Clinical isolates of *Klebsiella pneumoniae* and its virulence factors from a tertiary care hospital

Kalaivani Ramakrishnan[1*], Jenitha Johnsi P[2], Seetha K.S[3]

[1]Associate Professor, [2]Post Graduate, [3]Professor, Dept. of Microbiology, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth, Deemed to be University, Pillaiyarkuppam, Puducherry, India

***Corresponding Author: Kalaivani Ramakrishnan**
Email: kalaimicro21@gmail.com

Abstract
Introduction: *Klebsiella spp*. is well known hospital as well as community acquired bacterial pathogen. It is prone to cause severe pyogenic infections like bronchopneumonia, UTI, nosocomial infection, wound infection, septicaemia, meningitis, and rarely diarrhoea leading to marked fatality rate. *Klebsiella* spp. primarily attack immunocompromised individuals who are hospitalized and suffering from severe underlying diseases such as diabetes mellitus, chronic pulmonary obstruction and others. By acquiring various enzymes and virulence factors, it worsens patient's life. Thus we aimed to detect various virulence factors among the clinical isolates of *Klebsiella pneumoniae*.
Aim: To study the prevalence of various virulence factors among *Klebsiella pneumoniae* isolates.
Materials and Methods: Total of 115 clinical isolates of *Klebsiella spp*. from various clinical samples during January 2015 to June 2015 were included. Virulence factors includes biofilm formation, lipase, lecithinase, haemagglutination, protease, gelatinase, haemolysis and hypermucoviscosity were detected by standard technique.
Result: Out of 115 isolates, 55% and 45% of *Klebsiella pneumoniae* isolates were from female and male patients. Majority of isolates were isolated from urine samples (67%) followed by 27% exudate, sputum 19% and blood 17%. Among these, 79% showed biofilm formation, 58% haemagglutination and lipase formation, 56% lecithinase, 44% protease, 41% gelatinase production and 7% hypermucoviscosity. More than 3 virulence factors were observed in 93% of isolates.
Conclusion: Presence of multiple virulence factors increases pathogenic ability leading to therapeutic challenges. Among various virulence factors, biofilm formation, lecithinase and phospholipase was found to be the predominant virulence factors among the *Klebsiella pneumoniae* isolates.

Keywords: *Klebsiella pneumoniae*, Virulence factors, Gram negative bacteria, Phenotypic method, Virulence detection.

Introduction
The genus *Klebsiellae* is well known to cause nosocomial life threatening infections which commonly includes catheter associated urinary tract infection (CA-UTI), ventilator associated pneumoniae (VAP), surgical site infection (SSI), and central line associated bloodstream infection (CLABSI). Because of their ability to spread rapidly in the hospital environment, these bacteria tend to cause nosocomial outbreaks.[1] This pathogen is also very frequently isolated bug among community acquired infections.

It is a Gram negative, non-motile, bacillus. The principal pathogenic reservoirs for transmission of this potent pathogen could be from the gastrointestinal tract and also majority might be from hands of hospital personnel or healthcare workers.[2] As an opportunistic pathogen *Klebsiella pneumoniae* primarily attacks patients whose immunity is compromised by underlying illness such as diabetes mellitus, chronic pulmonary obstruction, patient underwent any major surgeries, etc.,.[3] It is also commonly found in the respiratory tract but its clinical significance is difficult to assess in patients whose immunity is compromised by underlying illness.[4,5]

When colonized or infected, not only by producing various beta lactamases enzymes [Extended spectrum beta lactamases (ESBLs), Carbapenemases (KPC producing *Klebsiella pneumoniae*) and AmpC beta-lactamase] it causes pathogenicity. Presence of various virulence genes which codes for virulence factors, allows it to attack immune system of mammalian cells that in turn causes lethal diseases. Some of these virulence factors are haemagglutination, haemolysin production, gelatinase, protease, phospholipase and lecithinase activity, hypermucoviscosity, biofilm formation, iron uptake, lipopolysaccharides formation, capsule synthesis, adhesions, etc.,

All these virulence factors have the potential to cause a wide variety of infectious diseases in patients with hospitalization as well as in community. Thus this study was to identify the various virulence factors among all our *Klebsiella pneumoniae* isolates from various patients.

