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Virulence Factors in Klebsiella pneumoniae: Detection by Multiplex PCR and Correlation with Clinical Patterns

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VIRULENCE FACTORS IN *KLEBSIELLA PNEUMONIAE*: DETECTION BY MULTIPLEX PCR AND CORRELATION WITH CLINICAL PATTERNS

Mémoire présenté pour l'obtention

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Virulence Factors in *Klebsiella Pneumoniae*: Detection by Multiplex PCR and Correlation with Clinical Patterns

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Virulence Factors in *Klebsiella pneumoniae*: Detection by Multiplex PCR and Correlation with Clinical Patterns

Rombaut Camille

Résumé

Klebsiella pneumoniae est une entérobactérie à Gram-négatif ubiquitaire dans l'environnement et commensale du tube digestif et de l'appareil respiratoire de l'homme. Cette bactérie provoque le plus souvent des infections nosocomiales comme des pneumonies, infections urinaires ou abdominales et des septicémies. Dans les années 1980, un nouveau variant nommé *Klebsiella pneuminae* hypervirulent est caractérisé en Asie du Sud-Est causant un syndrome d'abcès hépatique pyogénique. La particularité de cette souche est sa capacité à métastaser chez des sujets jeunes et en bonne santé. Cette hypervirulence lui est conférée par la présence de gènes dits facteurs de virulence. Déjà largement étudiés, peu d'associations claires entre ces facteurs et les données cliniques ont pu être établies à ce jour. Des PCRs multiplexes permettant de détecter certains de ces facteurs de virulence ont été mises au point à partir de celles décrites dans les articles de Turton et al. (2010) et Tang et al. (2010). Des collections de souches *Klebsiella pneumoniae* de différentes origines (Belgique, République Démocratique du Congo, Cambodge et Burkina Faso) ont été analysées avec ces PCRs. Les données cliniques relatives à chaque patient ont été collectées et mise en relation avec le profil de virulence de la souche correspondante. Une analyse approfondie de ces données a permis de confirmer de potentielles associations entre des tableaux cliniques particuliers et la présence de certains facteurs de virulence. Cependant, aucune nouvelle corrélation n'a pu être établie.

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Co-promoteur : X. De Bolle

Virulence Factors in *Klebsiella pneumoniae*: Detection by Multiplex PCR and Correlation with Clinical Patterns

Summary

Klebsiella pneumoniae is a Gram-negative *enterobacter* ubiquity in the environment and commensal of the intestinal and respiratory tract in human. This bacteria induces more often nosocomial infections as pneumonia, urinary or abdominal infections and septicemia. In the 1980's, a new variant of *Klebsiella pneumoniae* called hypervirulent has emerged in South-East of Asia causing a syndrome of pyogenic liver abscess. Its principal characteristic is the capacity to give metastasis in young and healthy patients. This hypervirulence comes from some particular genes said virulence factors. Already widely studied, few clear associations between factors and clinical data have been established nowadays. Multiplex PCRs allow to detect some virulence genes have been developed from these described in the publications of Turton et al. (2010) and Tang et al. (2010). Strains collections of *Klebsiella pneumoniae* from different origins (Belgium, Republic Democratic of Congo, Cambodia and Burkina Faso) have been analyzed with these updated PCRs targeting 12 virulence factors. The clinical data relatives to each patient were collected and linked with the virulence profile of the corresponding strain. A depth analysis of these data allowed confirming potential association between type of infections and the presence of some virulence factors already cited in the literature but had not established new relationship.

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Abbreviations

PCR: Polymerase Chain Reaction

DNA: Desoxyribonucleic Acid

cKP: classical *Klebsiella pneumoniae*

hvKP: hypervirulent *Klebsiella pneumoniae*

KPLA: *Klebsiella pneumoniae* liver abscess

bla: gene coding for β-lactamase

ESBL: Extended-spectrum β-lactamases

KPC: *Klebsiella pneumoniae* carbapenemase

CPS: capsular polysaccharides

LPS: lipopolysaccharides

RDC: Republic Democratic of Congo

rpm: rotation by minute

Tm: melting temperature

Maldi-tof: Matrix-assisted laser desorption/ionization - Time-of-flight mass spectrometry

ST: Sequence Type

CC: Clonal Complex

MLST: Multi-Locus Sequence Typing

RFLP: Restriction Fragment Length Polymorphism

WGS: Whole Genome Sequencing

Introduction

Introduction

1. Generalities

Klebsiella pneumoniae is an enteric Gram-negative bacillus from *Enterobacteriaceae* family, non-motile usually encapsulated extracellular pathogen ubiquitous in the environment (surface water, sewage, soil and on plants) and commensal resident of nasopharynx and gastro-intestinal tract in mammals including human. Because Gram-negative bacteria do not find good growth conditions on the human skin, *Klebsiella* are rarely found there. *Klebsiella pneumoniae* is the most pathogen specie of the *Klebsiella* gender for human. Moreover, this specie was initially known as a community-acquired pulmonary pathogen occurring mostly in chronic alcoholics. *Klebsiella pneumoniae* is now recognized as responsible of nosocomial infections involving pneumonia, septicemia and urinary tract, intra-abdominal or wound infections attacking primarily immunocompromised patients with underlying diseases, neonatology wards and intensive care units (Podschun et Ullman, 1998). In Europe as in the USA, approximately 8% of nosocomial infections are caused by *K. pneumoniae*. The transmission modes are skin contacts, contaminated surfaces or by food and water. A fecal transmission is also reported for some bacteremia cases. The bacteria adhere to epithelial cells of respiratory airways, gastrointestinal tract, urinary tract or endothelial cells before colonizing mucosis. The first symptoms are particularly aspecifics like fever, onset of chills and leukocytosis. Pneumonia, the most common infection caused by *K. pneumoniae*, is characterized by cough and thick expectorations of blood said currant jelly sputum. Advanced symptoms are abscesses and collapsed lungs with pulmonary vessels thrombosis risk. Diabetes mellitus is a well-known predisposing risk factor for severe complications (Lin et al., 2013). One explanation is that the neutrophil bactericidal function is positively associated with good blood glucose control but is impaired in diabetic patients and then increased susceptibility to infection. The infection control relies on the mastery of the transmission as respect of hygiene measures, hand washing or isolation of patients and also on efficient antibiotic prescriptions. However, the currently epidemic of antibiotic resistant bacterial infections is one of the greatest threats to human healthy according to the World Health Organization. Within this epidemic, more problematic pathogens have been collectively named ESKAPE (*Enterococcus Faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter*). They are the causative agents of the majority of hospital heavier infections because they easily "escape" the antibiotic treatment. Indeed, *K. pneumoniae* have accumulated plasmids that carry virulence and resistance genes increasing its survival ability in host (Ramirez et al., 2014).

2. Hypervirulent *Klebsiella pneumoniae*

In the mid-1980s, a new hypervirulent variant of *Klebsiella pneumoniae* (hvKP) has emerged causing an invasive syndrome of pyogenic liver abscess with a propensity for metastatic complications like meningitis, endophtalmitis, extrahepatic infections or bacteremia (Wang

et al., 1998; Shon et al., 2013). Metastatic risk in non-immunocompromised hosts is unusual for enteric Gram-negative bacilli and the ability of hvKP strains to cause life-threatening infections in young, healthy individuals is very intriguing (Ko et al., 2002). Although intestinal colonization is certainly a prerequisite for disease, the means of transmission, routes of acquisition and what features of hvKP enable metastatic spread remain unclear (Shon et al., 2013). Most common symptoms in patients with *K. pneumoniae* liver abscess (KPLA) are absolutely not differents from these observed in classical *K. pneumoniae* (cKP). A study didn't find any association between liver abscess and gender, age, previous antibiotic use or presence of underlying liver diseases (Ko et al., 2002). Contrariwise, some epidemiological studies have showed a higher incidence of disease at 55-60 years and a male dominance (Patel et al., 2014). Like for the cKP infections, diabete is a risk factor of the pyogenic liver abscess because of the lack of glycemic control. This distinctive form of *K. pneumoniae* infection was initially described in South-East Asia, especially in Taiwan and Korea (Wang et al., 1998). The reasons of this preponderance in Asia are unknown and pose questions of host genetic susceptibility. Both host and microbial factors are likely implied. Currently, hvKP is found in a variety of ethnic groups and spread worldwide (Turton et al., 2007; Pomakova et al., 2012). Since 2012, some cases have been reported in Belgium. *K. pneumoniae* became the most common cause of hepatic abscesses in Asia and maybe in North America during the past two decades (Qu et al, 2015). Moreover, the proportion of hvKP seems to increase over time (Li et al., 2014). Percutaneous aspiration or pigtail drainage of the abscess in combination with antibiotics seems the main therapeutic modality for management of KPLA (Lee et al., 2008). Fortunately, 87% of the cases show a favorable evolution and the mortality rate stays lower than 20%.

3. Resistance

Classical *K. pneumoniae* is being shown to exhibit resistance to the current antibiotics including carbapenemases. Carbapenems including imipenem, meropenem are often the last option for *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs) infections. The proportion of multiresistant strains continues to rise. For example, the KPC enzymes (*K. pneumoniae* carbapenemases) have spread across countries and continents (Figure 1). Bacteria producing KPC are only susceptible to a few antibiotics and there is high mortality rate among patients with bloodstream infections (Munoz-Price et al., 2013). In a Chinese study, among 28 carbapenem-resistant *K. pneumoniae* infections, 5 have been considered as hypervirulent. Among these 5 strains, 3 produced the carbapenemases KPC-2 and IMP-4 (Imipenem-resistant). However, these cases had provoked pneumonia, abdominal infection or sepsis but not liver abscess. Their hypervirulence stays to define more precisely (Zhang et al., 2015). Fortunately, most of the hvKP remain susceptible to antimicrobials with the exception of ampicillin which is a natural resistance of *K. pneumoniae*. Some speculations suppose that hvKP cannot acquire resistance-related plasmids or that some drug-resistant genes are lost when they become virulent. Further studies are required to confirm these hypotheses. Although antimicrobial-resistant and hypervirulent *K. pneumoniae* populations seem largely non-overlapping, some cases of multidrug resistant hvKP have

been reported (Zhang et al., 2015). Moreover, the coexistence and coevolution of resistance and virulence genes could be inherent to their genetic linkage in the same genetic determinants and accessory elements as plasmids, transposons or integrons (El Fertas-Aissani et al., 2012). This potential future confluence of hypervirulence and drug resistance in *K. pneumoniae* would complicate clinical practice. Its dissemination could be a disaster for the public health suggesting an urgent need to enhance clinical awareness and epidemiologic surveillance.

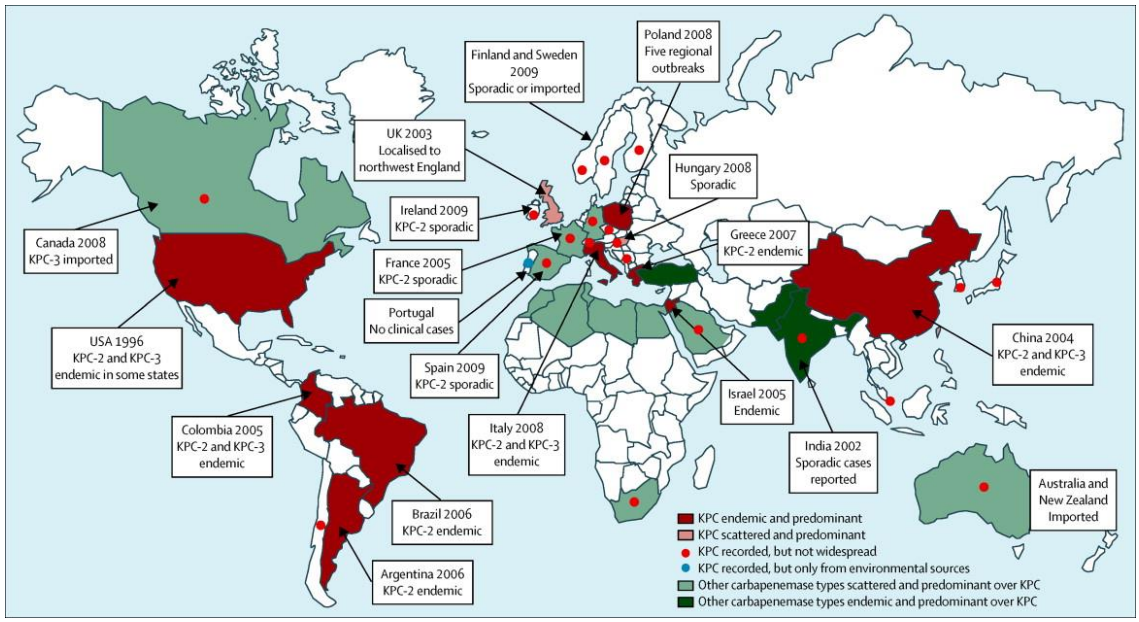


Figure 1. Epidemiological features of *K. pneumoniae* carbapenemase producers by country origin. Other carbapenemase types include VIM (Verona integron-encoded metallo β-lactamases), OXA-48 (oxacillinase) or NDM (New Delhi metallo β-lactamases).
Munoz-Price LS et al. (2013) Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. Lancet Infectious Diseases 13: 785-796.

4. Virulence factors

Mechanisms explaining the enhanced virulence of hvKP are always under investigation but known as dependent on virulence factors, essential molecules for the bacteria survival and propagation. The terms pathogenicity factors and virulence factors are often used as synonymous by different authors but a distinction exists. Indeed, "pathogenicity" designs the qualitative ability of bacteria to cause disease on a host whereas "virulence" is the quantitative measurement of the degree of damage caused to the host fitness (Podschun et Ullman, 1998). The term virulence factor will be used in this work. The ability to acquire new virulence genes is an evolutionary phenomenon from host-pathogen interactions and likely originates from horizontal gene transfer of virulence plasmids (Shon et al., 2013). These factors include fimbrial and non-fimbrial adhesins, iron scavenging systems, capsular polysaccharides, surface lipopolysaccharides, toxins and biofilm formation (Figure 2).

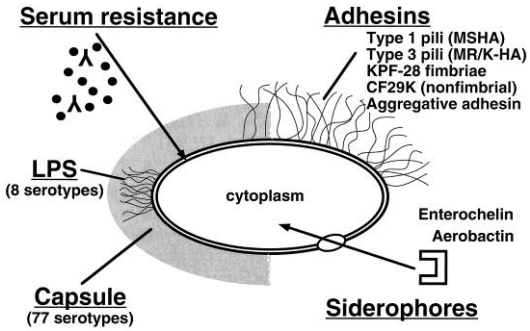


Figure 2. Virulence factors of *Klebsiella pneumoniae.*
Podschun R, Ullman U (1998) *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods and pathogenicity factors. Clinical Microbiology Reviews 11(4): 589-603.

4.1. Surface antigens

4.1.1. O antigens

First, two surface antigens play an important role in the virulence of *K. pneumoniae*. O antigens compose the liposaccharide (LPS) and K antigens compose the capsule. The LPS consisting of a proximal endotoxic lipid A, the medial core oligosaccharide and the distal O-polysaccharide antigen which prevents complement protein deposition and complement associated serum lytic activity (Cortés et al., 2002). In contrast of the large number of K antigens in *Klebsiella*, only nine LPS O groups have been discovered including the serotypes O1, O2, O2ac, O3, O4, O5, O7, O8 and O12. Among clinical isolates, the O1 antigen is the most common which is biosynthesized by the product of the *wb* gene cluster also considered as virulence factor of *K. pneumoniae*. Mutants for O1 production show decreased virulence with reduced bacterial colonization and decreased host inflammatory response. O1 antigen specific antiserum is able to limit the growth and reduce the bacterial dissemination of encapsulated *K. pneumoniae* O1 in a mouse model of septicemia. O1 antigen could be a useful vaccine candidate but would not provide protection against *K. pneumoniae* of the capsular type masking O1 (Hsieh et al., 2012).

4.1.2 K antigens

HvKP isolates have been associated with hyperproduction of CPS inducing hypermucoviscous phenotype distinguishable from the cKP thanks to a positive string test. It is a phenotypic test which is positive when a viscous string of > 5mm using a bacteriology loop to stretch a colony on agar plate is formed (Figure 3).

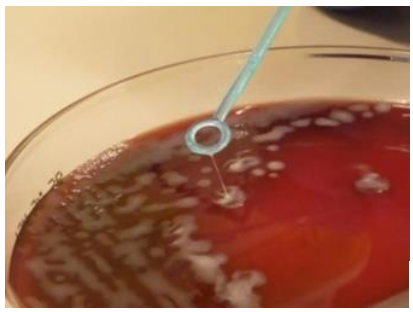


Figure 3. Positive string test on an hvKP from our laboratory.

