

Biofilm formation and extended spectrum *beta-lactamase* production in *Klebsiella pneumoniae* isolates from respiratory samples in a tertiary care hospital

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Abstract

Nosocomial and community acquired infections are being caused by *Klebsiella pneumoniae* (*K. pneumoniae*) worldwide. *K. pneumoniae* among gram negative organisms is an important bacterium responsible for site specific infection when they grow as biofilm. Survival of *K. pneumoniae* is facilitated by biofilm formation that makes it easier to multiply and spread to various sites. Extended-spectrum beta-lactamase (ESBL) producing *K. pneumoniae* isolates from respiratory samples were used to know the occurrence of biofilm formation conducted in a tertiary care medical college hospital in Mangalore, Dakshina Kannada District, Karnataka, India.

To know the occurrence of biofilm formation among extended-spectrum beta-lactamase (ESBL) producing *K. pneumoniae* isolates from respiratory samples a prospective study has been conducted in a tertiary care medical college hospital in Mangalore, Dakshina Kannada District, Karnataka, India. A total of 87 *K. pneumoniae* isolates from respiratory samples were characterized according to standard microbiological specific procedures. Screening was done for *K. pneumoniae* Biofilm formation in terms of their antibiotic sensitivity pattern by using standard Kirby Bauer disc diffusion method and presumptive ESBL production by double disk synergy test (DDST). Out of 87 clinical isolates from respiratory samples, 45 (51.72%) were found to be biofilm producers. 29 (33.33%) isolates were ESBL producers and all them produced biofilm. ESBL forming *K. pneumoniae* isolates had a significantly greater capacity to form strong biofilm (72.4%) than non ESBL producing *K. pneumoniae* isolates (27.58%).

Keywords: *Klebsiella pneumoniae*, Nosocomial infections, ESBL production, Kirby-Bauer, Biofilm formation.

Introduction

Hospital and community acquired infections are usually attributed to gram negative organisms mainly *K. pneumoniae* which is being the predominant bacteria involved in these infections with a increased tendency to form biofilm.¹ Nosocomial pathogens *K. pneumoniae* is one among the eight known bacteria to cause various infections with ability to form biofilm.² With the formation of biofilm, there is association of pathogenicity and chronicity of infections caused by this species.³ The ability of *K. pneumoniae* to form biofilm can be assessed by various methods such as tissue culture plate method, test tube method and congo red agar method.⁴

K. pneumoniae showing antibiotic resistance is a cause of concern because of ESBL and Carbapenemase producing bacterial strains are being isolated frequently from various part of the world.⁵ ESBLs are beta lactamase enzymes that hydrolyze beta lactam ring containing antibiotics such as penicillins and broad-spectrum cephalosporins. ESBL production could be tested making use of Double Disk Synergy Test (DDST) which is a cost effective laboratory diagnostic method. Studies have shown that there is an association between biofilm formation and production of ESBL in *K. pneumoniae* isolates from various clinical samples.⁶ The present study was conducted to know the local antibiotic resistance profile, ESBL production and biofilm formation among *K. pneumoniae* isolated from respiratory tract samples such as sputum, Bronchoalveolar Lavage (BAL) and Endotracheal

aspirate (ET) in a tertiary care hospital, Mangalore, Dakshina Kannada District, Karnataka.

Materials and Methods

Phenotypic identification *K. pneumoniae* from respiratory samples: A prospective study was conducted in the Department of Microbiology, Yenepoya Medical College and Hospital, Mangalore, Karnataka, India. A total of 87 isolates of *K. pneumoniae* obtained from various respiratory samples such as sputum, BAL and ETA were included in this study. Sputum samples quality was graded according to Bartlett's criterion.⁷ Clinical samples were inoculated on Mac Conkey's agar and 5% sheep blood agar and incubated overnight at 37°C. Agar plates were processed and identified to accesses bacterial colony growth based on criteria of morphology and biochemical reactions using standard microbiological tests.⁸ Isolates of *K. pneumoniae* isolates that were obtained as a pure and predominant growth from the above samples were included in this study.

