DOI 10.1007/s10517-022-05424-3
Bulletin of Experimental Biology and Medicine, Vol. 172, No. 5, March, 2022

REVIEWS

Molecular Epidemiology of Hypervirulent *K. pneumoniae* and Problems of Health-Care Associated Infections

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 172, No. 11, pp. 532-551, November, 2021
Original article submitted August 2, 2021

The review describes virulence factors of hypervirulent *K. pneumoniae* (hvKp) including genes determining its virulence and discusses their role in the development of health-care associated infections. The contribution of individual virulence factors and their combination to the development of the hypervirulence and the prospects of using these factors as biomarkers and therapeutic targets are described. Virulence factors of hvKp and "classical" *K. pneumoniae* strains (cKp) with no hypervirulence genes were compared. The mechanisms of biofilm formation by hvKp and high incidence of its antibiotic resistance are of particular importance for in health care institutions. Therefore, the development of methods for hvKp identification allowing early prevention of severe hvKp infection and novel approaches to abrogate its spreading are new challenges for epidemiology, infection diseases, and critical care medicine. New technologies including bacteriological and molecular studies make it possible to develop innovative strategies to diagnose and treat infection caused by hvKp. These include monitoring of both genetic biomarkers of hvKp and resistance plasmid that carry of virulence genes and antibiotic resistance genes, creation of immunological agents for the prevention and therapy of hvKp (vaccines, monoclonal antibodies) as well as personalized hvKp-specific phage therapies and pharmaceuticals enhancing the effect of antibiotics. A variety of approaches can reliably prepare our medicine for a new challenge: spreading of life-threatening health-care associated infections caused by antibiotic-resistant hvKp strains.

Key Words: *hypervirulent K. pneumoniae; infections; virulence factors; virulence genes*

Klebsiella pneumoniae is an opportunistic pathogen that can cause various infectious diseases. It easily colonizes human skin and mucous membranes, including the gastrointestinal tract and oropharynx, and penetrates other organs and tissues. *K. pneumoniae* can cause a wide range of infections, including pneumonia, urinary tract infections, bacteremia, and meningitis in hospital patients or immunocompromised individuals [6,87,99]. "Classical" *K. pneumoniae* (cKp) is a major cause of nosocomial pneumonia and urinary tract infections, one of the most frequently isolated gram-negative bacteria causing hospital-acquired infections worldwide [88,105]. The last decades, however, were marked by the emergence of a new type of pathogen, hypervirulent *K. pneumoniae* (hvKp), that quickly gained high clinical significance, because it caused infections with increased invasiveness in healthy immunocompetent hosts [47].

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The first cases of such infections were reported in Southeast Asia [80], further cases appeared in North America [58], the Middle East [2,91,100], Australia [133], and Europe [65]. In contrast to cKp, about half of all infections caused by hvKp occur in young healthy individuals [99,104,117,125]. Cases of neonatal sepsis caused by hvKp have also been described [5,93].

A typical feature of hvKp is the presence of multiple infection sites, and the infections themselves are disseminated, with a rapid development of life-threatening conditions [107]. HvKp pathogens cause sepsis with diffuse metastatic lesions [26,48], development of septic shock, and commonly result in lethal outcome [22,52,77,129]. HvKp bacteria can be the cause of Lemierre's syndrome, a rare severe illness characterized by oropharyngeal infection, septicemia, venous thrombosis, and metastatic foci [131].

Immunological studies have shown that compared to cKp, hvKp pathogens are more resistant to phagocytosis, neutrophil-mediated and complement-mediated cytotoxic activity, and NETosis — the process of neutrophil dying associated with neutrophil extracellular traps (NET) caused by the protrusion of large DNA fragments through the cell membrane [143]. hvKp strains demonstrate increased growth in human body fluids *ex vivo* and increased virulence in various models of infection compared to cKp strains [104,156].

Thus, hvKp is a dangerous pathogen with increased virulence capable of causing severe infections and sepsis; therefore, studying the molecular basis of the pathogenesis of infection caused by hvKp is a priority task.

This review presents an analysis of the data on the association of hvKp-specific virulence factors with the peculiarities of infection development.

Despite the fact that most patients with hvKp infection are young and have no comorbidities, the mortality rate among them is high and reaches 55% [78]. Clinical symptoms of hvKp are nonspecific and can include fever, chills, abdominal pain, nausea, and vomiting, and can also be due to metastatic infection [118]. The most common sites of metastasis are the eyes, lungs, and CNS [17]. One common complication of hvKp infection is bacteremia [70]. There are reports of patients with liver and spleen abscesses, severe infectious skin, soft tissue and bone lesions, including necrotizing fasciitis [11,14,32,44,71]. In rarer cases, hvKp affects the urinary system [70] (Fig. 1).

CAPSULE SEROTYPES

K. pneumoniae uses multiple strategies for growth and defense against the host's immune response. The capsule is formed by polysaccharides synthesized by all *K. pneumoniae* strains; it acts as a protective envelope, inhibiting phagocytosis, antimicrobial peptides, and host inflammatory responses [21,30].

