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Research Article

DETECTION OF *MRK* D GENE AND ANTIBIOGRAM OF BIOFILM PRODUCING *KLEBSIELLA PNEUMONIAE* FROM VARIOUS INDWELLING DEVICES

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Abstract- Indwelling medical devices are most vulnerable to microbial colonization. As a result, Device Related Infection has emerged as a new challenge in the medical research. In the present investigation total 414 multiple drug resistance gram negative bacilli were isolated in which 27.5% were associated with indwelling medical devices. 35% found to be *K. pneumoniae* isolates out of which 23 were detected as biofilm producing. Tracheostomy tips showed the presence of maximum number of *K. pneumoniae*. Highest sensitivity in biofilm producing strains was observed in Colistin followed by Tigecycline. Further, MrkD gene was also detected which is responsible for biofilm formation.

Keywords- *Biofilm, Fimbrae, Indwelling devices, MDR, MrkD*

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Introduction

Biofilms are structured layer of bacterial communities' adherent to abiotic or biotic surfaces enclosed within a self-produced exopolysaccharide matrix. Bacteria producing biofilms are responsible for antibiotic resistance due to restricted penetration of antibiotics into biofilm and expression of resistant genes, due to these causes Indwelling Medical Devices (IMDs) are most vulnerable to biofilm producing microbial colonizers [1]. Scant literature available in Indian subcontinent on indiscriminate use of antimicrobial agents and the superimposed complex nature of bacteria in biofilms colonizing Indwelling Medical Devices, have resulted in phenomenon of resistant device related infections (DRIs) [2].

Klebsiella pneumoniae is not only naturally present at low concentrations in the environment, but also in the gastrointestinal tract and natural cavities of humans and important opportunistic saprophytic pathogen which forms aggregates called "Biofilms" [3]. *K. pneumoniae* frequently causes urinary tract infections, septicemia, or pneumonia in immune compromised individuals. In fact, its considerable efficiency in colonization, accompanied by acquired resistance to antibiotics, has enabled *K. pneumoniae* to persist, grow and spread rapidly in healthcare settings.

The Infections caused due to this organism are severely devastating as the mortality rate is between 25% and 60%. Most clinical isolates of *K. pneumoniae* express Type-3 fimbrae which are known to promote biofilm formation both on biotic and abiotic surfaces [4]. The fimbrae are encoded by five genes in the same transcriptional order *Mrk* ABCDF and belong to the chaperone usher class of fimbrae. This operon comprising *Mrk* is known to produce the polypeptides required for assembly of structure to the surface of the bacterium and also produces *Mrk*D adhesin which is responsible to mediate adhesion to the collagen structures [3].

In the present study biofilm formation by *K. pneumoniae* in the various indwelling devices evaluated by Tube adherence and Microtitre plate methods. In addition, the presence of type 3 pili–encoding *Mrk*D gene fimbriae also investigated which is an important virulence factor for *K. pneumoniae*.

Materials and Methods

The present study was conducted after the clearance of Institutional Ethical committee and Research Committee of Adesh University, Bathinda (Punjab). A total 414 Gram negative bacilli were isolated, out of the device related and non-device related were differentiated, identified and were subjected to antibiotic susceptibility testing by Kirby Bauer disc diffusion method on Mueller Hinton Agar (MHA) and the zones were interpreted as per CLSI guidelines [5]. Out of these device related *K. pneumoniae* were then further investigated for Biofilm production.

Detection of Biofilm

Tube Adherence method (TA)

The quantitative assay for Biofilm formation was performed according to the method described earlier [6]. Glass tubes were filled with 3 ml of Brain Heart Infusion medium (Hi media, Mumbai) which were inoculated with a loopful of a pure culture of a strains of *K. pneumoniae* which was grown overnight from blood agar plate. After 48 hours of incubation at 37°C, the content of each tube was decanted. The tubes were then stained with 0.1% crystal violet for 8 min, followed by washing with phosphate buffer saline pH 7.2 for 5 min. A positive result was indicated by the presence of an adherent film of stained material. Slime production was not regarded as an indicative for the liquid-air interface. Tubes containing BHI only were included in the test as negative controls.