Antibiotic susceptibility testing: As per Clinical Laboratory Institute (CLSI) guideline, antibiotic susceptibility testing was done by using Kirby Bauer's disc diffusion method using Mueller- Hinton agar (MHA) plates conventionally.⁹ By using a suspension of *K. pneumoniae* adjusted to 0.5 McFarland turbidity standards, (1×10^8 cfu/ml) MHA plates were inoculated. The antimicrobial disks tested were ceftazidime (30µg), cefotaxime (30µg), ampicillin ((10µg), amikacin (30µg)

cefpodoxime (10µg), ciprofloxacin (5µg), netilmicin (30µg), piperacillin (100 µg), piperacillin-tazobactam (100/10µg), amoxicillin-clavulanic acid (20/10µg) and imipenem (10 µg). The plates were incubated overnight at 37°C. The zones of inhibition were measured and interpreted comparing with the standard measurement chart.

Detection of ESBL production by Double disk synergy test: Bacterium (*K. pneumoniae* isolates) that has showed resistance to III generation cephalosporins were tested for ESBL production by double disk synergy test (DDST) in accordance with CLSI guidelines.¹⁰ The disk containing amoxicillin – clavulanic acid was placed at the centre of the lawn culture made on Muller Hinton Agar (MHA) plate inoculated with each of the test isolates of *K. pneumoniae* found to be resistance towards any one or all the antibiotic disks of ceftazidime, cefotaxime and cefpodoxime. The discs of ceftazidime, cefotaxime and cefpodoxime each having a disc concentration of 30µg were placed central disc of amoxicillin – clavulanic with a distance of 30mm. The plate was incubated at 37°C for 24 hrs.

ESBL producer was considered in case of any enhancement of zone of inhibition between any one of the cephalosporin disks with the central disk the isolate.

Detection of biofilm formation: As described by Christensen et al., the tissue culture plate (TCP) assay was performed for the assessment of biofilm formation.¹¹ On an overnight culture, Trypticase Soy Broth (TSB) (10ml) containing 1% glucose was inoculated with a loopful of *K. pneumoniae* and was incubated at 37°C for 24 hours. This culture was later diluted with 1:100 with fresh medium. Those wells in the TCPs were later filled with 0.2ml of the diluted cultures individually and were later incubated at 37°C for 24 h. The contents were emptied after incubation and later the plates were tapped gently. By using phosphate buffer saline, the wells were washed in order to remove any free floating bacteria. Those biofilms which remained adherent to walls and as well as to the bottoms of the wells, were later fixed with 2% sodium acetate and stained with 0.1% crystal violet. De-ionised water was used to wash excess of strains followed by drying of the same. By ELISA method reader at wave length 570 nm, optical densities (OD) of stained adherent biofilms were obtained. Table 1 shows the criteria for interpretation of OD values.

Result and Discussion

This study included 87 isolates of *K. pneumoniae* isolated from various respiratory tract samples, out of which 76 (87.36%) were isolated from sputum samples. Table 2 shows the distribution of *K. pneumoniae* across various respiratory tract samples. Antibiotic sensitivity testing by Kirby Bauer disc diffusion test revealed high rates of resistance to ciprofloxacin, whereas the

bacterial isolates were sensitive to amoxicillin-clavulanic acid, piperacillin-tazobactam and imipenem. Table 3 represents the antibiogram of *K. pneumoniae* isolates. Among 87 isolates of *K. pneumoniae* 31 and 37 isolates were resistant to ceftazidime and cefotaxime respectively. DDST confirmed ESBL production among 29 (33.33%) of these isolates. 45 (51.72%) of the isolates of *K. pneumoniae* were biofilm producers. Among the ESBL producers 21 (72.41%) were strong biofilm producers and 12 (27.6%) were moderate biofilm producers. Among the 58 non-ESBL producers 9 (15.51%), 7 (12.06%) and 42 (72.4%) isolates were strong, moderate and non-biofilm producers respectively. Fig. 1 shows TCP assay for detection of biofilm formation by *K. pneumoniae* isolates cultured from the respiratory tract samples.

