

REVIEW



## Hypervirulence and hypermucoviscosity: Two different but complementary *Klebsiella* spp. phenotypes?

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### ABSTRACT

Since the hypermucoviscous variants of *Klebsiella pneumoniae* were first reported, many cases of primary liver abscesses and other invasive infections caused by this pathogen have been described worldwide. Hypermucoviscosity is a phenotypic feature characterized by the formation of a viscous filament  $\geq 5$  mm when a bacterial colony is stretched by a bacteriological loop; this is the so-called positive string test. Hypermucoviscosity appears to be associated with this unusual and aggressive type of infection, and therefore, the causal strains are considered hypervirulent. Since these first reports, the terms hypermucoviscosity and hypervirulence have often been used synonymously. However, new evidence has suggested that hypermucoviscosity and hypervirulence are 2 different phenotypes that should not be used synonymously. Moreover, it is important to establish that a negative string test is insufficient in determining whether a strain is or is not hypervirulent. On the other hand, hypervirulence- and hypermucoviscosity-associated genes must be identified, considering that these phenotypes correspond to 2 different phenomena, regardless of whether they can act in synergy under certain circumstances. Therefore, it is essential to quickly identify the genetic determinants behind the hypervirulent phenotype to develop effective methodologies that can diagnose in a prompt and effective way these hypervirulent variants of *K. pneumoniae*.

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### Introduction

In the mid-1980s, a new hypervirulent variant of *Klebsiella pneumoniae* was described for the first time in Taiwan.<sup>1</sup> This new variant of hypervirulent *K. pneumoniae* (hvKpn) differs from the classical *K. pneumoniae* (cKpn) in its ability to produce invasive infections even in apparently healthy adults. The hvKpn variants tend to spontaneously form abscesses (especially liver ones) and develop metastases (with or without secondary bacteraemia), particularly in the brain and eyes, from the primary sites of infection.<sup>2-4</sup> These clinical features, which are rarely caused by cKpn, together with a hypermucoviscous phenotype appearing when they are grown in various culture media (determined by a positive string test) are the hallmark of hvKpn variants.

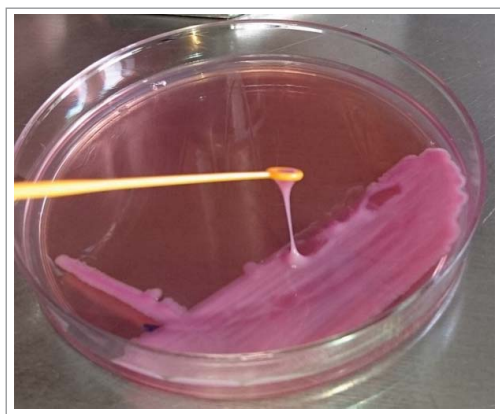
Since the hypermucoviscous clones of *K. pneumoniae* were described for the first time, they have been associated with hypervirulence, although no locus or loci have been clearly identified as being responsible for this trait. At first, capsular serotypes K1 and K2 were described as highly pathogenic to humans; they were

more frequently associated with this phenotype and characterized by producing mucous colonies. However, hvKpn clones with a hypermucoviscous phenotype and belonging to different capsular serotypes have been now described worldwide.<sup>5-7</sup> Hypermucoviscous clones belonging to the same capsular serotype do not produce a homogeneous pattern of infection despite sharing the same virulence factor profile.<sup>6,8</sup>

After the first cases of invasive infections caused by *K. pneumoniae* were reported, the hypermucoviscous phenotype was considered a marker of hypervirulence. This partnership is still widely accepted. Furthermore, it is common for these terms to be considered synonyms in the medical literature. However, is the hypermucoviscous phenotype an indicator of hypervirulence? Based on the information currently available, the aim of this review was to explore this question and identify unresolved questions that need to be investigated for a better understanding of hypermucoviscosity and hypervirulence.

### Hypermucoviscous and hypervirulent variants of *Klebsiella pneumoniae* (hmvKpn/hvKpn)

Since they were described for the first time, *K. pneumoniae* hypermucoviscous clones have been considered hypervirulent.<sup>9</sup> Like all capsulated bacteria, *K. pneumoniae* produces mucoid colonies in a nutritive medium. This mucoid phenotype differs from a hypermucoviscous phenotype in that hypermucoviscosity is defined by the formation of a viscous filament  $\geq 5$  mm after stretching a *Klebsiella* spp colony with a loop on an agar plate in the so-called string test (Fig. 1). Not all mucoid colonies of *K. pneumoniae* show a positive string test. This phenomenon highlights a distinct difference between mucoid capsular strains and the hypermucoviscous variants.<sup>10</sup> The first reports from the Far East indicated that these variants could produce invasive infections causing primary liver, prostate, bone, kidney, and lung abscesses; some cases of necrotizing fasciitis have also been described.<sup>2,4,11-13</sup> Metastatic complications to the brain and eyes from these primary sites of infection have also been documented.<sup>2,14-16</sup> Therefore, the invasive capability and the hypermucoviscous phenotype account for the main differences between the hvKpn and cKpn variants (Fig. 2).<sup>2</sup> However, the hypermucoviscous *K. pneumoniae* phenotype without a hypervirulence genotype has been poorly studied. The infections caused by these hypervirulent/hypermucoviscous *K. pneumoniae* (hvKpn/hmvKpn) variants have been observed in immunocompromised Asian patients and diabetics with poor glycemic control. Moreover, most of these strains belonged to the K1 serotype, the most prevalent one in the Far Eastern region.<sup>2,16,17,18</sup> In addition to the serotype K1/K2, other serotypes have also been associated with the hvKpn/hmvKpn phenotypes.<sup>6,19</sup> Similarly, hvKpn/hmvKpn strains are sensitive to several classes of antibiotics, except