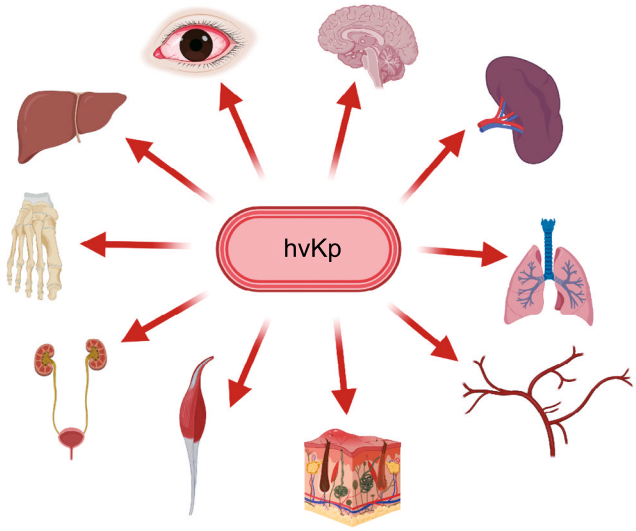

Fig. 1. Localization of infectious lesions caused by hypervirulent *K. pneumoniae*.

The genes controlling capsule formation are located in the chromosome and are largely conserved in all species, but the polysaccharide components of the capsule carry differences by which bacteria can be typed. The polysaccharide variants resulting from genetic differences are called K-antigens and are classified serologically. More than 130 types of capsules have been identified in *K. pneumoniae* [40,152].

The most common capsule loci associated with hvKp are K1 and K2, detected in 70% strains; the rest hvKp strains detect other serotypes: K2, K5, K16, K20, K54, and K57 [109,117]. In spite of the fact that serotypes K1 and K2 occur quite frequently in hvKp isolates, studies show that their presence is only partially responsible for hypervirulence [43,51,68].

HYPERMUCOVISCOSITY AND REGULATORS OF CAPSULE SYNTHESIS

Hypermucoviscosity (HMV) is a phenotypic feature of hvKp strains. For a long time, HMV was associated directly with hypervirulence and increased capsule synthesis associated with the expression of the *rmpA* gene [158]. The transcriptional regulator *rmpC* was shown to reduce the expression of the capsule synthesis (*cps*) gene cluster without affecting the HMV phenotype. This fact indicates that HMV phenotypes and enhanced capsule synthesis are independent phenomena [139]. At the same time, mutations that interfere with capsule synthesis lead to loss of HMV, which confirms the association between capsule formation and HMV.

For this reason, the associations of HMV with capsule synthesis and virulence determine the significance of the contribution of the link between *cps* and HMV expression to the transformation of the cKp strain into hvKp [132]. The ability to produce more capsular polysaccharide is mediated by a number of regulators that modulate capsule synthesis and/or transcription of capsular polysaccharide biosynthesis genes. The polymorphism of these genes demonstrates their role in *K. pneumoniae* survival. Figure 2 shows the effect of various transcriptional regulators on *cps* expression.

RmpA, rmpA2. The *rmpA* gene was first described in 1989 as a regulator of the mucoid phenotype located within the hvKp plasmid, along with *rmpA2*, another regulator of the cps gene [63,95,109]. Further analysis showed that the K1 serotype of hvKp from Asia was commonly associated with the *rmpA* chromosomal gene, whereas many hvKp strains contain only plasmid copies of *rmpA* and *rmpA2* [56]. More than half (55-100%) of hvKp strains express at least one copy of *rmpA* or *rmpA*2, whereas cKp contains only 7-20% [56,74], that makes *rmpA* commonly used diagnostic marker of hypervirulence [127]. These genes positively regulate the cps locus at the transcriptional level contributing to the HMV phenotype and hypervirulence [15,139].

Early studies of *rmpA* and *rmpA2* suggested that increased expression of the *cps* genes is not the basis for HMV [95]. Based on published data, the differences between capsule gene overexpression and HMV for the hvKp were analyzed. This analysis showed that HMV can be a more important attribute of virulence than the synthesis of a large number of capsular polysaccharides.

Although the presence of $rmpA^{+}/rmpA2^{+}$ and $magA^{+}$ genes combined with the HMV phenotype is a generally recognized marker of hvKp, there are other combinations $rmpA^{-}$/HMV^{+}, $rmpA^{-}/rmpA2^{+}$/HMV^{+}, and $rmpA^{+}$/HMV^{-} [17]. This exclusion demonstrates the diverse potential of *K. pneumoniae* capable of hypervirulence.

MagA. The increased level of capsule synthesis can be initiated in the absence of *rmpA* or *rmpA*2 by the chromosomal gene *magA* [157]. It has been shown that magA is specific to K1 strains representing a wzy-like polymerase required for capsule production [37]. Subsequently, the *magA* gene has been renamed wzy_K1, whereas other serotypes are associated with different *wzy* alleles [124].

RmpC. A study of the regulation of hvKp capsule synthesis revealed a new transcriptional regulator, *rmpC*. While *rmpA* apparently coordinates capsule production and the HMV phenotype, *rmpC* regulates only the capsule. Deletion of the *rmpC* gene resulted in decreased expression of *cps* gene promoters and capsule polysaccharide synthesis; similar changes were observed in the *rmpA* mutant. However, unlike the *rmpA* mutant, this strain remains hypermucoid. This fact indicates that capsule synthesis is not mandatory for the HMV phenotype [139].

