP causing pneumonia, septicemia, abdominal infection and sepsis.27

Laboratory screening method for detection of hvKP is by the string test which has a cut off of ≥5 mm for the formation of a viscous string. A modification with a cut-off of ≥10 mm has been proposed by Lee et al., 2010 to improve the specificity.28 Tan et al., found that the test sensitivity (90.5%) and specificity (63%-66%) remained the same with both the cut-off values.29 Although rmpA, rmpA2 and magA are associated with positive string test, in this study we found only three rmpA2 positives among the 27 string test positive. Isolates which are phenotypically hypermucoviscous but lack the rmpA and rmpA2 genes could be due to the presence of other genes producing the expression of the phenotype. Conversely, isolates with rmpA and rmpA2 genes which lack the phenotype may be due to mutations leading to truncated proteins30 or, as previously published in ESBL producers, resistance genes which are expressed over virulence genes.30 Other possible reasons could be the lack of another positive regulator for rmpA and the presence of negative regulators at post-transcriptional level. Further work investigating discrepancies between phenotypic and genotypic markers for hvKP is needed to allow the development of a formal definition of hypervirulent *K. pneumoniae*.

The community acquired hypervirulent *K. pneumoniae* syndrome is principally caused by the CC23 strains. In this study, MLST was performed only for 10 isolates and were found to belong to ST11, ST14, ST43 and ST231. These clonal types have previously been reported in carbapenem resistant *K. pneumoniae*; ST14 has been reported in NDM-1 producing isolate from India31 and ST231 in NDM *K. pneumoniae* in Spain.32 ST11 has also been reported among hypervirulent carbapenem resistant *K. pneumoniae* from China34 coding for KPC carbapenemase unlike the isolate in the present study which produced NDM. Since the isolates did not predominantly belong to a single clonal type, surveillance of antimicrobial resistance and virulence is needed in order to prevent new clones from acquiring resistance and virulence.

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**Table 3: Distribution of carbapenemase genes among the *K. pneumoniae* resistant to carbapenems**

<table>
<thead>
<tr>
<th>Carbapenemase genes</th>
<th>No. of positives (n=86)</th>
<th>p value</th>
<th>String test positives n=27</th>
<th>String test negatives n=59</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaNDM</td>
<td>31 (36%)</td>
<td>0.203</td>
<td>11 (41%)</td>
<td>20 (34%)</td>
</tr>
<tr>
<td>blaOXA48</td>
<td>23 (27%)</td>
<td>0.123</td>
<td>6 (22%)</td>
<td>17 (29%)</td>
</tr>
<tr>
<td>blaKPC, OXA48, blaNDM</td>
<td>23 (27%)</td>
<td>0.967</td>
<td>10 (37%)</td>
<td>13 (22%)</td>
</tr>
<tr>
<td>blaKPC</td>
<td>3 (3%)</td>
<td>0.134</td>
<td>0</td>
<td>3 (5%)</td>
</tr>
</tbody>
</table>

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**Fig. 1: Logistic regression for meropenem MIC and string positivity with mortality**
Conclusion

String test is a quick phenotypic test for detection of hvKp which aids prediction of disease severity and is independently associated with increased mortality in invasive CRKp health-care-acquired infections. High meropenem MIC is a significant risk factor for mortality. The combination of CRKp and string positive hvKp resulted in a mortality rate of 84.2%. It is important to monitor the prevalence of CR-hvKp among invasive isolates as they pose a public health threat in the treatment and management of infections. The combination of increased virulence and decreased susceptibility to antimicrobials is very challenging to treat and hence results in worse outcomes. Further work to define the hypervirulent strains with confirmatory markers are necessary since these strains are now seen globally. This will enable easier diagnosis and better management of the patients. Resistance and virulence are not restricted to a single clonal type in the present study reflecting on the diversity of clones present.

Ethics approval and consent to participate

Not applicable since this is a retrospective study in which the isolates were used without the patient identifier.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

We would like to thank Mr. Georgekutty Mathew, Department of Biostatistics, Christian Medical College, Vellore, India, for his assistance in statistical analysis.

References