Biofilm formation is a major virulence factor of *K. pneumoniae* and its role in several infections is well documented. Bacteria isolated from biofilms are known to exhibit higher levels of antibiotic resistance as compared to free growing bacteria. This resistance could be attributed to several factors such as: restricted penetration of the antibiotics into biofilms, expression drug resistance genes and decreased growth rate of bacteria.¹² In our study all the ESBL producing isolates of *K. pneumoniae* were strong biofilm producers which closely correlates with the findings of Lathamani et al., who reported biofilm production among 75.26% of the ESBL producing *K. pneumoniae* isolates included in their study. A study conducted by Pramodhini et al showed biofilm production among 55.9% of the isolates, whereas a study by Thiagarajan et al reported that 50% of the strains of *K. pneumoniae* were biofilm producers.^{13, 14} Whereas the study conducted by Ruchi et al has reported biofilm formation only among 18% of the strains of *K. pneumoniae*.¹⁵ This variation could be attributed to factors such as the type of hospital in which the study was conducted and the local antibiotic prescription practices etc. Our study highlights the prevalence of ESBL-producing *K. pneumoniae* in a tertiary care hospital in Mangalore.

Table 1: Interpretation of OD values for assessment of biofilm formation

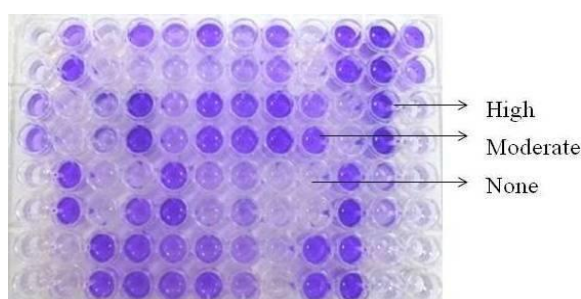
Mean OD values	Adherence	Biofilm formation
<0.120	None	None/Weak
0.120-0.240	Moderate	Moderate
>0.240	Strong	High

Table 2: Clinical source of *K. pneumoniae* isolates (N=87)

S.No	Respiratory Samples	n (%)
1	Sputum	76 (87.36)
2	Bronchoalveolar lavage (BAL)	5 (5.75)
3	Endotracheal tip (ET) aspirate	6 (6.89)

Table 3: The antibiotic susceptibility pattern of clinical isolates of *K. pneumoniae* (N=87)

Antibiotics	Sensitive n (%)	Intermediate n (%)	Resistant n (%)
Ampicillin	0	0	87 (100)
Amikacin	63 (72.41)	3 (3.45)	21 (24.14)
Ceftazidime	52 (59.77)	4 (4.59)	31 (35.63)
Cefotaxime	48 (55.17)	2 (2.29)	37 (42.53)
Cefpodoxime	51 (58.62)	3 (3.45)	33 (37.93)
Ciprofloxacin	24 (27.59)	2 (2.29)	61 (70.11)
Netilmicin	61 (70.11)	2 (2.29)	24 (27.59)
Piperacillin	58 (66.67)	1 (1.15)	28 (32.18)
Piperacillin-tazobactam	72 (82.76)	1 (1.15)	14 (16.09)
Amoxicillin-clavulanic acid	70 (80.46)	2 (2.29)	15 (17.24)
Imipenem	78 (89.66)	1 (1.15)	7 (8.06)

**Fig. 1: TCP assay showing occurrence of biofilm among *K. pneumoniae***

Conclusion

Pathogenic gram negative bacilli of family *Enterobacteriaceae* have been responsible for infections and multidrug resistance worldwide including India. Our study showed that many of the *K. pneumoniae* isolates from respiratory tract samples showed high antibiotic resistance for third generation Cephalosporins. There was strong biofilm formation by ESBL producing *K. pneumoniae*. Routine detection of ESBL-producing microorganisms is required to be performed by every laboratory using standard detection methods, so as to detect and control the spread of these infections. This also helps in prescribing appropriate therapeutic strategies. For the detection of ESBLs in a routine laboratory, the DDST is a simple, sensitive, and inexpensive test. However, there is a need to emphasize on the rational use of antimicrobials and strictly adhere to the concept of “reserve drugs” to minimize the misuse of available antimicrobials especially Carbapenems. This along with antimicrobial susceptibility surveillance and stringent infection control polices will go a long way in containing the spread of multidrug resistant bacteria in the hospitals and the community.

