



AKADÉMIAI KIADÓ

Acta Microbiologica et
Immunologica Hungarica

69 (2022) 4, 297–302

DOI:

[10.1556/030.2022.01908](https://doi.org/10.1556/030.2022.01908)

© 2022 The Author(s)

RESEARCH ARTICLE



*Corresponding author. Department of
Diagnostics and Public Health,
University of Verona, Strada Le Grazie
8, 37134 Verona, Italy.
E-mail: annarita.mazzariol@univr.it



Multiple detection of hypermucoviscous and hypervirulent strains of *Klebsiella pneumoniae*: An emergent health care threat

ANNA VENTURA, ELENA ADDIS, ANNA BERTONCELLI and
ANNARITA MAZZARIOL* 

Department of Diagnostics and Public Health, University of Verona, Verona, Italy

Received: October 15, 2022 • Accepted: October 31, 2022

Published online: November 22, 2022

ABSTRACT

This study focused on the characterization of 19 hypermucoviscous *Klebsiella pneumoniae* strains, that were identified from 26 hypermucosal strains. In order to identify hypermucoviscous strains of *K. pneumoniae*, the string test was applied. This phenotype is known in the literature as one of the virulence factors of this species together with the production of biofilm and other hypervirulence factor genes such as: *rmpA*, *rmpA2*, *iucA*, *iroB*, *peg-344*. We also investigated presence of *magA* gene that correlates with the hyper-production of capsule of K1 serotype. Of the strains under study, 13 out of 19 harboured at least one virulence factor.

Sequence type (ST) was determined in order to identify known high-risk clones or new emerging high-risk clones and their variability in a single clinical setting. Important STs found among these strains were ST65 and ST29. Carbapenem resistance was also investigated and 4 out of 19 strains harboured at least a carbapenemase: one strain harboured a KPC enzyme alone, one strain carried a KPC and an OXA-48 like, one strain produced OXA-48-like alone, and the last strain harboured two metallo- β -lactamases (VIM-1 and NDM-5) plus OXA-48-like. In particular, this latter strain belongs to ST383, which was recently reported in Northern Italy as a hypervirulent and XDR strain.

The global spread of hypervirulent *K. pneumoniae* is an important epidemiological issue that should be considered in diagnostic and therapeutic managements of patients with *K. pneumoniae* infections.

KEYWORDS

Hypervirulent *Klebsiella pneumoniae*, Hypermucoviscous *Klebsiella pneumoniae*, siderophores, aerobactin, salmochelin

INTRODUCTION

In recent years, *Klebsiella pneumoniae* has gained notoriety as an infectious agent due to its increase in number of serious infections and its increasing resistance to antibiotic therapy. Furthermore, additional genetic traits associated with hypervirulence, and antibiotic resistance have recently been identified that make *K. pneumoniae* infection an increasingly emergency problem [1].

It is important to differentiate between classical *K. pneumoniae* (cKp) strains that typically cause nosocomial infections, now found worldwide and hypervirulent (hvKp) strains that cause severe, community-acquired systemic infections in otherwise healthy individuals, an emergency that initially worried only Asian countries but it is now rapidly expanding [2].

In 1986, Liu et al. [3] reported the first seven clinical cases of invasive *K. pneumoniae* infection in community individuals who presented with a liver abscess in the absence of biliary tract disease and these *K. pneumoniae* strains were defined as hypervirulent. Notably, hvKp strains cause primary liver abscesses in patient populations that do not appear to have any underlying liver disease. In turn, liver abscesses can give rise to a number of other secondary infections due to hematogenous spread from the liver [4].

To date, there are four major classes of virulence factors that have been characterized well in *K. pneumoniae*: capsule, including the production of hypercapsule in *hvKp* strains; lipopolysaccharide (LPS); siderophores; and type 1 and 3 fimbriae. Siderophores play an important role in virulence, and *hv K. pneumoniae* encodes for salmochelin, aerobactin, other than enterobactin, and yersiniabactin, the last of which is also produced by *cKp* strains. Salmochelin is a form of c-glucosylated enterobactin encoded by the *iroA* gene. It is present in only about 2–4% of nosocomial *K. pneumoniae* strains but is much more prevalent in *hv K. pneumoniae* strains. Aerobactin is a citrate-hydroxamate siderophore encoded by the *iucA* gene; it is expressed rarely by classical nosocomial clinical isolates of *K. pneumoniae* but it is present in 93–100% of *hvKp* isolates [2].