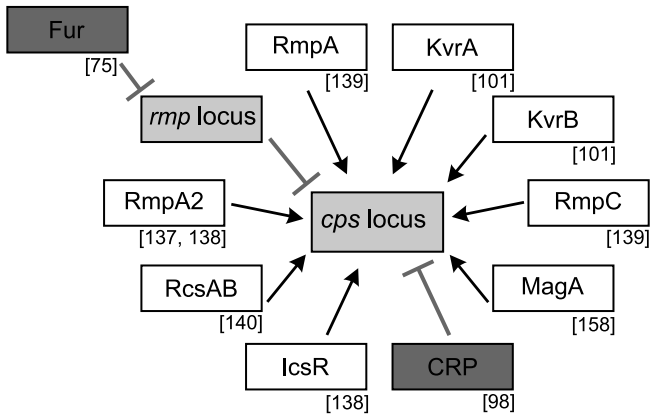

Fig. 2. Regulation of the expression of *cps* locus controlling the synthesis of capsular polysaccharide. Arrows indicate activators, lines indicate suppressors of locus activity.

KvrA and kvrB. New transcriptional regulators *kvrA* and *kvrB* have been described that activate fusion gene transcription and are required for HMV in hvKp strains. Strains with *kvrA* and *kvrB* deletions are known to be less virulent than wild-type strains [101].

Fur. The Fur regulator (an ferric uptake regulator) suppresses capsule synthesis in hvKp CG43 strains by reducing *rmpA* and *rmpA*2 expression; therefore, an enhancement of hvKp capsule synthesis in iron-deficient media, *e.g*. in the human body, is suggested [75].

IscR. IscR (iron-sulfur cluster regulator) is another iron-responsive transcriptional regulator that controls *cps* gene expression. IscR has been identified as a repressor of genes encoding proteins containing iron and sulfur ions [112]. IscR is active in iron binding and activates *cps* expression when iron levels are sufficient. In contrast, Fur carries out its repressive activity when *cps* is expressed when the iron level is sufficient and releases this repression when the iron level is low [138]. Thus, *K. pneumoniae* has acquired mechanisms ensuring capsule synthesis at a level that does not depend on iron availability and, at the same time, allowing changing the capsule production depending on iron concentration in the microenvironment.

cAMP receptor protein (CRP). CRP is a transcriptional regulator that suppresses *crp* gene expression. Studies of a strain of *K. pneumoniae* strain NTUH-K2044 showed that the mutant with the *Δcrp* deletion was characterized by increased HMV and ability to form biofilms and produce fimbriae [76,98]. Excess glucose in the external environment decreased

the CRP level, leading to increased *cps* gene expression [79].

RcsA, RcsB. Rcs-phosphorylation is an enzymatic reaction that triggers a complex signal transduction system in various gram-negative bacteria. The proteins involved in such reactions include sensory kinase RcsC, histidine phosphotransferase RcsD, reaction regulator RcsB, auxiliary protein RcsA, and outer membrane lipoprotein RcsF [140]. It was found that RcsB dimerizes with RcsA and binds a DNA sequence called RcsAB-box, which leads to activation of capsule gene expression in a number of bacteria. Direct regulation of genetic activity by RcsAB binding to DNA has been shown for the uppermost promoter of the three characterized capsule operons [146].

K. pneumoniae NTUH-K2044 capsule synthesis is also regulated by other genes that are not assigned to the known regulatory network containing the *argR* and *SapBCDF* ABC transporter genes. The *SapBCDF* gene determines the increased survival of bacterial cells in human serum but does not affect resistance to antimicrobial peptides. The *ΔSap* mutation resulted in a 2.5-fold increase in transcriptional expression of the *wcaG* capsular locus compared to the wild type. Sap-dependent induction of capsule gene expression occurs without *rcsA* involvement. The *argR* gene suppresses the transcriptional activity of arginine and other amino acid catabolism genes. The *ΔargR* mutants are characterized by a capsule with a slightly reduced thickness and thinner filaments than that of wild-type *K. pneumoniae*; the *ΔargR* mutation has been associated with a decreased virulence. The mechanisms by which *argR* and *SapBCDF* regulate capsule synthesis in different *K. pneumoniae* strains are not yet clarified [31].

Biofilm formation. *K. pneumoniae* can survive in the hospital environment for a long time by attaching to physical surfaces (medical devices, catheters, and ventilator devices) to form biofilms [94]. The similar capabilities of hvKp isolates contribute to their high virulence.

Biofilms are complex surface-associated bacterial communities, which form an extracellular matrix containing proteins, polysaccharides and DNA. Matrix composition provides protection from the immune system products and environmental influences. The texture and composition of the biofilm matrix are important for the regulation of biofilm density, which can be modulated depending on the alterations of the content of sugars and proteins in a microenvironment [3].

