Capsular typing and Analysis of Virulence Genes of multidrug resistant *Klebsiella Pneumoniae* and *Klebsiella oxytoca* from hospital-associated specimen in Nigeria.

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Abstract: *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae* and *Klebsiella oxytoca.* These isolates are important medical pathogen and responsible for some health problems especially nosocomial infections; pneumonia, septicaemia, respiratory tract and urinary tract infections. Capsule is an important virulence factor in *K. pneumoniae*. The aim of this study was to investigate the serotypes of *K. pneumoniae and K. oxytoca* and to detect the virulence genes. The clinical specimens were collected by standard methods from patients. The bacterial isolates were identified by conventional microbiological methods and the use of analytical profile index 20E (Bio-Mérieux, France) for the organisms. Molecular detection of the virulence genes was done by polymerase chain reaction and agarose gel electrophoresis. Capsule typing and virulence genes were characterized using PCR specific primers. PCR technique showed that thirteen (45%) isolates were positive for K1 gene, five (17%) for K2 gene and no isolates was amplified for K5 gene. The virulence *MagA* gene was present in 14 isolates, *rmpA* gene was detected in 10, *rmpA1* gene was detected in 3 while *wcaG* gene was detected only in 1 isolates. The results suggest that there are K1 and K2 serotypes associated with *magA*, *rmpA* and *wcaG* genes of the *K. pneumoniae* in this region.
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1. Introduction

Infections caused by *K. pneumoniae* can result in serious and life threating infections including pneumonia, urinary tract infections, intravascular line infections, soft tissue infections, intra-abdominal infections and bacteremia (Siu *et al*., 2014). *K. pneumoniae* is considered one of the most common Gram negative bacteria (Lin *et al*., 2010). A number of factors contribute to virulence and pathogenicity in *K. pneumoniae* such as capsular serotype, lipopolysaccharide, iron-scavenging systems and adhesions (Fuursted *et al*., 2012).

Clinically isolated *K. pneumoniae* strains usually produce a large amount of capsular polysaccharide (cps), which confers not only a mucoid phenotype to the bacteria but also resistance to engulfment by professional phagocytes or to serum bactericidal factors (Bach *et al*., 2000). The incidence of microbial infections has been increasing in the past few decades. This has led to the continuous and uncontrolled use of antimicrobial drugs for prevention and treatment in several parts of the world. This led to the emergence of specific drug and multidrug resistance among various strains of microorganisms including *K. pneumonia*e (Tanwar *et al*., 2014). Gram-negative bacteria have developed several mechanisms of resistance to currently used antimicrobials. One of the successful mechanisms for transmitting multiple-drug resistance among bacterial pathogens is horizontal transfer (Munoz-Price and Quinn, 2009). Capsular antigens are considered to be the ultimate virulence determinants. Among 77 capsular serotypes of *K. pneumoniae*, serotypes K1 and K2 are the most virulent ones in humans (Turton *et al*., 2008). Animal studies have shown that K1and K2 isolates are more virulent than other serotypes (Fang *et al*., 2004). Virulence gene *magA* (mucoviscosity associated gene) was identified in pathogenic strains from Taiwan causing liver abscess (Rahn *et al*., 1999; Wasfi *et al*., 2016). *magA* is described as a novel virulence factor responsible for the increased virulence of certain *K. pneumoniae* strains (Jazani *et al*., 2009; Pan *et al.,* 2008). On the other hand *rmp*A plays a minor role in virulence compared with the presence of serotype K1 or K2 (Rozalski, 2007). The aim of this study was to detect the virulence genes in *K. pneumoniae* strains and characterized their capsular serotypes using polymerase chain reaction.