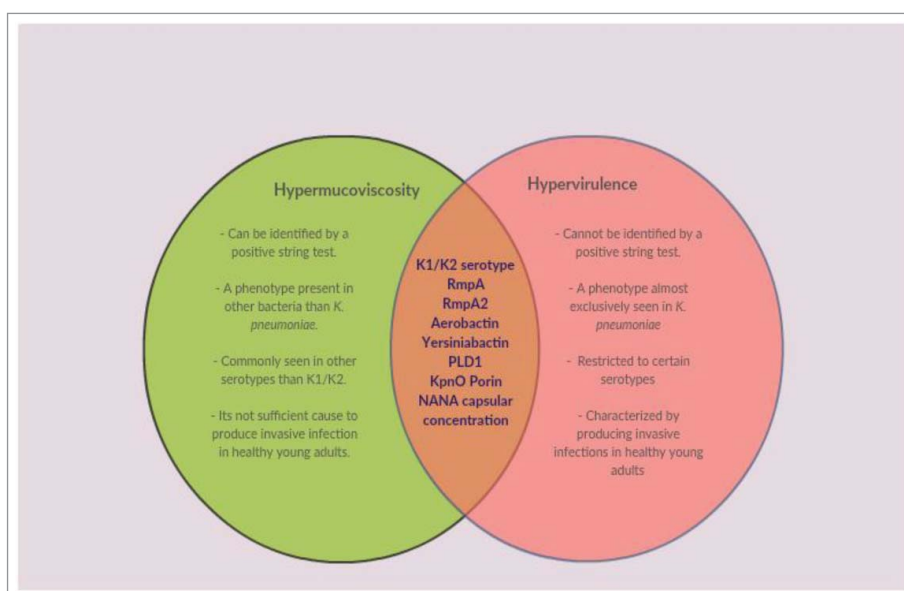


**Figure 1.** String test. A hypermucoviscous phenotype is seen when a viscous filament  $\geq 5$  mm is produced after stretching a *K. pneumoniae* colony with a loop on an agar plate (positive string test).

for ampicillin, to which *K. pneumoniae* is intrinsically resistant. Although the rate of mortality associated with these variants is low, the disabling consequences resulting from the invasion of the central nervous system and the eye are usually devastating.<sup>2</sup> Since most of hvKpn variants are sensitive to several classes of antimicrobials, the timely and prompt administration of appropriate antibiotics avoids the development of catastrophic disabilities and death.<sup>20</sup> The main differences between cKpn and hvKpn are summarized in Table 1.

Over the years, infections caused by hvKpn/hmvKpn *K. pneumoniae* have been detected all over the world. Unlike the first cases reported, the most affected people have been young adults without apparent immunodeficiency and people of Asian descent.<sup>6,21-26</sup> Infections in children have also been described,<sup>27,28</sup> and cases of invasive infections in animals have been reported.<sup>29,30</sup> Lee et al. (2016) tried to determine whether the development of invasive infection was due to genetic predisposition or geospecific strain acquisition. They analyzed 70 patients living in Singapore and belonging to 4 ethnic groups (Chinese, Malay, Indian and Caucasian) from which demographic and clinical profile information was recorded. In these groups, type 2 diabetes mellitus was the comorbidity more frequently found at a lower prevalence in the Chinese group. Lee et al. (2016) found that the Chinese had a higher probability of being infected and developing invasive infection by *K. pneumoniae* K1 serotype than did non-Chinese groups. In accordance with this result, the Chinese were rarely infected by non-K1 serotypes compared with non-Chinese ethnic groups. This particular result may be because the Chinese have a higher rate of intestinal carriage of *K. pneumoniae* K1 serotype compared with Malay, Indian and Caucasian groups. On the other hand, non-Chinese groups were more frequently infected by K2 and other non-K1 serotypes, but the development of invasive infection was associated with some comorbidity, especially type 2 diabetes mellitus. In conclusion, environmental factors (diet, water and direct contact with *K. pneumoniae* K1 carriers) that lead to the acquisition of *K. pneumoniae* with certain serotypes, genetic predisposition and comorbidities represent the principal factors determining the differential susceptibility to the development of invasive infection, independent of living in the same geographical area.<sup>31</sup>

Thus, hypervirulence in *K. pneumoniae* could be defined as the ability of the bacteria to produce invasive infections (metastatic dissemination) after a primary focus of infection in apparently healthy adults. The emergence of multidrug-resistant strains with hvKpn/