Microtitre plate (MTP) Method

Organism was isolated from fresh agar plates and inoculated in Brain Heart Infusion broth for 24 hours. The cultures were diluted 1:100 with fresh BHI broth. Wells of a sterile 96-well flat-bottomed plastic tissue culture plate (Genaxy) were filled with 200 µl of bacterial suspension in Brain Heart Infusion broth and incubated at 37°C for 24 hours. Negative control wells contained broth only. The plates were covered and incubated aerobically for 24 hours at 37°C. Then the content of each well was washed three times with 200 µl of PBS with pH 7.2 to remove free floating bacteria.

Biofilm formed by bacteria adherent to the wells were fixed with 2% sodium acetate and stained by 0.1% crystal violet. Excess stain was washed by deionized water and later air dried. The absorbance of each well was measured at 570 nm using ELISA reader. The experiment was done in triplicates [7].

For the purpose of comparative analysis of test results, the adherence capabilities of the test strains were classified according to [8]. Strains were classified as follows:

O.D value of Biofilm Formation	
Less than 0.120	Non Biofilm producer
In the range of 0.120 – 0.240	Moderate Biofilm Producer
Greater than 0.240	Strong Biofilm producer

Detection of Type-III Pili encoding gene

Total 15 strains were selected randomly which were positive by both TA and MTP method for biofilm production and tested for the presence of *Mrk* D gene. The DNA was isolated manually by lab standardized protocol. Bacteria were grown overnight in blood agar (BA) medium and was inoculated in Brain heart infusion (BHI) broth and incubated at 37 °C for 24 hrs. The bacterial growth was suspended in eppendorf and after centrifugation at 8000 rpm the pellet was suspended in 10 mM Tris EDTA with addition of lysozyme (20 mg/ml) and 10% SDS. The mixture was mixed vigorously for 2 min followed by addition of Proteinase K (10 mg/ml) and further incubated for 1-3 hrs at 56°C. If cells were not lysed incubation was proceeded overnight. After the addition of 5M NaCl, it was mixed well and incubated at 65°C for 10 min. Then Chloroform: Isoamyl alcohol (24:1) was added after cooling of the tubes and mixed well for 1 min. followed by centrifugation at 5000 rpm for 10 min at 25°C. The aqueous phase was transferred into fresh eppendorf and PCI (25:24:1) was added. After proper mixing the final volume was centrifuged at 5000 rpm for 10 min and aqueous phase was transferred to fresh tube. After the addition of 0.6 volumes isopropanol, the eppendorfs were incubated at -20 °C for 2 hrs or overnight followed by centrifugation at 8000 rpm for 5 min and washed the DNA pellet with 70% ethanol twice and after evaporation of alcohol the DNA was suspended in 10mM TE buffer.

PCR amplification of Type-III fimbriae encoding gene

PCR reaction was performed in a Biorad T100 Thermal cycler. The conserved primers for *Mrk* D gene were selected from previously reported study (14), which was synthesized from Chromous Biotech (Bangaluru). For the amplification of the *Mrk* D gene the forward primer was 5'-TTCTGCACAGCGGTCCC-3' and reverse primer 5'- GATACCCGGCGTTTTCGTTAC-3' were used. The reaction mixture of 25 µl consisted of 12.5 µl 2X master mix (SRL India), 3 µl of 10 pmoles each of the two primers, 3 µl DNA and final volume was made up with sterilized MilliQ water to 25 µl. Amplification was done by initial denaturation at 94ºC for 5 minute, followed by 34 cycles of denaturation at 94ºC for 1 minute, annealing temperature of primers was 52ºC for 1 min and extension at 72ºC for 2 minutes. The final extension was conducted at 72ºC for 8 minutes.