Currently, *magA* is defined as an essential gene for the formation of the biosynthesis of K1 capsular polysaccharides but it is not an independent specific virulence gene in strains of *K. pneumoniae* that cause liver abscesses [5].

Clinical laboratories are currently still in the midst of studying the differentiation between *hvKp* and *cKp*. Increased capsule production and aerobactin production have been identified as specific virulence factors for the hypermucoviscous phenotype; in addition to these elements, it has been shown that several biomarkers and quantitative production of siderophores accurately predict *hvKp* strains [6].

Klebsiella pneumoniae is known for its propensity to collect resistance plasmids. Extensive use of carbapenems has led to the emergence and rapid spread of carbapenemase-producing strains. The types of carbapenemases prevalent in *K. pneumoniae* are: *K. pneumoniae* carbapenemase (KPC)-type enzymes; New Delhi metallo- β -lactamase (NDM)-type enzymes; and oxacillinase (OXA)-type enzymes, mainly OXA-48 [7].

The aim of this study is the identification of hypermucoviscous and hypervirulent strains through the molecular and phenotypic characterization of 19 isolates of multidrug-resistant (MDR) and non-MDR *K. pneumoniae*. This work stems from an ongoing health emergency, since the failure to recognize the increasingly dangerous factors of hypervirulence and resistance could lead to underestimate the risky circulation of these strains.

MATERIALS AND METHODS

Bacterial strains

The study included a collection of 26 strains of *K. pneumoniae* that have been isolated at Microbiology and Virology Hospital Service in Verona, Italy in the year 2021 and collected from blood cultures and abscesses during routine clinical analysis. All strains were identified by Maldi-tof Vitek MS (BioMérieux, France).

Antimicrobial susceptibility determination

Antimicrobial susceptibility tests for carbapenems were performed by disc diffusion method following the EUCAST

(European Committee on Antimicrobial Susceptibility Testing) guideline. The results were interpreted using the breakpoints table of *Enterobacterales* on EUCAST's website [8].

String test

The string test is a simple, rapid, and readily available screening method that confirms a known virulence factor of *K. pneumoniae*, the hypermucoviscous phenotype. Furthermore, not all mucoid strains of *K. pneumoniae* show a positive string test, therefore a colonial mucoid appearance does not equate to a hypermucoviscous phenotype [9]. This phenomenon highlights a clear difference between the classic capsular mucoid strains and the hypermucoviscous variants [10]. The test is positive when there is formation of a viscous string of in > 5 mm in length when a loop is used to stretch the colony on an agar plate [11].

Hypervirulence factor detection

Hypervirulence factors of hypermucoviscous strains were detected by Polymerase Chain Reaction (PCR) using primers and thermal profiles reported earlier [12]. The marker genes used to distinguish *hvKp* from *cKp* are: *rmpA/rmpA2* encoding regulator of mucoid phenotype (*hypermucoid*); *iucA* gene in the locus of aerobactin siderophore; *iroB* gene in the locus of salmochelin siderophore; and *peg-344*, which is a putative transporter. To determine the presence of *magA* gene, a PCR was performed using primers and conditions already reported [13].

Carbapenemase detection

The CARBA NP phenotypic test [14] was used for the rapid detection of carbapenemase production in *Enterobacterales*. Molecular characterization of carbapenemase genes (*bla_{VIM}*, *bla_{IMP}*, *bla_{NDM}*, *bla_{KPC}* and *bla_{OXA-48}*) was performed by PCR [15].

Multilocus Sequence Typing (MLST)

MLST analysis was conducted through simplex PCRs for seven different housekeeping genes as described on the Institute Pasteur website [16, 17]. The MLST PCR products were purified through the QIAquick PCR Purification Kit and then sequenced at Eurofins Genomics (Germany, Ebersberg) in order to obtain sequence-typing (ST).