**Figure 2.** Venn diagram showing differences and similarities between hypermucoviscous and hypervirulent phenotypes. New evidence suggests that hypermucoviscosity and hypervirulence are 2 different phenomena. The green region shows the main characteristics of the hypermucoviscous phenotype that differ from those of the hypervirulent phenotype. The intersection highlights the elements that have been strongly associated with both phenotypes.

hmvKpn phenotypes is a cause for concern.<sup>32-35</sup> Siu et al. (2014) have shown that the transferability of plasmidic *bla*<sub>KPC</sub> (KPC-2 and KPC-3) into a virulent *K. pneumoniae* K2 serotype isolated from a liver abscess is possible. Despite the plasmid harboring carbapenemase, the recipient strain conserved its virulence attributes and gained a multidrug-resistant phenotype.<sup>36</sup> Zhang et al. (2015) have shown the emergence of a K1 hypervirulent strain of *K. pneumoniae* harboring *bla*<sub>KPC-2</sub> carbapenemase in China.<sup>35</sup> The hypervirulent K1 *K. pneumoniae* strain harboring plasmid-mediated colistin resistance *mcr-1* gene has also been identified, which is even more alarming.<sup>37</sup> So far, the number of strains showing multidrug-resistant and hypervirulent phenotypes has constantly increased.

### **Virulence factors behind the hypervirulent/hypermucoviscous phenotype: From polysaccharide capsule to nowhere?**

Due to the characteristic hypermucoviscous aspect of the colonies produced by these hvKpn variants, it was proposed that the polysaccharide capsule was the main virulence factor responsible for the hypervirulent phenotype.<sup>38</sup> As observed in all encapsulated bacteria, the polysaccharide capsule represents a major virulence factor in *K. pneumoniae*. The capsule acts as a protective shield that protects bacteria from phagocytosis by immune cells, avoiding the bactericidal action of complement and antimicrobial peptides by preventing its deposition on the bacterial surface. Furthermore, the capsule blocks the

**Table 1.** Main differences between cKpn and hvKpn strains.

Feature	cKpn	hvKpn
Infections	Urinary tract infections, pneumonia, bloodstream infections, wound surgical infections and meningitis, mainly in alcoholics, elderly adults, patients undergoing invasive procedures or have significant comorbidities and immunocompromised patients. These infections are mainly nosocomial-acquired	Liver, lungs, kidney, bones, prostate abscesses and necrotizing fasciitis have been already described. Invasive infections to eye and central nervous system from this primaries sites of infection even in healthy adults. These infections are usually acquired in community.
Antimicrobial susceptibility	Multidrug-resistant strains represent the main threat in this variants of <i>K. pneumoniae</i> . Strains harboring extended-spectrum $\beta$ -lactamases (ESBLs) and carbapenemases genes are a clear example.	The first cases of these variants showed a susceptibility phenotype to several classes of antimicrobials. Worryingly, hypervirulent and multi-drug resistant strains have now been described. hvKpn strains harboring ESBLs, carbapenemases or <i>mcr-1</i> genes have been described.
Virulence-associated genes	Mainly enterobactin, aerobactin, yersiniabactin and salmochelin.	<i>rmpA</i> , <i>rmpA2</i> , aerobactin, yersiniabactin, <i>pld1</i> , KpnO porin and higher content of capsular sialic acid

bacterial opsonization with antibodies and prevents the activation of the innate immune response.<sup>39-41</sup> In accordance with this mechanism, acapsular variants of *K. pneumoniae* seem to be less virulent than capsular ones.<sup>40,41</sup> Moreover, the polysaccharide capsule is responsible for the appearance of mucus (but not necessarily the hypermucoviscous phenotype) when the microorganism is grown in different culture media.<sup>2</sup>

So far, 78 serotypes have been described, of which K1, K2, K5, K20, K54 and K57 have been identified as the most frequent, pathogenic and prevalent in human infections caused by *K. pneumoniae*. Among them, serotype K1 is predominant in Asia, and serotype K2 has been more frequently isolated in America and Europe; these serotypes are the most pathogenic to humans.<sup>42</sup> The genes involved in the synthesis and transport of the capsular antigen in *K. pneumoniae* are coded at the *cps* locus. Among others, this locus contains the genes required for the polymerization and translocation of the capsular antigen, and this operon is located within the bacterial chromosome.<sup>43-45</sup>

The *magA* gene ("mucoviscosity-associated gene A") was identified as the gene responsible for the hypermucoviscous phenotype by means of site-directed mutagenesis studies of the genes present in the locus.<sup>46,47</sup> Subsequent studies showed that the *magA* gene encodes a polysaccharide polymerase enzyme that is specific to capsular serotype K1 and is responsible for the formation of the characteristic structure of the capsule.<sup>48</sup> It was later discovered that this polymerase is specific to each of the currently known 78 capsular serotypes. Therefore, mucoviscosity-associated gene *magA* was renamed as *wzy\_K1*, and this nomenclature was applied to the remaining serotypes.<sup>49</sup> For example, the polymerase gene specific to serotype K2 is called *wzy\_K2*, the polymerase gene specific to serotype K3 is called *wzy\_K3*, and so forth.<sup>48,50</sup> This polymerase is essential for the synthesis of the capsule. Deletion of the *wzy* gene prevents the production of the polysaccharide capsule.<sup>51</sup> These features, along with the classical limitations of serotyping, have turned molecular typing by *wzy*-PCR into the gold standard for the characterization *K. pneumoniae* clinical isolates.<sup>52,53</sup> However, strains with the hypervirulent/hypermucoviscous phenotype that do not belong to the K1 serotype (*wzy\_K1* (*magA*)-negative) have also been described.<sup>54</sup> Therefore, another virulence gene (or genes), different from those present in the *cps* cluster, could be associated with the hypervirulent/hypermucoviscous phenotype.