2. Materials and Methods

Seventy-seven *K. pneumoniae* and twenty-eight *K. oxytoca* isolates were recovered from patients at Obafemi Awolowo University Teaching Hospital of which ethical clearance approval was obtained from

the Research and Ethics committee of the institution. Informed consent was obtained from all subjects. The specimens were collected aseptically from all patients (sputum and throat swab samples). The sputum samples were collected into well-labelled sterile, wide mouthed glass bottles with screw cap tops. Using a sterile cotton swab, the inner surface of the infected throat was swabbed gently and then were transported to the laboratory. The bacterial isolates were identified using morphological, microscopy and biochemical tests following standard procedures described by Sharma (2005). The identity of the isolate was confirmed using Analytical Profile Index 20E kit (BioMerieux, Inc., France) following the manufacturer's instruction.

Antibiotic susceptibility testing of *Klebsiella* sp was performed according to the Kirby-Bauer's disk diffusion method (Bauer *et al*., 1966). The antibiotic discs (Oxoid Ltd, Basingstoke, Hampshire, England) of varying and specific concentrations were used for the tests and they include; cefotaxime (30 ug), piperacillin (10 ug), augmentin (2 ug), cefoxitin (30 ug), ceftazidime (30 µg), cefuroxime (30 µg), ofloxacin (5 µg), cefixime (5 ug), imipenem (10 ug), gentamicin (10 ug), ciprofloxacin (5 ug) and nitrofurantoin (300 ug). These discs were firmly placed on the surface of the culture plates using a sterile forceps and incubated in an inverted position at 37 °C for 18 h. Resistance profiles of the isolates were determined by measuring the diameter of zones of inhibition of each antibiotic on the bacterial isolates between 16-18 h of incubation and comparing these zones of inhibition with CLSI (2013).

The multiple antibiotic resistant isolates were randomly selected based on their antibiotic susceptibility profile.

The isolates were investigated for the presence of virulence genes mucoviscosity-associated gene A (*magA*) and regulation of capsular polysaccharide synthesis (*rmpA*), capsular fucose synthesis (*wcaG*) and *rmpA*1 by polymerase chain reaction (Table 1). The PCR product was separated by agarose gel electrophoresis.

The isolates were typed and identified by molecular detection of capsular polysaccharide gene (K1, K2 and K5) using PCR specific primers following the instruction of the manufacturer. The molecular investigations of the *Klebsiella* serotypes include the extraction of DNA templates of the isolates by boiling method, preparation of the PCR reaction mixture using appropriate primers, standard PCR reaction in a thermocycler, agarose gel electrophoresis, bands visualization by ultraviolet trans-illumination and bands photography.

Polymerase Chain Reaction (PCR) was performed in a total volume of 25 µl containing 2.5µl of both the forward and the reverse primers, 12.5 µl master mix, 2.5 µl free water nuclease and 5 µl of the extracted DNA (as DNA template), then DNA amplification was carried out with the thermal cycler. The lyophilized oligonucleotide forward and reverse primers were prepared according to the manufacturing company. Polymerase chain reaction was performed for 30 cycles. After the completion of electrophoresis, the molecules in the gel were viewed by UV-transilluminator (Chuang *et al*., 2006).

3. Results

Table 1: Primers used for the Detection of Virulence Genes in *Klebsiella* Isolates

Markers	Oligonucleotide Sequences 5'- 3'	Size of amplicon	Thermal cycler conditions
*Mag*A- F *Mag*A -R	TAGGACCGTTAATTTGCTTTGT GAATATTCCCACTCCCTCTCC	1282	95°C for 4 min 94°C for 30sec 57°C for 30sec 30 cycles 72°C for 30sec 72°C for 5min
rmpA-F *rmpA*-R	GCAGTTAACTGGACTACCTCTG GTTTACAATTCGGCTAACATTTTTCTTTAAG	553	95°C for 2.5 min 94°C for 30sec 55°C for 1min 30 cycles 72°C for 30sec 72°C for 7min
*rmpA*1-F *rmpA*1-R	CTGTGTCCACATTGGTGGG GATAGTTCACCTCCTCCTCC	448	95°C for 2.5 min 94°C for 30sec 55°C for 1min 30 cycles 72°C for 30sec 72°C for 7min
wcaG- F *wcaG* –R (Turton *et al*., 2010)	GGTTGGKTCAGCAATCGTA ACTATTCCGCCAACTTTTGC	169	95°C for 15 min 94°C for 30sec 58°C for 90 sec 35 cycles 72°C for 90sec 72°C for 10min

