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Phenotypic Characterization of Virulence Determinants in Colistin-Resistant *Klebsiella pneumoniae* Clinical isolates

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Abstract

Klebsiella pneumoniae is an important opportunistic pathogen causing a broad spectrum of healthcare-associated infections. The development of colistin-resistant strains is an alarming phenomenon given that colistin is often the antibiotic of choice for the treatment of multidrug resistant infections. Assessing the pathogenic potential of these resistant isolates requires knowledge about their virulence profile. This study sought to characterize the phenotypic virulence factors of colistin-resistant *K. pneumoniae* isolates and examine their relationship with potential increased pathogenicity. A cross sectional study of 22 colistin resistant *K. pneumoniae* isolates from clinical specimens. Colistin resistance was determined using broth microdilution according to CLSI guidelines. Various virulence factors such as hypermucoviscocity, biofilm formation, gelatinase production, serum-resistance, cell-surface hydrophobicity and type 1-fimbriae were screened. Chi-square or Fisher's exact test was used for statistical analysis, with significance defined at p 61 years) and intensive care environments, with endotracheal aspirates being the most common source (50%). Presence of serum resistance in all isolates (100%) and presence of type 1 fimbriae in 86.36% showed significant association with a p-value < 0.001. Gelatinase production (63.63%) and biofilm formation (54.54%) were moderately common but not significant. 9.09% isolates manifested hypermucoviscocity and cell surface hydrophobicity which was statistically significant (p < 0.001). The study showed that immune evasion and adhesion mechanisms were one of the major role in promoting colistin resistance especially serum resistant, fimbriae expression in *K. pneumoniae* strains. The hypermucoviscocity was less prevalent, suggesting that hypervirulence phenotype is not highly associated with it. These finding highlight the importance of ongoing monitoring for growth and spread, as well as additional molecular analysis to address how they relate to resistance versus virulence.

Keywords: *Klebsiella pneumoniae*; Colistin; Virulence factor; Biofilm; Hypermucoviscosity; Serum resistance; Cell surface hydrophobicity

1. Introduction

Klebsiella pneumoniae is an opportunistic pathogen that causes a wide range of clinical disease including urinary tract infection, pneumonia, liver abscess and bloodstream infection [1]. The increasing prevalence as a cause of health care-associated infections is attributable to dissemination of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains in both clinical setting as well as in environment [2]. Among these, colistin resistance, a last-resort antibiotic that is commonly used for treatment poses a major therapeutic challenge and has been associated with increased morbidity and mortality [3].

The pathogenic mechanism of *K. pneumoniae* is mediated by numerous virulence factors that promote colonization, immune evasion and persistence inside the host [4]. Key virulence determinants including capsular polysaccharide, lipopolysaccharide (LPS), fimbriae and siderophores that play a part in the survival of the bacteria and disease progression [5]. The capsule serves as an important feature of protection from complement-mediated killing and phagocytosis and is also associated with hypermucoidy that is associated with hypervirulence [6].

Biofilm development is another important phenotypic trait that aids the maintenance of microorganisms on biotic and abiotic surfaces especially medical devices [7]. These provide a protected environment which can impact on resistance to drugs and host immune mechanisms. These molecules sequester iron ions from the host environment required for bacterial growth. Siderophore dependent iron acquisition systems such as enterobactin and aerobactin enable more efficient bacterial growth in low-Iron habitats found throughout host environments.

Recent reports suggest that emerging high-risk clones of *K. pneumoniae* carry a combination of antimicrobial resistance and virulence through co-evolved or even synergized mechanisms. There is still a lack of understanding how these other virulent traits (other than antibiotics resistances) can help in increasing the pathogenic potential of the specific clonal strain. To facilitate a detailed understanding of how resistance contributes to disease severity and treatment response, phenotypic characterization of virulence factors can be performed.

Despite emerging concern regarding colistin resistance, very limited data are available on the phenotypic virulence profile of colistin-resistant *K. pneumoniae* isolates in clinical settings. Hence, the present study is aimed at unraveling the phenotypic virulence determinants in clinical isolates of colistin-resistant *K. pneumoniae* and also evaluating their association with potential hyper-pathogenicity.