In vitro biofilm production

The production of biofilm was also analysed through the crystal violet test performed on a microtiter plate, and the optical density was measured in the plate at 550 nm through a fluorescence microplate reader. The results obtained were then subjected to the analysis described by Stepanovic et al. [18].

RESULTS AND DISCUSSION

To date *K. pneumoniae* is recognized as an urgent threat to human health due to the emergence of multidrug-resistant



Table 1. Virulence factors, antimicrobial susceptibility, carbapenemases and sequence types of the 19 hypermucoviscous strains under study

Strains	String test	Carba NP	Carbapenemases	ST	Hypervirulence factors	magA	Biofilm	Kirby-Bauer Disk Diffusion Susceptibility		
								Imipenem	Meropenem	Ertapenem
AMP 3291	+	-	ND	65	<i>iucA, iroB, peg-344, rmpA, rmpA2</i>		Strong	S	S	S
AMP 3678	+	-	ND	86	<i>iucA, iroB, peg-344, rmpA, rmpA2</i>		Strong	S	S	S
AMP 3679	+	-	ND	65	<i>iucA, iroB, peg-344, rmpA, rmpA2</i>		Strong	S	S	S
AMP 3680	+	-	ND	1,224	None		Strong	S	S	S
AMP 3681	+	-	ND	45	None	+	Strong	S	S	S
AMP 3682	+	-	ND	ND	None	+	Strong	S	S	S
AMP 3683	+	-	ND	380	<i>iucA, iroB, peg-344, rmpA, rmpA2</i>	+	Strong	S	S	S
AMP 3866	+	-	ND	29	<i>iucA, iroB, rmpA, rmpA2</i>		Strong	S	S	S
AMP 3872	+	-	ND	17	None		Strong	S	S	S
AMP 3875	+	+	KPC	35	None		Strong	R	I	R
AMP 3876	+	-	ND	86	None		Strong	S	S	S
AMP 3877	+	-	ND	29	<i>iucA, iroB, peg-344, rmpA, rmpA2</i>		Strong	S	S	S
AMP 3878	+	-	ND	10	None	+	Strong	S	S	S
AMP 3880	+	-	ND	65	<i>iucA, iroB, peg-344, rmpA, rmpA2</i>		Strong	S	S	S
AMP 3881	+	-	ND	111	None		Strong	S	S	S
MDR 444	+	+	OXA-48-like	395	None	+	Strong	S	I	R
MDR 1682	+	+	VIM-1	383	<i>rmpA2</i>		Strong	R	R	R
			NDM-5							
			OXA-505							
MDR 2145	+	+	KPC	101	None		Strong	R	R	R
			OXA-48-like							
MDR 3605	+	-	ND	307	<i>rmpA2</i>	+	Strong	S	S	S

ND = Not Determined; S = Susceptible; R = Resistant; I = Susceptible at increase dosage

ST = sequence type



strains associated with hospital outbreaks as well as due to hypervirulent strains associated with severe community-acquired infections. Therefore, the convergence of virulence and resistance genes could potentially soon lead to the emergence of invasive, untreatable infections caused by *K. pneumoniae* [1–4].

All 26 *K. pneumoniae* isolates were selected in this study on the basis of their hypermucosal aspect and were analysed through the string test screening method. Strains with a string >5 mm were classified as a hypermucoviscous (*hmv*) phenotype; this was shown in 19 out of the 26 strains. The incorporation of the string test into the daily practice of microbiological surveillance could lead to more appropriate diagnostic tools. At the same time, we must remember that non-*hmvKp* positive strains can also be highly virulent when organisms possess hypervirulence genes [19].

These 19 hypermucoviscous strains were further investigated for virulence factors, and MLST was performed in order to check for high-risk clones; all data are summarized in Table 1.

All 19 strains, following the Stepanovic criteria for classification [18], resulted in strong biofilm producers. This factor will contribute to these strains' tolerance of antibiotics [2].