Hypermucoviscosity depends of the expression of different K antigens. Eighty two K antigens have been identified and 77 of them form the basis of an internationally recognized serotyping scheme (Sturve et al., 2005). A thickened CPS acts as a physical barrier between immunostimulatory bacterial products such as fimbriae or LPS and the host's immune system. Evasion of innate immunity is possible via interferences of CPS by inhibiting Toll-like receptor 4 that decreases the induction of TNF-α, essential for the prevention of bacterial invasion. The capsule impedes also the adhesion to invade epithelial cells by *K. pneumoniae* (Wu et al., 2009). These capsular characteristics induce resistance to phagocytosis by polymorphonuclear neutrophils and protection against bactericidal serum complement (especially C3b) which cannot easily reach the bacterial cell membrane (Podschun et Ullmann, 1998). These resistance capacities could allow *K. pneumoniae* survival in the blood to reach the liver through the portal vein and cause primary abscess (Fang et al., 2004). Previous studies established that more than one-third of patients with hypermucoviscous *K. pneumoniae* bacteraemia will develop invasive infections. Mucoidity is now considered as a virulence factor of *K. pneumoniae* infection but its clinical significance has not been fully elucidated (Lee et al., 2006). This phenotype has never been associated with a particular type of infections. Nevertheless, the prevalence of *K. pneumoniae* without the hypermucoviscous phenotype in numerous cases suggests that strains not hypermucoviscous have emerged as etiologic in the formation of tissue abscesses (Lin et al, 2011).

In Gram-negatives and Gram-positives, the majority of cell-surface polysaccharides are made via the Wzx/Wzy-dependent pathway including enterobacterial O and K antigens (Dang et al., 2006). Into the intern membrane, the flippase Wzx transfers the preformed oligosaccharide subunit across the plasma membrane and the K-antigen polymerase Wzy links each subunit to form the whole polysaccharide antigens (Yeh et al., 2010) (Figure 4). *magA* (mucovsicosity-associated gene A) is chromosomal, specific of the K1 gene cluster and encodes a *wzy*-type capsular polysaccharide polymerase essential for the formation of a protective exopolysaccharide web. *magA* is the particular name of *wzy* in K1 strains (Islam et al., 2014). Mutations in *magA* have been shown to result in CPS deficiency and avirulence in a mouse model of septicemia (Fang et al., 2004). The same region is merely called *wzy* in the *cps* gene cluster of strains with other serotypes. K1 is now considered as the most common serotype isolated from patients with KPLA followed by catastrophic septic ocular or central nervous system complications (Fang et al., 2007). But multiple surveys suggest that serotype K1 is infrequent among *K. pneumoniae* isolates from North America, Europe and Australia but is the most common in Taiwan (Fang et al., 2007). The expression of K2 seems more prevalent in *K. pneumoniae* causing purulent infections outside the liver (Ku et al., 2008). In Europe and the North of America, K2 serotype is responsible from 5 to 20% of nosocomial infections. Other K antigens often mentioned in a virulence context are K5, essentially presents in animals, K20, K54 and K57 (Figure 5).

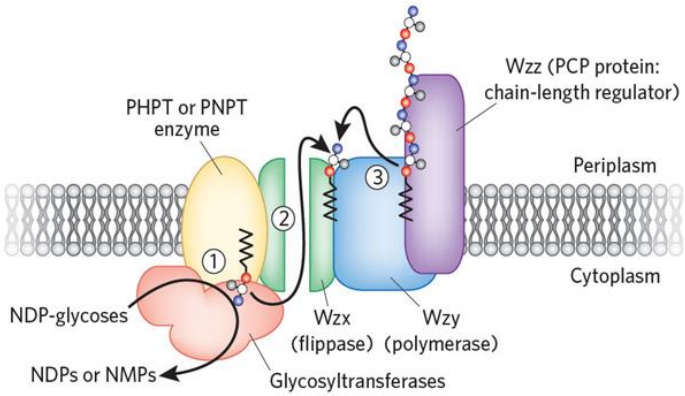


Figure 4. Component and proposed activities Wzy-dependent polysaccharide biosynthesis pathway. NDP-glycoses: nucleotide diphospho-linked sugar donors/PHPT: polyisoprenyl-phosphate hexose-1-phosphate transferase/PNPT: polyisoprenyl-phosphate *N*-acetylhexosamine-1-phosphate transferase/PCP: polysaccharide copolymerase family member (Wzz is a O-antigen polysaccharide of *Salmonella enterica* and *Escherichia coli.)*

Whitfield et al. (2010) Polymerase: Glycan chain lenght control. Nature Chemical Biology 6: 403-404.

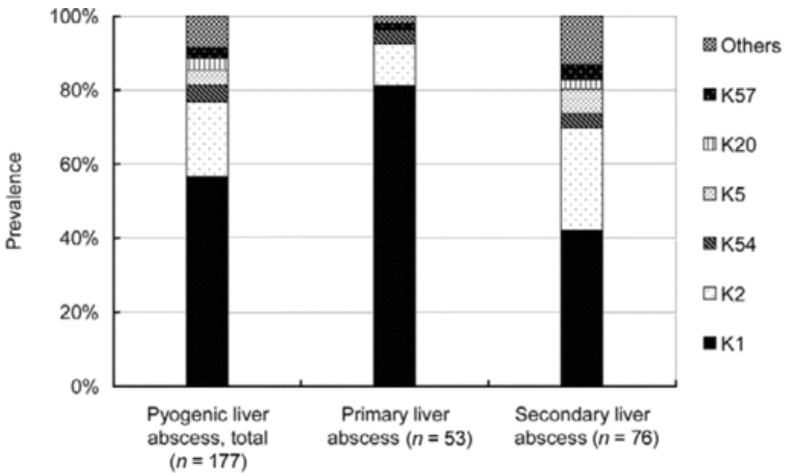


Figure 5. Genotype distribution of *Klebsiella pneumoniae* strains by type of pyogenic liver abscesses.

Fang CT, Lai SY, Yi WC, Hsueh PR, Liu KL, Chang SC (2007) *Klebsiella pneumoniae* genotype K1: an emerging pathogen that causes septic ocular or central nervous system complications from pyogenic liver abscess. Clinical Infectious Diseases 45: 284-293.

4.2. *rmpA*

In 2006, a plasmid-borne *rmpA* (regulator of mucoid phenotype A) gene was also proposed as important in virulence. It is a transcriptional activator for synthesis of extracapsular polysaccharides participating in hypermucoviscous phenotype (Yu et al., 2006). It activates the chromosomal *cps* transcription (Yu et al., 2006). However, the functional role of the encoding protein RmpA remains unknown (Cheng et al., 2010). *rmpA* is likely linked with *terW-iutA-silS* loci of the plasmid pLVPK. Due to this correlation, *rmpA* could be a marker of KPLA without being directly responsible for virulence (Tang et al., 2010). It could be just co-inherited with the adjacent virulence genes carried by the plasmid (Hsu et al., 2011). It seems that capsular serotypes K1 and K2 are more involved in virulence than *rmpA* in KPLA (Yeh et al., 2007) although this claim remains controversial. Indeed, another study shows that K1/K2 isolates that were neither hypermucoviscous nor positives for *rmpA* are avirulents

while hypermucoviscous non K1/K2 isolates positives for *rmpA* are highly virulents (Yu et al., 2008). These results seem indicate that *rmpA* is linked with the hypermucoviscous phenotype that could induce virulence without K1 or K2 antigens.

4.3. Siderophores

Another way to improve the bacterial invasiveness is secretion of siderophores, high-affinity iron chelators essential for the bacteria growth and survival. This iron scavenging system attracts extracellular ferric iron (Fe^{3+}) which has more affinity for iron than the host iron-associated glycoproteins. In tissues extremely poor in free iron, siderophore specific receptors in the outer membrane allow the entry of iron into bacteria (Figure 6). Three types of siderophore may be found in *K. pneumoniae*: aerobactin, a hydroxamate siderophore whose receptor is encoded by *iutA*, enterobactin (Ent), the prototypical catecholate siderophore and yersiniabactin (Ybt), a phenolate-type siderophore. *iutA* is part of the *iuc*ABCD*iutA* operon consisting of 5 genes responsible for synthesis and transport of the aerobactin, previously reported for *Escherichia coli*, *Salmonella* and *Shigella* spp. (Hou et al., 1999). The coded protein, IutA is the outer membrane receptor responsible for the uptake of ferric aerobactin (Bouchet et al., 1994). It seems that hvKP produces quantitatively more biologically active siderophores increasing affinity of iron and resistance to host factors than those produced by cKP strains (Russo et al., 2011). Strong association found between genes coding receptors of aerobactin and the *rmpA* gene suggests that these two virulence factors might be genetically coupled on a large virulence plasmid (Yu et al., 2007). The expression of siderophores during infection allows providing an adaptive advantage because of its flexibility in responding to various environmental stimuli (Lin et al., 2011).

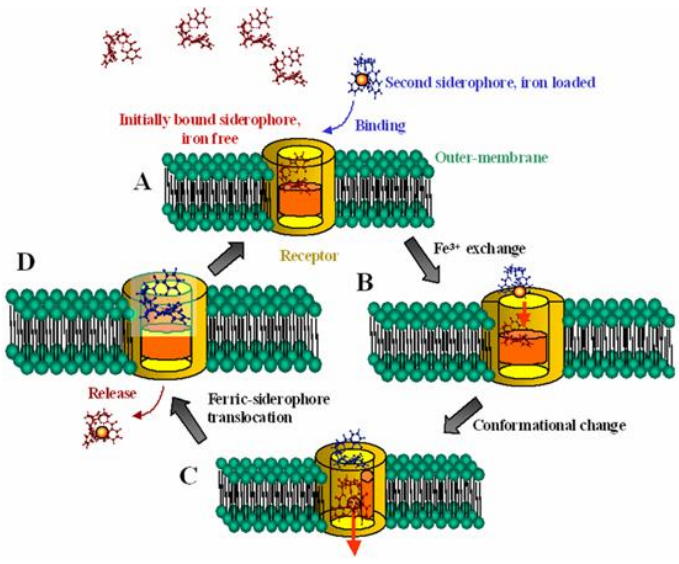


Figure 6. Proposed model of the siderophore shuttle iron exchange mechanism for iron transport in Gram-negative bacteria.
Stintzi A et al. (2000) Microbial Iron Transport via a Siderophore Shuttle: A Membrane Ion Transport Paradigm. Proceedings of the National Academy of Science of the USA 97, 10691-10696.

4.4. *wcaG*

Another virulence factor less known but targeted by the Multiplex PCR of Turton et al. (2010) is *wcaG*. This gene is implied in the biosynthesis of capsular fucose of *K. pneumoniae* causing liver abscesses but not in those causing urinary infections. The products of *wcaG* genes are responsible for converting mannose to fucose. Usually, the macrophages mannose receptors and surface lectin are able to recognize microorganisms to mediate phagocytosis. The conversion in fucose on the capsule leads to rare association with *K.pneumoniae* and then immune invasion (Wu et al., 2008). The protein encoded is WcaG, the enzyme GDP-L-fucose synthase responsible for GDP-fucose synthesis (Wu et al., 2009) (Figure 7). Evidences are growing that fucose is an important factor in bacterial pathogenicity even if no mechanism has been suggested about the specific functions of fucose biosynthetic genes (Ho et al., 2011).

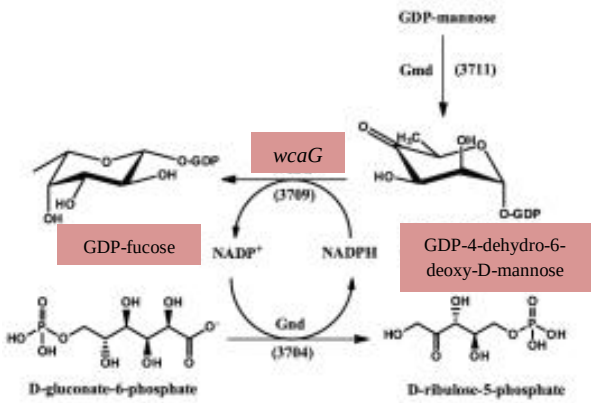


Figure 7: Biosynthetic pathway of GDP-fucose.
Ho JY et al. (2011) Functions of some capsular polysaccharide biosynthetic genes in *Klebsiella pneumoniae* NTUH K-2044. PLoS One 6(7).

4.5. pLVPK loci

Chen et al. (2004) determined the entire DNA sequence of pLVPK, a 219kb virulence plasmid from *K. pneumoniae* of Clonal Complex 23 (CC23), an invasive K2 strain causing pyogenic liver abscess. They showed that the loss of this plasmid resulted in a loss of colony mucoidy, the ability to synthetize aerobactin and a 1000 fold decrease of virulence. Tang et al. (2010) studied the involvement of pLVPK in virulence and its clinical significance in abscess formation. Five genetic loci were used for the detection of pLVPK derivatives including *terW, rmpA, iutA, silS* and *repA*. A multiplex PCR assay was developed to investigate the prevalence of those loci into abscess formations in hepatic and non-hepatic sites. *rmpA* and *iutA* have been already described above.

4.5.1. *terW*

terW is a tellurite resistance gene. Oxyanions of tellurium like tellurite (TeO_3^{2-}) are toxic to most microorganisms, particularly Gram-negative bacteria. The tellurite strong oxidizing ability might interfere with many cellular enzyme processes but mechanisms of tellurite

resistance are not clearly established (Coral et al, 2006). Elemental tellurium (Te^0) is insoluble and precipitates in black deposits in some bacterial-selective growth media. A typical human body contains > 0.5g of Te, mostly in bone exceeding the level of all other trace elements in humans except for iron, zinc and rubidium (Chasteen et al., 2009). Five genetics tellurite resistance determinants usually plasmidics have been characterized in Gram-negative bacteria including the *ter* operon (terZABCDEF) encoding TerB, *K. pneumoniae* tellurite-resistance proteins which precise functions are unknown (Chiang et al., 2008). Development of resistance is possible only in highly polluted environments and most human pathogens are never exposed to such compounds. However, tellurium compounds are used in some medical devices, in tooth-filling material in dentistry, in batteries or in electronic components. Bacteria could acquire new plasmids which encode tellurite resistance in hospital sewage, an ideal environment for developing a bacterial heavy metal resistance (Coral et al., 2006).

4.5.2. *silS*

The *sil* operon is responsible for silver resistance in a number of medically important Gram-negative pathogens. The silver cation Ag^+ has for centuries been employed as an antimicrobial agent but since recent years silver is routinely incorporated into various products like paints, deodorants and clothing. This increasing use has induced resistance development and compromised its clinical utility. The *sil* operon comprises apparently 7 structural genes and 2 genes encoding a cognate two-component regulatory circuit (*silS* and *silR*). Proteins encoded by *sil* operon could mediate silver resistance by restricting the accumulation of silver in the cell through thanks active efflux transporter SilCFBA (Figure 8). In strains silver resistants, derepression of transporter expression occurs owing to amino acid substitutions within the cognate sensor kinase SilS. In the absence of the stimulus Ag^+ expression of these transporters is ordinarily repressed. The maximal rate at which these transporters are able to expulse silver is not alone sufficient to counteract silver ingress into the periplasm. Consequently, for a resistant phenotype manifestation, the effect of efflux must be increased by mechanisms that act to restrict the accumulation of free silver ions in the periplasm. SilE would be implied in the reduction of the periplasmic concentration of silver ions through sequestration but at to high concentrations, SilE protein becomes exceeded leading to toxic silver concentration (Randall et al., 2015).

(a)

(b)

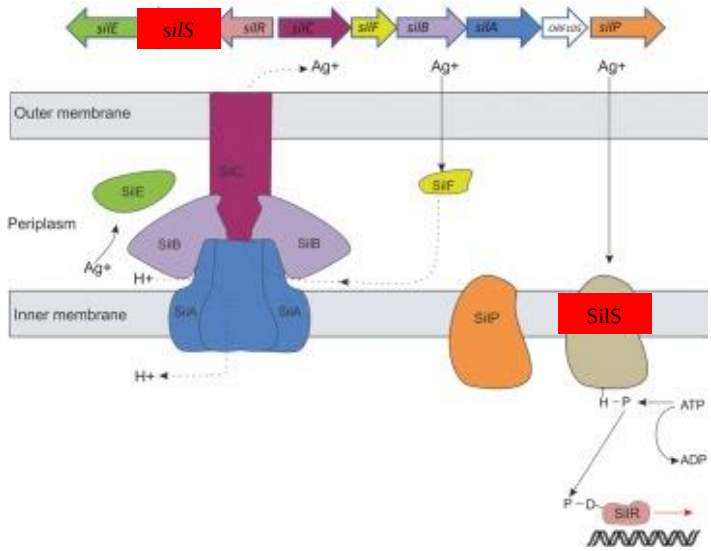


Figure 8. Sil system and its proposed role in silver resistance in Gram-negative bacteria (a) Genetic architecture of the *sil* operon (b) Organization and function of the Sil system where dashed lines represent proposed interactions of Ag^{+}/H^{+} with Sil proteins.

Randall CP et al. (2015) Silver resistance in Gram-negative bacteria: a dissection of endogenous and exogenous mechanisms. Journal of Antimicrobial Chemotherapy 70: 1037-1046.

sequence similarity to a number of plasmid replication proteins of other enteric bacteria. Strains *repA* positives carry their plasmid in an extra-chromosomal form while *repA* negative isolates have pLVPK genes into their chromosome (Chen et al., 2004). Tang et al. (2010) concluded that these pLVPK-derived *terW-rmpA-iutA-silS* loci may predispose patients to abscess formation caused by various K isolates with different genetic backgrounds. Their study shows the first strong indication that loci derived from this virulent plasmid, other than a particular capsular type, have also a role in hvKP infections.