Agarose Gel Electrophoresis

1.2% agarose with ethidium bromide was used to analyze 8 µl of the amplified reaction mixture with 1 kb DNA ladder by gel electrophoresis at 8 V/cm and the reaction product was visualized under Gel doc (Biorad Gel DocTM EZ Imager).

Results

A total 414 MDR Gram negative bacilli were isolated out of which 114 (27.5%) MDR Gram negative bacilli were isolated from various devices. Out of these 40 (35.08%) strains of *K. pneumoniae* were isolated from various devices [Table-1]. A total of 23 strains of *K. pneumoniae* (57.5%) were found to be biofilm producing strains [Fig-1]. Maximum number of *K. pneumoniae* was isolated from Tracheostomy tips followed by Urinary catheter tips [Table-2]. Central line tips and endotracheal tips showed the similar number of isolates. The antibiogram of Non Biofilm producing strains showed more sensitivity to various drugs as compared to various Biofilm positive strains [Fig-2]. The panel of drugs which were tested for the antibiotic sensitivity of *K. pneumoniae* included: cefuroxime, cefoxitin, cefotaxime, ceftazidime, cefperazone, cefixime, cefipime, amoxycillin+clavulanic acid, gentamycin, amikacin, ciprofloxacin, levofloxacin, aztreonam, pippercilin+tazobactam, cotrimoxazole, chloramphenicol, imipenem, meropenem, ertapenem, doripenem, polymyxin-B, colistin and tigecycline. Biofilm positive strains were sensitive to only chloramphenicol, amoxycillin+clavulinic acid, gentamycin, amikacin, levofloxacin, aztreonam, pippercillin+tazobactam, imipenem, meropenem, ertapenem, doripenem, polymyxin B, colistin and tigecycline. Non-Biofilm producers were also sensitive to cotrimoxazole, cefepime, ciprofloxacin and ceftazidime besides the above-mentioned drugs.

This antibiogram showed the high resistance levels of biofilm producing strains. Several risk factors which favoured Biofilm production was that most of the patients were immunocompromised and stayed in hospital for more than 15 days. Duration of the devices implanted inside the patients played a significant role in the production of Biofilms by *K. pneumoniae* as the prolonged implantation of the device lead to resistant biofilm production. Highest sensitivity in Biofilm producing *K. pneumoniae* strains was observed in colistin (78.26%), followed by tigecycline 73.91%. Imipenem was 69.56% sensitive and polymyxin B was 60.86%. Least sensitivity in Biofilm producing *K. pneumoniae* strains was observed in levofloxacin (8.69%) [Fig-2]. In addition, the genotyping of *K. pneumoniae* strains was also done. The DNA was isolated manually from the most promising strains of *K. pneumoniae* and 12 strains showed the presence of *Mrk* D gene [Fig-3].

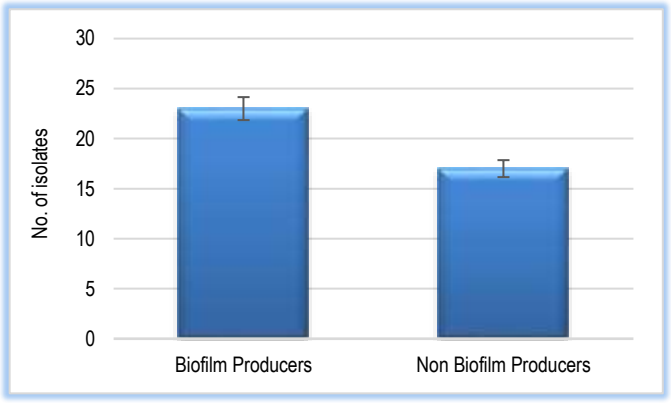

Fig-1 Biofilm Production by MDR *K. pneumoniae* from various Indwelling Medical devices