2. Material and Methods

2.1 Study Design and Bacterial Isolates

This cross-sectional study was conducted on clinical isolates of *Klebsiella pneumoniae* obtained from various clinical specimens, including blood, urine, sputum, and wound samples, collected from patients attending a tertiary care hospital. Isolates were identified using standard microbiological techniques, including colony morphology, Gram staining, and biochemical tests.

2.2 Antimicrobial Susceptibility Testing

Colistin susceptibility was determined using the broth microdilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Isolates with minimum inhibitory concentration (MIC) ≥2 μg/mL were considered colistin-resistant [8].

2.3 Phenotypic Detection of Virulence Factors

2.3.1. Hypermucoviscocity test

The string test for hypermucoviscocity was determined according to Lin et al., 2011 [9]. All the strains were inoculated on 5% sheep blood agar plates and incubated at 37°C over night. A standard bacteriological loop was used to stretch a mucoviscous string. The formation of a viscous string ≥5 mm long, representing the characteristic of hypermucoviscocity and regarded as a positive string test.

2.3.2. Biofilm Formation Assay

The biofilm forming ability of all isolates was determined as described by Stepanovic et al., (2000) with some modification [10]. We defined the cutoff of OD (ODc) for the micro titter plate test as three standard deviations. Isolated were classified as follows: OD < ODc – non adherent, ODc< OD < 2X ODc –weakly adherent, 2 ODc< OD < 4X ODc – moderately adherent and 4X ODc< OD– strong adherent.

2.3.3. Gelatinase production

Single colony from overnight grown pure culture was stabbed into tubes containing 12% gelatin in 0.8% Nutrient Broth (Difco Labs, Detroit, MI). Tubes were incubated at 37˚C for 48 and 60h and then placed into the refrigerator for approximately 30 min. Extent of liquefaction of gelatin indicated extent of gelatinase production by the organism.

2.3.4. Serum Resistance Assay

Bacteria were diluted to $2x10^6$ cells/ml in physiological saline. Twenty five microliters of bacterial suspensions and 75 µl of normal human serum (NHS) were put into micro titer trays, mixed, and incubated at 37°C. Viability was determined immediately and after 1, 2 and 3h of incubation. After mixing, samples were taken and serial dilutions were spread plated on brain heart infusion agar for colony counts. Responses were graded from 1 to 6 according to Hughes et al., (1982) [11]:

Interpretation of serum resistance test

2.3.5. Hydrophobicity test

Bacterial hydrophobicity is assessed by hydrocarbon-xylene (BATH) method according to Wojnicz et al., (2007) [12]. All isolates were grown in LB broth and harvested by centrifugation at 5000 rpm for 5 min. The harvested cells were washed in sterile phosphate buffer saline (pH 7.4) and suspended in the same buffer. Three milliliter of bacterial culture was vortexed with 1ml of xylene for 60S and left for 30 min. After the sample had separated in to two phase, optical density of the aqueous phase was measured at 470 nm. The degree of hydrophobicity was expressed as the percentage decrease in optical density of lower aqueous phase compared with that of cell suspension without xylene. 2.3.7.2 Cell damage factors

2.3.6. Type 1 Fimbriae

The presence of type 1 fimbriae (mannose-sensitive hemagglutination [MSHA]) at the bacterial cell surface was assessed using commercial baker's yeast (Saccharomyces cerevisiae) suspended in phosphate-buffered saline (5 mg dry weight per ml) as described by Hennequin et al., (2009) [13]

2.3. Statistical Analysis

Data were analyzed using statistical software GraphPad Prism. A p-value <0.05 was considered statistically significant.

3. Result

A total of 22 isolates of colistin-resistant *Klebsiella pneumoniae* were assayed. The majority of the isolates were from male patients (54.54%), while 45.45% of the isolates were from female patients. Among clinical specimens, endotracheal aspirates had the highest proportion (50.00%), followed by urine samples (27.27%) whereas bloodstream and wound-associated isolates were comparatively less common (9.09%) each. Almost two-thirds (68.18%) of them were older than 61 years, calling attention to their higher susceptibility. Most of the isolates were recovered from inpatients (IPD 45.45%) and intensive care units (ICU/CCU 40.90%) while lesser percentage had their origins in outpatient settings (13.63%) (Table1).