4.6. Other virulence factors

Of course, a lot of other virulence factors exist but are not targeted by Multiplex PCRs in this work. First, *entB* is an enterobactin biosynthesis gene with a siderophore function but also implicated in the biofilm formation exactly as *fyuA* coding for a ferric yersiniabactin uptake receptor (El Fertas-Aissani et al., 2012). Indeed, the enterobactin are siderophores the most often present in *K. pneumoniae* strains. The siderophore yersiniabactin is biosynthesized by *irp* (iron regulatory protein) and *ybtT, E, S* coding for thioesterases. The transport of the complex iron-siderophore into bacteria is performed by an extramembranair receptor coded by *fuyA* and transmembranair receptors coded by *ybtP, Q*. The yersiniabactin production is regulated by *ybtA*, a transcriptional activator of the receptor and synthesis genes (Lawlor et al., 2007). Genes to synthestize yersiniabactin *irp*, *aerobactin iuc*, *salmochelin iro* and *kfu* genes are all implied in the iron transport. Kfu protein is more prevalent in hvKP compared with cKP and shows to be a factor for virulence in mice after intragastric infection playing a role in the intestinal colonization. The disruption of *tonB* encoding for a protein requisite for uptake of siderophores, hemin and ferric citrate resulted in decreased virulence in mouse models (Shon et al., 2013). Only a combination of these siderophores allowing iron importation from multiple sources is needed for optimal systemic virulence of hvKP. A system alone is sufficient for bacterial colonization but not for virulence (Hsieh et al., 2008).

rmpA expression is repressed by *fur* (ferric uptake regulator), the global regulator for the expression of iron acquisition systems (Lin et al., 2011). Fur serves as repressor of at least eight iron acquisition systems in *K. pneumoniae* of the CC43 (complex clonal) at different levels.

Another virulence factor found frequently in KPLA is *allS*, included in a 22kb chromosomal region encoding the activator of the allantoin regulon. *allS* is responsible for the anaerobic assimilation of allantoin as the unique source of carbon, nitrogen and energy under aerobic or anaerobic conditions. Allantoin is the product of the acid uric uricolysis by the bacteria thanks to an uricase. *allS* appears to be totally specific for the strain K1 but not universally present in the genome of these strains (Chou et al., 2004).

A bacterial biofilm is an aggregate of bacteria contained within a matrix of surface polysaccharides, proteins and DNA. They enhanced resistance to host defenses and antimicrobials, increasingly recognized as important virulence factors. The hvKP have been shown to produce biofilm with gene products similar to those identified in cKP. One study has even observed that hvKP produced more biofilm than cKP. However, the mechanism responsible for increased biofilm formation in hvKP has not yet been defined (Shon et al., 2013).

5. Typing methods

Recent genomic characterizations established that the genomic background rather than the hypermucoviscous phenotype or the serotype defines hvKP pathogenicity (Russo et al., 2011). Consequently, different typing methods are proposed to compare *K. pneumoniae* strains. Capsule is still recognized as an important virulence factor in infection due to hvKP. Foremost, K serotyping allows comparisons of strains from different geographical locations at different period of times. This phenotypic method is difficult to apply clinically because preparation and maintenance of antisera are not easy. Moreover, interpretation of the results may be subjective (Ayling-Smith et Pitt, 1990). Another major drawback is the large number of potential cross-reactions occurring among the 77 capsular serotypes. Development of molecular methods enables determination of isolates without the use of antisera was needed. One of them is the PCR- restriction fragment length polymorphism (RFLP) analysis of the capsular antigen cluster (*cps*), responsible for K antigens synthesis, by digestion with a restriction enzyme (Brisse et al., 2004). A database of reference profiles (C-patterns), based on the results of *cps* PCR-RFLP, has been initiated to determine the K serotype for *K.pneumoniae* clinical strains more easily and discriminatory. However, this technique can be problematic because requires amplification of a very large product. The implementation of new C-patterns in the database is needed to improve the rate of success (Brisse et al., 2004).

Turton et al. (2008) proposed an alternative approach using Multiplex PCR for serotype-specific targets K1, K2 and K5 within *cps* cluster. In 2010, K20, K54, K57, *wcaG* and *rmpA* were added in this multiplex (Figure 9). This *cps* genotyping can provide an accurate

molecular diagnosis for highly pathogenic strains that is inexpensive and possible to apply in clinical microbiology laboratories. PCR takes little time and allows easy comparisons of virulence profiles of *K. pneumoniae* large collections.

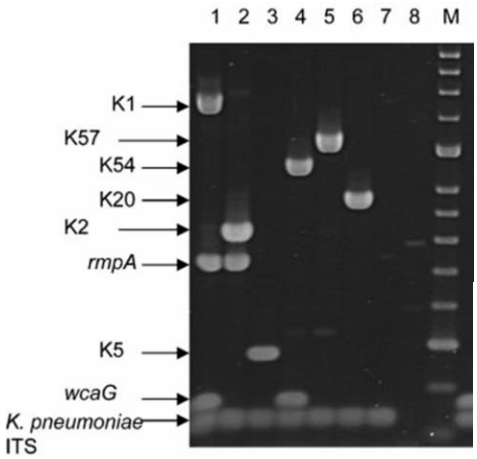


Figure 9. Results of K1, K2, K5, K20, K54, K57, *rmpA* and *wcaG* Multiplex PCR. (ITS: internal transcribed spacer 16S-23S).
Turton JF et al. (2010) PCR characterization and typing of *Klebsiella pneumoniae* using capsular type-specific, variable number tandem repeat and virulence gene targets. Journal of Medical Microbiology 59: 541-547.

To evaluate the involvement of pLVPK in *K. pneumoniae* virulence and its clinical significance, Tang et al. (2010) have screened 207 clinical isolates with a Multiplex PCR detecting pLVPK-derived genetic loci *terW-iutA-rmpA-silS* (Figure 10).

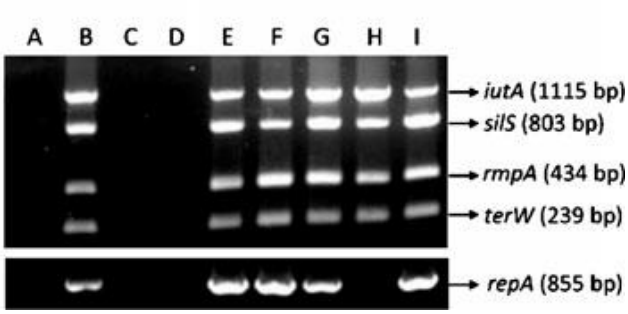


Figure 10. Results of *terW-iutA-rmpA-silS* Multiplex PCR.
Tang H-L et al. (2010) Correlation between Klebsiella pneumoniae carrying pLVPK-derived loci and abscess formation. European Journal of Clinical Microbiology & Infectious Disease 29: 689-698.

Multi-Locus Sequence Typing (MLST) is a nucleotide typing method based on PCR amplification followed by sequencing of 7 housekeeping genes. It is a tool for strain phylogeny and large-scale epidemiology (Liao et al., 2014). A Sequence Type (ST) is determined in function of which alleles are present for these 7 genes. This typing process has showed high discriminatory power (96%), reproducibility and provides unambiguous data useful for the epidemiology of *K. pneumoniae* isolates (Diancourt et al., 2005). A high number of different genotypes were disclosed by MLST even if the population of *K. pneumoniae* is characterized by a low level of nucleotide polymorphism. Studies suggest that homologous recombination has more impact on sequence evolution than mutations (Brisse et al., 2009). For example, the MLST scheme has highlighted the dissemination of the ST23 clone in emerging liver abscesses among K1 strains found in three continents (Turton et al., 2007). Contrariwise, the composition of ST types among K2 is quite variable with a majority

of ST65 and ST86. However, the correlation between STs and types of infection is not at all clearly defined. STs are gathered into clonal complexes (CC) defined as groups for which MLST profiles showed only one allelic mismatch with at least one other member of the group (Bialek-Davenet et al., 2014). It seems that classification in clonal groups is a better predictor of virulence gene content than their K-types (Brisse et al., 2009). However, MLST is unable to draw clear discontinuities between CC. The molecular epidemiology of *K. pneumoniae* determined with MLST is quite versatile and more information is needed.

Rapid bioinformatics tool and genomic data may differentiate more precisely CC. The Whole genome sequencing (WGS) could enable rapid acquisition of medically relevant informations. Indeed high-throughput sequencing promises to revolutionize medical microbiology and molecular epidemiology (Bialek-Davenet et al., 2014). WGS is improving constantly since some years allowing reductions in the results turnaround time from days to hours and decreases the cost of sequencing for a bacterial genome. Current genotyping methods have a limited resolution because they analyze only small regions of the genome whereas WGS provides a maximum of genetic informations. However, WGS is likely not suitable for the routine identification of pathogens because of actual tests are much cheaper and fast enough for the clinical need. WGS would be useful when standard tests fail to find the causative bacterium because it is a new variant leading to false negative results for example. A lot of technical advances and studies for each pathogen are required to determine if routine use of WGS would be cost-effective (Köser et al., 2012).

The main concern about *K. pneumoniae* and more precisely about hvKP is no longer mortality but catastrophic morbidity and disability caused by irreversible complications. Rapid detection and better understanding of virulence factors of *K. pneumoni*ae would help clinicians to give earlier diagnostic, adapted antibiotic treatments and then improving clinical outcomes. Indeed, the timing of appropriate antimicrobial therapy is an important factor to increase the survival rate. Rapid diagnostic is then helpful to prevent exacerbation of disease (Chou et al., 2004). The advances in the genotyping method including better reproducible Multiplex PCR could provide a rapid molecular diagnosis for the most important virulence factors of *K. pneumoniae*.

Materials and methods

Materials and methods

1. Bacterial isolates

Isolates strains *Klebsiella pneumoniae* were selected from CHU Dinant-Godinne laboratory collections. They were conserved at -80°C in glycerol 100%. The extracts in which the presence of virulence genes was previously determined, were used in PCR developments as positive controls (Table 1):

- Isolates from CCUG (Culture Collection, University of Göteborg, Sweden), reference hypervirulent *K. pneumoniae* strains and one from CHU Sart-Tilman (Liège, Belgium).
- Isolates from Erasme Hospital (Brussel, Belgium) and Cambodian blood cultures.
- Four isolates collected in 2014 by the CHU Dinant-Godinne CNR (Centre National de Référence pour la recherche de résistance aux antibiotiques: *Enterobacteriaceae, Pseudomonas, Acinetobacter*) including one isolate from the hospital ZNA (Ziekenhuis Netwerk Antwerpen, Belgium) and 3 from Erasme Hospital, CHU St-Pierre (Brussel, Belgium) and VUB (Vrije Universiteit Brussel, Belgium).

Table 1. Reference *K. pneumoniae* strains.

Reference strains	**Laboratory**	**Isolation year**	**Site of isolation**	**Virulence genes**	**Underlying disease**
31615	CCUG	Unknown	Bronchial washings	K1, *rmpA, wcaG, iutA, terW*	Unknown
31617	CCUG	Unknown	Unknown	K2, *rmpA, silS, terW, repA*	Unknown
416	CCUG	Unknown	Unknown	K5, *iutA, terW*	Unknown
60507	CCUG	1950 (Denmark)	Urine	K20	Unknown
60508	CCUG	2000 (Denmark)	Urine	K54, *wcaG, terW*	Unknown
60509	CCUG	1951 (Denmark)	Sputum	K57	Unknown
M006834	CHU Sart-Tilman	Unknown	Unknown	K1,*rmpA, wcaG, allS, iutA, terW, silS*	Unknown
25014	Erasme	2013	Blood	K1, *rmpA wcaG, iutA, silS, terW*	Triple bypass multicomplicated by septicemia
24902	Erasme	2013	Blood	K1, *rmpA wcaG, iutA, silS, terW*	Triple bypass multicomplicated by septicemia
24842	Erasme	2013	Blood	K1, *rmpA wcaG, iutA, silS, terW*	Triple bypass multicomplicated by septicemia
25097	Erasme	2013	Blood	K2	Septicemia (pyelonephritis)
24917	Erasme	2013	Blood	K2	Septicemia (urinary infection)
24905	Erasme	2013	Blood	K2, *rmpA, iutA*	Septicemia (cholangitis post renal transplantation)
24739	Erasme	2013	Blood	K2	Septicemia (pyelonephritis)
25172	Erasme	2013	Blood	K57	Septicemia (cholangitis); Liver abscess
24705	Erasme	2013	Blood	K57, *rmpa, iutA, silS, terW, repA*	Septicemia post transhepatic cholangiography
24692	Erasme	2013	Blood	K57, *rmpa, iutA, silS, terW, repA*	Septicemia post transhepatic cholangiography
SHCH408	Cambodia	2008	Blood	K2, *rmpA*	Unknown

CNR20140475	ZNA	2014	Blood	K1, *rmpA, wcaG*	Unknown
CNR20140549	Erasme	2014	Punction liquid	K1, *rmpA, wcaG, silS, iutA, terW*	Unknown
CNR20141030	CHU St-Pierre	2014	Blood	K1, *rmpA, wcaG*	Septicemia (liver abscess; HIV+)
CNR20140913	Ziekenhuis VUB	2014	Hepatic punction	K2, *rmpA*	Recurrent liver abscess

Clinical strains *K. pneumoniae* from different origins have been gathered in order to be analyzed with our virulence genes Multiplex PCRs. Clinical patient files of CHU were collected from the program Omnipro.

- Thirty isolates from blood cultures identified as *K.pneumoniae* by the CHU routine microbiology laboratory and isolated from February 2010 to August 2013.
- Ten isolates from clinical blood culture of the project PARADIS (*P*CR *A*nd *RA*pid *D*etection *I*ntegrated *S*ystem) of the CHU collected since December 2014.
- Six isolates from the project FEAR (*F*ighting *E*nterobacteriacea *A*ntibiotic *R*esistance) of the CHU.
- Two hundred forty three *K. pneumoniae* strains from blood culture isolates collected from 2007 to 2014 in the Republic Democratic of Congo (RDC).
- Fifty-two isolates from different types of samples from Cambodia.
- Eight isolates from Burkina Faso collected in potable water.

2. Species identification

All our isolates were identified to the species by mass spectrometry using Microflex MALDI-TOF Biotyper 2.0 (*M*atrix *A*ssisted *L*aser *D*esorption *I*onization - *T*ime *O*f *F*light) (Bruker Daltonics, Billerica, USA). First, the surface of the investigated colony was touched with a sterile loop and the small amount of sample applied on a MSP 96 target polished steel plate (Bruker Daltonics). The deposited bacteria were overlaid with 1µL of HCCA matrix, a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile- 2.5% trifluoroacetic acid. The first well didn't contain any bacteria as negative control. A BTS standard *Escherichia coli* was deposited on the second and third well as positive controls. The plate was let at room temperature to allow cocrystallization with the sample before to be put into the MALDI-TOF mass spectrometer. According to the manufacturer, the identification was considered valid whenever the score value was ≥2.

3. Culture conditions and nucleic acid extraction

Colonies were subcultured on Trypticase Soy Agar (TSA; bioMérieux, Marcy l'Etoile, France) non selective medium and incubated at 37°C overnight. DNA was released from bacteria by heat extraction at 99°C for 10 min in dry bath incubator FB15101 (Fisher Scientific, Pittsburg, USA) of one colony suspended in 200µL of PCR grade water (B Braun

Medical S.A., Diegem, Belgium), vortexing followed by centrifugation (13000 rpm for 5 min). The supernatant was transferred in a new PCR tube and considered to be a crude DNA extract.

4. Multiplex PCR and electrophoresis

The end-point PCR is a classical detection method based on the amplification of a specific DNA fragment followed by amplicons revelation by electrophoresis. In order to validate the PCR described by Turton et al. (2010), each PCR was carried out using the Qiagen® Multiplex PCR kit (Qiagen, Hilden, Germany) composed of *Taq* PCR MasterMix. This latter is a ready-to-use solution including pre-optimized concentrations of HotStarTaq® DNA Polymerase, Multiplex PCR Buffer (3mM $MgCl_2$, KCl, $NH_4(SO_4)_2$, a Qiagen synthetic factor MP increasing the local concentration of primers at the template and stabilizing specifically bound primers) and 200µM dNTPs mix ultrapure quality (Qiagen, Hilden, Germany). PCR amplifications were performed in 25µL volumes containing 12.5µl MasterMix, 0.2µM of each forward and reverse primer (Table 2) and 2µL crude DNA extract. Applied Biosystems®2720 Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), with a maximum ramp rate of 2,6°C/s, was used in the following amplification conditions:

- Initial denaturation step at 94°C for 15 min.
- 40 cycles at 94°C for 30 s, annealing at 58°C for 90 s, 72°C for 90 s.
- Final extension at 72°C for 10 min.

PCR products were separated by capillary gel electrophoresis using a QIAxcel® instrument (Qiagen, Hilden, Germany) providing fully automated separation of the amplicons according to their size. Each sample is automatically loaded into an individual capillary of a precast gel cartridge, the QIAxcel DNA Screnning Cartidge here, which separates fragment from 15bp to 5kb thanks to combination of 1.5 ml of QX Alignment marker and 50µl of QX DNA Size marker. The resolution method preinstalled AM320 was used with 5kV of injection voltage, 10s of injection time, 6kV of separation voltage and 320s of separation time. Finally, electronic data were transferred to BioCalculator software providing both an electropherogram and a gel image.