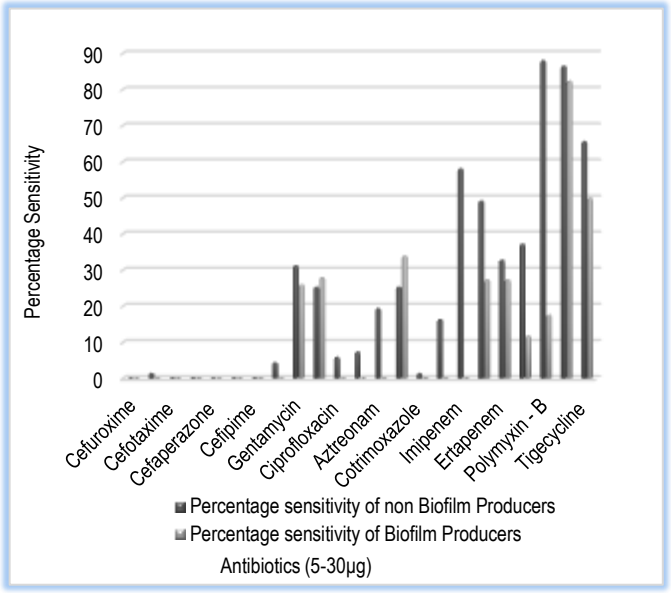

Fig-2 Representation of antibiogram of *Klebsiella pneumoniae*

Discussion

Biofilm formation being a cause of sustained infections is becoming a menace in the hospital settings due its high co-relation with antibiotic resistance, hence it has become a notable cause of severe nosocomial infections which are onerous to treat.

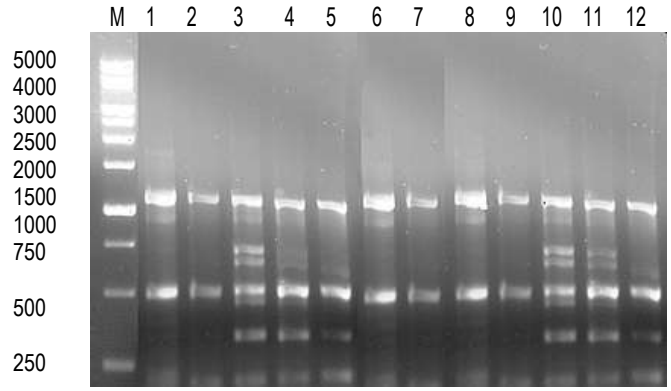

Fig-3 Agarose gel electrophoresis (1.2%) of PCR amplified product of *Mrk*D gene, Lane M: 5000 bp ladder, Lane 1-12: The PCR amplicon sizes for *Mrk*D gene (Lane: 1,2,6,7,8,9) 1069bp, 465bp, (Lane: 3,10,11) 1069bp, 670bp, 610bp, 465bp, 300bp and (Lane: 4,5,12) 1069bp, 465bp, 300bp respectively.

The persistence of this organism in the hospital settings may be partly due to its adherence capability to different surfaces. Influence of capsule formation, quorum sensing and extended-spectrum beta-lactamases encoding plasmids are some of the major factors responsible for biofilm formation in *K. pneumoniae* which have been documented till now [9]. Microbial biofilms are present on the luminal surface of endotracheal and tracheostomy tubes of all patients ventilated in the ICU and form within hours of tracheal intubation, becoming abundant at 96 hours. Whilst the exact sequence of tube colonization and infection is unclear, it is thought that the microbial biofilm may act as a reservoir of pathogens causing recurrent infections. In the year 1999, Adair and colleagues showed that 70% of patients with VAP had the identical pathogen isolated from their tracheal tube and lower respiratory tract [10].