One hundred and thirty-eight *Klebsiella* isolates were recovered. Figure 1 shows the distribution of *Klebsiella* isolates in which *K. pneumonia* had the highest frequency followed by K. oxytoca while the least was found in *K. rhinoscleromatis*. All the isolates were multiple antibiotic resistant showing resistance to three or more different classes of antibiotics. All were resistant to piperacillin and susceptible to imipenem, ciprofloxacin and ofloxacin. However, none of the isolates were intermediately resistant to chloramphenicol, pipiracillin and imipenem, while few *Klebsiella* isolates displayed intermediate resistance to augmentin, ceftazidime, streptomycin and other antibiotics used. Table 1 shows the primers of K1, K2 and K5 antigens were used for the typing of *K. pneumoniae* and *K. oxytoca* into K1 group, K2 group, and K5 group. It was found that K1 was amplified in only 13 isolates and K2 in 5 isolates, while K5 was not amplified in any of the isolates. It was seen that the prevalence of K1 isolates was higher than K2 isolates (Figure 5). Among the MAR *Klebsiella* isolates, fourteen (48%) harboured *magA* gene of molecular weight of 1280bp. Ten (34%) of the isolates were amplified for *rmpA* gene with molecular weight of 553bp while 10% and 3% isolate harboured *rmpA1* and *wcaG* gene respectively (Figure 2).

The agarose gel electrophoresis of the amplification product coding for K1 serotype at 1283bp and K2 serotype at 646bp in *K. pneumoniae* and *K. oxytoca* is shown in Figure 3.

Figure 4 shows agarose gel electrophoresis of the amplification product coding for *MagA* gene (1280bp), *rmpA* gene (553bp), *wcaG* gene (169bp) and *rmpA*1 gene (448bp) in selected multiple antibiotic resistant *K. pneumoniae* and *K. oxytoca*.