The most important surface structures of *K. pneumoniae* involved in the process of biofilm formation are pili, capsule polysaccharides, the quorum sensing (QS) system, other polysaccharides, and adhesins [13,19,151]. Pili mediate stable adhesion; previously, it was thought that type I pili did not play a significant role in biofilm formation. However, later it was found that both type I and type III pili contributed to it. Thus, genes *fimA*, *fimH*, and *mkrD* found simultaneously in 6 *K. pneumoniae* isolates are directly related to biofilm formation [28,122]. One of the genes that control biofilm formation, the gene for lipoprotein *YfgL* (BamB), was involved in the transcriptional expression of type I pili, which is essential for the expression of *K. pneumoniae* antiphagocytic activity *in vivo* [53].

Capsule polysaccharides affect the biofilm structure and intercellular interactions. Thus, the *treC* gene is critical for capsule synthesis and biofilm formation, two processes that develop through trehalose utilization [151]. Secretory polysaccharides and other adhesins (pgaABCD and bcsA) also play a role in the physical attachment of bacteria to the surface, which enhances biofilm formation by *K. pneumoniae* bacteria [12,142]. Given the dynamics of biofilm formation and the variability of the environmental stimuli, the bacterium must have the ability to rapidly change gene expression. The regulation of bacterial transcription is carried out by the QS system, which coordinates the signals and responses that control gene expression in the microbial population. QS is a well-known mechanism involved in the process of biofilm formation. A study of the type II QS system in *K. pneumoniae* revealed the role of *luxS* in the synthesis of the AI-2 autoinducer [4,160]. It is interesting that *luxS* was detected in 98% of the *K. pneumoniae* isolates forming biofilms [113]. In the *luxS*-mutant *K. pneumoniae* mutant, changes in biofilm architecture were observed: less surface coverage and reduced macrocolony formation [13]. Another study showed that mutations of the *luxS* gene and the autoinducer AI-2 transport systems did not affect the expression of the *wzi*, *wza*, and *wzx* genes controlling the expression of capsular polysaccharides, but caused an increase in the expression of *wbbM* and *wzm*, associated with LPS synthesis. In *K. pneumoniae* cells AI-2 seems to act as a regulator of biofilm formation and LPS synthesis [24].

In a number of studies, the biofilm phenotype was associated with a capsule [33] and/or fimbriae [111], while the absence of a capsule has been shown to enhance biofilm formation [57]. Thus, under laboratory conditions, hvKp strains form biofilms more efficiently than cKp isolates [151]. At the same time, no differences were found between HMV and non-HMV *K. pneumoniae* isolates in biofilm formation [120]. HvKP strains NTUH-K2044 and KpL1 were shown to form biofilms with gene products similar to cKp [102]. These include genes for capsular polysaccharides, LPS, pilin, carbohydrate metabolism genes, and type II QS genes. Studies of the hvKP1 strain revealed genes presumably encoding glutamine synthetase and

the succinyl-CoA synthase α-subunit that contribute to biofilm formation [60].

SIDEROPHORES

The ability to accumulate iron is essential for bacterial growth and reproduction; this property plays a crucial role in the development of infection [145]. The host organism contains a number of iron-binding proteins, which serve to retain iron and limit its availability to pathogens [10]. In turn, in order to utilize iron ions from the iron-binding proteins of the host, *K. pneumoniae* produces high-affinity, low-molecular-weight iron chelate molecules, the siderophores [50]. The siderophores accumulate the host iron cations due to their higher affinity as compared to the iron-binding proteins of the body, and then along with the bound cations the siderophore molecules are acquired back by the bacterial cell via specific receptors [41,89]. Bacteria hvKp produce four types of siderophore molecules: yersiniabactin (Ybt), enterobactin (Ent), salmochelin (Iro), and aerobactin (Iuc). The latter two are specific for hvKp and are undetectable in cKp [51,55,109].

Yersiniabactin is an important virulence factor of cKp, but its role in hvKp has not been elucidated. Analysis of 2500 *K. pneumoniae* genomes demonstrated that both *iro* and *iuc* loci were detected together in hvKp strains. This suggests that salmochelin contributes to the pathogenesis of infection caused by hvKp [65]. Although the data do not support a role for salmochelin in the development of systemic infection, in combination with microcin E492 it can provide a competitive advantage in gastrointestinal colonization [109,110,125]. Additional analysis of 97 genomes of the CG23 hvKp clonal group revealed the *ybt*, *iuc*, and *iro* loci in almost all genomes [64].

Aerobactin (Iuc) is considered to be the most important siderophore system of hvKp providing systemic infection [108]. Aerobactin has been shown to be the only siderophore required for isolated hvKp strains in laboratory experiments; it accounts for about 90% of siderophore activity [110].

The presence of several siderophores in hvKp strains suggests that other siderophores also play a role in pathogen colonization and the development of systemic infections. However, their activity can be inhibited by innate immunity factors. For example, the antibacterial peptide LL-37 is capable of binding aerobactin [162]. Another innate immunity factor, lipocalin-2 protein, effectively binds enterobactin, preventing its return to the cell and thus protecting the macroorganism [39]. It is assumed that the high binding efficiency of lipocalin and enterobactin reduces the role of the latter in the development of systemic infection [108].

ADDITIONAL VIRULENCE FACTORS

Allantoin metabolism. Allantoin serves as a source of nitrogen for various bacterial species and as a source of nitrogen and carbon for *K. pneumoniae* [18,23]. It is a metabolic intermediate for purine degradation by various organisms, including microbes [136]. In hvKp isolates, allantoin metabolism is controlled by the allantoinase *allB*, negative regulator allR, transcriptional activator *allS*, and allantoinpermease *ybbW* enzyme genes [116].