In the present study maximum colonization (41.6%) of the endotracheal tubes was by *K. pneumoniae* which showed similarity with the study conducted by Sands *et al*. (2017) that reported that infections like ventilator associated pneumoniae caused by *K. pneumoniae* was 15% prevalent and its prognosis was negatively affected by MDR organisms present in biofilms [11]. The considerable efficiency of colonization of *K. pneumoniae* associated with the acquired resistance to antibiotics, has enabled *K. pneumoniae* to persist and spread rapidly in healthcare settings. Surgical site infections along with soft tissue infections caused by *K. pneumoniae* are some of the common causes that lead to bacteremia [12].

The virulence factors playing an important role in the severity of *K. pneumoniae* infections are capsular polysaccharides, type 1 and type 3 pili (also called as fimbriae). These are the factors which are involved in aggregative adhesions and high pathogenicity. Type 3 fimbriae are the one which are responsible for biofilm formation on the indwelling devices [13]. Type 3 fimbriae are encoded by the *Mrk* gene cluster type 3 fimbriae and have been established to play a significant role in *K. pneumoniae* biofilm formation [14]. The Type 3 fimbriae are encoded by *Mrk* D gene, and was detected by the PCR method. Out of the 79 biofilm producing strains in *K. pneumoniae*, 15 most promising strains were selected and 12 strains showed the presence of this gene showing that it is responsible for biofilm production in *K. pneumoniae* bacterial strains [Fig-3]. Recently, in another study done by Shakib *et al* molecular detection of the various virulence factors in *K. pneumoniae* was observed and was found that *Mrk* D gene is one of the second highest prevalent virulence gene (65.7%) leading to high antibiotic resistance [24].

In another study reported by Hornick *et al*. [15] that *K. pneumoniae* IA565 possesses two *Mrk* gene clusters that are located on a plasmid and the chromosome and also investigated that plasmid borne *Mrk* D is 978 bp in length and chromosomal *Mrk* D is 581 bp. Hornick *et al*. (1991) also studies that the *Mrk* genes of the fimbrial gene cluster are highly conserved regardless of whether they are present on the chromosome or a plasmid in *K. pneumoniae*. On the contrary, Sebghati *et al*. (1998) reported that plasmid-borne determinant, *Mrk*D1P, and the chromosomally borne gene, *Mrk*D1C, are not genetically related. In the present study we selected the primers which were reported by Sebghati *et al*. [16] that derived them from the sequences which were not associated with plasmids.

The amplicon of *Mrk*D gene in present study were in the range from 500 and 1000 bp whereas the reported *Mrk*D gene in the study done by Sebghati *et al* with same primers was 581 bp. Whereas the band which was near approximately 1000 bp was in accordance with plasmid borne 978bp, was different as primers used in that study were reported as conserved for chromosomal *Mrk*D. Since, manual method of DNA isolation was adopted in the present study it was observed that in addition to chromosomal band an additional plasmid band was also reflected. This shows that primers are not conserved for chromosomal, but same gene sequence can also be present in the plasmid also which is in accordance with both Sebghati *et al* and Hornick *et al*. The presence of the genes on both chromosome and plasmids can be related to the fact that the type 3 fimbriae have undergone evolutionary divergence.

This Investigations leading to the molecular biology of these naturally occurring *Mrk*D polypeptides could provide information on the nature of the receptors recognized by type 3 fimbriae and needs to be explored more so that their origin can be recognized and since type 3 fimbriate Enterobacteria are frequent opportunistic pathogens of immunocompromised individuals [17], the analysis of its role can help in preventing many nosocomial hospital acquired infections.