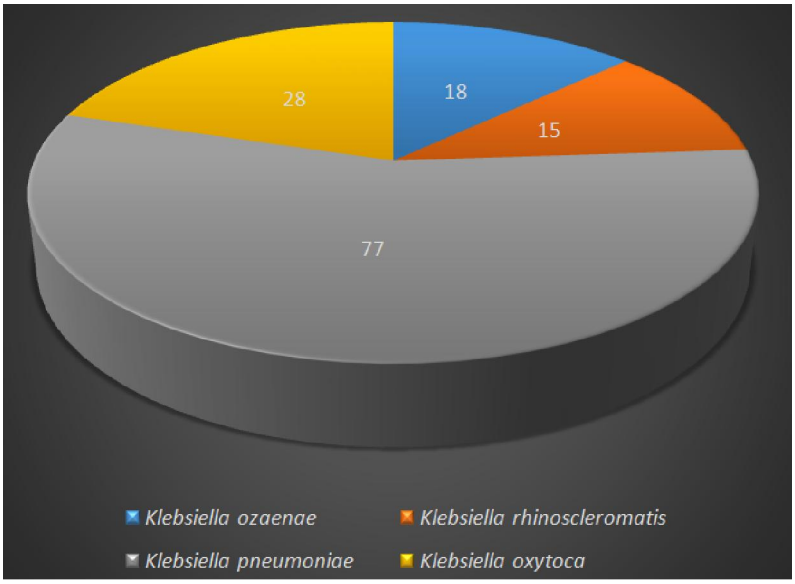

Figure 1: Distribution of *Klebsiella* species

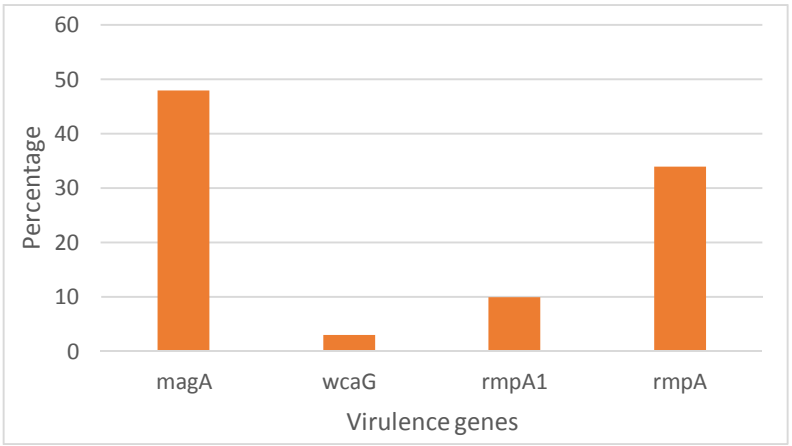

Figure 2: Molecular Detection of Virulence Genes in *Klebsiella* species

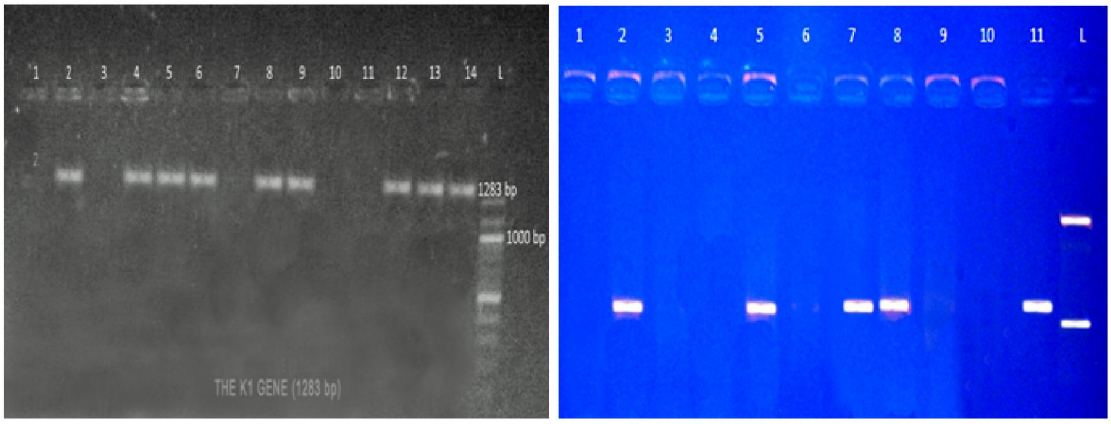

Figure 3: Agarose gel Electrophoresis of the Amplification Product Coding (A.) K1 serotypes (1283bp) (B.) K2 serotypes (646bp) gene in selected multiple antibiotic resistant *K. pneumoniae* and *K. oxytoca*