Table 2. Turton Multiplex PCR primers.

Target	**Primer**	**Sequence (5'-3')**	**Tm* (°C)**	**Product size (bp)**	**Reference**
Capsular type K1	MagAF1 MagAR1	GACCCGATATTCATACTTGACAGAG GCAATGGCCATTTGCGTTAG	59.7 67.2	1283	Fang et al. (2004)
Capsular type K2	K2wzy-F1 K2wzy-R1	GACCCGATATTCATACTTGACAGAG CCTGAAGTAAAATCGTAAATAGATGGC	63.5 64.3	641	Turton et al. (2008)
Capsular type K5	K5wzxF360 K5wzxR639	TGGTAGTGATGCTCGCGA CCTGAACCCACCCCAAT	64.4 65.2	280	Turton et al. (2008)
Capsular type K54	wzxK54F wzxK54R	CATTAGCTCAGTGGTTGGCT GCTTGACAAACACCATAGCAG	61.8 62.2	881	Fang et al. (2007)
Capsular type K57	wzyK57F	CTCAGGGCTAGAAGTGTCAT	58.5	1037	Fang et al. (2007)

	wzyK57R	CACTAACCCAGAAAGTCGAG	59.0		
Capsular type K20	wzyK20F wzyK20R	CGGTGCTACAGTGCATCATT GTTATACGATGCTCAGTCGC	63.7 59.9	741	Fang et al. (2007)
rmpA	rmpAF rmpAR	ACTGGGCTACCTCTGCTTCA CTTGCATGAGCCATCTTTCA	63.9 64.0	516	Nadasy et al. (2007)
wcaG	wcaGF wcaGR	GGTTGGKTCAGCAATCGTA ACTATTCCGCCAACTTTTGC	65.1 62.9	169	Turton et al. (2010)
K. pneumoniae 16S ITS**	K.pneumoniae Pf K.pneumoniae Pr1	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	63.5 62.4	130	Liu et al. (2008)

* Melting Temperature
**Internal transcribed spacer

Turton JF, Perry C, Elgohari S, Hampton CV (2010) PCR characterization and typing *Klebsiella pneumonia* using capsular type-specific, variable number tandem repeat and virulence gene targets. Journal of Medical Microbiology 59: 541-547.

In addition, to investigate the distribution of pVLPK-derived loci in *K. pneumoniae* isolates, we used the multiplex PCR described by Tang et al. (2010) using genomic DNA as the template and specific primer for each locus targeted. The Qiagen® Multiplex PCR Kit had been applied again. PCR amplifications were performed in 25µL volume containing 12.5µl MasterMix, 0.2µM of each forward and reverse primer (Table 3) and 2µL crude DNA extract. Amplification, performed on Applied Biosystems® 2720 Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), held in the following conditions:

- Initial denaturation at 95°C for 10 min.
- 35 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 45 s, 72°C for 90 s.
- Final extension at 72°C for 10 min.

Final extension was not reported in the Tang et al. (2010) article but we did one at 72°C during 10 minutes such as routine PCRs in the laboratory. The amplicons were separated by capillary gel electrophoresis using QIAxcel® instrument (see above).

Table 3. Tang Multiplex PCR primers.

Target	**Primer**	**Sequence 5'-3'**	**Product size (bp)**
terW	terWF terWR	ATGCAATTAAACACCAGACAG GATGTCATAGCCTGATTGC	239
iutA	iutAF iutAR	ACCTGGGTTATCGAAAACGC GATGTCATAGCCTGATTGC	1115
rmpA	rmpAF rmpAR	ACGACTTTCAAGAGAAATGA CATAGATGTCATAATCACAC	434
silS	silSF silSR	CATAGCAAACCTTCCAGGC ATCGGCAGAGAAATTGGC	803
repA	repAF repAR	GGCCAATGATAACAATCAG GAATGACCAGTACATAATCC	855
K. pneumoniae 16S	K. pneumoniae Pf K. pneumoniae Pr1	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130

Tang HL, Chiang MK, Liou WJ, Chen YT, Peng HL, Chiou CS, Liu KS, Lu MC, Tung KC and Lai YC (2010) Correlation between *Klebsiella pneumoniae* carrying pLVPK-derived loci and abscess formation. Eur J Clin Microbiol Infect Dis 29: 689-698.

5. Hypermucoviscosity phenotype detection

Some hvKP variants display a hypermucoviscous appearance of colonies grown on an agar plate. This phenotype is defined semi-quantitatively by a positive string test. When a bacteriology loop stretching bacterial colonies on TSA agar plate is able to generate a viscous string longer than 5mm, the strain is string test positive and defined as hypermucoviscous.

6. Antibiotic susceptibility testing

Antibiograms for Gram-negative bacteria have been carried out on strains from RDC, Cambodia and Burkina Faso to determine at which antibiotics each strain was potentially resistant. Antibiotic sensitivity test was done by a disc diffusion method according to CLSI guidelines (Clinical and Laboratory Standards Institute). Bacteria were cultivated on Mueller-Hinton gelose before deposition of disc charged with different antibiotics (Bio-Rad, Marnes-la-Coquette, France) which diffuse into the gelose and inhibit the bacterial growth by formation of an inhibition zone centered on the disc.

With a sterile loop, colonies were touched and mixed in 2 ml of sterile solution of NaCl 0.85% (bioMérieux, Marcy l'Etoile, France) in order to obtain a suspension of 0.5 McFarland measured by photometer Densimat (bioMérieux, Marcy l'Etoile, France). A swab was soaked in the bacterial suspension and inoculated on the Mueller-Hinton gelose (bioMérieux, Marcy l'Etoile, France) homogeneously. Subsequently, one loop soaked in the suspension was inoculated on TSA gelose as purity control. The 16 antibiotics rack for bacillus Gram-negative was applied on the Mueller-Hinton. After approximately 18-24h of incubation at 37°C, the inhibition zone diameters were measured by the Sirscan and classified in sensitive (green), intermediate (yellow) or resistant (red) according to international recommendations (CLSI). Sirscan2000 (i2a, Montpellier, France) is an Automatic Agar Reader-Incubator for **susceptibility** testing. After exportation of the results to the Laboratory information system of GLIMS, the inhibition diameters were controlled visually and manually adjusted.

Antibiotics for Gram-negative antibiograms:

AM: Ampicillin 10 µg
ATM: Aztreonam 30 µg
CXM: Cefuroxim 30 µg
TEM: Temocillin 30 µ
CAZ: Ceftazidim 30 µg
AMC: Amoxicillin + Clavulanic Acide 30 µg
FEP: Cefepim 30 µg
TZP: Piperacillin 100 µg + Tazobactam 10 µg

MEM: Meropenem 10 µg
CTX: Cefotaxim 30 µg
FOX: Cefoxitin 30 µg
ETP: Ertapenem 10µg
CIP: Ciprofloxacin 5 µg
SXT: Trimethoprim-sulfamethoxazole 25 µg
AN: Amikacin 30 µg
GM: Gentamicin 10 µg

7. Whole Genome Sequencing Softwares

Softwares for detection of virulence and resistance genes have been sought and tested on *K. pneumoniae* whole genome of NCBI database. Concerning the virulence, two relevant softs were held:

1) *Virulence Factors of Pathogenic Bacteria*
    - http://www.mgc.ac.cn/VFs/main.htm
    - Choose "Search" in the site menu.
    - In the category "Blast search", choose "Regular BLAST".
    - Choose "Program blastn" and "Database DNA sequences from VFDB core dataset (R1)".
    - Upload the file of the sequence to analyze recorded in FASTA format.
    - Push on « Search ».
    - Results obtained: blast ID, length, start position and percentage of identity.

2) *Institut Pasteur MLST and whole genome MLST databases*
    - http://bigsdb.web.pasteur.fr/
    - Choose the Database "*Klebsiella pneumoniae*".
    - Select "Sequences and profiles database public".
    - In the category "Query Database", select "Sequence query - query an allele sequence".
    - Choose "virulence genes" in the category "locus/scheme" and locus in "order results by".
    - Upload the file in FASTA format.
    - Push on "Submit".
    - Results obtained: name of allele, length, start and end position.

The same work was performed for the resistance genes detection and 3 softs were retained:

1) *The Comprehensive Antibiotic Resistance Database*
    - http://arpcard.mcmaster.ca/
    - Choose "Tools" in the site menu and select "BLAST".
    - Copy the whole nucleotid sequence in "Enter query sequences here in Fasta format".

- Choose “Program blastn” and “Database Resistance Genes” before push on “Basic search”.
- Results obtained: description, maximum identity and e-value.

2) *ARDB-Antibiotic Resistance Genes Database*
   - http://ardb.cbcb.umd.edu/
   - Choose the Database “Resistance Type”.
   - Put the genome complete in “Input”.
   - Push on “Search”.
   - Results obtained: resistance type, resistance profile and description.
3) *ResFinder* (Zankari et al., 2012)
   - http://genomicepidemiology.org/
   - Select in the category “Phenotyping”: Identifcation of acquired antibiotic resistance genes ResFinder.
   - Select “All” in antimicrobial configuration, “98%” in threshold for % ID, “60%” in minimum length and “Assembled Genome/Contigs” in type of your reads.
   - Press “Isolate File” to load the sequence in FASTA format.
   - Push on “Upload”.
   - Results obtained: resistance genes, % of identity, query length, contig, and its position, predicted phenotype and accession number.

Results

Results

1. Validation Multiplex PCRs for virulence factors

In the first part of this work, 5 Multiplex PCRs have been updated from these described by Turton et al. (2010) and Tang et al. (2010). Indeed, the laboratory team had already tried to reproduce them with problems of robustness. The principal modifications implemented during the validation were the removal of the Q solution (factor MP in the PCR buffer of Qiagen®) and the increase of 5 amplification cycles in the 2 PCRs. Finally, we obtained 5 Multiplex to target 12 virulence factors which were used for all our future analyzes:

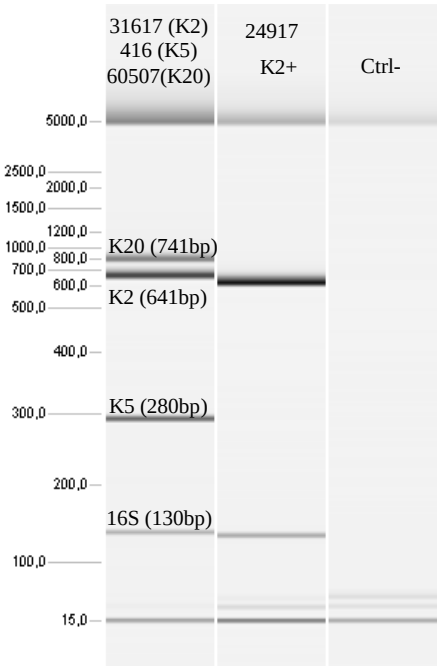


Figure 11. Multiplex PCR targeting K2, K5, K20 and 16S (M1).

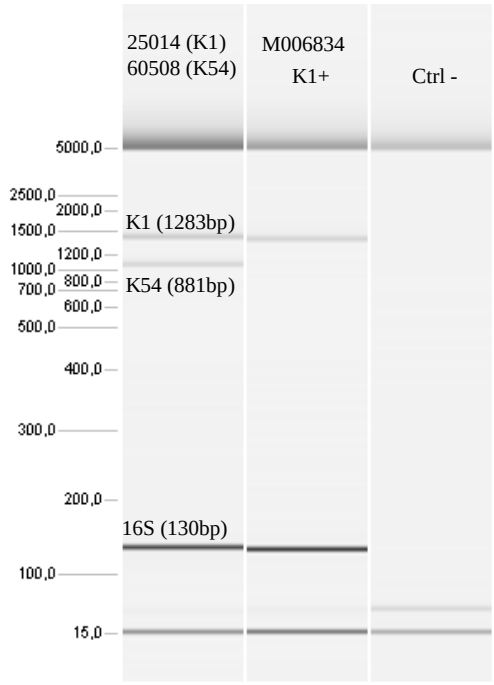


Figure 12. Multiplex PCR targeting K1, K54 and 16S (M2).

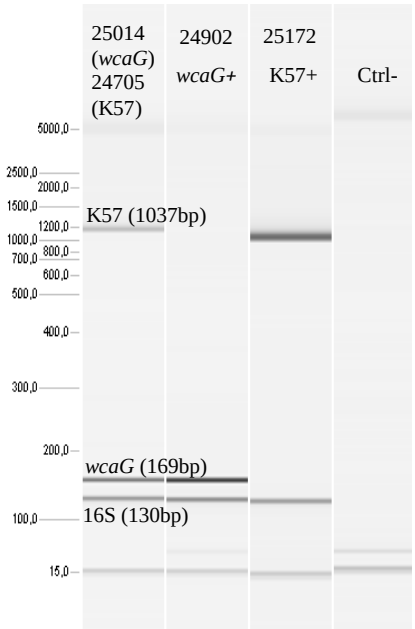


Figure 13. Multiplex PCR targeting K57, *wcaG* and 16S (M3).

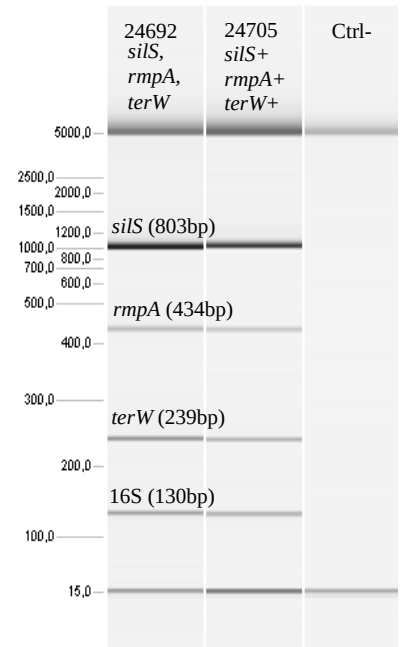


Figure 14. Multiplex PCR targeting *silS*, *terW*, *rmpA* and 16S (M4).

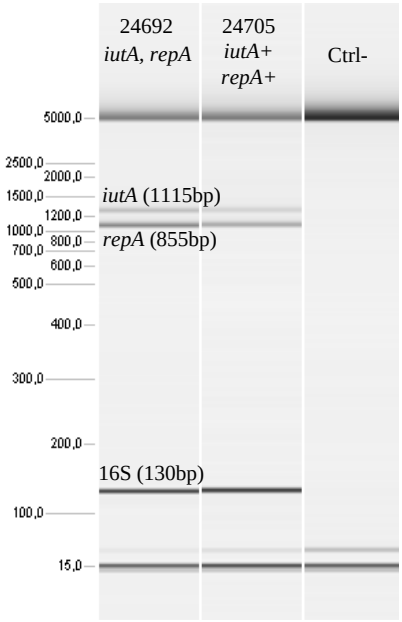


Figure 15. Multiplex PCR targeting *iutA*, *repA* and 16S (M5).

2. Detection of virulence factors in clinical *K. pneumoniae*

2.1. Clinical isolates from the CHU Dinant-Godinne

Thirty *K.pneumoniae* samples from blood cultures conserved by the CHU Dinant-Godinne clinical laboratory have been tested with the 5 Multiplex PCRs. These samples have been selected among strains identified by the routine microbiology laboratory between February 2010 and August 2013. Ten isolates from the study PARADIS and 6 from the study FEAR were added at our collection. *K.pneumoniae* presence in blood of not immunocompromised patients indicated bacteria with higher invasiveness capacity having already overcome the immune system. Among a total of 46 strains, 20 contain at least one virulence factor.

Table 4. Positive results of 5 PCRs obtained among 46 clinical samples from CHU Dinant-Godinne.