Capsular polysaccharide (CPS) in *Klebsiella* is similar to *Escherichia coli* group I CPS in primary structure and biosynthesis mechanisms.<sup>55</sup> In *E. coli*, the 2-component system (TCS) RcsBCD plays an important role as a

regulator system for the synthesis of colonic acid, which simultaneously functions as a model for group I CPS biosynthesis. In this system, protein RcsC acts as a sensor kinase, which is autophosphorylated when it senses environmental stimuli. It subsequently transfers the stimuli to RcsD, a phosphotransfer histidine-containing protein, which is located in the inner membrane. Later, the signal is relayed to RcsB, a cytoplasmic response regulator protein. When RcsB is phosphorylated, it interacts with RcsA, which acts as an auxiliary transcriptional regulator. Finally, RcsA binds to promoters in the *cps* cluster, and then the biosynthesis of colonic acid capsule is activated.<sup>55,56,57</sup>

In *K. pneumoniae*, the mucoid phenotype A2 regulator (*rmpA2*) gene and the truncated form of the mucoid phenotype A regulator (*rmpA*) gene apparently act as positive transcriptional regulators of the capsular polysaccharide synthesis; thus, they code for the counterparts of the RcsA protein found in *E. coli*, leading to the association of capsular hyperproduction with hypermucoviscosity and hypervirulence.<sup>56</sup> Both can be encoded either in a chromosome or in a plasmid, and more than one copy can be found in a bacterial cell. Hsu et al.<sup>58</sup> studied a K1 *K. pneumoniae* strain harboring 2 *rmpA* genes (plasmidic and chromosomal) and one copy of *rmpA2* (plasmidic).<sup>58</sup> Through isogenic gene deletions and complementation experiments, they found that only plasmidic *rmpA* was able to increase the expression of *cps* genes and CPS production. On the other hand, *cps* expression was inhibited by plasmidic *rmpA2* but not chromosomal *rmpA*. Surprisingly, the authors found that although the plasmidic *rmpA* promoted *cps* gene expression and increased CPS production, the plasmidic  $\Delta$ *rmpA* mutants did not show any differences in serum resistance, proliferation in non-immune human serum or virulence in a murine model.<sup>59</sup> A high prevalence of both genes, combined or alone, was associated with strains that had a hypervirulent/hypermucoviscous phenotype.<sup>56,58,60</sup> For example, plasmid pLVPK (219-Kb) harbors the *rmpA* and *rmpA2* genes, as well as others that code for virulence factors, such as several siderophore systems and genes associated with resistance to tellurite, copper, silver and lead. This virulence plasmid has been associated with virulent strains of *K. pneumoniae*.<sup>61</sup> Likewise, numerous studies have reported that the presence of some of these transcriptional regulators, regardless of the capsular serotype, is strongly associated with hypervirulent/hypermucoviscous *K. pneumoniae*.<sup>58,62</sup> However, similar to the *wzy\_K1* gene, the strains lacking the *rmpA/rmpA2* genes with the hypervirulent/hypermucoviscous phenotype were also described.<sup>6,46</sup> Moreover, strains positive for the *rmpA* and *rmpA2* genes and without a hypermucoviscous phenotype or capsular serotypes

K1 or K2 were also isolated.<sup>6</sup> Some authors suggest that frameshift mutations (insertion or deletion) in the *rmpA/rmpA2* genes in these strains can produce altered RmpA/RmpA2 proteins that lead to a non-hypermucoviscous phenotype.<sup>63</sup> Therefore, hypervirulent/hypermucoviscous *K. pneumoniae* strains that do not belong to capsular serotypes K1 or K2 and that may or may not harbor the *rmpA/rmpA2* genes represent a great enigma regarding the genetic factor or factors responsible for its particular phenotype.

Sialic acid, also known as N-acetylneuraminic acid (NANA), is a monosaccharide that is present in the *K. pneumoniae* polysaccharide capsule. Recent studies have shown that the hypervirulent/hypermucoviscous variants of *K. pneumoniae* have a significantly higher concentration of sialic acid in their capsular extracts than do those strains that do not display the hypermucoviscous phenotype.<sup>64</sup> Moreover, these strains have a phagocytosis-resistance phenotype, in contrast to those strains with smaller concentrations of sialic acid in their capsules and without a hypermucoviscous phenotype. Sialic acid in the *K. pneumoniae* polysaccharide capsule inhibits the infected host's innate immune response by modulating the inflammatory responses. In addition, the depletion of sialic acid in the capsules produced by *K. pneumoniae* may result in a loss of hypermucoviscosity and, therefore, a decrease in the resistance to phagocytosis by neutrophils.<sup>64</sup> Given that only a small number of studies have analyzed the concentration of sialic acid present in the capsules of these hypervirulent/hypermucoviscous *K. pneumoniae* variants, it would be interesting to study a larger number of hypermucoviscous strains to determine whether the concentration of sialic acid plays a fundamental role in the expression of the hypervirulent phenotype.