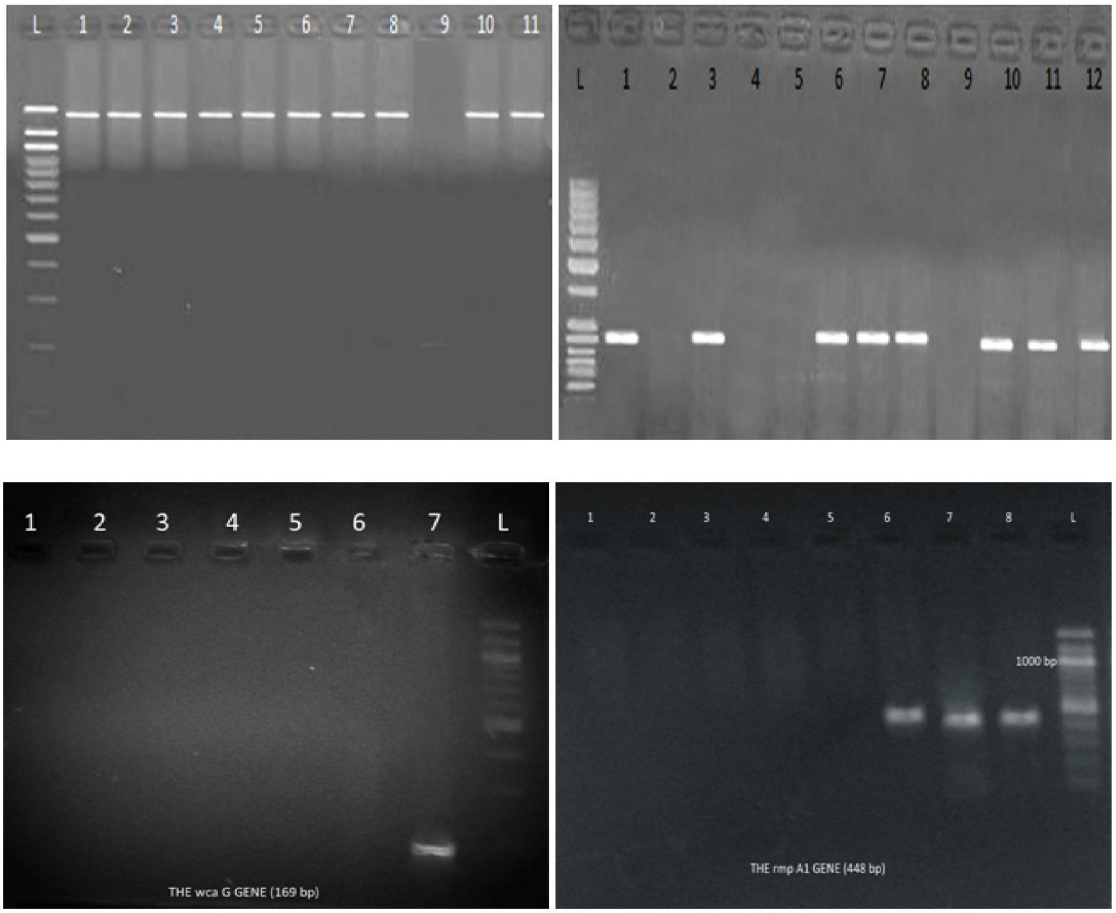

Figure 4: Agarose gel Electrophoresis of the Amplification Product coding (A.) *MagA* gene (1280bp), (B.) *rmpA* gene (553bp), (C.) *wcaG* gene (169bp) and (D.) *rmpA*1 gene (448bp) in selected multiple antibiotic resistant *K. pneumoniae* and *K. oxytoca*

Lane L, molecular size marker expressed in base pairs. Lanes A1-A8, A10-A12 - positive isolates with amplified genes, A9 -negative isolates. Lanes B1, 3, 6-8, 10-12 -positive isolates with amplified genes