The five main loci, namely *iroB*, *iucA*, *peg-344*, *rmpA*, and *rmpA2*, which according to the literature can differentiate and therefore define a hypervirulent strain with respect to the classical forms of *K. pneumoniae*, have been selected. Through molecular characterization, it was found that seven strains of the study displayed at least 4 hypervirulence genes. According to what is reported in the literature, a *K. pneumoniae* strain is defined as hypervirulent if it has at least four of the aforementioned genes [12].

Figure 1 reports the distribution of virulence genes in the strains under study.

However, it should also be taken into account that the strains MDR 1682 and MDR 3605 carry the *rmpA2* gene alone, which according to the ECDC risk assessment [1] is one of the major factors of hypervirulence.

According to the PCR results for the *magA* gene, which is responsible for the capsular serotype K1, 6 out of 19 strains (31.6%) resulted positive. This result is consistent with literature, which reports that K1 capsular serotype together with K2 are the two types of capsule mostly related to hypervirulence [10], since capsule serotyping is a very long procedure that includes different type K and type O antigens, these strains cannot be defined with certainty as K1 serotype. However, this test can still be a good start in the *K. pneumoniae* capsule serotyping procedure.

Carbapenemase-producing strains were screened through the CarbaNP test, and only 4 out of 19 strains resulted positive (21.05%). All CarbaNP-positive strains were subjected to molecular analysis through various PCRs. Carbapenemases were detected in different combinations: KPC alone; OXA-48 alone; KPC and OXA-48 together; and one strain harboured VIM-1, NDM-5, and OXA-48-like. The last is considered also a potential pathotype since it harbours the *rmpA2* gene, while the other three strains harbouring carbapenemases did not carry *rmpA2*. The three carbapenemases harboured by the MDR 1682 strain were sequenced and found to be VIM-1, NDM-5, and OXA-505 enzymes. NDM-5 is a variant recently found in Italy, in *Escherichia coli* strains isolated in Tuscany [20].

MLST was performed in order to identify ST linked to the *hmv Kp* pattern, and to check if ST43 was present as indicated by the ECDC [1]. We have not found ST43;

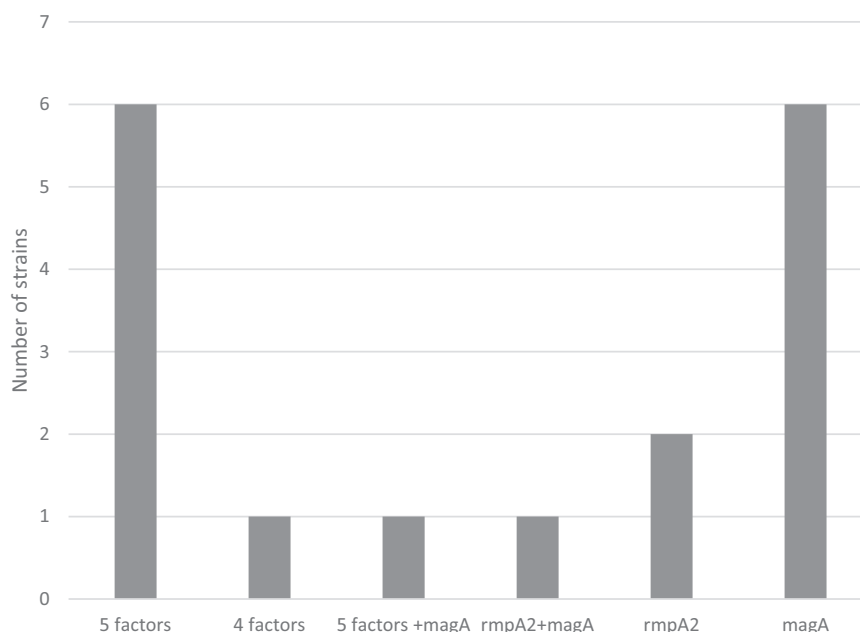


Fig. 1. Virulence factor distribution among the strains in this study



however, we were able to detect other STs present in our hospital setting that can act as emerging clones. Noteworthy also are the multiple STs that we found among *hmv* strains and harbouring hypervirulence factors. Three strains were characterized as ST65, which, according to the literature, is precisely linked to the hypervirulence of the K2 capsular serotype strains and has caused severe and fatal infections in clinical setting in China [21]. Two strains, on the other hand, belong to ST29, which is commonly associated with extended-spectrum β -lactamase-encoding genes (in particular, *bla*_{CTX-M-15}), but rarely has carbapenemase genes [22]. The AMP 3678 strain was identified as ST86, which corresponds to the ST found in the first case of community-acquired pneumonia due to an *hmv* strain of *K. pneumoniae* [23]. The strain AMP 3683 showed ST380, which is considered an emerging ST in severe community-acquired infections [24].