As was noted above, the genetic factors associated with the synthesis of the polysaccharide capsule were the first analyzed. However, the role of the virulence factors described in cKpn was also studied. Among these, the siderophore system was the most analyzed. Siderophores play an important role in the ability of *K. pneumoniae* to cause invasive disease in human hosts with a functional immune system.<sup>65</sup> Russo et al. (2014) found that hypervirulent/hypermucoviscous variants of *K. pneumoniae* produced more siderophores than did the classical (not hypermucoviscous) ones.<sup>65</sup> In addition, they found that aerobactin was the most-produced siderophore under certain experimental conditions and that the increase in siderophore production was not related to gene copy number.<sup>65</sup> Moreover, they found that aerobactin was the primary factor in a conditioned medium that increased the growth/survival of bacteria in human ascites or serum. An increase in the virulence of an *in vivo* infection of a mouse pulmonary challenge model was also

identified.<sup>66</sup> It is noteworthy that these effects were not observed in the case of salmochelin, enterobactin and yersiniabactin siderophores.<sup>65,66</sup> These data suggest that aerobactin plays an important role as a fundamental virulence factor in the development of the hypervirulence of *K. pneumoniae*. *In silico* studies performed by Holt et al. (2015) showed a higher prevalence of siderophores in the invasive variants of *K. pneumoniae* than in the classical *K. pneumoniae*. In this study, yersiniabactin was the most prevalent siderophore found in the hypervirulent/hypermucoviscous variants of *K. pneumoniae*. Unlike the findings made by Russo et al. (2014), in relation to aerobactin, Holt et al. (2015) found that yersiniabactin showed no significant association between any of the other siderophores and the expression of the invasive phenotype.<sup>67</sup>

Searching for the genetic determinants behind the hypervirulent phenotype has identified new factors associated with virulence. A phospholipase D family protein (PLD1) encoded by the *pld1* gene that is located within a type 6 secretion system (T6SS) locus has been described by Lery et al (2014). These authors found that, in an intranasal murine model of infection,  $\Delta pld1$  mutants were avirulent in comparison with wild-type strains, while virulence was restored in complemented strains.<sup>68</sup> In concordance, phospholipase D family proteins have been identified as key factors for virulence in *Legionella pneumophila* because they promote host cell invasion and consequent bacterial dissemination.<sup>69</sup> Interestingly, an *in silico* analysis of *pld1* in 171 *Klebsiella* genomes found that this gene was widely distributed in *K. pneumoniae subsp rhinoscleromatis*, the only intracellular subspecies of the *Klebsiella* genus.<sup>68</sup>

In bacteria, porins constitute one of the major proteins of the outer membrane (OM). These also called OM proteins (OMPs), which interact with the bacterial environment and act as pores through which certain molecules can diffuse. Moreover, these proteins have been implicated in bacterial pathogenesis. A novel OMP, the *KpnO* porin, was identified by Srinivasan et al (2012). These authors found that  $\Delta kpnO$  strains produced less capsular polysaccharide, which was reflected in non-mucoid and smaller colonies in comparison with the hypervirulent wild-type strains. In addition, using a *Caenorhabditis elegans* infection model, the authors found that *kpnO* mutants were less virulent than the hypervirulent wild-type strains. Complemented strains recovered virulent and hypermucoviscous phenotypes.<sup>70</sup> As we observed, a large number of virulence factors have been associated with the hypervirulent phenotype; thus, hypervirulence seems to be a consequence of a complex interplay of several genetic determinants rather than a phenomenon originated by a single gene.

### Hypervirulence-associated clonal groups

Bialek-Davenet et al. (2014) analyzed 694 highly conserved genes using a core-genome multilocus sequence typing scheme (cgMLST). These authors reported that serotype K1 hypervirulent variants belonged to clonal group 23 (CG23), where ST23 was predominant. Serotype K2 hypervirulent variants, on the other hand, belonged to the following clonal groups: CG86 (the most widely distributed), CG65, CG375 and CG380. These results suggest that invasive infection-causing serotype K2 isolates are more genetically diverse than serotype K1 ones.<sup>71</sup> Interestingly, these researchers found that tellurite resistance (due to the presence of the *terW* gene) is strongly associated with CG23, CG65 and CG86.<sup>72</sup> Globally, CG23 (associated with the K1 serotype) is the most prevalent CG associated with hypervirulence, followed by CG86. To date, all these clonal groups have been described as causal agents of invasive infections around the world.<sup>17,71</sup>