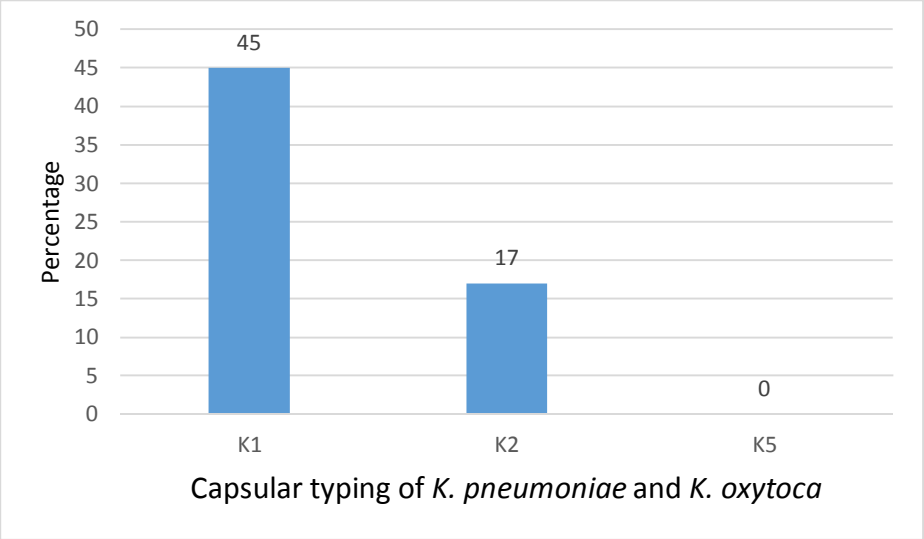

Figure 5: Distribution of Capsular Serotypes in *Klebsiella* isolates

4. Discussions

Infections such as hospital and community acquired infections are caused by *K. pneumoniae* and other species of *Klebsiella*. *K. pneumoniae* infection is often treated with beta-lactam and cephalosporin antibiotics, but beta-lactam and cephalosporin antibiotics are one of the most drug used to combat these infections which are becoming resistant that created a major crisis in medical practice or clinic in the last two decades (Amin *et al*., 2009; Amraie *et al*., 2014)

However, the high percentage of K1 antigens from the isolates maybe highly virulent than others K serotypes (Turton *et al*., 2008). This results is in agreement with (Kyong and Jae, 2008) who found that K1 serotype is striking and that the general prevalence of the K1 serotype is significantly higher.

However, All *K. pneumoniae* revealed mucoid phenotype regardless of genotype. Genotyping of *K. Pneumoniae* and *K. oxytoca* is important to know the prevalence of bacterial genotypes particularly K1 and K2 which are present in our area. Serologically, *K. pneumoniae* belonging to serotypes K1 and K2 which are the most common types that are highly virulent (Hansen *et al*., 2002). According to the results obtained in this study, only eighteen isolates were found to be positive for either K1 or K2 gave an indication that these isolates are highly virulent than the other (Mizuta *et al*., 1983).

This study disagrees with the report of Whitfield and Roberts, (1999) which indicated that K2 was more predominant than K1 in human infections but is very rarely identified in the natural environment. Many studies have shown that *K. pneumoniae* isolates and other species belong to serotypes K1 and K2 which are the most virulent (Jazani *et al*., 2009).

This result is in agreement with Rozalski, (2007) who showed that strain of *K. pneumoniae* with capsule such as (K1, K2) was virulent to human whereas serotypes without capsule are less virulent or without virulence.

The presence of *mag*A gene in *K. pneumoniae* in clinical samples is important. Therefore *mag*A gene is used as a marker for the diagnosis of invasive *K. pneumoniae* infections. These results shows that *mag*A gene can be seen in *K. pneumoniae* capsules with high viscosity as it has the highest frequency. This gene can act as a pathogenicity island and increase the virulence of the bacteria. The presence of this gene in samples without any antibiotic treatment may cause patient's death (Struve *et al*., 2005). The *mag*A is a chromosomal gene which plays an important role in serious infection of *Klebsiella* such as septicemia, bacteremia, and pneumonia as well as lung and liver abscesses (Chan et al., 2005; Chung et al., 2007).

rmpA and *rmpA1* virulence genes were detected by using PCR markers, *rmpA* was present in 10 isolates and absent in nineteen isolates, also *rmpA1* was found in three isolates. *RmpA* gene encodes for the regulation of mucoid phenotype which may be located on bacteria chromosome or on plasmid and so the absence of such genes may be related to where these genes are located in *Klebsiella* genome (Brisse, 2004).

Previous studies had shown that strains carrying *rmp*A were related with the hypermucoviscosity, and had a significant correlation with liver abscess and lung, neck, psoas muscle, or other focal abscess (Amraie *et al*., 2014).