Among seven strains with five or four hypervirulence genes, we found four STs, most of them already correlated with hypervirulence and considered as emerging clones. This was a surprising and worrying result, since these strains are more widespread than is generally believed and they can easily combine with multidrug resistance.

Another important finding is that the strain harbouring the three carbapenemases belong to ST383. At the beginning of 2022, NDM-1/5- and OXA-48-co-producing, extensively drug-resistant hypervirulent *K. pneumoniae* strains were detected in Northern Italy, more specifically at the San Raffaele hospital in Milan, and these strains were ST383 [25].

Among the strains that do not harbour the virulence factors taken into consideration, we find ST101 and ST307. These STs have been identified in Southern Italy in serious bloodstream infections and are emerging high-risk carbapenems-resistant clones of *K. pneumoniae*. Furthermore, these clones appear to be potentially extremely virulent [26].

In conclusion, the global spread of *hvKp* is an important epidemiological change that should be considered in the diagnostic and therapeutic management of patients with *K. pneumoniae* infections. Unfortunately, there is high concern about the possibility of a further combination of virulence and resistance, thus leading to severe and untreatable infections in healthy individuals, which would be extremely difficult to manage.

The emergence of these high-risk clones and the global spread of these strains has left physicians with very few treatment options. Therefore, it is essential to have new protocols for strengthened screening control in order to limit the spread of multidrug-resistant and hypervirulent *K. pneumoniae* strains. New strategies need to be further explored as therapeutic options in the treatment of infections caused by both classical and hypervirulent strains of *K. pneumoniae*.

Conflict of interests: None.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

1. European Centre for Disease Prevention and Control. Risk assessment: emergence of hypervirulent *Klebsiella pneumoniae* ST23 carrying carbapenemase genes in EU/EEA countries. ECDC website; March 2021.
2. Paczosa MK, Mecsas J. *Klebsiella pneumoniae*: going on the offense with a strong defense. Clin Microbiol Rev 2016; 80: 629–61.
3. Liu YC, Cheng DL, Lin CL. *Klebsiella pneumoniae* liver abscess associated with septic endophthalmitis. Arch Intern Med 1986; 146: 1913–16.
4. Russo TA, Marr CM. Hypervirulent *Klebsiella pneumoniae*. Clin Microbiol Rev 2019; 32: e00001–19.
5. Yeh KM, Chang FY, Fung CF, Lin JC, Siu LK. *magA* is not a specific virulence gene for *Klebsiella pneumoniae* strains causing liver abscess but is part of the capsular polysaccharide gene cluster of *K. pneumoniae* serotype K1. J Med Microbiol 2006; 55: 803–4.
6. Russo TA, Olson R, MacDonald U, Beanan J, Davidson BA. Aerobactin, but not yersiniabactin, salmochelin, or enterobactin, enables the growth/survival of hypervirulent (hypermucoviscous) *Klebsiella pneumoniae* *ex vivo* and *in vivo*. Infect Imm 2015; 83: 3325–33.
7. Piperaki ET, Syrogiannopoulos GA, Tzouveleki LS, Daikos GL. *Klebsiella pneumoniae*: virulence, biofilm and antimicrobial resistance. Ped Infec Dis J 2017; 36: 1002–5.
8. https://www.eucast.org/clinical_breakpoints.
9. Pomakova DK, Hsiao CB, Beanan JM, Olson R, MacDonald U, Keynan Y, et al. Clinical and phenotypic differences between classic and hypervirulent *Klebsiella pneumoniae*: an emerging and under-recognized pathogenic variant. Eur J Clin Microbiol Infect Dis 2012; 31: 981–9.
10. Catalán-Nájera JC, Garza-Ramos U, Barrios-Camacho H. Hypervirulence and hypermucoviscosity: two different but complementary *Klebsiella* spp. Phenotypes? Virulence 2017; 8: 1111–23.
11. Walker KA, Miller VL. The intersection of capsule gene expression, hypermucoviscosity and hypervirulence in *Klebsiella pneumoniae*. Curr Opin Microbiol 2020; 54: 95–102.
12. Patro LPP, Sudhakar KU, Rathinavelan T. K-PAM: a unified platform to distinguish *Klebsiella* species K- and O-antigen types, model antigen structures and identify hypervirulent strains. Sci Rep 2020; 10: 16372.
13. Yeh KM, Kurup A, Siu LK, Koh YL, Fung CP, Lin JC, et al. Capsular serotype K1 or K2, rather than *magA* and *rmpA*, is a major virulence determinant for *Klebsiella pneumoniae* liver abscess in Singapore and Taiwan. J Clin Microbiol 2007; 45: 466–71.
14. Nordmann P, Poirel L, Dortet L. Rapid detection of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis 2012; 18: 1503–7.
15. Dallenne C, Da Costa A, Decré D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important β -lactamases in Enterobacteriaceae. J Antimicrob Chemother 2010; 65: 490–5.
16. <http://www.pasteur.fr/mlst> Genotyping of pathogens and public health, Institute Pasteur, Paris, France.
17. Diancourt L, Passet V, Verhoef J, Grimont PAD, Brisse S. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J Clin Microbiol 2005; 43: 4178–82.



18. Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Cirković I, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *J Pathol Microbiol Immunol* 2007; 115: 891–9.
19. Hagiya H, Watanabe N, Maki M, Murase T, Otsuka F. Clinical utility of string test as a screening method for hypermucoviscosity-phenotype *Klebsiella pneumoniae*. *Acute Med Surg* 2014; 1: 245–6.
20. Bibbolino G, Di Lella FM, Oliva A, Lichtner M, Del Borgo C, Raponi G, et al. Molecular epidemiology of NDM-5-producing *Escherichia coli* high-risk clones identified in two Italian hospitals in 2017–2019. *Diagn Microbiol Infect Dis* 2021; 100: 115399.
21. Zhang Y, Wang X, Wang Q, Chen H, Li H, Wang S, et al. Emergence of tigecycline nonsusceptible and IMP-4 carbapenemase-producing K2-ST65 hypervirulent *Klebsiella pneumoniae* in China. *Microbiol Spectr* 2021; 9: e01305–21.
22. Liu L, Feng L, Wei L, Xiao Y, Zong Z. KPC-2-producing carbapenem-resistant *Klebsiella pneumoniae* of the uncommon ST29 type carrying OXA-926, a novel narrow-spectrum OXA β -lactamase. *Front Microbiol* 2021; 12: 701513.
23. Hirai J, Sakanashi D, Kinjo T, Haranaga S, Fujita J. The first case of community-acquired pneumonia due to capsular genotype K2-ST86 hypervirulent *Klebsiella pneumoniae* in Okinawa, Japan: a case report and literature review. *Infect Drug Res* 2020; 13: 2237–43.
24. Hentzien M, Rosman J, Decré D, Brenkle K, Mendes-Martins L, Mateu P. Seven hypervirulent ST380 *Klebsiella pneumoniae* septic localizations. *Med Mal Infect* 2017; 47: 171–3.
25. Lorenzin G, Gona F, Battaglia S, Spitaleri A, Saluzzo F, Trovato A, et al. Detection of NDM-1/5 and OXA-48 co-producing extensively drug-resistant hypervirulent *Klebsiella pneumoniae* in Northern Italy. *J Glob Antimicrob Resist* 2022; 28: 146–50.
26. Loconsole D, Accogli M, De Robertis AL, Capozzi L, Bianco A, Morea A, et al. Emerging high-risk ST101 and ST307 carbapenem-resistant *Klebsiella pneumoniae* clones from bloodstream infections in Southern Italy. *Ann Clin Microbiol and Antimicrob* 2020; 19: 24.