When studying the *K. pneumoniae* genes whose transcription is increased in hvKp strains as compared to cKp, an operon containing genes involved in allantoin metabolism was identified. Further experiments demonstrated the dependence of hvKp on this operon when using allantoin as the sole source of nitrogen under aerobic conditions. Deletion of *allS*, an activator of the operon involved in this process, led to a significant decrease in the virulence of the hvKp strain in an *in vivo* model [18]. According to some data, the allantoin operon is present in liver abscess-associated strains in higher copy numbers compared to cKp [20]. The *allS* gene has been shown to be present in 50-100% of hvKp isolates possibly determining the invasiveness [35,157].

LPS. Both cKp and hvKp strains synthesize LPS consisting of the core oligosaccharide O-antigen and lipid A. These components are encoded by genes in the *wb*, *waa*, and *lpx* gene clusters, respectively [99]. LPS is one of the key factors in the development of *K. pneumoniae*-induced sepsis, and its various modifications play a role in immune modulation during infection [90]. LPS has previously been shown to protect bacterial cells from phagocytosis, complement-mediated bactericidal activity, and antimicrobial peptides [117].

In HMV strains of *K. pneumoniae* NTUH-K2044 and *K. pneumoniae* ATCC 43816, three *arn* operon genes responsible for the modification of LPS lipid A by l-Ara4N were identified: *arnF*, *arnE*, and *arnD*. The *arnE* and *arnF* genes encode a flippase, which translocates the modified arabinose across the cell membrane, while *arnD* is involved in its biosynthesis. The LPS-modifying mutations identified in the study affect capsule conservation or capsule biosynthesis. In both strains, *arn* gene mutations reduced capsulation that is independent on modification by lipid A, because other genes in the operon did not affect capsule formation, and l-Ara4N-dependent modification of lipid A does not occur in cells grown under these experimental conditions (Luria-Bertani broth) [31].

Peg-344. Another gene that increases the virulence of hvKp, *peg-344*, is mapped to the virulence plasmid and is widely distributed among hvKp strains. The *peg-344* gene product is thought to act as a transporter

located in the inner membrane. In *in vivo* models, *peg-344* is required to achieve maximum virulence, but it does not contribute to systemic infection [7,109].

Colibactin. Another feature of hvKp is its ability to produce the genotoxin colibactin. It is synthesized as part of the secondary metabolism by non-ribosomal peptide synthetases, polyketide synthases, and other enzymes encoded by the *pks* gene [34]. The *pks* locus was detected in the majority (66-100%) of K1 serotype isolates tested. The proportion of other genes associated with hvKp virulence (*rmpA*, *iutC*, and *ybtA*) has been shown to be higher in pks^+ isolates than in pks^- cells [61,66]. Inactivation of colibactin synthesis in a pks^+ isolate of K1 ST23 hvKp strain reduces its ability to colonize the intestine and spread to other organs in an *in vivo* model [85]. It was shown that genotoxicity of *K. pneumoniae* 1084 is associated with activity of the *clbA* gene, because its deletion led to a decrease in colibactin synthesis but could be restored back to the wild-type level by transcomplementation with the clbA plasmid. In addition, in BALB/c mice infected with *K. pneumoniae* 1084, increased DNA damage was observed in liver parenchymal cells compared to the isogenic mutant *ΔclbA* [61]. Although the exact mechanisms by which colibactin contributes to hvKp pathogenesis are unknown, it probably supports colonization of hvKp strains by promoting their spread in the body [65].

Phospholipase D. The gene encoding the phospholipase D family protein (PLD1) located at the locus of the type VI secretion system has been shown to be expressed *in vivo* and control the lipid composition of bacterial membranes and is a component of the hvKp virulence [72].

The kvgAS signaling system. A number of hvKp genes (*mrkC*, *moaR*, and *kva15*) that control signaling functions are also contribute to colonization of the intestine and/or possible invasion through the intestinal barrier in *in vivo* models. Twenty-eight mutants characterized by reduced growth and survival in liver and spleen samples after intragastric administration were identified [132]. In individual testing, 8 mutants caused no increase in lethality after injection compared to 100% lethality in the parental hvKp CG43 strain (ST86, K2 serotype). The mutant genes included the loci encoding proteins: the putative fimbrial protein type III (mrkC), uracil permease (kva28), the two-component kvgA-kvgS regulatory system that promotes capsule formation [62], the transcriptional regulator kva15 of the LuxR family, and two hypothetical proteins kva7 and kva21. Nevertheless, after intraperitoneal injection into mice, mutations were just as lethal as in bacteria of the CG43 strain, indicating that these mutations inactivated genes important for colonization and/or invasion of the gut [117].