Materials and Methods
In a tertiary care hospital, Pondicherry, during January 2015 to June 2015, a total of 115 consecutive *klebsiella pneumoniae* clinical isolates, isolated from various clinical samples like, sputum, endotracheal aspirates, pus, tissues, wound swabs, urine, blood and CSF were included in this study. Repeat *klebsiella pneumoniae* isolates from the same patient were excluded. All these *klebsiella pneumoniae* isolates were studied for their presence of various virulence factors phenotypically. The following virulence factors like protease activity, gelatinase, production, lipase production, lecithinase, haemolysin production, hypermucoviscosity and biofilm formation were tested for all our *Klebsiella pneumoniae* isolates by standard methods as follows.

Methodology

Protease Activity

Protease activity assay was carried out to check for the presences of *klebsiella pneumoniae* strains that were able to degrade casein. With molten Nutrient agar (NA), autoclaved Skimmed milk was added with a final concentration of 1.5% and skimmed milk agar pates were made. From each *Klebsiella pneumoniae* clinical isolates single colony was picked up from the primary NA plate and 'spot' inoculated on 1.5% skimmed milk agar. At 37° C plates were incubated for 72 hours. Protease production was identified by formation of clear zone around the colonies.[5-7]

Gelatinase Activity

The ability to degrade gelatin (Gelatinase production) by *klebsiella pneumoniae* isolates were assessed using plate assay method. Nutrient agar plates with 3% gelatine were prepared and the tests isolates were spot inoculated. Inoculated plates were incubated at 37 °C for 16 hours followed by 4°C for 5 hours. Gelatinase producing isolates were identified by demonstrating a zone of turbid halo around the colonies.[8,9]

Lecithinase and Lipase Activity

This assay was carried out to check for the production of phospholipase C (or) lecithinase and lipase. *Klebsiella pneumoniae* isolates grown overnight on nutrient agar were inoculated onto Egg yolk agar and incubated at 37°C. Following 24 hours of incubation, the plates were flooded with- saturated aqueous solution of copper sulphate and were allowed to stand for 20 minutes, excess solution got drained. After few minutes of drying, the interpretation was made. Zone of clearance around the colony was considered as positive for lecithinase production and the greenish blue colour of opalences indicated lipolysis synthesis. [10-12]

Haemolysis

The haemolysis assay was carried out to determine the haemolytic activity of *klebsiella pneumoniae* isolates.[12] Test isolates were inoculated in 5% Sheep blood agar, following overnight incubation at 37°C presence / absence of haemolysis surrounding the colonies were documented.[12,13]

Hypermucoviscosity

Hypermucoviscosity was identified by viscous string test. Formation of string greater than 5mm in length was considered as presence of hypermucoviscosity among the test isolates.[14-16]

Haemagglutination Assays

Using 3% of "O" positive red blood cells, presence /absence of haemagglutinin, (clumping factor) was done in microtitre plates. Mannose sensitivity was identified using 2% mannose sugar.[9,13,17,18]

Biofilm Formation

The ability of bacterial strains to form biofilm was detected using microtitre plate method. In triplets, 200µl of overnight broth culture was transferred onto microtiter plates. Following 24 hours incubation, in each wells 25 µl of 1% crystal violet was added and incubated for 15 minutes. Wells were washed three times with phosphate- buffered saline, and the ethanol was added to dissolve the strain. OD values were taken at 540nm and interpretations were made. [19,20]

Result

Out of 115 *Klebsiella pneumoniae* isolates, 55% (63) of the isolates were isolated from female and 45% (52) were from male patients with various clinical infections. Sample wise distribution of all these 115 isolates showed, 37% were of urinary isolates, 27% from wound swabs, respiratory isolates 19% and blood isolates 17%. Following age wise distribution of isolates from various patients, majority of them were from patients with age between 20-35 years followed by others (Table 1). Among various virulence methods tested, majority of *Klebsiella pneumoniae* isolates (79%) showed biofilm formation, 58% of isolates showed lipase and haemagglutination production, 49% were mannose sensitive, 53% as mannose resistant and 56% showed lecithinase formation followed by others (Table 2). Among these *Klebsiella pneumoniae* test isolates, a maximum of 84% (97) isolates showed more than three phenotypic virulence factors positive and 3% from the total showed only one test positive.