Conclusion

Klebsiella infections are often considered as a paradigm of hospital-acquired infections. The indiscriminate use of antibiotics has revealed a considerable increase in outbreaks caused by organisms resistant to antimicrobial drugs, such as KPC-producing *K. pneumoniae*.

This study has revealed the presence of *magA*, *rmpA* and *wcaG* genes in *Klebsiella* isolates recovered from patients with LRTI and serotypes K1 and K2 detected have not been previously reported in the study area.

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References

1. Amin A, Ghumro PB, Hussain S, Hameed A. Prevalence of antibiotic resistance among clinical isolates of *Klebsiella pneumoniae* isolated from a tertiary care Hospital in Pakistan. Malaysian J Microbiol. 2009;5:81–86.
2. Amraie H, Shakib P, Rouhi S, Bakhshandeh N, Zamanzad B. Prevalence assessment of *mag*A gene and antimicrobial susceptibility of *Klebsiella pneumoniae isolated* from clinical specimens in Shahrekord, Iran. Iran J Microbiol. 2014;6(5): 311–316.
3. Bach S, De Almeida A, Carniel E. The *Yersinia* high-pathogenicity island is present in different members of the family *Enterobacteriaceae*. FEMS Microbiol. Lett. 2000;1:83-28-35.
4. Bauer AW, Kirby WM, Sherris JC, Turch M. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 1966;36(3): 493-496.
5. Brisse S, Issenhuth-Jeanjean S, Grimont PA. Molecular serotyping of *Klebsiella* species isolates by restriction of the amplified capsular antigen gene cluster. J. Clin. Microbiol. 2004;2: 3388–3398.
6. Chan KS, Chen CM, Cheng KC, Hou CC, Lin HJ, Yu WL. Pyogenic liver abscess: A retrospective analysis of 107 patients during a 3-year period. Infect Dis. 2005;58:366–368.
7. Chuang YP, Fang, CT, Lai SY, Chang SC, Wang JT. Genetic determinants of capsular serotype K1 of *K. pneumoniae* causing primary pyogenic liver abscess. *Journal of Infectious Disease* 2006;193: 645-654.
8. Chung DR, Lee SS, Lee HR, Kim HB, Choi HJ, Eom JS, et al. Emerging invasive liver abscess caused by K1 serotype *Klebsiella pneumoniae* in Korea. *J Infect*. 2007;54:578–583.
9. CLSI-Clinical Laboratory Standards Institute. (2013). Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement, CLSI document 2013, vol. 30. Villanova, PV: CLSI; 2013, M100-S20.
10. Fang CT, Chuang YP, Shun CT, Chang SC Wang JT. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. J. Exp. Med. 2004;199:697–705.
11. Fang CT, Lai SY, Yi WC, Hsueh PR, Liu KL, Chang SC. *Klebsiella pneumoniae* genotype K1: an emerging pathogen that causes septic ocular or central nervous system complications from pyogenic liver abscess. *Clin. Infect. Dis.* 2007;45:284–293. doi: 10.1086/519262.
12. Fuursted K, Scholer L, Hansen F, Dam K, Bojer MS, Hammerum AM, Dagnæs-Hansen F, Olsen A, Jasemian Y, Struve C. Virulence of a *K. pneumoniae* strains carrying the New Delhi metallo-beta-lactamase-1 (NDM-1) Microbes Infection 2012;14: 155-158.
13. Hansen DS, Skov R, Benedi JV, Sperling V, Kolmos HJ. *Klebsiella* typing: pulsed-field gel electrophoresis (PFGE) in comparison with O: K-serotyping. Clin. Microbiol. Infect. 2002;8:397–404.
14. Jazani NH, Ghasemnejad- Berenji H, Sadegpoor S. Antibacterial effects of Iranian Menthapulegium essential oil on isolates of *Klebsiella* sp. Pakistan. Journal of Biological Sciences. 2009;12(2):183-185.
15. Kyong RP, Jae HS. Evidence for Clonal Dissemination of the Serotype K1 *Klebsiella pneumonia* Strain Causing Invasive Liver Abscesses in Korea. J. Clinic. Microbiol. 2008;7: 4061–4063.
16. Lin WH, Wang MC, Tseng CC, Ko WC, Wu AB, Zheng PX, Wu JJ. Clinical and Microbiological characteristics of *K. pneumoniae* isolates causing community-acquired urinary tract infections. Infection, 2010;38:459-464.
17. Mizuta K, Ohta M, Mori M, Hasegama T, Nakashima I, Kato N. Virulence for mice of *Klebsiella* strains belonging to the O1 group: relation to their capsule (K) types. Infect. Immun. 1983;40: 56-61.
18. Mohammad SA, Jawad KT, Esraa HK. Molecular characterization of capsular polysaccharide genes of *Klebsiella pneumoniae*