Identification year	Ward	Modified Turton PCRs			Modified Tang PCRs		String test
		M1	M2	M3	M4	M5	
2010	Cardiology	neg	K54	*wcaG*	neg	neg	neg
2010	Day hospital	neg	neg	neg	*silS*	neg	neg
2010	Internal medicine	neg	K54	*wcaG*	*silS*	neg	neg
2010	Hematology	neg	neg	neg	*silS*	neg	neg
2011	Vasculary	K2	neg	neg	*rmpA*	neg	pos
2011	Internal medicine	neg	neg	neg	*terW*	*repA*	neg
2011	Day hospital	neg	neg	neg	*terw*	neg	neg
2011	Emergency	K2	neg	neg	*rmpA*	*iutA*	pos
2012	Gastro-enterology	neg	K54	*wcaG*	neg	neg	neg
2013	Intensive care	K2	neg	neg	neg	neg	neg
2015	Hematology	neg	neg	neg	*silS*	neg	neg

2015	Gastroenterology	neg	neg	neg	*silS*	neg	neg
2015	Hematology	neg	neg	neg	*silS*	*repA*	neg
2015	Emergency	neg	neg	neg	*silS*	neg	neg
2015	Gastroenterology	neg	neg	neg	*silS*	neg	neg
2015	Emergency	K20	neg	neg	neg	*iutA*	neg
2015	Pneumology	neg	neg	*wcaG*	neg	neg	neg
ND	ND	neg	neg	neg	*silS*	neg	neg
2012	VUB (Grèce)	neg	neg	neg	*silS*	neg	neg
2014	France	neg	neg	neg	*silS*	neg	neg

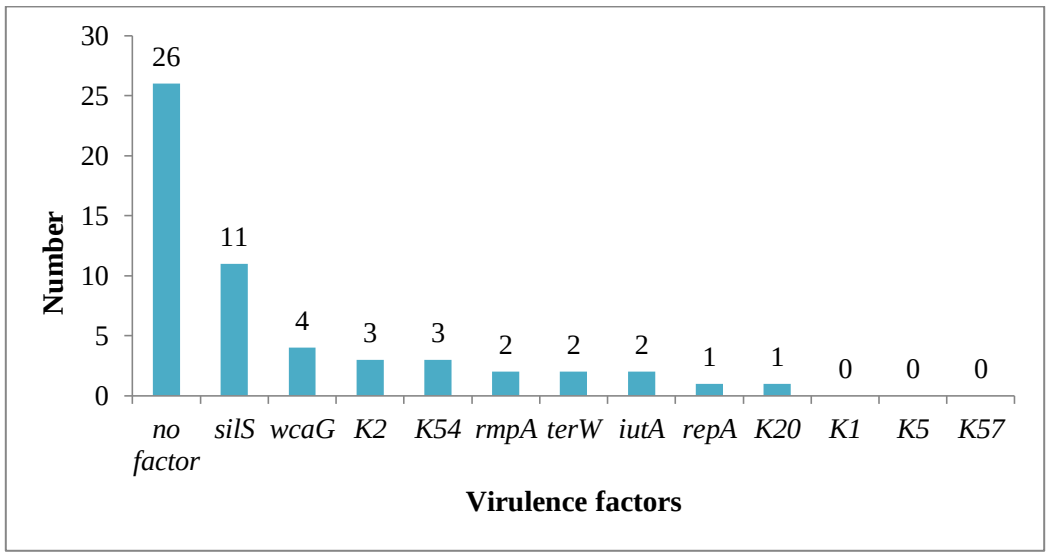


Figure 16. Results of 5 Multiplex PCRs on 46 clinical samples from CHU Dinant-Godinne.

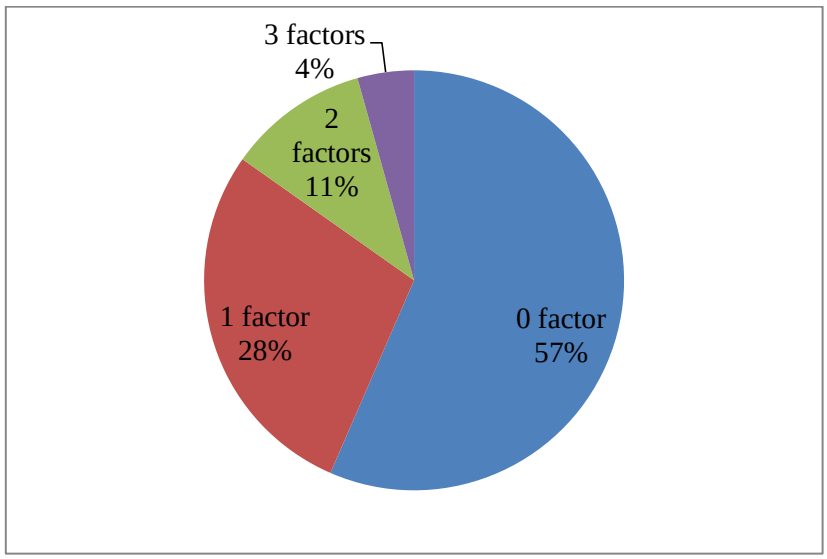


Figure 17. Distribution of the isolates in function of the presence of 1, 2 or 3 virulence factors.

Table 5. Clinical characteristics of strains with at least one virulence factor.

Age, year (sex)	**Virulence factor(s)**	**Underlying disease(s)**	**Infection origin**	**Infection site(s)**	**Outcome**
76 (F)	K54, *wcaG*	Ventricular tachycardy	Urinary tract	Sepsis	Favourable
62 (M)	silS	Chronic renal insufficiency	ND	Sepsis	Dead
81 (F)	K54, *wcaG*, *silS*	Lung carcinoma	ND	Sepsis	Favourable for infection but metastasis
70 (M)	*silS*	Myeloblastic leukemia	ND	Lung Sepsis	Hepatic and renal insuffisance
60 (M)	K2, *rmpA*, ST+	Vascular stenosis Diabetic	ND	Lung Sepsis	Favourable
68 (F)	*terW*	Diabetic type 2 HTA Hypercholesterolemia Alcoholism	ND	Sepsis Urinary tract Pyelonephritis	Relapse of the infection after 3 months
82 (M)	*terW*	Multiple myeloma Vascular bypass	Urinary	Sepsis	Favourable
85 (M)	K2, *rmpA*, *iutA*, ST+	Bronchopneumonia Lung carcinoma Smoking	ND	Sepsis Empyema	*K. pneumoniae* wound infection 5 months later
54 (M)	K54, *wcaG*	Spastic tetraplegy Gastrostomy	ND	Sepsis Urinary tract	ND
53 (M)	K2	Hepatic, renal and pulmonary insufficiencies Ethylic cirrhosis Smoking	ND	Sepsis Peritonitis Hepatitis	Dead
61 (M)	*silS*	Myeloblastic leukemia Diabetic type 2 Smoking	Urinary	Sepsis Lung Intestinal tract	ND
51 (F)	*silS*	HTA treated	Lithiasic angiocholitis	Sepsis	Favourable
74 (F)	*silS*, *repA*	Multiple myeloma HTA treated HTAP Hepatic and renal insufficiency	Urinary	Sepsis Lung	Dead
88 (F)	*silS*	Severe chronic renal insufficiency Vascular bypass and pacemaker	ND	Sepsis	Dead
63 (M)	*silS*	Chronic ethylic pancreatitis Portal hypertension Splenomegaly Smoking	ND	Sepsis	ND
80 (F)	K20, *iutA*	Renal insufficiency	Pulmonary	Sepsis	ND
76 (F)	*wcaG*	Lung adenocarcinoma	Urinary	Sepsis, lung	Favourable
86 (F)	*silS*	ND	ND	ND	ND
ND	*silS*	ND	ND	ND	ND
53 (M)	*silS*	ND	ND	Sepsis, lung	ND

ST+: string test positive; HTA: arterial hypertension; HTAP: pulmonary hypertension; ND: not determined

Table 6. Clinical characteristics of strains without virulence factor.

Age (sex)	**Underlying disease(s)**	**Infection site(s)**	**Outcome**
85 (F)	Renal insufficiency Diabetic	Sepsis	ND
96 (F)	Angiocholitis	Sepsis	ND
81 (F)	Carcinoma pulmonary BPCO Smoking	Sepsis	Favourable
81 (M)	Renal insufficiency Pneumonia Cardiac decompensation	Sepsis	Favourable
91 (M)	Pulmonary decompensation Decompensated cardiopathy BPCO Colic adenocarcinoma	Urinary, sepsis	Favourable
93 (M)	Peritonitis Renal insufficiency FA	Sepsis with urinary origin	Dead
86 (F)	Renal insufficiency	Sepsis Pyelonephritis	ND
31 (M)	Lymphoblastic lymphoma Splenomegaly	Sepsis with urinary origin	Dead
82 (M)	Angiocholitis Diabetic	Sepsis Bronchopneumonia	Weakness
76 (F)	Veinous insufficiency Obesity Oedema in inferior members FA	Sepsis with endovascular prosthesis origin	Favourable
69 (F)	Angiocholitis Ascitis	Sepsis	Dead
84 (F)	Angiocholitis Pancreatic adenocarcinoma	Sepsis with angiocholitis origin	Favourable
78 (F)	Pulmonary adenocarcinoma	Sepsis with urinary origin	Dead because of septicemia
81 (F)	Hilair carcinoma Renal insufficiency	Sepsis with central catheter origin	ND
55 (F)	Hepatic canal cholangiocarcinoma	Sepsis with angiocholitis origin (prosthesis)	ND
56 (F)	Lithiasic angiocholitis Smoking Past alcoholism	Sepsis probably with lithiasic origin	Favourable
69 (M)	Splenomegaly Cellulite of inferior members Diabetic type 2 HTA and morbid obesity Past smoking and alcoholism	Sepsis with central catheter origin	Favourable
80 (M)	Renal insufficiency with kyst Urinary sepsis in the past	Sespsis with urinary catheter origin	Favourable
78 (M)	Renal insufficiency Hyperthyroidy	Sepsis	Favourable
64 (M)	*Staphyloccocus aureus* cellulite in left foot and right arm Diabetic type 2 Chronic renal insufficiency	Sespsis with central catheter origin	Favourable after amputation
69 (M)	Chronic lymphoid leukemia with chemotherapy	Sepsis	Dead

	Pneumopathy		
82 (M)	Enteroid adenocarcinoma Pneumopathy	Sepsis	ND
FEAR*	ND	ND	ND
FEAR*	ND	ND	ND
47 (F) FEAR*	Toxicomania	Urinary tract	ND
FEAR*	ND	ND	ND

BPCO: broncho-pneumopathy chronic obstructive; HTA: hypertension arterial; FA: fibrillation auricular; ND: not determined; FEAR*: strains from the FEAR study for which medical files were not available.

First, about the gender, 47% of the positive cases for one or multiple virulence factors were from women and 53% from men. Among negative strains, 52% came from women and 48% from men. No association can hence be done between the gender and infections by strains with virulence factors. The mean age of patients with *K. pneumoniae* carrying virulence factors was 69 years while these of patients with strain without virulence factor was 74 years.

Among isolates carrying virulence factors, 3 came from patients diabetics type 2 (15%). This percentage is lower compared to previous studies in which the results gave 61% of diabetics among patients with liver abscess (Fang et al., 2007), 29% among community-acquired bacteremia (Ko et al., 2002), 38% among community-acquired bacteremia (Peirano et al., 2013), 49% of liver abscesses in China (Qu et al., 2015). The other underlying diseases cited in several publications are smoking, cardiovascular or pulmonary diseases, alcoholism, biliary tract disease, renal, hepatic insufficiency and others but in lower proportion (Ko et al., 2002; Ku et al., 2008; Qu et al. 2015; Siu et al., 2012). Among all our isolates from the CHU, the most underlying diseases in patients with *K. pneumoniae* infections are by decreasing order: 28% of renal insufficiency, 26% of cardiopathy, 21% of pulmonary disease, 15% of smokers, 10% of alcoholics and only 6% of liver disease. In the literature, pneumonia and urinary infections are the most frequent infection sites of hypermucovsicous *K. pneumoniae*. It was confirmed in our study where the sites of infection inducing sepsis were 28% urinary, 17% pulmonary and 10% intestinal. However, these numbers are probably underestimates because of the lack of information about infection sites for some patients. The rate of mortality obtained according to medical records (Omnipro) is 19.5%. Nevertheless, some dead outcomes are not related to the *K. pneumoniae* infection and then not considered as a negative outcome after treatment. The hvKP infections are associated with a significant mortality rate ranging from 3 to 42% (Shon et al., 2013). However, the major problem with the hvKP is not so much an increased mortality rate but the more severe sequels induced (Yu et al., 2007).

2.2. Strains from Republic Democratic of Congo

We received from the Institute of Tropical Medicine (Anvers, Belgium) 243 *K. pneumoniae* collected in RDC in different health districts of the country. Their identification has been confirmed by Maldi-TOF and 18 strains were not *K. pneumoniae* and not further analyzed. The 5 Multiplex PCRs and antibiograms for bacilli Gram-negatives were carried out on 225 *K. pneumoniae*.

Table 7. Positive results of 5 PCRs on 225 *K. pneumoniae* clinical samples from RDC.

Year	Virulence factors	String test	Age (sex)	Province	Underlying disease(s)	Resistance
2010	K2, *silS*	neg	<1 (M)	KINSHASA	Other	CTX-M G1
2011	K2, *silS, terW*	neg	<1 (M)	KINSHASA	Other	CTX-M G1
2011	K2, *silS, terW*	neg	4 (F)	KINSHASA	Urinary tract	CTX-M G1
2010	K2	neg	45 (M)	KINSHASA	Pneumonia	CTX-M G1
2008	K20	neg	3 (F)	EQUATEUR	Pneumonia	NO ESBL
2008	K20	neg	<1 (F)	KINSHASA	Other	NO ESBL
2010	K20	neg	45 (M)	KINSHASA	Pneumonia	CTX-M-like
2009	K20	neg	6 (F)	KINSHASA	No	CTX-M G1
2008	K20, *silS*	neg	7 (F)	BANDUNDU	No	NO ESBL
2007	K20, *silS*	neg	69 (M)	KINSHASA	No	ESBL
2011	K20, *silS*	neg	ND (M)	BAS CONGO	Other	CTX-M G1
2009	K54, *wcaG*	neg	<1 (M)	ORIENTALE	Other	NO ESBL
2007	K54	neg	47 (F)	BAS CONGO	No	NO ESBL
ND	K54	neg	ND (ND)	ND	ND	NO ESBL
2014	K57, *silS*	neg	<1 (M)	BAS CONGO	Typhoid fever	CTX-M G1
2014	K57	neg	7 (M)	BAS CONGO	Typhoid fever, malaria	CTX-M G1
2008	K57	neg	40 (M)	KINSHASA	Other	CTX-M G1
2009	K57	neg	<1 (M)	ORIENTALE	Malaria, pneumonia	NO ESBL
ND	K57, *silS, rmpA, terW, iutA*	pos	ND	ND	ND	NO ESBL
ND	K57, *silS, rmpA, terW, iutA*	pos	ND	ND	ND	NO ESBL
ND	K57	neg	ND	ND	ND	CTX-M G1
2007	*wcaG, repA*	neg	74 (F)	KINSHASA	No	CTX-M G1
2009	*wcaG*	neg	<1 (F)	ORIENTALE	Meningitis	CTX-M G1
2009	*wcaG, silS*	neg	12 (F)	BAS CONGO	No	NO ESBL
2009	*wcaG*	neg	<1 (ND)	KINSHASA	Other	NO ESBL
2008	*wcaG*	neg	3 (F)	KINSHASA	No	NO ESBL
ND	*wcaG, silS*	neg	ND (ND)	ND	No	NO ESBL
2010	*wcaG*	neg	<1 (M)	ORIENTALE	Other	CTX-M G?
2011	*wcaG, terW, repA*	neg	5 (F)	BAS CONGO	No	CTX-M G1
2012	*wcaG*	neg	1 (M)	BAS-CONGO	Other	NO ESBL
2008	*silS, repA*	neg	<1 (M)	KINSHASA	Meningitis, other	CTX-M G1
2008	*silS*	neg	21 (F)	KINSHASA	Urinary tract	NO ESBL
2008	*silS*	neg	21 (F)	KINSHASA	Urinary tract	NO ESBL
2008	*silS*	neg	<1 (F)	KINSHASA	Other	CTX-M G1