Table 1: Age wise distribution of *Klebsiella pneumoniae*

Age variation	Distribution of isolates based on age of the patients (n=115)
0-19	23 (20%)
20-35	36 (31%)
36-55	29 (25%)
56-65	10 (9%)
More than 66	17 (15%)

Table 2: Distribution of *Klebsiella pneumoniae* isolates positive for various virulence factors

S. No	Phenotypic detection of various virulence factors	*Klebsiella pneumoniae* isolates showed positive virulence factors (%) (n=115)
1.	Biofilm	79.1% (91)
2.	Lipase	58.2% (67)
3.	Haemagglutination	58.3% (67)
4.	Lecithinase	55.7% (64)
5.	Protease	44.3% (51)
6.	Gelatinase	40.9% (47)
7.	Haemolysis	22.6% (26)
8.	Hypermucoviscosity	6.9% (8)

Discussion

In addition to various drug resistant mechanisms, *Klebsiella pneumoniae* possess various virulence factors which are proven to initiate greater lethal effects. It proportionally leads to high morbidity among infected population. Detection of various virulence factors plays important tool to identify the virulence potential of the pathogen. Biofilm formation was observed from majority (79%) of this study isolates. Recently, 100% biofilm formation was documented with *Klebsiella pneumoniae* isolates which was very close

to our findings.[21] Similarly, Gharrah et al., reported non-biofilm production only in one ESBL producing and in two non ESBL producing *Klebsiella pneumoniae* isolates out of their 100 study isolates.[22]

The enzyme lipase digests the host cellular lipid for its nutritional demand. Lecithinase and phospholipase are enzymes released by bacteria which have the ability to destroy host tissues. In contrast to our isolates (58%), Gharrah et al., also encountered 6% and 10% lipase production among their ESBL and non-ESBL producers.[22] Close to our findings, recently, Alam et al., from Lucknow reported 76.9% lipase formation.[23] Lecithinase production among *Klebsiella pneumoniae* was found to be directly associated with skin and soft tissue infections and systemic infections. A study done by Singh et al., with 208 *Klebsiella pneumoniae* isolates, 16 (7.69%) was found to produce lecithinase activity.[24] Interestingly our 55.7% (64) *Klebsiella pneumoniae* isolates showed Lecithinase production which might predict more pathogenic ability among these isolates. None of the *Klebsiella* isolates from Brazil reported lecithinase production.[25]

Haemaggutination was observed among 58% of this current study *Klebsiella pneumoniae* isolates. Among the two types of most common fimbrial and nonfimbrial adhesins (hemagglutinins) produced by *Klebsiella pneumoniae*, mannose sensitive (MS) type 1 was observed among 49% and mannose-resistant (MR) type 3 was notice in 53% of our stains by microtitre plate method. Strains with MS type of fimbriae were known to be more pathogenic and isolates with type 3(MR) were known to bind human endothelial cells, epithelial cells of genitourinary and respiratory system.[26,27] A study by Sahly H et al., showed that out of their 152 non-ESBL producing strains 32.8% showed type I or type 3 fimbriae and only 17.2% of ESBL producing *Klebsiella* isolates did so.[28] Stahlhut S.G. et al concluded that for characterization of virulence factors and epidemiological analysis, detection of fimbrial expression is significant. [29]

Enzyme gelatinase hydrolyses gelatin and has the ability to hydrolyse collagen in subcutaneous tissue during wound infections.[9] In the present study, protease and gelatinase production was observed among 44% and 41%. Study by Taher et al., with 22 *Klebsiella* isolates, reported very significant percentage of protease production, which was less similar to our findings.[30] Very less similar to our findings, isolates from Alam M et al., from Uttar Pradesh documented 24% and 84% protease production, 20% and 58% gelatinase formation.[23]

Pereira et al., reported that none of their klebsiella isolates showed haemolytic activity.[25] But in contrast, a significant percentage of our isolates showed haemolytic activity. *Klebsiella pneumoniae* strains producing hypermucoviscosity were known to cause abscess formation in distant sites especially in brain, eyes, liver, poor visual outcome, retinal dysfunction and secondary bacteraemia. *K. pneumoniae* strains with hypermucoviscosity were considered as hypervirulent.[31,32]

Regarding hypermucoviscosity Gharrah et al., documented highest of 62% among non-ESBL producers when compared to ESBL producers (4%).[22] Another study from Brazil reported 6.7% hypermucoviscous phenotypic *Klebsiella pneumoniae* isolates which was very less similar to our findings.[25] Study by Ahmed Abduljabbar et al., documented 62.5% presence of hypermucoviscosity among their *Klebsiella pneumoniae* along with capsule formation.[21] In contrast, only 7% of our *K. pneumoniae* isolates were found to produce hypermucoviscosity.