References

1. Vuotto C, Longo F, Balice MP, Donelli G, Varaldo PE. Antibiotic resistance related to biofilm formation in *K. pneumoniae*. *Pathogens* 2014;3:743-758.

2. Chhibber S, Nagand D, Bansal S. Inhibiting biofilm formation by *Klebsiella Pneumoniae* B5055 using an iron antagonizing molecule and a bacteriophage. *BMC Microbiology* 2013;13:174-81.
3. Azzopardi EA, Ferguson EL, Thomas DW. The enhanced permeability retention effect: a new paradigm for drug targeting in infection. *J Antimicrob Chemother* 2013;68(2):257-74.
4. Stepanovic S, Vukovic D, Dakic I, Savic B, Svabio-Vlahovic M. A modified microtiter-plate test for quantification of Staphylococcal biofilm formation. *J Microbiol Methods* 2000;40:175-179.
5. Low YM, Yap PS, Abdul Jabar K, Ponnampalavanar S, Karunakaran R, Velayuthan R, et al., The emergence of carbapenem resistant *Klebsiella pneumoniae* in Malaysia: correlation between microbiological trends with host characteristics and clinical factors. *Antimicrob Resist Infect Control* 2017;6:5-6.
6. Lathamani K, Kotigadde S. Biofilm Formation and its Correlation with ESBL Production in *Klebsiella pneumoniae* Isolated from a Tertiary Care Hospital. *International J Sci Res* 2016;5 (2):1059-62.
7. Bartett, R.C.1974. *Medical microbiology: quality cost and clinical relevance*, p.24-31. John Wiley and sons, Inc., New York.
8. Washington Jr W, Stephan A, William J. Eds., *Koneman's Color Atlas and Text book of Diagnostic Microbiology*, Lippincott Williams & Wilkins, 6th edition, 2006.
9. Clinical Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Twenty fifth ed. CLSI document M100S Wayne, PA: CLSI; 2015 CLSI (Kirby bauer)
10. Tsering DC, Das S, Adhiakari L, Pal R, Singh SK. Extended Spectrum Beta-lactamase Detection in Gram-negative Bacilli of Nosocomial Origin. *J Glob Infect Dis*. 2009;1:87-92.
11. Christensen GD, Simpson WA, Younger JA, Baddour LM, Barrett FF, Melton DM, et al. Adherence of coagulase negative Staphylococci to plastic tissue cultures: A quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol* 1985;22:996-1006.
12. Mathur, S Singhal, S Khan, D J Upadhyay, T Fatma, A Rattan. Detection of biofilm formation among the clinical isolates of *Staphylococci*: An evaluation of three different screening methods. *Indian Journal of Medical Microbiology* 2006;24:25-29.

13. Pramodhini Subramaniaer, S Umadevi, Shailesh Kumar, Selvaraj Stephen. Determination of correlation between biofilm and ESBL producers of Enterobacteriaceae. *Scholar's Research Journal* 2012;2:2-6.
14. Thiyagarajan Santhanamari, Jamal Alruwailiand Sathish Kumar. In vitroinhibition of ESBL positive Multidrug resisting Uropathogenicbacteria using *Coleus forskohlii*. *Int. J.Curr. Microbiol. App. Sci* 2014;3:431-444
15. Ruchi A Tayal, Sujata M Baveja, Anuradha S De. Analysis of biofilm formation and antibiotic susceptibility pattern of uropathogens in patients admitted in a tertiary care hospital. *International Journal of Health and Allied Sciences* 2015;4:247-252.