Antibiotic resistance of *K. pneumoniae*

Health care associated infections due to multidrug resistant gram negative bacilli (MDR GNB) and methicillin resistant Staphylococcus aureus is one of the leading causes of Hospital acquired infections (HAI). MDR-Pseudomonas aeruginosa, MDR-*Acinetobacter baumannii* and Enterobacteriaceae family producing extended-spectrum beta lactamases (ESBL), metallo beta lactamases (MBL) and carbapenemases, have been implicated in severe HAIs and their occurrence has increased steadily. Treatment of biofilm producing microorganisms has become a major problem now-a-days as the antibiotics are becoming extremely resistant to biofilm producers than to the non-biofilm producers. In the current ongoing study, a comparison has been done between antibiotic sensitivity of biofilm producing and non-biofilm producing microorganisms. In biofilm producing *K. pneumoniae* cephalosporins, aminoglycosides, few of the carbapenams and flouroquinolones were resistant and the most effective drug came out to be Tigecycline and Colistin [Fig-2]. These results were slightly contradictory as compared to the study done by Ruchi *et al*. in 2016 that stated that Amikacin was 65% which was an effective drug against biofilm producing *K. pneumoniae*. In the biofilm producing *K. pneumoniae* strains gentamycin 26%, amikacin 28%, piperacillin+ tazobactam 34%, Cotrimoxazole, chloramphenicol, ciprofloxacin, levofloxacin, imipenem came out with 0% sensitivity, meropenem and ertapenem were 27.45% sensitive and doripenem with just 11.46% sensitivity. Similarly, a study done by Cernohorska and Votava, (2004) [18] also tested the efficacy of different antibiotics including piperacillin+tazobactam, cefoperazone, ceftazidime, cefepime, meropenem, ciprofloxacin and amikacin, and came out with the results that adherent bacterial populations exhibited reduced antimicrobial susceptibility with respect to their planktonic counterpart. MDR bacteria are resistant to almost all other currently available antibiotics hence the class of polymyxin antibiotics is increasingly considered as the final option of antibiotic therapy [19]. The most important domain in the LPS among three domains is lipid A among them which serves as a hydrophobic anchor with tight packing of fatty acyl chains. Divalents like Ca^{2+} and Mg2+ serve as a bridge between the adjacent LPS molecules to stabilize monolayer [20]. It is believed that polymyxin kills bacteria through membrane lysis as it inserts its hydrophobic N-terminal fatty acyl chain and D-Phe6-L-Leu7 (polymyxin B) or D-Leu6-L-Leu7 (polymyxin E) segment into Outer membrane (OM). This insertion weakens the packing of adjacent lipid A, which reduces the expansion of OM monolayer. This makes the destabilized areas which makes the entry of polymyxin entry easy. Eventually, it facilitates the formation of destabilized areas through which polymyxin crosses OM which finally destroys the physical integrity of phospholipid bilayer of inner membrane (IM) through membrane thinning destabilizes the interface leading to IM lysis and cell death [21].

Tigecycline is aglycylcycline derivative of tetracycline, which is bacteriostatic in nature and effective against both Gram- positive as well as Gram-negative organisms. This can be used against multidrug resistant organisms [22,23]

Table-1 *Total strains of K. pneumoniae from various Indwelling Devices*

SN	Devices	Total isolates	Biofilm Positive	Percentage
1	Tracheostomy tips	14	8	45%
2	Endotracheal tips	8	4	25%
3	Central line tips	6	3	15%
4	Urinary catheter tips	12	3	15%