### Hypermucoviscosity and hypervirulence: Two different but (at times) complementary phenotypes?

In 2014 in China, Luo et al. (2014) analyzed 51 non-repetitive *K. pneumoniae* clinical isolates causing primary liver abscesses (PLAs).<sup>73</sup> The samples were obtained from the drainage of these PLAs. Patients from both sexes were included in the study, and their mean age was 53 y. Most isolates belonged to the K1 serotype, accounting for 39% (20/51) of the isolates; 31% (16/51) belonged to the K2 serotype, and 29% (15/51) belonged to non-K1/K2 isolates. Interestingly, approximately 29% (15/51) of the clinical isolates causing PLAs did not show the characteristic hypermucoviscous phenotype that was associated with the hypervirulent *K. pneumoniae* variant. Almost all clinical isolates belonging to the K2 serotype (93.8% (15/16)) displayed the hypermucoviscous phenotype. Nevertheless, 70% (14/20) of the clinical isolates belonging to the K1 serotype showed the hypermucoviscous phenotype, whereas only 46.7% (7/15) of the non-K1/K2 clinical isolates showed a positive string test. In accordance with other studies, *K. pneumoniae* clinical isolates belonging to the K1 serotype were grouped into clonal complex 23 (CC23); those belonging to the K2 serotype were grouped into CC65, and non-K1/K2 isolates were grouped into minor clonal complexes.<sup>67,71,74</sup> In addition, virulence genes, such as *rmpA* and aerobactin, *kfu*, and *allS* siderophore genes showed a diverse distribution among all clonal complexes. Overall, these results suggest that the hypervirulent phenotype is not dependent on hypermucoviscosity. Therefore, hypervirulence should be defined not only by the phenotype but also by the genotype and the clinical characteristics of the infection. In other words,

hypervirulence goes beyond a capsular serotype and a positive string test.<sup>73</sup>

Similar results were reported in Spain, where Cubero et al. (2015) analyzed 878 cases of bacteremia caused by *K. pneumoniae*.<sup>6</sup> Only 5.4% (53/878) of the isolates collected from 50 patients displayed the hypermucoviscous phenotype identified through string test. Among these, 16/53 (30.2%) were K1 serotype and *rmpA*-gene positive, 12/53 (22.6%) were non-K1 serotype and *rmpA* gene-positive, and 25/53 (47.2%) were non-K1 serotype and *rmpA* gene-negative. Cases of bacteraemia due to hypermucoviscous were more frequent in men with a mean age of 67 y and were community acquired. The vast majority of these patients had an underlying disease. Moreover, these researchers identified that some *rmpA* gene-positive strains belonging to the K1 serotype did not display the hypermucoviscous phenotype. Nevertheless, these strains were able to produce an invasive infection. Surprisingly, they found a small number of non-hypermucoviscous isolates that did not harbor *rmpA* genes and did not belong to the K1 serotype causing liver abscesses, a type of infection that is commonly restricted to K1/K2, *rmpA*-positive and hmvKpn isolates. These results were in agreement with the observations reported by Luo et al. (2014) in the sense that hypermucoviscosity detected through the string test is not enough to render an isolate hypervirulent.<sup>73</sup> According to these findings, hypermucoviscosity and hypervirulence are 2 separate entities, and a hypervirulent phenotype is only determined by genetic traits, in particular (but not solely) by the transcriptional regulator of the *cps* cluster, *rmpA*. Cubero et al. (2015) also identified *rmpA*-negative invasive strains belonging to the diverse capsular serotypes.<sup>6</sup>

In Japan, Togawa et al. (2014) studied 83 bloodstream infection cases caused by *K. pneumoniae*.<sup>75</sup> Thirty-eight percent (32/83) of the isolates were classified as community-acquired infections and 61% (51/83) as hospital-acquired infections. These authors reported that the mucoid phenotypes (colonies with a higher degree of mucoviscosity than other cKpn but with a negative string test) and hypermucoviscous phenotypes were more prevalent in the community-acquired *K. pneumoniae* isolates. As expected, septic shock was more frequently observed in isolates with mucoid and hypermucoviscous phenotypes than in non-hypermucoviscous isolates. However, no statistically significant differences were observed when mucoid isolates were compared with non-mucoid ones. Interestingly, a statistically significant association was seen between liver abscess formation and the mucoid phenotype. Surprisingly, liver abscess formation was not significantly associated with hypermucoviscosity. This finding is contrary to the idea that the hypermucoviscous phenotype is strongly associated with the development of

liver abscesses.<sup>75</sup> This phenomenon, which is rarely described, needs to be studied more deeply for a better understanding of the bacterial genetic determinants and the host factors that could be responsible for the hypervirulent phenotype in *K. pneumoniae*.

Recently in China, Wu et al (2017) conducted a retrospective study to investigate the phenotypic and genotypic characteristics of 76 *K. pneumoniae* strains causing bloodstream and at least one another site infection during the same period of infection. Of these 76 strains, 28 were considered as hypervirulent and the remaining 48 as classic variants. Because there is no consensus about the definition of hypervirulence, the authors used 3 microbiological characteristics that have been associated to it: A positive string test, a positive PCR amplification of *rmpA* gene and a positive PCR amplification of aerobactin gene. Those strains with at least 2 of these indicators were considered as hypervirulent. They found that 2 of 28 strains classified as hypervirulent did not show the hypermucoviscous phenotype. However, up to 90% of them were positive to *rmpA* and aerobactin genes. Interestingly, 2 strains classified as classic variants of *K. pneumoniae* harbored *rmpA* gene which usually has been associated only to hypervirulent variants. Although none of them expressed the hypermucoviscous phenotype and only less than 10% harbored the aerobactin gene. Regarding to the development of metastasis (invasive infection), which was defined by the authors as infections occurred at 2 or more different infection sites in one patient with bacteremia and without injury or former invasive medical procedure around the infection sites. Two metastatic infection cases were identified in homo-cKpn (cKpn strains isolated from different specimens sources from the same patient with the same PFGE pattern). Surprisingly, with respect to metastasis development, no differences were observed between the homo-hvKpn and the homo-cKpn strains.<sup>76</sup>