Table1: Clinical characteristics of Colistin-Resistant *Klebsiella pneumoniae* isolates

Property	Colistin resistant *Klebsiella pneumoniae* N=22 (%)
Gender	
Male	12 (54.54%)
Female	10 (45.45%)
Clinical sources	
Urine	6 (27.27%)
Blood	2 (9.09%)
Endotracheal Aspirate	12 (50.00%)
Wound Swab/Pus/TT Swab	2 (9.09%)
Age group	
20-30 years	1 (4.54%)
31-50 years	1 (4.54%)
51-60 years	5 (22.72%)
>61 years	15 (68.18%)
Hospital sites	
ICU/CCU	9 (40.90%)
IPD	10 (45.45%)
OPD	3 (13.63%)

The distribution of virulence factors among colistin-resistant *Klebsiella pneumoniae* isolates (N = 22) revealed variable expression patterns. Serum resistance was observed in all isolates (100%), showing a highly significant association ($p < 0.001$). Similarly, type 1 fimbriae were detected in 86.36% (19/22) of isolates, also demonstrating strong statistical significance ($p < 0.001$) as shown in Table 2.

Gelatinase production was present in 63.63% (14/22) of isolates, while biofilm formation was observed in 54.54% (12/22); however, these factors did not show statistically significant associations ($p = 0.20$ and $p = 0.83$, respectively). In contrast, hypermucoviscocity and cell surface hydrophobicity were detected in only 9.09% (2/22) of isolates each, yet both exhibited statistically significant differences ($p <$

0.001). Overall, serum resistance and fimbriae adhesion emerged as the predominant virulence traits among the studied isolates (Table 2).

Table2: Phenotypic Virulence Factors in Colistin-Resistant *Klebsiella pneumoniae*

Virulence Factor	Colistin resistant *Klebsiella pneumoniae* (N=22)		
	Positive n (%)	Negative n (%)	p-value
Hypermucoviscocity	2 (9.09)	20 (90.91)	<0.001
Biofilm production	12 (54.54)	10 (45.45)	0.83
Gelatinase production	14 (63.63)	8 (36.36)	0.20
Serum Resistance	22 (100.00)	0 (0.00)	<0.001
Cell Surface Hydrophobicity	2 (9.09)	20 (90.91)	<0.001
Type 1 fimbriae	19 (86.36)	3 (13.63)	<0.001

Discussion

Emergence of colistin-resistant *Klebsiella pneumoniae* strains poses a severe global public health challenge, especially in hospital settings due to the presence of multidrug resistant (MDR) and extensively drug-resistant (XDR) strains. As previously observed, a great proportion of the isolates in the present study originated from either intensive care units or elderly patients (>61 years) highlighting that older age, hospital stay and surgery/invasive procedures are important risk factors for *K. pneumoniae* [14, 15, 16]. The preponderance of respiratory samples, notably endotracheal aspirates, also reinforces the potential clinical significance of ventilator-associated pneumonia as a prominent manifestation of MDR *K. pneumoniae* disease [17, 18].

A striking result of this study is the universal presence (100%) of serum resistance among colistin resistant isolates, underscoring its pivotal role in immune evasion. Survival from complement-mediated lysis via serum resistance promotes dissemination to systemic sites. Complement resistance was found to be strongly associated with invasive infections and clinical outcome in recent studies [19]. The significant portion of serum resistance observed in our study infers that colistin-resistant strains have a survival advantage in the host.

Type 1 fimbriae was present in 86.36% of isolates and exhibited a statistically significant association. Adhesins involved in fuzzy nanowire and dynamic filament formation can link to the biophysics of pathogenesis by promoting bacterial adherence on host epithelial cells during infections, especially those located in the respiratory and urinary tracts. Fimbrial expression is a critical factor for colonization and biofilm initiation in uropathogenic *E. coli* [20, 21, 22]. Notably, the high prevalence seen in this study is consistent with recent genomic studies showing that adhesion-related genes are highly conserved in the population of *K. pneumoniae* high-risk clones [23, 24].