Fimbriae. cKp and hvKp possess type 1 (mannose-sensitive) and type 3 (mannose-resistant) fimbriae. Type 3 fimbriae are well-studied virulence factors of bacteria mediating enhanced biofilm formation on abiotic surfaces; however, little is known about their role in hvKP strains [111]. Seven new clusters of fimbrial genes — *kpa*, *kpb*, *kpc*, *kpd*, *kpe*, *kpf*, and *kfg* — were identified in the hvKP NYH-2044 genome, and cKp fimbriae are largely associated with serotype K1 hvKP [148]. Genes encoding virulence determinants for type 3 fimbriae (*mrkA*, *mrkB*, *mrkE*, *mrkF*, *mrkI*, and *mrkJ*), as well as genes of the efflux and regulator system (*acrR*, *envR*, *fis*, *marA*, *marR*, *ramA*, *ramR*, *sdiA*, *soxR*, and *soxS*) were found in hvKp isolate KPHU468 [92]. It was shown that type 3 fimbriae activity and biofilm formation in *K. pneumoniae* CG43 both depend on iron availability and are positively controlled by Fur suggesting that type 3 fimbriae can be less important for hvKp infection *in vivo* under iron-limited conditions [149].

NON-SPECIFIC VIRULENCE FACTORS

Porins. A number of outer membrane proteins, including OmpA, peptidoglycan-associated lipoprotein (Pal), and murein-lipoprotein (LppA), contribute to *K. pneumoniae* virulence. When mutant strains of *K. pneumoniae* NTUH-20444 *pal*, *lppA*, and *ompA* were intraperitoneally injected, only *lppA* and *pal* mutants showed increased resistance to killing and phagocytosis, disruption of the outer membrane permeability barrier and increased sensitivity to bile salts. The *lppA*-mutant strain had a reduced ability to activate Toll-like receptor 4 (TLR4). The *pal* and *lppA* mutants with low virulence retained intact K1 and O1 antigens that induced antibody production in mice indicating the utility of such mutant strains for vaccine development [54].

In addition, a new porin, KpnO, was discovered in *K. pneumoniae* NTUH-2044. The mutant with the *ΔkpnO* deletion produced less capsular polysaccharide and killed *Caenorhabditis elegans* more slowly than wild-type strains [121].

Efflux pumps. Efflux systems represent an important *K. pneumoniae* virulence factor determining its increased antibiotic resistance. AcrAB pumps are under the control of the operon acRAB of the *acrR* gene encoding the AcrAB repressor. While *acrA* encodes a periplasmic lipoprotein anchored to the inner membrane, *acrB* binds the outer membrane protein TolC, which belongs to a family of proteins required for the elimination of compounds. Studies have demonstrated the role of AcrAB pumps in the resistance of *K. pneumoniae*: increased expression of the efflux pump genes reduces the sensitivity to antibiotics increasing the pathogenicity of the strain [130,150].

ANTIBIOTIC RESISTANCE OF HVKP

During the first few decades after appearance of hvKp, the widespread sensitivity of hvKp to antibiotics allowed effective treatment without complications. Early reports on antibiotic resistance of hvKp showed very low levels of resistance: less than 5% of hvKp bacterial isolates produced extended spectrum β-lactamase (ESBL) genes, and only 2% or less were resistant to the antibiotics [36,59]. However, in recent years, there are more and more reports of multidrug-resistant (MDR) hvKp, which is of serious concern [45,115].

There are two different mechanisms of multidrug resistance of hvKp (MDR-hvKp). According to the first one, hvKp strains acquire antimicrobial resistance genes or plasmids through horizontal transfer and become MDR-hvKp. Such strains are referred to as MDR-hvKp type I. For example, two carbapenemase plasmids were isolated together from strain K2 ST86 MDR-hvKp: plasmid IncN, carrying bla_{NDM-1}, and plasmid IncFIIK, carrying bla_{KPC-2} [81]. In another mechanism, strains with MDR-hvKp can result from the transfer of a pLVPK-like virulence plasmid to the classical MDR strain of *K. pneumoniae* forming the MDR-hvKp type II. A study in China described a fatal outbreak caused by an ST11 strain that had acquired the pLVPK-like virulence plasmid [42]. MDR-hvKp type I strains possess the same virulence determinants as the hvKp strains described above, while the hypervirulence of MDR-hvKp type II strains is mainly due to overproduction of capsule and siderophores resulting from the acquisition of virulence plasmid determinants, including *rmpA/rmpA2*, *iut*, and *iro* [161].

Resistance to various antibiotics. Colistin (polymyxin E) is the key component of combination antimicrobial therapy used to treat severe *K. pneumoniae* infections resistant to carbapenems [69]. However, recent studies have shown that some strains acquire resistance to colistin through LPS modification [8], and the frequency of such colistin-resistant isolates is gradually increasing [69,86]. For example, one HMV *K. pneumoniae* isolate with the carbapenemase-encoding plasmid KPC-3, which also exhibited heteroresistance to colistin, was first described in the United States [147]. The number of reports on bacterial resistance to β-lactam antibiotics is rapidly growing [83,114,119]. After the discovery of ESBL in *K. pneumoniae* between 1990 and 2000, it became the major ESBL pathogen in outbreaks of nosocomial infection. In all hospitals in Iraq and Spain, of the clinical strains of *K. pneumoniae* 40% of the strains belong to ESBL [9]. Another study showed that among tigecycline-non-susceptible (TNS) strains, 19.4% were hvKp strains. Changes in the *ramA* region are a mechanism for the development of tigecycline resistance *in vivo* in the hvKp strain [16]. Hyperexpression of the efflux pump genes *AcrAB-TolC* and *OqxAB* and changes in the expression level of their regulators *ramA*, *ramR*, *rarA*, and *acrR*) can also lead to the development of resistance to tigecycline [97]. A study of 35 hvKp serotype K1 (K1-hvKp) isolates collected from a Chinese hospital during 2017 demonstrated the prevalence of plasmid-mediated quinolone resistance (PMQR) genes. A total of 18 (51.4%) isolates had PMQR genes; the most frequently detected gene was *qnrS1* (37.5%), followed by *aac(6')-Ib-cr* (15%) and *qnrB4* (2.5%) [82]. The PMQR genes contain the *qnr* gene, which encodes a family of proteins protecting DNA gyrase and topoisomerase IV from quinolone inhibition. The *qnrS1*, *qnrD*, *qnrB*, and *oqxAB* efflux system genes can be detected in MDR strains of *K. pneumoniae* [126].