Conclusion

The expression of virulence factor in multidrug resistance *Klebsiella pneumoniae* isolates can cause various life threatening infections. Majority of our isolates showed presence of multiple virulence factors. Among *Klebsiella pneumoniae* isolates biofilm formation was found to be the predominant virulence factor. Majority of them showed lipase, haemagglutination, lecithinase and protease formation. Detection of virulence factors might help in management process, as presence of more virulence factors increases pathogenic ability leading to therapeutic challenge.

Conflict of Interest: None.

References

1. Williams, P., and J. M. Tomas. The pathogenicity of *Klebsiella pneumoniae*. *Rev Med Microbiol* 1990;1:196–204.
2. Montgomerie, J. Z. Epidemiology of *Klebsiella* and hospital-associated infections. *Rev Infect Dis* 1979;1:736–53.
3. Podschun, R., and U. Ullmann. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* 1998;11:589–603.
4. Kaul S, Brahmadathan KN, Jagananati M, Sudarsanam TD, Pitchamuthu K, Abraham OC, John G. One Year Trends in the Gram Negative Bacteria antibiotic susceptibility patterns in a Medical Intensive care unit in South India. *Indian J Med Microbiol* 2007;25:230-5.
5. Podschun R, Pietsch S, Holler C, Ullmann U. Incidence of *Klebsiella species* in surface waters and their expression of virulence factors. *Appl Environ Microbio* 2001;67(7):3325-3327.
6. Nehad A, Taher, Batool A. A. Baqir, Fatima. R Abdul. A Study on the Effect of Ethidium Bromide on Virulence Factors (Protease and Biofilm Formation) by *Klebsiella Pneamoniae* isolated from Different Clinical Sources. *Historical Res Lett* 2016;(30):1-6.
7. Alam M, Imran M. Screening and Potential of Gram Negative Bacterial Isolates for their Extracellular Enzymatic Activities Isolated from the Hospital Aquatic Environment. *J Basic Clin Pharma* 2018;9:41-5.
8. Aneja KR. Experiments in Microbiology Plant Pathology and Biotechnology. New Age International Pvt. Ltd., Publishers, New Delhi, India 2003. 4th edn.
9. Cevahir N, Demir M, Kaleli I, Gurbuz M, Tikvesli S. Evaluation of biofilm production, gelatinase activity, and mannose resistant hemagglutination in *Acinetobacter baumannii* strains. *J Microbiol and Imunnol Infect* 2008;41(6):513-8.
10. Esselmann TM, Liu PV. Lecithinase production by Gram negative bacteria. *J Bacteriol* 1961;81(6):939-45.