in Iraq. *International Journal of Current Microbiology and Applied Science* 2014;3(7) 224-234.
19. Munoz-Price LS, Quinn, JP. The Spread of *Klebsiella pneumoniae* Carbapenemases: A Tale of Strains, Plasmids, and Transposons. *Clinical Infectious Diseases* 2009;49:1739–1741, doi: 10.1086/648078 (2009).
20. Pan YJ, Fang HC, Yang HC, Lin TL, Hsieh PF, Tsai FC, Keynan Y, Wang JT. Capsular polysaccharide synthesis regions in Klebsiella pneumoniae serotype K57 and a new capsular serotype. J. Clinical Microbiology 2008;46: 7-9.
21. Rahn A, Drummelsmith J, Whitfield C. Conserved organization in the cps gene clusters for expression of *Escherichia coli* group (1) K antigens: relationship to the colanic acid biosynthesis locusand the cps genes from *Klebsiella pneumoniae*. Bacteriol 1999;181: 2307–231.
22. Rozalski A. Potential virulence factors of *Klebsiella pnemouniae* bacilli Microbiol. Mol. Biol. 2007;61:65-89.
23. Sharma R, Sharma CL, Kapoor B. Antibacterial resistance: current problems and possible solutions. Indian J. Med. Sci 2005;59, 120-129.
24. Siu LKK, Huang DB Chiang T. Plasmid transferability of KPC into virulent K2 serotype K. pneumoniae. BMC Infectious Diseases 2014;14: 1-6.
25. Struve C, Bojer M, Nielsen FM, Hansen DS, Krogfelt KA. Investigation of the putative virulence gene *mag*A in a worldwide collection of 495 *Klebsiella* isolates: *mag*A is restricted to the gene cluster of *Klebsiella pneumoniae* capsule serotype K1. J Med Microbiol. 2005;54:1111–1113.
26. Tanwar J, Das S, Fatima Z, Hameed S. Multidrug Resistance: An Emerging Crisis. *Interdisciplinary Perspectives on Infectious Diseases* 2014;7, doi: 10.1155/2014/541340 (2014).
27. Turton JF, Hatice B, Siu LK, Mary EK, Tyrone LP. Evaluation of multiplex PCR for detection of serotypes K1, K2 and K5 in *Klebsiella*spp. and comparison of isolates within these serotypes. Fems Microbiol Lett 2008;284, 247–252.
28. Turton JF, Perry C, Elgohari S, Hampton CV. PCR characterization and typing of *Klebsiella pneumoniae* using capsular type-specific, variable number tandem repeat and virulence gene targets. *J. Med. Microbiol.* 2010;59, 541–547. doi: 10.1099/jmm.0.015198-0.
29. Wasfi R, Elkhatib WF, Ashour HM. Molecular typing and virulence analysis of multidrug resistant *Klebsiella pneumoniae* clinical isolates recovered from Egyptian hospitals. *Sci. Rep.* 2016;6:38929; doi: 10.1038/srep38929 (2016).
30. Whitfield C, Roberts IS. Assembly and regulation of expression of capsules in *Escherichia coli.* Mol. Microbiol. 1999;31:1307–3019.

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