The combined manifestation of virulence and antimicrobial resistance of hvKp isolates, their wide distribution and ability to cause disease in healthy humans is a global problem that must be taken into account in infection control and patient treatment. There is an urgent need to select appropriate antibiotic therapy, as well as to develop alternative therapies for hvKp infection (monoclonal antibodies, pharmacomodulation of antibiotic resistance, antibacterial peptides, hvKp-specific bacteriophages).

DIAGNOSTIC AND IDENTIFICATION OF HVKP

With the rapid hvKp spreading throughout the world, the need to pay more attention to the assessment of the virulence of clinical isolates of *K. pneumoniae* is becoming apparent. An increasing number of hvKp strains are being isolated, described, and sequenced. The use of proteomics makes it possible to reveal the molecular mechanisms of the interaction between the bacteria and the host organism and the emergence of resistance to antimicrobial drugs [141]. Phenotypic and genomic analysis, including multiloci sequencing, will make it possible to identify factors contributing to the development of hypervirulence. This should definitely facilitate the understanding of the causes of this phenomenon and provide the information necessary for vaccine development [103,155,159]. In addition, this information is needed to assess the risks associated with hvKp strains. Genetic variants, which can serve as genetic markers associated with HMV and hypervirulence in hvKp strains, can aid to virulence mechanisms clarification [106]. The capsule and siderophores are predominant virulence factors that play a major role in the HMV phenotype of hvKp. However, the molecular mechanisms of HMV have yet to be studied, because hypervirulent strains without HMV phenotype are often identified. Additional virulence factors include LPS carrier proteins, fimbriae,

signaling systems and metabolic pathways, and outer membrane proteins whose functions in hvKp require further investigation. For a long time, only capsular serotypes, HMV, served as the main factors for hvKp identification, which resulted in a significant number of hvKp isolates being overlooked. A study of potential genetic biomarkers for use in identifying hvKp isolates showed that the presence of *peg-344*, *iroB*, *iucA*, *rmpA/rmpA2*, *mrkD*, *wcaJ*, *pgaA*, *fimA*, *fimH*, and *treC* and quantitative assessment of siderophore production can act as predictors of hvKp [28,45,109,154]. Importantly, the combination of biomarkers can be considered as a diagnostic tool for hvKp identification in the hospital, surpassing in accuracy and speed the so-called "string" test currently in use [68,73].

DIRECTIONS FOR CONTROLLING THE SPREAD OF INFECTION CAUSED BY HVKP

Traditional and new ways to control hvKp infections include several strategies: control and surveillance of the infection localization, use of antimicrobial therapy, phage therapy, and biofilm-destroying agents, development of vaccines [25,108]. As hvKp strains often cause abscesses, source control by radiological methods and abscess drainage are the key aspects of treatment. The HMV phenotype of hvKp can lead to the formation of extremely viscous fluid in the abscess. Abscesses smaller than 5 cm can only be treated with antimicrobial therapy. Imaging can be used to assess the response to therapy and determine its duration [108,128]. In addition, hvKp reinfection or recurrence has been reported to develop months or years after the completion of treatment, so long-term follow-up is necessary [117].

One of the main ways to combat infections caused by hvKp is the use of antibiotics. Controlled trials evaluating the efficacy of different antimicrobials against hvKp infection have not been conducted, due to the difficulty of performing differential tests by clinical microbiology laboratories to distinguish between cKp and hvKp strains. The use of genetic biomarkers allowing accurate identification of hvKp strains would help to solve this problem [46].

Some foci of hvKp infection can be poorly accessible for antimicrobial agents. In this case, ceftriaxone and meropenem are used in CNS infections; in prostatic infections, fluoroquinolones: trimethoprim/sulfamethoxazole or fosfomycin are used; in ocular infections a combination of systemic and local therapy (cefazolin, ceftazidime, aminoglycosides, and imipenem) is advisable [108]. In some cases, local antibiotic perfusion (CLAP) has been suggested as a part of infection control maintaining a constant concentration of antibiotics in the foci of infection for a long time with less invasiveness and fewer systemic complications. Therapy included intraosseous antibiotic perfusion and intramedullary antibiotic perfusion [49].