ND	*silS*	neg	ND	ND	ND	CTX-M G
2008	*silS*	neg	6 (M)	KINSHASA	Urinary tract	NO ESBL
2010	*silS*	neg	2 (M)	ORIENTALE	Malaria	CTX-M G1
2009	*silS*	neg	<1 (F)	KINSHASA	Other	NO ESBL
2009	*silS*	pos	<1 (F)	BAS CONGO	Pneumonia, urinary tract, other	ND
2014	*silS*	neg	1 day (M)	BAS-CONGO	Typhoid fever, malaria, skin infection, other	NO ESBL
2014	*silS*	neg	6 (F)	BAS-CONGO	No	CTX-M G1
2008	*silS*	neg	<1 (M)	KINSHASA	Other	CTX-M G1
2007	*silS*	neg	2 (F)	KINSHASA	ND	NO ESBL
2010	*silS*	neg	<1 (M)	KINSHASA	Malaria, other	CTX-M G1
2010	*silS*	neg	23 (M)	BAS CONGO	Malaria	NO ESBL
2010	*silS*	neg	<1 (F)	KINSHASA	ND	CTX-M G1
2010	*silS*	neg	<1 (F)	KINSHASA	Other	CTX-M G1
2010	*silS*	neg	43 (F)	KINSHASA	Meningitis	CTX-M G1
2010	*silS*	neg	<1 (F)	KINSHASA	Malaria	CTX-M G1
2011	*silS*	neg	ND (M)	BAS CONGO	Meningitis, urinary tract	CTX-M G1
2011	*silS*	neg	<1 (F)	BAS CONGO	Meningitis	CTX-M G1
2010	*silS*	neg	<1 (F)	KINSHASA	Other	CTX-M G1+OXA-1
2011	*silS*	neg	<1 (M)	KINSHASA	Other	CTX-M G1+OXA-1
2010	*silS*	neg	<1 (M)	KINSHASA	Other	CTX-M
2010	*silS*	neg	14 (M)	ORIENTALE	Other	NO ESBL
2010	*silS*	neg	<1 (M)	EQUATEUR	Other	CTX-M G?
2009	*silS*	neg	<1 (M)	KINSHASA	ND	CTX-M G1
2011	*silS*	neg	7 (F)	BAS CONGO	ND	CTX-M G1
2011	*silS*	neg	3 (F)	BAS CONGO	ND	CTX-M G1
2011	*silS*	neg	<1 (M)	KINSHASA	Other	NON ESBL
2011	*silS*	neg	ND (ND)	ORIENTALE	ND	CTX-M G1
2011	*silS*	neg	1 (F)	ORIENTALE	Pneumonia	CTX-M G1
2011	*silS*	neg	55 (M)	KINSHASA	Other	CTX-M G1
2011	*silS*	neg	55 (M)	KINSHASA	Other	CTX-M G1
ND	*silS*	neg	ND (ND)	ND	ND	NO ESBL
2011	*silS, repA*	neg	71 (M)	KINSHASA	Pneumonia, urinary tract	NO ESBL
2011	*silS, repA*	neg	71 (M)	KINSHASA	Pneumonia, urinary tract	NO ESBL
2012	*silS, repA*	neg	<1 (M)	KINSHASA	Neonatal infection	CTX-M G1
2012	*silS, repA*	neg	<1 (M)	ORIENTALE	Pneumonia	CTX-M G1
2008	*silS, terW, repA*	neg	33 (M)	KINSHASA	Urinary tract, other	CTX-M G1
2008	*terW*	neg	<1 (M)	KINSHASA	Other	CTX-M G1
2008	*silS, terW, repA*	neg	<1 (M)	KINSHASA	Meningitis, other	CTX-M G1
2008	*silS, terW, repA*	neg	<1 (M)	KINSHASA	Other	CTX-M G ?
2008	*terW*	neg	<1 (F)	KINSHASA	ND	CTX-M G1
2008	*terW*	neg	<1 (F)	KINSHASA	Meningitis, other	CTX-M G1
2008	*silS, terW, repA*	neg	<1 (M)	KINSHASA	Other	CTX-M G1
2008	*terW*	neg	<1 (F)	KINSHASA	Other	CTX-M G1
2008	*silS, terW, repA*	neg	18 (F)	KINSHASA	Other	NON ESBL
2008	*silS, terW,repA*	neg	11 (F)	KINSHASA	ND	CTX-M G1
2007	*terW*	neg	<1 (F)	KINSHASA	Other	CTX-M G1
2008	*terW*	neg	<1 (F)	KINSHASA	Other	CTX-M G1
2008	*terW*	neg	<1 (M)	KINSHASA	Other	CTX-M G1
2008	*terW, repA*	neg	2 (F)	KINSHASA	ND	CTX-M G1
2007	*terW, repA*	neg	<1 (ND)	KINSHASA	Meningitis, other	CTX-M G?
2008	*terW, repA*	neg	41 (F)	KINSHASA	Other	CTX-M G1
2009	*terW, repA*	neg	41 (F)	KINSHASA	Other	CTX-M G1
2009	*terW*	neg	3 (M)	KINSHASA	Other	CTX-M G1
2008	*terW*	neg	<1 (M)	KINSHASA	Meningitis, other	CTX-M G1
2008	*terW, repA*	neg	<1 (F)	KINSHASA	Other	CTX-M G1
2008	*terW, repA*	neg	<1 (F)	KINSHASA	Other	CTX-M G1

2008	*terW*	neg	63 (M)	ORIENTALE	Meningitis, urinary tract	BLSE TEM SHV
2014	*terW*	neg	58 (F)	KINSHASA	ND	ND
2007	*terW*	neg	9 (F)	KINSHASA	ND	CTX-M G1
2007	*silS, terW, repA*	neg	<1 (F)	KINSHASA	Meningitis	CTX-M G1
2008	*terW, repA*	neg	ND (ND)	ND	ND	CTX-M G1
ND	*terW,repA*	neg	<1 (M)	ORIENTALE	Meningitis	CTX-M G1
2008	*terW*	neg	<1 (M)	KINSHASA	Meningitis	CTX-M G1
2008	*terW*	neg	8 (M)	KINSHASA	Other	NO ESBL
2008	*terW*	neg	<1 (M)	KINSHASA	Meningitis	NO ESBL
2009	*silS, terW, repA*	neg	ND (ND)	ND	ND	CTX-M G1
ND	*terW, repA*	neg	34 (F)	KINSHASA	Meningitis, other	CTX-M G1
2009	*terW, repA*	neg	<1 (M)	KINSHASA	Other	CTX-M G1
2009	*terW, repA*	neg	<1 (M)	KINSHASA	Other	CTX-M G1
2009	*silS, terW, repA*	neg	ND (ND)	BAS CONGO	Other	CTX-M G?
2008	*terW, repA*	neg	<1 (F)	BAS CONGO	Other	CTX-M G?
2008	*silS, terW,repA*	neg	2 (M)	BAS CONGO	Other	CTX-M G?
2008	*sils, terW, repA*	neg	14 (M)	BAS CONGO	Other	CTX-M G?
2008	*silS, terW, repA*	neg	ND (ND)	ND	ND	ND
ND	*terW,repA*	neg	19 (M)	BAS CONGO	Other	CTX-M G?
2008	*silS, terW, repA*	neg	5 (M)	BAS CONGO	ND	CTX-M G?
2008	*terW*	neg	7 (M)	KINSHASA	ND	CTX-M G ?
2007	*terW*	neg	6 (M)	KINSHASA	Typhoid fever, urinary tract	CTX-M G1
2010	*silS, terW*	neg	1 (F)	KINSHASA	Malaria	ND
2011	*silS, terW*	neg	3 (F)	ORIENTALE	Other	CTX-M G1
2011	*terW, repA*	neg	7 (M)	KINSHASA	Malaria	CTX-M G1
2011	*terW, repA*	neg	<1 (M)	KINSHASA	Meningitis	CTX-M G1
2011	*terW*	neg	1 (M)	ORIENTALE	Malaria	CTX-M G1
2011	*terW*	neg	<1 (F)	ORIENTALE	Pneumonia	CTX-M G1
2011	*terW*	neg	<1 (F)	ORIENTALE	Malaria, urinary tract	AmpC DHA1+ESBL
2011	*terW*	neg	2 (F)	ORIENTALE	Urinary tract	CTX-M G1
2011	*terW*	neg	1 (M)	ORIENTALE	Malaria	CTX-M G1
2011	*terW*	neg	<1 (F)	ORIENTALE	Other	CTX-M G1
2011	*terW*	neg	3 (F)	ORIENTALE	Malaria	CTX-M G1
2011	*terW*	neg	3 (F)	ORIENTALE	Meningitis	CTX-M G1
2011	*terW*	neg	3 (M)	ORIENTALE	Malaria	CTX-M G1
2011	*terW*	neg	4 (F)	ORIENTALE	Malaria, meningitis	CTX-M G1
2011	*terW*	neg	<1 (M)	ORIENTALE	Other	CTX-M G1
2011	*terW*	neg	1 (M)	ORIENTALE	Other	CTX-M G1
2011	*terW*	neg	<1 (M)	ORIENTALE	Meningitis	CTX-M G1
2011	*terW*	neg	1 (M)	ORIENTALE	Malaria	CTX-M G1
2011	*terW, repA*	neg	<1 (M)	KINSHASA	Other	CTX-M G1
2011	*terW*	neg	<1 (M)	KINSHASA	Typhoid fever	CTX-M G1
2011	*terW*	neg	4 (F)	ORIENTALE	Other	CTX-M G1+OXA-1
2011	*terW*	neg	4 (M)	ORIENTALE	Malaria	CTX-M G1
2011	*silS, terW*	neg	ND (ND)	ND	ND	CTX-M G1
2008	*repA*	neg	<1 (M)	KINSHASA	Other	CTX-M G1

CTX-M: cefotaximase-Munich; OXA: oxacillinase; TEM: name of the first patient affected by this resistant strain; SHV: sulhydril-varaible; AmpC: plasmid mediated AmpC β-lactamase.

PS: type of ESBL was determined by Pr. Glupczynski on basis of antibiograms.

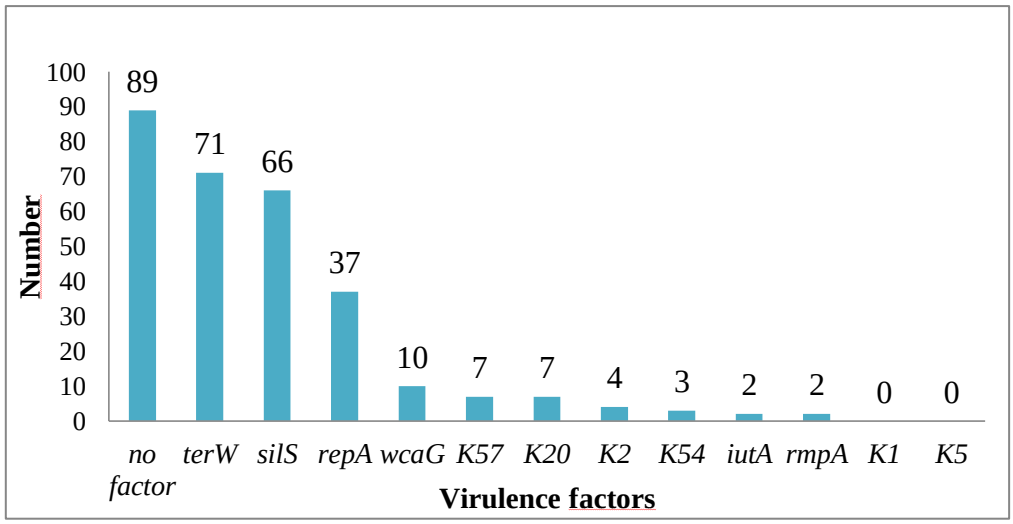


Figure 18. Results of 5 Multiplex PCRs on 225 *K.pneumoniae* clinical samples from RDC.

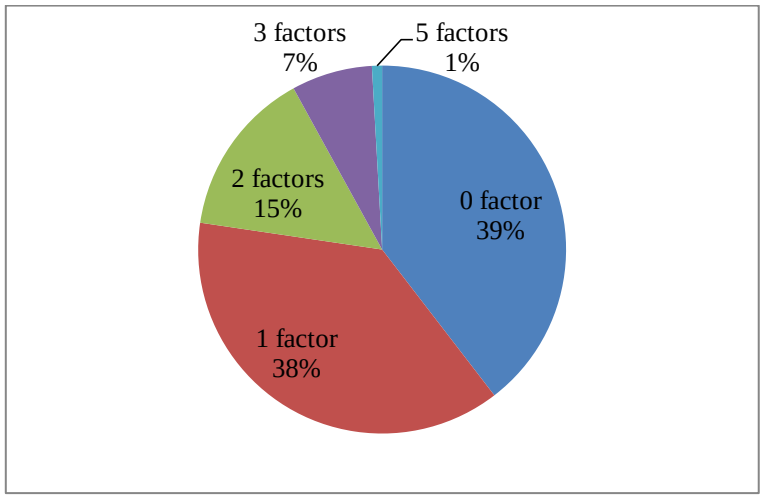


Figure 19. Distribution of the isolates according to their number of virulence factors.

First, we can observe that the majority of the cases come from the urban region of the capital Kinshasa and Bas Congo. It could be explained by a density of population higher than in the other regions of RDC with a rate of transmission more important. Forty-five of the *K. pneumoniae* came from Bas Congo (west of the country) with 21 strains positives for virulence factors (46,6%). One hundred twenty-five came from Kinshasa including 75 positives (60%). Only 1 positive was collected in Bandundu and 1 negative in Kantaga (south). Three came from the Equateur region (north-west) with 2 positives (66%) and 35 are from the Oriental region including 27 positives (77%). All the strains carrying the genes *silS* and *terW* are concentrated in the geographic areas of Kinshasa, Bas Congo and Oriental region. However, few numbers of isolates were collected in the other part of the country then no conclusion can be done. The RDC (mostly near Kinshasa) contains 8.5% of the global reserves in tellure in its ground (+/- 1700 tones). It could explain the high concentration of the strains *terW* positives in this region. The country is also known for its mines of silver

explaining also the big proportion of strains positives for *silS*. Tellure and silver, presents in the environment in important quantity, induce the development of resistance among cKP.

Figure 20. Location of regions where the strains were collected (www.rfi.fr/pays/republique-democratique-congo-chronologie-dates-carte-geographie-demographie-economie-chiffres) consulted the 10/11/2015.

According to analyses of informations obtained by the IMT, any association could be established between the presence of particular virulence factor and age, sex, type of infection, antibiograms or hypermucoviscosity. About resistance, the most interesting is that the 2 strains positives for 5 virulence factors (K57, *rmpA*, *silS*, *terW*, *iutA*), supposed to be very virulent, are not ESBL. It is in accordance with the literature where the hvKP are described as less resistant than cKP. However, all our strains positives for K2 are ESBL CTX-M group1 which is very worrisome. Among the strains with virulence factors, 78% are ESBL and among those without virulence gene 31.5% are ESBL. There is no association between the presence of ESBL and particular virulence factors.

The types of underlying disease found in patients are malaria, meningitis, pneumonia, urinary infections, typhoid fever and neonatal infection (Figure 21).

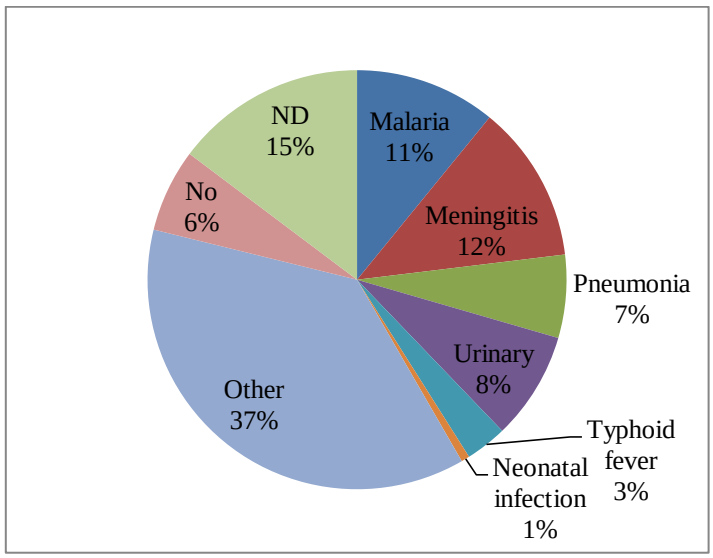
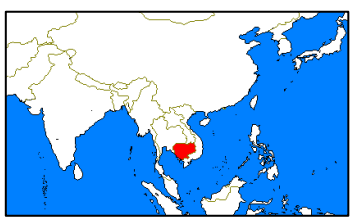


Figure 21. Percentages of underlying diseases among strains with virulence factors.

The next observations concern only strains with at least one virulence factor. Among the cases of malaria, 88% of the isolates are positives for *silS* and/or *terW* and 66,6% came from the region Oriental of RDC. All the patients are 4 years maximum except one of 23 years. Indeed, the majority of malaria infections in Africa reaches the children younger than 5 years. As regards of meningitis, 97,4% are *silS, terW* and these are all ESBL CTX-M. The majority of the patients are children younger than 1 year (68%). Meningitis is one of the worst metastatic infections and indicates an infection by bacteria particularly virulent. Among only 10 cases of pneumonia, there are strains ESBL as far as no ESBL. All the ages are reached. Five cases of typhoid fever were reported, all by children younger than 7 years. Four on five are *silS* or *terW* and CTX-M group1. Thirteen patients of all ages suffered of urinary tract infections with 100% of the strains being *silS* and/or *terW,* ESBL as far as no ESBL. Just one case of neonatal infection was diagnosed affected with a strain positive for *silS, repA* and CTX-M group1. Ten patients didn't present underlying disease with a proportion of strain *silS* or *terW* lower than in the other categories. Unfortunately, the underlying diseases were not determined in 23 cases. For example, the 2 strains positives for 5 factors (K57, *rmpA, silS, terW* and *iutA*) were provided without information about the type of infection. Consequently, we cannot conclude if these strains carrying the maximum of virulence factors observed are especially virulent clinically. In the strains CTX-M, 90% are *silS* and/or *terW*. However, a potential link between this resistance and these factors has never been highlighted in the literature. After these observations, the general trend is that a lot of infections are caused by *K. pneumoniae* positives for *silS* and *terW*. It seems that these 2 factors are particularly associated with virulence in RDC.

The principal difference between our Belgian strains and the Congolese is that we found 61% of *K. pneumoniae* positives for at least one virulence factor in RDC strains and 43% in Belgium. A big difference is the proportion of strains carrying *terW* and *silS* in RDC possibly because of richer grounds in tellure and silver. These factors could help the bacteria to survive in the environment. On the other side, any strain with serotype K1 or K5 are found in the 2 countries while strains with the serotype K1 are reported in Asia. In RDC, 69.7% of the patients suffering of *K. pneumoniae* infections are children while none among Belgian patients where *K. pneumoniae* touch mainly the elderly. Indeed, the youngest patient in Belgium is 31 years. It could be explained by better sanitary control of the children health in Western countries than in Africa.

2.3. Strains from Cambodia



The Institute of Tropical Medicine sent us 52 *K. pneumoniae* clinical isolates from Cambodia. The identification was confirmed thanks to Maldi-TOF spectrometry. Only 1 was an *Escherichia coli* and 51 others were really *K. pneumoniae*. They came not exclusively

from blood cultures. Some have been collected in ascitis, urines, cerebrospinal fluid, tong smear, soft tissues or pus. These strains were analyzed with the 5 Multiplex PCRs to search potential virulence factors and by antibiograms to determine the resistance type.

Table 8. Positive results of 5 PCRs on 51 *K. pneumoniae* from Cambodia.