Hypermucoviscosity has not only been described in *K. pneumoniae* clinical isolates but also in *Klebsiella quasipneumoniae* subsp. *similipneumoniae* (initially classified as *K. pneumoniae* through automated methods) with a hypermucoviscous phenotype by Garza-Ramos et al. (2016). This identification was performed using whole genome sequencing and a subsequent analysis of the *rpoB* gene, and it was confirmed by average nucleotide identity (ANI). The string test was positive, and the isolate was identified as an extended-spectrum  $\beta$ -lactamase producer encoding the extended-spectrum  $\beta$ -lactamase (ESBL) SHV-12. Both *in silico* analysis and PCR assays showed negative results for the *rmpA* and *rmpA2* genes. Regarding the “classical” virulence factors, this strain was positive for enterobactin, urease, UDP galacturonate 4-epimerase, glycosyltransferases, pili, and several

adhesins.<sup>77</sup> Simultaneously, a previous study (personal communication, August 2015) analyzed a hypermucoviscous K2 serotype *K. pneumoniae* obtained from liver abscess drainage that was positive for the *rmpA* and *rmpA2* genes. The PCR assay to detect the *terW* gene was also positive. Furthermore, Southern hybridization showed that this strain harbored 2 copies of the *rmpA* gene: one was located in the bacterial chromosome, and the other one was plasmidic. Southern hybridization against the *rmpA2* gene showed its plasmidic location. Subsequent whole genome sequencing indicated that this strain harbors the virulence plasmid pLVPK. Siderophore-associated genes *iucA*, *iroB* and *entB* were also detected *in silico* and by PCR. Finally, yersiniabactin gene *irp2* was not present in this strain. Once identified, these 2 clinical isolates with a hypermucoviscous phenotype were subject to an analysis of virulence through resistance to non-immune human serum, biofilm formation, and survival rate assays in a murine model by intraperitoneal inoculation of  $1 \times 10^4$  CFU/mL of both strains. Despite its hypermucoviscous phenotype, the *K. quasipneumoniae* subsp. *similipneumoniae* isolate was highly sensitive to the non-immune human serum; it was sensitive to neutrophil phagocytosis and was unable to kill the infected mice after a 14-day follow-up (personal communication, August 2015). On the other hand, the K2 serotype *K. pneumoniae* clinical isolate with a hypermucoviscous phenotype was resistant to the components of the non-immune human serum; it evaded neutrophil phagocytosis and killed mice 2 d after inoculation (personal communication, August 2015). No differences were observed with respect to biofilm production: both hypermucoviscous isolates were classified as non-producers, which is consistent with previous studies.

Moreover, other studies have described the hypermucoviscous phenotype in the *Klebsiella* genus. Arena et al. (2015) reported the whole genome sequence of a *Klebsiella quasipneumoniae* subsp. *quasipneumoniae* clinical isolate displaying a hypermucoviscous phenotype. This strain was isolated from a bloodstream infection, and it had a new capsular *wzi* allele. *In silico* screening of virulence genes showed the absence of the *rmpA* and *rmpA2* genes. However, this strain harbored other virulence genes, such as the *allABCDRS* operon (associated with allantoin metabolism), the *kfuABC* system, and the *mrkABCDFHIJ* fimbriae cluster.<sup>78</sup> Cases of PLAs and subsequent endophthalmitis produced by *K. quasipneumoniae* subsp. *quasipneumoniae* have also been described. Brisse et al. (2016) reported the whole genome sequence of a *K. quasipneumoniae* subsp. *quasipneumoniae* isolate that showed a *cps* gene cluster associated with a capsular serotype K1. Interestingly, this virulent strain harbored a

76-Kb DNA genomic island, which is closely related to the integrative and conjugative element (ICE) ICEKp1, a mobile genetic element described previously in the hypervirulent/hypermucoviscous strain of *K. pneumoniae* NTUH-K2044. The mobile genetic element contained the *rmpA* gene and genes encoding for the siderophores yersiniabactin and salmochelin. These data suggest that the natural horizontal acquisition of gene clusters associated with interspecies virulence in the *Klebsiella* genus is a concerning possibility. In addition, the clusters *mrkABCDFHIJ* and *fimABCDEFGH* (encoding type III and type I fimbriae, respectively) and the *iutA* gene (encoding the aerobactin receptor) were also identified in the bacterial genome.<sup>79</sup> In these 2 reports, both *K. quasipneumoniae* subsp *quasipneumoniae* strains were susceptible to several classes of antimicrobials, and only  $\beta$ -lactamase *bla*<sub>OKP</sub> (a  $\beta$ -lactamase characteristic of *K. quasipneumoniae* subsp *quasipneumoniae*) was detected.<sup>78-80</sup>