Biofilm formation was observed in 54.54% of the isolates but did not reach statistical significance. Biofilms are resilient multilayered colonies of bacteria that significantly augment bacterial survival on medical devices and drive antimicrobial resistance owing to poor antibiotic diffusion into the biofilm structure as well as horizontal gene transfer. However, the present study found a non-significant association suggesting that biofilm formation may not play a direct role in colistin resistance. Similar observations have being reported by Shadkam et al. (2021) and Vuotto et al. (2017) [25, 26] suggesting that resistance and biofilm formation might be regulated as independent traits.

However, moderate prevalence was shown by gelatinase production detected in 63.63% of isolates. While the role of gelatinase in tissue invasion and nutrient acquisition is well established, its contribution to *K.*

pneumoniae pathogenicity remains less clearly defined than that of other virulence factors. The lack of statistical significance in the present study indicates that gelatinase is perhaps not a key determinant of virulence among colistin-resistant strains, in agreement with recent phenotypic studies [27].

Harboring hypermucoviscosity, an identification of hvKP isolates, was only detected in 9.09% of the samples which is rather peculiar. Such a low prevalence indicates that in the current cohort, colistin resistance is independent of hypervirulent phenotypes. Hypermucoviscosity is classically associated with capsular overproduction and the expression of virulence genes including rmpA and magA [28, 29]. Emerging strains carrying both resistance and virulence determinants were revealed in recent studies to be convergent [30, 31]. The low frequency observed in this study may suggest that convergence is still limited or occurring only to a certain degree in the studied context.

Although statistically significant, cell surface hydrophobicity was detected in a low frequency (9.09%). Because hydrophobicity impacts bacterial adhesion and host tissue interaction, it may play some role in *K. pneumoniae* pathogenicity but less prominently than other factors. This low prevalence is consistent with prior reports indicating hydrophobicity is not a prominent virulence factor in clinical isolates [32, 33].

This study indicates that immune evasion (serum resistance) and adhesion (type 1 fimbriae) are the major virulence mechanisms in colistin-resistant *K. pneumoniae*. Other classical hypervirulence-associated traits, including hypermucoviscosity, were rarely observed, reflecting a dissociation of resistance and hypervirulence in this cohort. Confirming this observation, recent genomic and epidemiological studies have demonstrated that MDR strains are more frequently driven by fitness/survival strategies over true virulence [30, 34]. This study has some limitations as well, small sample size and lack of molecular characterization of virulence and resistance genes. Future work combining genotypic screening and whole-genome sequencing will elucidate the antimicrobial resistance interacts with virulence.

Conclusion

The current study underscores the increasing clinical importance of colistin resistant *Klebsiella pneumoniae* as a stubborn nosocomial pathogen, and extends to older and ill patients. Overall, these findings indicate that serum resistance and type 1 fimbriae are the principal virulence determinants of the isolates tested here, highlighting the significance of immune evasion and adherence strategies in their pathogenicity. On the contrary, classical virulence characteristics including hypermucoviscosity and cell surface hydrophobicity were rarely found, implying a minimal linkage between colistin resistance and hypervirulent phenotypes in the population studied. Death by biofilm and gelatinase production in moderate prevalence as well, but not statistically significant may suggest that these mechanisms are not major driving forces of virulence among colistin-resistant strains. In summary, this study indicates that colistin-resistant *K. pneumoniae* is not more virulent than its wild type but seems to be well adapted for survival in the host as a non-virulent strain. These data highlight an essential need for ongoing surveillance of antimicrobial resistance and robust phenotypic and molecular characterization to further delineate the complex evolving relationship between resistance and virulence. Genomic approaches in future studies focusing on mechanisms explaining the convergence of resistance and pathogenicity should be performed to guide effective infection control and therapeutic strategies.

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