Reference	**Date**	**Origin**	**M1**	**M2**	**M3**	**M4**	**M5**	**String test**	**Resistance**
COL2015386	2013	Blood	neg	neg	neg	*rmpA*	neg	pos	WT
COL2015387	2013	Ascitis	neg	neg	neg	*rmpA*	neg	pos	WT
COL2015388	2013	Urine	K20	neg	neg	*rmpA, silS, terW*	*iutA, repA*	neg	WT
COL2015390	2013	Blood	K2	neg	neg	neg	neg	neg	WT
COL2015391	2013	Blood	K2	neg	neg	*silS*	neg	neg	WT (cipro and SXT resistance)
COL2015393	2013	Blood	neg	neg	K57	*rmpA, terW*	*iutA*	pos	WT
COL2015394	2013	Urine	neg	K54	*wcaG*	*silS*	*iutA*	neg	WT
COL2015397	2014	Blood	neg	neg	K57	*rmpA, silS, terW*	*iutA, repA*	pos	WT
COL2015398	2014	Urine	K2	neg	neg	*silS*	neg	neg	NO ESBL (SHV-1 like)
COL2015399	2014	Blood	neg	neg	K57	*rmpA, terW*	*iutA*	pos	NO ESBL (SHV-1 like)
COL2015400	2014	Urine	K2	neg	neg	*rmpA*	neg	pos	NO ESBL SHV-1 hyperproduced or SHV-1 like
COL2015402	2014	Blood	K2	neg	neg	*rmpA, silS, terW*	*iutA, repA*	pos	NO ESBL (SHV-1 like)
COL2015404	2014	Pus	K2	neg	neg	neg	neg	neg	ESBL+CTX-M G1?
COL2015405	2014	Blood	K2	K54	*wcaG*	*rmpA, silS, terW*	*iutA*	neg	NO ESBL (SHV-1 like)
COL2015406	2014	Blood	neg	neg	neg	*silS*	neg	neg	ESBL + CTX-M G?
COL2015407	2014	Urine	neg	neg	*wcaG*	*silS*	*repA*	neg	ESBL + CTX-M G1?
COL2015408	2014	Ascitis	neg	neg	neg	*rmpA*	neg	pos	NO ESBL (SHV-1 like)
COL2015410	2014	Urine	neg	neg	neg	*silS*	neg	neg	ESBL + CTX-M G1?
COL2015411	2014	Blood	neg	neg	neg	*silS*	neg	neg	ESBL + CTX-M G1
COL2015412	2014	Blood	neg	neg	neg	*rmpA*	*iutA*	neg	NO ESBL (SHV-1 like)
COL2015413	2014	Blood	neg	neg	neg	*silS*	neg	neg	ESBL+ CTX-M G1

COL2015416	2014	Blood	K2	neg	neg	*rmpA*	*iutA*	neg	NO ESBL (SHV-1 like)
COL2015417	2015	Blood	K2	neg	neg	*silS, rmpA, terW*	*iutA, repA*	pos	NO ESBL (SHV-1 like)
COL2015418	2015	Urine	neg	K1	*wcaG*	*silS, rmpA, terW*	*iutA*	neg	ESBL + CTX-M G1
COL2015420	2015	Tongue smear	neg	neg	neg	*terW*	*repA*	neg	NO ESBL (SHV-1 like)
COL2015421	2015	Soft tissue	K20	neg	neg	*silS*	neg	neg	NO ESBL (SHV-1 like)
COL2015422	2015	Urine	K20	neg	neg	*silS*	*repA*	neg	NO ESBL (SHV-1 like)
COL2015423	2015	Blood	K20	neg	neg	*rmpA*	*iutA*	neg	NO ESBL (SHV-1 like)
COL2015424	2015	Pus	K2	neg	neg	*terW*	neg	neg	ESBL + CTX-M G1
COL2015425	2015	Blood	K5	neg	neg	*rmpA*	*iutA*	pos	NO ESBL (SHV-1 like)
COL2015429	2015	Pus	K20	neg	neg	neg	neg	neg	ESBL + CTX-M G1
COL2015431	2015	Urine	K20	neg	neg	*rmpA*	*iutA*	neg	NO ESBL (SHV-1 like)
COL2015432	2015	Urine	neg	neg	neg	*silS*	neg	pos	NO ESBL (SHV-1 like)
COL2015433	2015	Cerebrospinal fluid	neg	K1	*wcaG*	*silS, rmpA, terW*	*iutA*	pos	NO ESBL (SHV-1 like)
COL2015434	2015	Blood	K20	neg	neg	*rmpA*	*iutA*	neg	NO ESBL (SHV-1 like)
COL2015435	2015	Blood	K2, K5	neg	neg	*rmpA*	neg	pos	NO ESBL (SHV-1 like)
COL2015436	2015	Blood	neg	K1	*wcaG*	*silS, rmpA, terW*	*iutA*	pos	ESBL + CTX-M G1 probably
COL2015437	2015	Blood	neg	K1	*wcaG*	*silS, rmpA, terW*	*iutA*	pos	ESBL+ CTX-M G1

WT: wild-type (ampicillin for *K. pneumoniae*); SXT: trimethoprim-sulfamethoxazole; SHV: sulhydril-varaible; AmpC; CTX-M: cefotaximase-Munich.

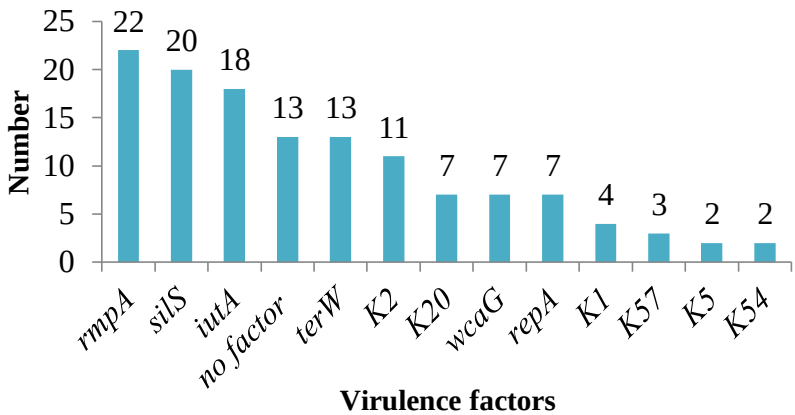


Figure 22. Results of 5 PCRs on 51 *K. pneumoniae* clinical samples from Cambodia.

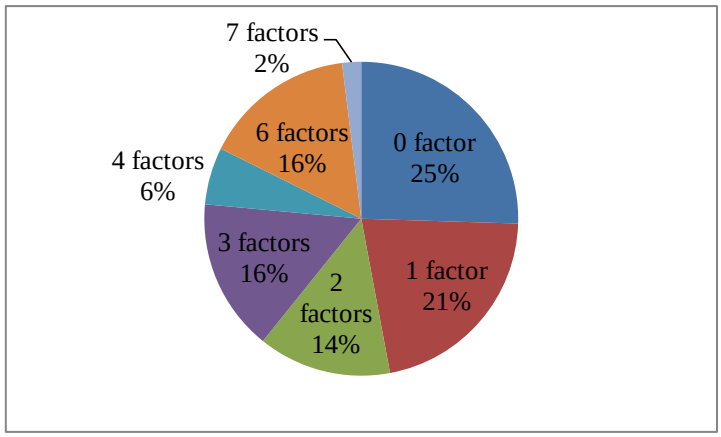


Figure 23. Distribution of the isolates according to their number of virulence factor.

Seventy four percent of Cambodian strains are positives for at least one factor. It is more than in Belgium and RDC respectively 43% and 61%. Moreover, when maximum 3 factors are found in a same Belgian strain and maximum 5 factors in a Congolese strain, strains with 6 or 7 factors were detected in the collection from Cambodia. The Asiatic origin risk factor, very often cited in the literature, seems to be confirmed here.

2.4. Strains from Burkina Faso

The ITM sent us 8 isolates from Burkina Faso isolated from potable water. Identification as *K. pneumoniae* was confirmed by the MALDI-TOF. The detection of virulence factors was realized by the 5 Multiplex PCRs and the resistance by antibiograms.

Table 9. Results of 5 PCRs on 8 *K. pneumoniae* environmental strains from Burkina Faso.

Reference	Date	Origin	M1	M2	M3	M4	M5	String test	Resistance
COL2015438	2013	Water	neg	neg	neg	*silS*	neg	neg	ESBL + CTX-M G1
COL2015439	2013	Water	K20	neg	neg	*silS*	neg	neg	ESBL + CTX-M G1
COL2015442	2013	Water	neg	neg	neg	*silS*	neg	neg	ESBL + CTX-M G1
COL2015443	2013	Water	neg	neg	neg	*silS*	neg	neg	ESBL + CTX-M G1
COL2015460	2013	Water	K20	neg	neg	*silS*	neg	neg	ESBL + CTX-M G1
COL2015464	2013	Water	K20	neg	neg	*silS*	neg	neg	ESBL+ CTX-M G1
COL2015465	2013	Water	K20	neg	neg	*silS*	neg	neg	ESBL + CTX-M G1
COL2015466	2013	Water	K20	neg	neg	*silS*	neg	neg	ESBL + CTX-M G1

These strains came from the environment contrariwise of these analyzed until now coming from clinical isolates. The first observation showed that 5 strains were K20 and all were positives for the factor *silS*. Silver seems to be present in high quantity into the ground of this country possibly selecting the development of resistance to silver.

2.5. Comparison of virulence factors between the different countries

2.5.1. Serotype K1

NB: all the percentage cited below represent the proportion among the total number of strains analyzed for each country (with and without virulence factor).

- Belgium: none
- RDC: none
- Cambodia: 4 (7.8%)
- Burkina Faso: none

According to literature, the serotype K1 is more present in Asia and rare in Europe. That is confirmed here with strains K1 positives only found in Cambodia. Patients infected with strains K1 have a risk significantly higher to develop ocular infections (endophtalmitis) or central nervous system infections than strains non K1 (19% vs. 5%) (Fang et al., 2007). Our results count neither liver abscess nor ocular and nervous infections among our isolates K1. Interestingly, the 4 strains are also *wcaG, rmpA, silS, terW* and *iutA* meaning carriers of 6 virulence factors. Three of them are hypermucoviscous. Then, these isolates combine a phenotype mucous, the more prevalent serotype for primary liver abscess, resistance to silver and tellurite and the presence of a siderophore. It would be very interesting to obtain clinical informations about these particular patients to confirm or invalidate the direct association between K1 and liver abscess. Unfortunately, we had not access to these data.

2.5.2. Serotype K2

- Belgium: 3 (6.5%)
- RDC: 3 (1.3%)
- Cambodia: 11 (21.5%)
- Burkina Faso: none

On 3 of Belgian isolates K2, 2 were associated with *rmpA* and string test positives. Both presented a pulmonary infection and the strain only K2 presented an intestinal infection. It is concordant with the fact that K2 is more often associated with non-hepatic infections. About the Cambodian strains, one K2 was also K54 and another was also K5. Then, a same *K. pneumoniae* could express several CPS. For Cambodia, 54% of the K2 positives were also *rmpA* and 36% *iutA*.

2.5.3. Serotype K5

- Belgium: none
- RDC: none
- Cambodia: 2 (3.9%)
- Burkina Faso: none

Among the Cambodian *K. pneumoniae*, for the first time in this work, 2 strains were positives for the serotype K5 both also positives for *rmpA* and with a string test positive. This rare serotype appears really associated with hvKP. However, it is present in very low frequency.

2.5.4. Serotype K20

- Belgium: 1 (2%)
- RDC: 1 (0.4%)
- Cambodia: 7 (13.7%)
- Burkina Faso: 5 (62.5%)

In our Belgian strains, just 1 strain was K20 in the same time *iutA* and giving pulmonary infections. Only 1 K20 associated with *silS* was found among the strains from RDC. About Cambodian strains, 4 strains K20 are *rmpA* but none has positive string test. According our results, this serotype seems not to be associated with the hypermucoviscous phenotype. Curiously, K20 is the unique serotype found in 5 strains from Burkina Faso, all string test negatives and *silS* positives. The explanation of why only the serotype K20 was found in these strains is not known. The frequency of a serotype is probably not dependent of geographic location because of the differences between the two Africans countries.

2.5.5. Serotype K54

- Belgium: 3 (6.5%)
- RDC: 1 (0.4%)
- Cambodia: 2 (3.9%)
- Burkina Faso: none

About Belgium, the 3 strains K54 are also *wcaG*. This association is observed for the 2 Cambodian K54. The combination of these 2 factors has been already reported in one article and is confirmed here (Turton et al., 2010). These patients presented different underlying diseases and favourable outcomes.

2.5.6. Serotype K57

- Belgium: none
- RDC: 4 (1.7%)
- Cambodia: 3 (5.8%)
- Burkina Faso: none

Among the 4 Congolese K57, 2 are *rmpa, silS, terW, iutA* and string test positives. Moreover, the 3 Cambodian K57 are also *rmpA, terW, iutA* and hypermucous. In the literature, this phenotype has been reported as associated with *rmpA* most of the time (Hsu et al., 2011). *K. pneumoniae* belonging to serotype K57 can induce community-acquired pyogenic liver abscess. Then, they can be considered as hvKP (Pan et al., 2008). But it is present in very low frequency in our collections.

2.5.7. wcaG

- Belgium: 4 (8.6%)
- RDC: 2 (0.8%)
- Cambodia: 7 (13.7%)
- Burkina Faso: none

About Cambodian strains, 3 *wcaG* positives are K1 and 2 are K54. In Belgium, 3 are associated with K54. The frequent association between *wcaG* and K1 or K54 seems confirmed here.

2.5.8. rmpA

- Belgium: 2 (4%)
- RDC: 2 (0.8%)
- Cambodia: 22 (43%)
- Burkina Faso: none

rmpA is the gene systematically associated with the hypermucoviscosity phenotype. Our 2 Belgian strains string test positives were indeed *rmpA* positives as 14 Cambodian strains on 15 isolates string test positives. That is confirmed for the 2 Congolese strains *rmpA*. Ninety-four percent of our strains with a phenotype hypermucoviscous carry *rmpA* which is consistent with the literature.

2.5.9. silS

- Belgium: 10 (21.7%)
- RDC: 64 (28.4%)
- Cambodia: 20 (39%)
- Burkina Faso: 8 (100%)

Isolates positives for *silS* are the most frequent in our collection from the 4 countries. This gene can be associated with all other factors.

2.5.10. terW

- Belgium: 2 (4%)
- RDC: 71 (31.5%)
- Cambodia: 13 (25.4%)
- Burkina Faso: none

Only 2 Belgian strains were *terW* positives both induced urinary infection in patients. In the 3 countries, this factor is very often associated with *silS*.

2.5.11. iutA

- Belgium: 2 (4%)
- RDC: 2 (0.8%)
- Cambodia: 18 (35%)
- Burkina Faso: none

All the *iutA* positives in Cambodia strains are also *rmpA* except one. Indeed, this association between siderophores and genes of viscosity is already reported in the literature.

2.5.12. repA

- Belgium: 2 (4%)
- RDC: 37 (16.4%)
- Cambodia: 7 (13.7%)
- Burkina Faso: none

In RDC collection, 3 strains *repA* positives but negatives for all others factor were obtained. This gene codes for the plasmid pLVPK origin of replication but also for replication origins of others plasmids. These 3 isolates probably didn't carry pLVPK because were negatives for the pLVPK loci implied in virulence (*terW, silS, rmpA, iutA*). In Cambodia, only 7 strains are positives for *repA* always associated with others factors then containing probably pLVPK.

2.5.13. Hypermuciviscosity

- Belgium: 2 (4%)
- RDC: 6 (2.6%)
- Cambodia: 15 (29.4%)
- Burkina Faso: none.

The Belgian strains string test positives were also K2 and *rmpA* while among isolates from RDC, the string test positives were never K2 but carried others genes (K57, *rmpA, terW, silS* and *iutA*). That could indicate that the hypermucoviscous phenotype is not always associated with the presence of K2 and/or *rmpA*. Other genes have to be implied in the expression of this phenotype. However, in the Cambodian strains, 93% of the mucous strains are also *rmpA*.

3. Synthesis on all isolates

To conclude this part, a table synthetizes the most important informations among our results.

Table 9. Principal results obtained after 5 Multiplex PCRs on strains from the 4 countries.

Country	**Number of samples**	**Positives (%)**	**Maximum of factor**	**String test positives (%)**	**K1 or K2 (%)**	***rmpA* (%)**
Belgium	46	43	3	4	6.5	4
RDC	225	61	5	0.2	1.7	0.8
Cambodia	51	74.5	7	29	29	43
Burkina Faso	8	100	2	0	0	0

4. Analysis by whole genome sequencing softwares

4.1. Virulence

Different free access softwares were tested in order to detect virulence factors into *K. pneumoniae* whole genomes. *Virulence Factors of Pathogenic Bacteria* and *Institut Pasteur MLST and whole genome MLST databases* have been retained as relevant and tested with whole genome sequences of *K. pneumoniae* from the database NCBI. Curiously for a same genome, different genes were found. However, some genes are common in the 2 softs. The laboratory doesn't get a whole genome of *K. pneumoniae*. Whole genomes from the NCBI database were used.