The hypermucoviscous phenotype has also been observed in *Klebsiella variicola*. *K. variicola* is a Gram-negative rod bacterium closely related to *K. pneumoniae* and *K. quasipneumoniae* species. It was initially described as endophytic and pathogenic to humans and subsequently identified as a symbiont in leaf-cutting ants. In the last year, *K. variicola* has been broadly described as a human pathogen in different parts of the world.<sup>81-85</sup> Garza-Ramos et al. (2015) sequenced the whole genome of a *K. variicola* hypermucoviscous clinical isolate obtained from the sputum of an elderly man in Mexico. The *in silico* search of the *rmpA*, *rmpA2* and *wzy\_K1* (*magA*) genes was negative, but the search for virulence genes, such as those involved in iron capture systems (*ironN*, *iutA*, *entB* and *kfuABC*), bacterial metabolism (*uge*, *ureA* and *wabG*) and adhesion (*mrkABCDFHIJ*) was positive. A 100% nucleotide identity between this strain and the serotype harboring the *wzc*-932 variant was also found.<sup>86</sup> Currently, hypermucoviscosity has been described even in other bacterial genera, particularly in the uropathogenic *Escherichia coli* (UPEC).<sup>87</sup> Therefore, these results could indicate that the hypermucoviscous phenotype might be a feature that can be acquired horizontally through a mobile genetic element. However, the results found in the *Klebsiella* species suggest that hypermucoviscosity *per se* is not enough to confer hypervirulence to a bacterial strain and that other bacterial factors are required for the development of hypervirulence.

Over time, new evidence has arisen suggesting that the string test is not an effective means of determining whether an isolate is hypervirulent. However, we cannot discard the string test as a probe that give us a warning signal that indicates the possibility that a clinical isolate

could be hypervirulent. Until now, string test together with the clinical data and molecular probes, for example the PCR identification of *rmpA* gene, represent the best preliminary way to detect and diagnose infections produced by hypervirulent isolates. Therefore, considering the most important advances in this field, we propose that the hypermucoviscous phenotype is not a reliable indicator of hypervirulence. We also postulate that the search for the genetic traits responsible for the hypervirulent and hypermucoviscous phenotypes should be made considering these phenotypes as 2 different phenomena that may act as 2 synergistic phenotypes (only in some cases) to produce strains that display a hypervirulent phenotype.

### Concluding remarks/Future perspective

It has long been widely accepted that *K. pneumoniae* isolates displaying a hypermucoviscous phenotype are responsible for causing invasive infections in apparently healthy people that are rarely produced by enteric Gram-negative microorganisms. In addition, due to the apparent association between this phenotype and the clinical manifestations of the infection, these variants have been called hypervirulent. In medical literature, the terms hypervirulent and hypermucoviscous are commonly used as synonyms. Moreover, a positive string test has been considered as indicative of hypervirulence. However, recent findings show that in at least 3 *Klebsiella* spp bacterial species, a hypermucoviscous phenotype *per se* is not sufficient to induce a hypervirulent state, and the identification of hypervirulent *K. pneumoniae* variants that do not express the hypermucoviscous phenotype but still can cause invasive infections in healthy people is concerning. Thus, the absence of hypermucoviscosity is not an appropriate way to exclude hypervirulence. Molecular tests to detect virulence factors, such as aerobactin, yersiniabactin and the *rmpA/rmpA2* genes associated with this phenotype, and the clinical manifestations of the disease are necessary. However, a simple molecular test is not enough to detect hypervirulence.

Several studies have indicated the K1 and K2 capsular serotypes (despite the fact that hypervirulence is associated with clones rather than with serotypes<sup>74</sup>) or transcriptional regulators, such as the *rmpA/rmpA2* genes, and even to some siderophores, such as aerobactin and yersiniabactin, as the responsible factors for the hypervirulent phenotype. However, several studies have reported the existence of non-K1/K2, *rmpA/rmpA2*-negative strains with a different distribution of siderophores that cause invasive infections. Therefore, until the genetic determinants associated with hypervirulence are clearly identified, we cannot assert or rule out that a clinical



isolate is hypervirulent. Next-generation DNA sequencing techniques, bioinformatics and transcriptomics represent powerful tools that could help us identify the elements underlying the hypervirulence and hypermucoviscous phenotypes. All of these methodologies could also help us understand whether (under certain circumstances) these phenotypes act synergistically or whether they definitely correspond to 2 different entities. Since hypermucoviscosity is not a perfect marker of hypervirulence, the available literature concerning this theme would be imperfect too.

On the other hand, most available studies have focused on the analysis of bacteria. Few studies have analyzed host factors that could favor the development of the invasive infection, for example, age and health status. The in-depth study of the host-associated factors, such as the major histocompatibility complex (MHC) variants that are more frequently associated with such infections, eating habits, nutritional status, and gut microbiota composition, could provide important information to enhance our understanding of the hypervirulence phenomenon. The rapid spread of these hypervirulent variants around the world and the emergence of multidrug-resistant hypervirulent strains require us to act quickly in such a way that we can appropriately face this great threat to global public health. Finally, the evidence exposed in this review suggests that hypermucoviscosity and hypervirulence are 2 different phenotypes.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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