ORIGINAL ARTICLE

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.[1] Infections with *K. pneumoniae* are usually hospital-acquired and occur primarily in patients with impaired host defences[2] including solid tumours, haematological malignancies, liver cirrhosis and diabetes mellitus.[3] 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%.[4-6]

Recent years have also brought a rise in incidence of a hypermucoviscous/ hypervirulent variant of *K. pneumoniae* (hvKp) associated with liver abscess.[7] 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.[7] 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), *entB* (enterobactin), *kfu* (mediating ferric iron uptake); *allS* (allantoin metabolism) and *mrkD* (type3 fimbriae mediating attachment to extracellular matrix).[8]

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.[9-13]

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Table 1: List of primers used in the study

Gene	Forward primer (5′- 3′)	Reverse primer (5′-3′)	Reference
bla_{IMP}	GGAATAGAGTGGCTTAAYTCTC	GGTTTAAYAAAACAACCACC	16
bla_{VIM}	GATGGTGTTTGGTCGCATA	CGAATGCGCAGCACCAG	19
bla_{NDM}	CACCTCATGTTTGAATTCGCC	CTCTGTCACATCGAAATCGC	16
$bla_{OXA-48\ like}$	TATATTGCATTAAGCAAGGG	CACACAAATACGCGCTAACC	16
bla_{KPC}	TGTCACTGTATCGCCGTC	CTCAGTGCTCTACAGAAAACC	18
bla_{SPM}	AAAATCTGGGTACGCAAACG	ACATTATCCGCTGGAACAGG	17
rmpA	TGTTAACATGCAAGGAAATG	ATTGCAGCACTGCTTGTT	This study
rmpA2	CTTTATGTGCAATAAGGATGTT	CCTCCTGGAGAGTAAGCATT	10
magA	GGTGCTCTTTACATCATTGC	GCAATGGCCATTTGCGTTAG	10

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 carbapenamase genes which included bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, $bla_{OXA\ 48\text{-}like}$ and bla_{KPC} as described by Poirel *et al.*, 2011, Dallene *et al.*, 2010, Yigit *et al.*, 2001 and Ellington *et al.*, 2007.[14-17] 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.[10] *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://bigsdb.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 carbapenamase 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 2 µg/ml (n=11), and the third of resistant isolates with MIC between ≥4 µg/ml to ≤16 µg/ml (n=11). Since a significant proportion of the isolates in the study had MIC of ≥16 µg/ml (n=53), a fourth group considered these isolates that were highly resistant to meropenem.

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

	Numbers (%)
Sex	
Male	56 (65)
Female	30 (35)
Age	
Range: 0.002 to 80 yrs	-
Median: 37.5yrs	
Immune status	
Immunocompromised	45 (52)
Other co-morbidities	28 (33)
No comorbidities	13 (15)
Nature of infection	
Health-care Acquired	79 (92)
Community Acquired	5 (6)
Unclear	2 (2)
Source of infection	
Neutropenic sepsis	29 (33)
Abdominal sepsis	20 (23)
Respiratory sepsis	15 (16)
Unknown	9 (10)
Central/peripheral line	6 (7)
Neonatal sepsis	5 (6)
Skin and soft tissue infection/ bone/joint	4 (5)

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

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

Carbapenamase genes	No. of positives (n=86)	p value	String test positives n=27	String test negatives n=59
bla_{NDM}	31 (36%)	0.203	11 (41%)	20 (34%)
$bla_{OXA48\ like}$	23 (27%)	0.123	6 (22%)	17 (29%)
$bla_{NDM\ and\ OXA48\text{-}like}$	23 (27%)	0.967	10 (37%)	13 (22%)
$bla_{NDM,\ OXA48\ like\ and\ VIM}$	3 (3%)	0.134	0	3 (5%)
$bla_{NDM\ and\ VIM}$	3 (3%)		0	3 (5%)
$bla_{VIM\ and\ OXA48\ Like}$	3 (3%)		0	3 (5%)

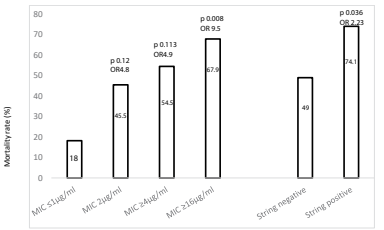

Fig. 1: Logistic regression for meropenem MIC and string positivity with mortality

they lacked *magA* gene. The results of the multiplex PCR for carbapenamase genes are tabulated in Table 3. bla_{NDM} was the most common gene expressed by the isolates followed by $bla_{OXA48\ like}$, 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 ≤1μg/ml is 20% but is double this for patients with MIC of 2μg/ml. Mortality rates in patients with MIC ≥4 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 enzymes[22,23] as seen in this study while in Europe KPC is the major carbapenamase 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 >32μg/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≥16μg/ml (*p* 0.008) was a significant risk factor, immunosuppression (*p* 0.533) was not. Nature and source of infection as mentioned in Table2 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[33]; 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, septicaemia, abdominal infection and sepsis.[27]

Laboratory screening method for detection of hvKp is by the string test which has a cut off of ≥5mm for the formation of a viscous string. A modification with a cut-off of ≥10mm 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 proteins[30] 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 India[31] and ST231 in NDM *K.pneumoniae* in Spain.[32] ST11 has also been reported among hypervirulent carbapenem resistant *K. pneumoniae* from China[34] 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.

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.

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