Table 10. Example of virulence factors into "*Klebsiella pneumoniae str. Kp52.145, chromosome, complete genome"* (genome 1) found by the 2 softs *Virulence Factors of Pathogenic Bacteria* and *Institut Pasteur MLST and whole genome MLST databases.*

Genes	Functions	Implication in virulence
fuyA	Pesticin/yersiniabactin receptor protein	Biofilm formation (El Fertas-Aissani et al., 2012)
irp1,2	Yersiniabactin biosynthetic protein HMWP1,2	Synthesis and regulation of the yersiniabactin siderophore (Lawlor et al., 2007)
ybtA	Transcriptional regulator	Synthesis and regulation of the yersiniabactin siderophore
ybtE	Yersiniabactin siderophore biosynthetic protein	Synthesis and regulation of the yersiniabactin siderophore
ybtP	Lipoprotein inner membrane ABC-transporter	Synthesis and regulation of the yersiniabactin siderophore
ybtQ	Inner membrane ABC-transporter	Synthesis and regulation of the yersiniabactin siderophore
ybtS	Putative salicylate synthetase	Synthesis and regulation of the yersiniabactin siderophore
ybtT	Yersiniabactin biosynthetic protein YbtT	Synthesis and regulation of the yersiniabactin siderophore
ybtU	Yersiniabactin biosynthetic protein YbtU	Synthesis and regulation of the yersiniabactin siderophore
ybtX	Putative signal transducer	Signal transduction
ssb	ssDNA-binding protein controls activity of RecBCD nuclease	$bla_{\text{OXA-48}}$ gene from the plasmid pOXA-48a *(Poirel et al., 2012)*
fur	Transcriptional repressor of iron-responsive genes	Ferric uptake repressor
sitA	Salmonella iron transporter	Regulator of *fur* (Huang et al., 2012)
soxS	Transcriptional activator of superoxide response	Transcriptional regulators associated with multidrug resistance (Veleba et al., 2012)

Table11. Number of virulence genes found by the 2 softs *Virulence Factors of Pathogenic Bacteria (BLAST)* and *Institut Pasteur MLST and whole genome MLST databases (BIGS)* and them in common.

Results	Genome 1	Genome 2	Genome 3	Genome 4	Genome 5	Genome 6	Genome 7
BLAST	100	100	49	100	76	100	40
BIGS	36	15	1	11	1	3	21
Common	15	11	0	8	0	0	1

Genome 1: *Klebsiella pneumoniae str. Kp*52.145, chromosome, complete genome
Genome 2: *Klebsiella pneumoniae subsp. rhinoscleromatis* strain SB3432, complete genome
Genome 3: *Klebsiella pneumoniae* SB3193 genomic scaffold, KpST82scacor00008, whole genome shotgun sequence
Genome 4: *Klebsiella pneumoniae* CG43 plasmid pLVPK, complete sequence
Genome 5: *Klebsiella pneumoniae* SB3193 genomic scaffold, KpST82scacor00011, whole genome shotgun sequence
Genome 6: *Klebsiella pneumoniae* SB3193 genomic scaffold, KpST82scacor00012, whole genome shotgun sequence
Genome 7: *Klebsiella pneumoniae subsp. pneumoniae* T69 genomic scaffold, SB4536_2858, whole genome shotgun sequence

Table 12. Virulence genes in common into the whole genomes 2 to 7.

Genome number	Virulence genes
2	*iroC* (ABC transport protein), *iutA, iucA* (IucA protein), *iucB* (IucB protein), *iucC* (IucC protein), *iucD* (lysine 6-monooxygenase IucD), *iroC* (ABC transport protein), *ssb, fur, soxS, soxR* (redox-sensing transcriptional activator SoxR)
3	None
4	*iroC, iroB, iroD, iroN, iutA, iucA, iucB, iucD*
5	None
6	None
7	*ureB* (urease β-subunit UreB, urea amidohydrolase)

4.2. Resistance

Three reliable softs in free access on Internet have been selected: *The Comprehensive Antibiotic Resistance Database*, *ARDB-Antibiotic Resistance Genes Database* and *ResFinder*. Again, different results were obtained for resistance genes.

Table 13. Example of resistance genes into "*Klebsiella pneumoniae str. Kp52.145, chromosome, complete genome"* (genome 1) found by the 2 softs *The Comprehensive Antibiotic Resistance Database*, *ARDB-Antibiotic Resistance Genes Database* and by *ResFinder*.

Genes	Function	Implication in resistance
acrB	AcrAB multidrug resistance efflux pump/ aminoglycoside, glycylcycline macrolide, β-lactam acriflavin	Cross-resistance to cefoxitin, quinolones, and chloramphenicol (Bialek-Davenet et al., 2011)
mdt	multidrug resistance efflux pump/ deoxycholate fosfomycin	Resistance of several antibiotics (Andersen et al., 2015)
emrd	multidrug resistance efflux pump	Resistance of several antibiotics (Andersen et al., 2015)
oqxA, B (only by *ResFinder*)	quinolone resistance genes	Ciprofloxacin resistance (Wong et al., 2014)

Table 14. Number of resistance genes obtained by the 2 softs *The Comprehensive Antibiotic Resistance Database (CARD) and ARDB-Antibiotic Resistance Genes Database (ARDB)* and them in common.

Results	Genome 1	Genome 2	Genome 3	Genome 4	Genome 5	Genome 6	Genome 7
ARDB	79	142	79	96	76	79	142
CARD	51	51	51	51	51	51	51
Common	3	3	5	2	4	6	1

NB: number of each genome corresponds to the same analyzed with softs detecting virulence factors.

Table 15. Resistance genes in common into the whole genomes 2 to 7.

Genome number	Resistance genes
2	*acrB, mdt, emrD*
3	*emrA, mdt, macB* (macrolide-specific efflux system), *tet* (tetracycline efflux pump), *acrA*
4	*van* (VanA type vancomycin resistance operon genes), *mdt*
5	*acrA/B, mdt, tolC* (Multidrug resistance efflux pump), *bacA (*Undecaprenyl pyrophosphate phosphatase)
6	*mdt, mdfA, macB* (Macrolide-specific efflux system), *tet, cml* (chloramphenicol efflux pump), *acr*
7	*emr*

Discussion

Discussion

1. Context

The patients infected by hypervirulent *Klebsiella pneumoniae* are healthy and suffer from community-acquired KPLA and serious metastatic infections resulting from bacteraemic dissemination. The initially described liver abscess is just one of many primary infections due to hvKP including pneumonia, endophthalmitis, meningitis, extra hepatic abscess at variable sites and necrotizing fasciitis (Shon et al., 2013). Despite the fact that a higher incidence of diseases was observed among Asian ethnicity, males between 55 and 60 years and diabetics, hvKP infections reach all age groups of patients not suffering from diabete mellitus or any other co-morbidities (Siu et al., 2012; Shon et al. 2013). Microbiological diagnoses of *K.pneumoniae* from blood cultures or liver abscesses with viscous appearance suggest an invasive strain and must be notified to clinician as soon as possible (Siu et al., 2012). Increasingly PCR assays targeting different virulence genes have been already designed allowing rapid, reproducible and sensitive detection.

2. Virulence factors detection

The first objective of this work was to develop a reliable molecular method to detect virulence factors among *K. pneumoniae* clinical strains. The technique of Multiplex PCR has been chosen for its facility of execution and its routine use in our laboratory. One PCR described by Turton et al. (2010) targeting the genes K1, K2, K5, K20, K54, K57, *rmpA* and *wcaG* has been updated. Amplify all the targets in unique PCR was inconclusive. Finally, three Multiplex were obtained, the first targeting K2, K5, and K20 (M1), the second K1 and K54 (M2) and the third K57 and *wcaG* (M3). Integration of *rmpA* was attempted but unsuccessful in all the different primer mix tested. It has been decided that *rmpA* would be targeted by the Multiplex PCR described by Tang et al. (2010). This publication used a unique Multiplex PCR to detect the genes *silS, terW, rmpA, iutA* and *repA* carried by the plasmid pLVPK. Two Multiplex PCRs were designed, the first targeted *silS, terW* and *rmpA* (M4) while the second detected *iutA* and *repA* (M5). A total of 330 strains have been tested with these 5 Multiplex of which 46 from Belgium, 225 from RDC, 51 from Cambodia and 8 from Burkina Faso. Two hundred twenty-one positives strains were obtained meaning 63.4% of all the isolates. This proportion suggests that virulence factors are largely spread among the general *K. pneumoniae* population. In accordance with the literature, the highest number of factors detected was found in strains from Cambodia, a South-East Asiatic country very touched by hvKP.

3. Principal observations

1) All our strains string test positives are also *rmpA*. That confirms the evidence that *rmpA* is an important risk factor of having a hypermucoviscous phenotype (Yu et al., 2007). Conversely, the strains *rmpA* positives are not automatically hypermucoviscous. Then, the hypermucoviscosity phenotypic test called string test is not enough specific to detect all the strains with virulence factors. Detection by PCR is necessary to confirm the presence of virulence factor as *rmpA*.
2) Ninety percent of all *iutA* positive strains were associated with the factor *rmpA*. It is in accordance with literature that reported frequent combination of *rmpA* and siderophores.
3) In the Turton article, their strains *wcaG* positives were often associated with the serotypes K1 or K54. Our results confirmed this trend with all the Belgian strains K54 and the Cambodian K54 or K1 being also *wcaG* positives. However, it is not the case for the Congolese strains. Moreover, *wcaG* was not associated with the phenotype hypermuciviscous (Turton et al., 2010). The presence of this factor seems not to be involved in the development of hypermucoviscosity which is logical because *wcaG* doesn't participate in the thickening of the bacterial capsule.
4) The 4 K1 obtained were Cambodian and also positives for *wcaG, silS, rmpA, terW* and *iutA*. This results corroborate the propensity of K1 strains to carry others virulence factors and to be spread in Asia.
5) The serotype K5 was detected into 2 strains from Cambodia, also *rmpA* and string test positives. K5 is more often incriminated in epidemics in animals particularly in horses (Sharma et al., 2014). This could be an explanation of the extremely low prevalence (0.5%) in our specimens.
6) The higher proportion of strains with the serotype K20 was found among *K. pneumoniae* from Burkina Faso which were collected in potable water well. These waters could have been contaminated by animals before being transferred to humans or inversely. No conclusion can be done about the original reservoir of this serotype or its exact role in virulence.
7) The most prevalent factor in our total collection is, without doubt, *silS* not associated with particular other factors except frequent combination with *terW*. *silS* and *terW* acquire their resistance respectively from the silver and tellurite of the environment. Silver resistance is easily selected in Gram-negative bacteria in vitro suggesting that there would be benefit to improve surveillance of this resistance in the clinic with greater control over use of silver-containing products (Randall et al., 2015). The increasing applications of tellurium in electronics, optics, batteries and mining industries have indirectly led to increased environmental contamination and then the development of naturally occurring tellurite-resistance (Chasteen et al., 2009). Silver and tellurium are increasingly presents in the hospitals that could probably promote the patients contamination during hospitalization causing nosocomial infections.

4. Comparison with a previous study

The previous study "Detection of virulence factors in *Klebsiella pneumoniae* isolated from deep seated infections in Belgium and in Cambodia, and in multidrug resistant KPC-producing isolates" leading by CHU Dinant-Godinne and Hospital Erasme ULB aimed to assess the occurrence rate of hvKP isolates originating from different collections, including strains recovered from bloodstream infections in Belgium and in Cambodia, as well as from Belgian multidrug-resistant KPC-producing *K. pneumoniae* isolates. Seventy-six *K. pneumoniae* from bloodstream infections were collected in which 41 came from Belgian university Hospital and 35 from Cambodia. Among Belgian isolates, 17% of the *K. pneumoniae* isolates carried at least one virulence factor compared to the 43% of positives strains in our Belgian collection. In Cambodian isolates, 31% of their isolates contained genes of virulence compared to 75% of positives in our Cambodian strains.

Our results for Belgian strains have shown any strain K1, K5 or K57 unlike this previous study. That demonstrates that the groups of patients are too small to represent the variety of virulence factors present in Belgium. This study concluded that hypervirulence-associated factors are present in Belgian bacteraemic *K. pneumoniae* isolates but multicentric studies should be carried out to assess the overall prevalence of those isolates.

5. Associations with clinical data

Another problem is the lack of clear associations known between clinical manifestations and the presence or absence of particular genes (Ku et al., 2008). However, some correlations have been already established in function of *K. pneumoniae* serotypes. The serotype K1 is most of the time found in liver abscesses whereas K2 is often associated to abscesses extra-hepatic. A lot of studies aimed to determine whether the different manifestations of infection could be correlated with differences in host and/or bacterial characteristics. In the article of Yu et al. (2007), 49% of isolates from patients with community-acquired pneumonia and 50% of isolates from patients with other invasive syndromes possessed a serotype K1 or K2. Moreover, the mucoid phenotype was present in 93% of strains from patients with invasive diseases. Community-acquired pneumonia was due to mucoid strains in younger patients without serious underlying diseases while nonmucoid strains predominated in older patients with comorbidities. All their isolates from Taiwan or South-Africa inducing liver abscess, meningitis or endophthalmitis were hypermucoviscous. However, there was no association between deaths and hypermucoviscosity. The mucus phenotype was highly correlated with presence of the gene *rmpA* itself often associated with aerobactin producers. In this study, strains with K1 or K2 serotypes, mucoid phenotype and capable of aerobactin production are rarely found to cause severe infections outside Taiwan and South-Africa.

According to Qu et al. (2015), there is no significant relationship between the microbiological and clinical characteristics in East China. Neither the serotypes and *rmpA* genotypes nor the STs were associated with metastatic infections and prognosis of *K.*

pneumoniae liver abscess. Among 45 strains KPLA in East China, K1 was the dominant phenotype (69%) followed by K2 (20%). More patients infected with cKP tend to have a history of diabete mellitus or drinking than them affected by hvKP. But again, there was no significant difference of complications or prognosis between patients infected with hvKP and cKP (Qu et al., 2015). Another study asserted that diabete mellitus and *K. pneumoniae* with mucoid phenotype were significantly associated with distinctive invasive syndromes (Lin et al., 2013). However, others variables like age, gender, presence of uraemia, malignancy, neutropenia, prior surgery and inappropriate initial antibiotic therapy were unrelated to the development of invasive syndromes (Lee et al., 2006).

The finding of a hvKP ST23 causing liver abscess in a Danish patient with none of the normal predisposing factors (diabete or alcoholism) and without travel in Asia or known connection to persons of Asiatic origin can have two possible explanations. First, this particular strain is endemic circulating all times in low numbers in the community. Secondly, this patient was part of an infectious chain with the ST23 clone which was not elucidated at the time. The association of most cases from USA and Europe with Asian origin or travel history is in favour of the second possibility. Consequently, hvKP clones could develop into an important worldwide health problem (Gundestrup et al., 2014).

6. Perspectives: WGS

The software *Institut Pasteur MLST and whole genome MLST databases* also called BIGSdb-Kp have been created by Bialek-Davenet et al. (2014) to enable rapid extraction of medically and epidemiologically relevant informations from genomic sequences of *K. pneumoniae*. Although drug-resistant and virulent populations were largely no-overlapping, isolates with combined virulence and resistance features were detected with this informatics tool. Indeed, genes encoding resistance to β-lactams by $bla_{\text{CTX-M-15}}$, quinolones and aminoglycosides were detected in 2 hvKP isolates from Vietnam and Madagscar. Their results show that the bad prospect of dual-risk *K. pneumoniae* strains, combining virulence and antimicrobial resistance genes, is becoming a reality. This freely accessible database represents a novel useful informatic tool for monitoring the emergence of high-risk clones (Bialek-Davenet et al., 2014). However, it is recommended using at least 2 different softs in order to compare the results and keep only the genes found by several softs. These virulence and resistance genes obtained consist in a first basis of work before whole genome sequencing. They could be compared and completed with genes detected by WGS before to be added in international databases.

Indeed, WGS could extract more virulence-associated genomic features than PCR just by using bioinformatics tool to sequence a whole bacterial genome in 24 hours at a cost increasingly weak. A few numbers of strains with capsular serotype K1 or K2 have been already wholly sequenced. The relevant informations on the resistance and virulence could be useful for the diagnostic and be obtained by a unique technique internationally comparable. WGS is currently available in fundamental research centers but their

introduction in clinical routine need to overcome some challenges like the interpretation of high amounts of data obtained requiring new analytical softwares (Didelot et al., 2012). Another problem is the fact that the presence of a gene did not imply its expression. This could be kept in mind while analysis detection of DNA and not RNA.

7. Conclusion

A large number of knowledge remains to discover about this highly virulent pathogen. An increased understanding of the epidemiology, reservoirs, acquisition and the routes of entry may enable prevention of diseases. Moreover, it is now clear that all hvKP are not string test positives and inversely a phenotype hypermucoviscous not always indicates a hvKP. The development of a more objective diagnostic test is requisite to reliably identify hvKP. It will also allow knowing the full spectrum of infectious syndromes and their incidence especially outside Asia where hvKP are still relatively rare. A critical question is why do hvKP strains have the propensity for metastatic spread, a capacity highly unusual for enteric gram negative bacteria. If nothing is done to control this new pathogen, hvKP strains will likely acquire extreme antimicrobial resistance in the near future. The scenario could become truly frightening (Shon et al., 2013).

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