11. Cappuccino JG, Sherman N. Microbiology - A laboratory manual 1996;159-201.
12. Panus. E, Chifiriuc M. B., Bucur M. Virulence, pathogenicity, antibiotic resistance and plasmid profile of *Escherichia coli* strains isolated from drinking and recreational waters, in 17th European Congress of Clinical Microbiology and Infectious Diseases and 25th International Congress of Chemotherapy, 2008.
13. Mishra M, Thakar Y S, Pathak A A. Haemagglutination, haemolysin production and serum resistance of Proteus and related species isolated from clinical sources. *Indian J Med Microbiol* 2001;19:5-11.
14. Hagiya H, Watanabe N, Maki M, Murase T, Otsuka F. Clinical utility of string test as a screening method for hypermucoviscosity-phenotype *Klebsiella pneumoniae*. *Acute Med Surg* 2014;1(4):245-6.
15. Lee HC, Chuang YC, Yu WL, Lee NY, Chang CM, Ko NY, et al. Clinical implications of hypermucoviscosity phenotype in *Klebsiella pneumoniae* isolates: association with invasive syndrome in patients with community-acquired bacteraemia. *J Intern Med* 2006;259:606–14.
16. Grange JM. Mycobacteria and human disease. Edward Arnod. *London* 1988;67(12).
17. Vagarali M., Karadesai S., Patil C., Metgud S., Mutnal M. Haemagglutination and siderophore production as the urovirulence markers of uropathogenic *Escherichia coli*. *Indian J Med Microbiol* 2008;26(1):68–70.
18. Vandekerchove D., Vandemaele F., Adriaensen C. Virulence-associated traits in avian *Escherichia coli*: comparison between isolates from colibacillosis-affected and clinically healthy layer flocks. *Vet Microbiol* 2005;108:1-2:75–87.
19. Stepanovic S, Vukovic D, Dakic I, Savic B, and Svabio-Vlahovic M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods* 2000;40(2),175–9.
20. Nabajit Deka. Comparison of Tissue Culture plate method, Tube Method and Congo Red Agar Method for the detection of biofilm formation by Coagulase Negative Staphylococcus isolated from Non-clinical Isolates. *Int J Curr Microbiol App Sci* 2014:3(10);810-5.
21. Ahmed Abduljabbar Jaloob Aljanaby, Aaa Hassan Abdulhusain Alhasani. Virulence factors and antibiotic susceptibility patterns of multidrug resistance Klebsiella pneumoniae isolated from different clinical infections. *Afr J Micobiol Res* 2016:10(22);829-43.
22. Mustafa Muhammad Gharrah, Areej Mostafa El-Mahdy, and Rasha Fathy Barwa. Association between Virulence Factors and Extended Spectrum Beta-Lactamase Producing *Klebsiella pneumoniae* Compared to Nonproducing Isolates. Interdisciplinary Perspectives on Infectious Diseases 2017;1-14.
23. Alam M and Imran M. Screening and Potential of Gram Negative Bacterial Isolates for their Extracellular Enzymatic Activities Isolated from the Hospital Aquatic Environment. *J Basic Clin Pharm* 2018;9(1):41-5.
24. Singh B.R., Sharma V.D., Chandra R. Detection, prevalence, purification and characterization of lecithinase of *Klebsiella pneumoniae*. *Indian J Exp Biol* 1999(37):925-32.
25. Pereira S.C.L., Vanetti M.C.D. Potential virulence of *Klebsiella sp*. isolates from enteral diets. *Braz J Med Biol Res* 2015;48(9):782-9.
26. Hornick, D. B., B. L. Allen, M. A. Horn, and S. Clegg. 1991. Fimbrial types among respiratory isolates belonging to the family *Enterobacteriaceae*. *J Clin Microbiol* 1991;29:1795-1800.
27. Maayan M C., I Ofek, O. Medalia, M. Aronson. Population shift in mannose-specific fimbriated phase of *Klebsiella pneumoniae* during experimental urinary tract infection in mice. *Infect Immun* 1985;49:785–9.
28. Sahly H, Navon-Venezia S, Roesler L Hay. A, Carmeli. Y, Podschun R, Hennequin. C, Forestier. C, I. Ofek. Extended-Spectrum _-Lactamase Production Is Associated with an Increase in Cell Invasion and Expression of Fimbrial Adhesins in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 2008;3029–4.
29. Steen G. Stahlhut, Carsten Struve and Karen A. Krogfelt Klebsiella pneumoniae type 3 fimbriae agglutinate yeast in a mannose-resistant manner. J Med Microbiol 2012;61:317–22.
30. Taher N.A., Baqir B.A.A, Abdul F.R.A study A Study on the Effect of Ethidium Bromide on Virulence Factors (Protease and Biofilm Formation) by *Klebsiella Pneamoniae* isolated from Different Clinical Sources. *Historical Res Lett* 2016;30:1-6.
31. Brandt J. Wiskur, Jonathan J. Hunt, and Michelle C. Callegan. Hypermucoviscosity as a virulence factor in experimental *klebsiella pneumoniae* endophthalmitis. *IOVS* 2008;49(11).
32. Juan Carlos Catalán-Nájera, Ulises Garza-Ramos & Humberto Barrios- Camacho. Hypervirulence and hypermucoviscosity: Two different but complementary *Klebsiella* spp. phenotypes?, *Virulence* 2017;8(7):1111-23.

How to cite this article: Kalaivani Ramakrishnan, Jenitha Johnsi P, Seetha K.S. Clinical isolates of Klebsiella pneumoniae and its virulence factors from a tertiary care hospital. *Indian J Microbiol Res* 2019;6(2):109-12.