A frequent problem with antibiotic use is the emergence of MDR-hvKp strains. To predict their spreading, it is important to consider the environmental and molecular barriers preventing the acquisition of MDR plasmids. The mechanism of capsule synthesis regulation can act as such a barrier [153]. In addition, there is evidence that in some cases phage therapy along with antibiotics is effective against hvKp-induced infections, and can also be used further to block the emergence of carbopenem-resistant hvKp strains [3,144]. In domestic studies, the possibility of developing several phages active against antibiotic-resistant Kp has been recently demonstrated [163].

An important area of hvKp control is the creation of bioconjugate vaccines. Genetically engineered strains of *E. coli* were used to produce a bivalent bioconjugate vaccine against K1/K2 Kp, which demonstrated its effectiveness *in vivo* [38]. The use of monoclonal antibodies was also effective. In mice colonized with hvKp, administration of monoclonal antibodies significantly reduced the spread of hvKp from the intestine to the mesenteric lymph nodes and organs [29].

Another promising direction in the fight against the spread of hvKp is the use of compounds that prevent the formation or destroy already existing biofilms. Such compounds include QS inhibitors (furans, pyridines, phenylacyl alkaloids, and fatty acids), enzymes, synthetic polymers, antimicrobial peptides (polymyxin, polyalanine), metals (copper, gold), and combinations of these different classes of molecules to achieve a synergistic effect [25]. However, the efficacy of these agents available in the arsenal of domestic and world medicine with respect to hvKp remains virtually unstudied.

The treatment of infections caused by hvKp consists of the long-term use of one or more antimicrobial agents; however, as in the treatment of other infections, this strategy is not sufficiently effective. Translational studies of hvKp biomarkers, especially in combination with the determination of MDR genes and their distribution in different populations (primarily, in patients at medical institutions), can expand the possibilities of creating clinically relevant strategies to prevent and treat life-threatening infections caused by hvKp.

CR-hvKp isolates from patients with nosocomial infections were obtained and characterized [123]. Complete genome sequencing demonstrated that 8 of 9 isolates possessed plasmids carrying carbopenem resistance genes along with hypervirulence factors; four types of in hybrid plasmids were identified. In 2020, an analysis of 15 HMV-isolates of *K. pneumoniae* from

patients with cancer demonstrated a certain degree of kinship between the *K. pneumoniae* plasmid ST147, acting as a virulence gene donor, and the plasmid described in the United Kingdom [67]. BLAST analysis also showed a high degree of relatedness between the two hybrid plasmids and the plasmids identified in the Czech Republic and the United Kingdom. In addition, it was found that one of the hybrid plasmids carried a new metallo-β-lactamase variant of New Delhi (NDM), which differs from NDM-1 by replacing one amino acid. However, this fact did not provide significant evolutionary advantages to the hybrid plasmid compared to NDM-1. Hybrid plasmids are a factor of increased danger, because they carry resistance genes as part of the virulence plasmid [67]. The discovery of structurally similar plasmids in geographically distant regions indicates that hybrid plasmids carrying virulence and resistance genes simultaneously are much more common than previously thought.

The development of molecular methods for monitoring such hybrid plasmids in medical institutions and creating means of blocking their spread is an urgent task in the molecular epidemiology of healthcare-associated infections (HAIs). It is not yet known whether recently isolated Kp-specific bacteriophages [163] are effective against hvKp strains carrying hybrid plasmids. It is hoped that personalized approaches to phage therapy, especially for nosocomial pneumonia associated with *K. pneumoniae* and characterized by high mortality, will lead to significant progress in combating the spread of this life-threatening infection in medical institutions.

At the same time, as studies have shown, it should be taken into account that nosocomial strains of *K. pneumoniae* are characterized not only by high frequency of β-lactamase production and resistance to ampicillin in most isolates, but also by resistance to bacteriophages [1]. Therefore, the path to developing clinically effective bacteriophages that are sufficiently specific and active against hvKp could be difficult.

Another strategy for combating hvKp can involve targeting changes in the antibiotic sensitivity of the pathogen. Recent studies on dormant forms of opportunistic pathogenic bacteria (persisters) capable of surviving antibiotic therapy due to antibiotic tolerance [27,84] have confirmed that such cells can be a new target in the fight against antibiotic resistance [134]. Antibiotic-resistant persistent forms are often found among nosocomial strains of pathogens [134], including *K. pneumoniae* [1]. It was also possible to show an increase in the sensitivity of antibiotic-resistant strains of *K. pneumoniae* to antibiotics due to the effect of resorcinols — plant compounds capable of preventing the formation of antibiotic-tolerant persister forms of the bacteria. Thus, the use of an experimental model of sepsis induction in mice using a strain of *K. pneumoniae* strain with MDR revealed high adjuvant activity of the resorcinol derivative used *in vivo* for polymyxin [96]. Subsequent *in vitro* experiments on the effect of antibiotics without and with the drug on the growth of various bacteria, including *K. pneumoniae*, confirmed the ability of the latter in combination with each of the 12 clinically used antibiotics to significantly (up to 50 times) reduce their minimum inhibitory concentration [96].

Thus, the variety of new currently developed approaches can prepare the world community for the new challenge — the spreading of life-threatening HAI caused by antibiotic-resistant strains of hvKp.

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