Table-2 *Classification of Biofilm production by K. pneumoniae*

SN	Type of device	Tube adherence method	Type of biofilm producer	Microtitre plate method	Type of biofilm producer
1	Endotracheal tip	++	Moderate producer	0.123	Moderate producer
2	Endotracheal tip	Negative	Non producer	0.12	Moderate producer
3	Endotracheal tip	-	Non producer	0.113	Non producer
4	Endotracheal tip	++	Moderate producer	0.138	Moderate producer
5	Endotracheal tip	Negative	Non producer	0.122	Moderate producer
6	Endotracheal tip	Negative	Non producer	0.117	Non producer
7	Endotracheal tip	Negative	Non producer	0.173	Moderate producer
8	Tracheostomy tip	++	Moderate producer	0.161	Moderate producer
9	Tracheostomy tip	Negative	Non producer	0.12	Moderate producer
10	Tracheostomy tip	++	Moderate producer	0.109	Non producer
11	Tracheostomy tip	++	Moderate producer	0.127	Moderate producer
12	Tracheostomy tip	Negative	Non producer	0.095	Non producer
13	Tracheostomy tip	++	Moderate producer	0.151	Moderate producer
14	Tracheostomy tip	++	Moderate producer	0.186	Moderate producer
15	Tracheostomy tip	++	Moderate producer	0.166	Moderate producer
16	Tracheostomy tip	++	Moderate producer	0.102	Non producer
17	Tracheostomy tip	Negative	Non producer	0.141	Moderate producer
18	Tracheostomy tip	++	Moderate producer	0.133	Moderate producer
19	Tracheostomy tip	++	Moderate producer	0.132	Moderate producer
20	Tracheostomy tip	+++	Strong producer	0.249	Strong producer
21	Urinary catheter tip	++	Moderate producer	0.141	Moderate producer
22	Urinary catheter tip	++	Moderate producer	0.15	Moderate producer
23	Urinary catheter tip	Negative	Non producer	0.088	Non producer
24	Urinary catheter tip	Negative	Non producer	0.091	Non producer
25	Urinary catheter tip	Negative	Non producer	0.071	Non producer
26	Urinary catheter tip	Negative	Non producer	0.101	Non producer
27	Urinary catheter tip	Negative	Non producer	0.118	Non producer
28	Urinary catheter tip	Negative	Non producer	0.093	Non producer
29	Urinary catheter tip	Negative	Non producer	0.07	Non producer
30	Urinary catheter tip	Negative	Non producer	0.12	Moderate producer
31	Urinary catheter tip	Negative	Non producer	0.109	Non producer
32	Urinary catheter tip	Negative	Non producer	0.88	Non producer
33	Central line tip	Negative	Non producer	0.105	Non producer
34	Central line tip	Negative	Non producer	0.122	Moderate producer
35	Central line tip	Negative	Non producer	0.191	Moderate producer
36	Central line tip	+++	Strong producer	0.245	Strong producer
37	Central line tip	++	Moderate producer	0.153	Moderate producer
38	Central line tip	Negative	Non producer	0.089	Non producer
39	Tracheostomy tip	++	Moderate producer	0.151	Moderate producer
40	Central line tip	++	Moderate producer	0.195	Moderate producer

Conclusion

The opportunistic pathogen, *K. pneumoniae*, can give rise to severe diseases, typically nosocomial infections, such as septicemia, pneumonia, UTI and soft tissue infections. *K. pneumoniae* are often considered as a paradigm of hospital-acquired infections. Nosocomial *K. pneumoniae* infections continue to be a heavy burden on the economy and on the life expectancy of patients in developed countries. Thus, further progress in the prevention of hospital-acquired infections will require new approaches to infection control. The increasing evidence on the ability of *K. pneumoniae* to form biofilm, mostly on medical devices and the recent data supporting the correlation of such a behavior with the antibiotic resistance acquisition should alert even more regarding the hazard of this pathogen in hospital settings.

Application of Research: Exploration of the virulence factors and the study of new mechanisms to control them could be an important way to counteract *K. pneumoniae* nosocomial infections. In particular, the biofilm mode of growth makes bacteria up to 1,000-times more resistant to antibiotic therapy. Management of such infections is now a big challenge, as they lead to persistent and resistant infections. Hence, newer methods can be incorporated in the diagnostic clinical laboratory to demonstrate the biofilm production ability of microbes.

Research Category: Antibiotic sensitivity

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Study area / Sample Collection: Centre for Interdisciplinary Biomedical Research, Adesh University, Bathinda, 151101, Punjab, India

Strain name: *Klebsiella pneumoniae*

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