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To evaluate the drug resistance pattern in various antibiotics with biofilm-producing capability of Klebsiella pneumoniae

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***Abstract*---**Aim: Objective of study was to evaluate the drug resistance pattern in various antibiotics with the biofilm-producing capability of *Klebsiella pneumoniae*. Methods: *Klebsiella pneumoniae* was isolated for identification from total of 83 OPD and IPD patients by analysing colony morphology, microscopic examination, and performed biochemical testing. Antibiotics susceptibility Tests and biofilm-producing capacity was done by the Kirby-Bauer disk diffusion method and adherence quantitative assays. Chi-square test was used and $P < 0.05$ was measured as statistically significant. Results: Out of 83 isolates, 47 (56.6%) were Biofilm Producer and 36 (43.4%) were non-biofilm producers by tissue culture method, and tube method 62.7% and 37.3% respectively. Among biofilm producer and non-biofilm producer isolates, *Klebsiella pneumoniae* had the most Resistant to Ceftazidime (100%) & (61.0%), followed by Ceftriaxone (56.0%) & (95.7%) respectively. The association between biofilm and antibiotic resistance was checked to be statistically significant for aminoglycosides, fluoroquinolones, Cephalosporin's, Imipenem, Meropenem, Amoxyclav, and Piperacillin Tazobactam. No resistance was observed against Colistin and Polymyxin B. Conclusion: The

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strain *Klebsiella pneumoniae* isolated from different samples showed drug resistance to all broad-spectrum antibiotics whereas there is no drug resistance was found with narrow-spectrum drugs. This study supports the use of narrow-spectrum antibiotics for most of infections.

***Keywords*---**Klebsiella pneumoniae, biofilm producer, broad spectrum antibiotics, narrow spectrum antibiotics, drug resistance.

Introduction

Klebsiella pneumoniae is a gram-negative, ubiquitous, non-motile, facultatively anaerobic, and small rod-shaped bacterium[1] which is also an opportunistic pathogen causing many clinical outcomes including UTIs, Bacteremia, meningitis, skin and soft tissue infections, hospital, and community-acquired pneumonia[2]. *Klebsiella pneumoniae* forms biofilm on indwelling plastic devices, such as catheters and endotracheal tubing, and then colonize human tissue. One of the early stages of *Klebsiella pneumoniae* airway infections may also comprise biofilm formation[3]. The fimbriae and capsule are protuberant structural components of the *Klebsiella pneumoniae* cell surface and have a significant role in biofilm formation[4]. Biofilm is a microorganism derived sessile community that is considered by cells that are irreversibly attached to a substratum or interface with each other and embedded in a matrix of self-produced extracellular polymeric substances[5,6]. Biofilm producer *Klebsiella pneumoniae* could be resistant to a wide range of antibiotics[7]. Prominently, it has been stated that about 40% of biofilm-producing *Klebsiella pneumoniae* were improved not only from urine, blood, sputum, and wound swabs[8]. Precise physiological and genetic relations within the biofilm are causing a dramatic increase in antimicrobial-resistant agents[9].

Previous reports have depicted numerous associations between antibiotic resistance and Biofilm formation. Some studies have shown that increased resistance was correlated with high biofilm production[10,11], On the other hand, few researches reported that biofilm formation decreased in high resistance isolates[12,13]. It depicts that the association between antibiotic resistance and biofilm formation is presently not clear and requires more investigation[14, 15].

Materials and Methods

This is a prospective study in which a total number of 83 isolates of *Klebsiella pneumoniae* were obtained for aerobic culture and sensitivity from different IPDs & OPDs of the National Institute of medical science (NIMS) Hospital Jaipur Rajasthan from, January 2020 to June 2020.

Sample type and collection methods

Various samples including pus, urine, sputum, ET, and blood were collected from an outpatient department and indoor patient department. Clinical Samples were stored in the sterile container. These clinical samples were further investigated in

the microbiology laboratory. All clinical specimens were inoculated on MacConkey agar and Blood except urine specimens that were plated on Cysteine Lactose Deficient Medium as per the standard bacteriological measures. The culture plates were incubated at 35 °C for 24–48 h. The growth isolates were identified based on colony morphology, pigmentation, odor, and their unique biochemical tests[16].

Standard antibiotics

Antibiotic susceptibility test[17]: it was performed on Muller Hinton agar by Kirby Bauer disc diffusion method following CLSI guidelines. Imipenem (10µg), Meropenem (10µg), Colistin (10 µg), Ciprofloxacin (5µg), Ceftazidime (30µg), Polymyxin B (300 units), Piperacillin-Tazobactam (100/10), Ceftriaxone (30µg), Gentamycin (10µg), Amoxicillin-Clavulanic acid (30µg), Cefotaxime (30µg) were tested as common antibiotics for all samples.

Detection of biofilm production

Biofilm of all the Klebsiella pneumoniae isolates were detected using Tube method and Tissue culture plate method. These methods indicated the qualitative and quantitative study of biofilm production respectively. All the isolates were categorized based upon the biofilm formation abilities.

Tube method

BHI broth with 2% sucrose (10 ml) was inoculated with a loopful of microorganisms from overnight culture plates and incubated for 24 h at 37°C. The tubes were then decanted and washed with PBS (pH 7.3) and dried. Dried tubes were then stained with crystal violet (0.1%) for half an hour. Excess stain was removed, tubes were then dried and observed for biofilm formation. Biofilm formation was considered positive at the moment that a visible film lined the wall and bottom of the tube. Tubes were examined, and the amount of biofilm formation was scored as absent and present[18].

Tissue culture plate method

Isolates from fresh agar plates were inoculated in brain heart infusion (BHI) broth with 2% sucrose and incubated for 18–24 h at 37°C in a stationary condition. The broth with visible turbidity was diluted to 1 in 100 with a fresh medium. Individual wells of flat-bottom polystyrene plates were filled with 0.2 ml of the diluted cultures, and only broth served as a control to check sterility and nonspecific binding of the medium. These plates were incubated for 24 h at 37°C. After incubation, the content of the well was gently removed and then were washed 4 times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating “planktonic” bacteria. Biofilms formed by adherent “sessile” organisms in the plate were fixed with sodium acetate (2%) for half an hour and stained with crystal violet (0.1% w/v) for half an hour. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent bacterial cells usually formed a biofilm on all side wells and were uniformly stained with crystal violet. Optical densities (OD) of stained adherent

bacteria were determined with a micro–Enzyme Linked Immunosorbent Assay auto reader at a wavelength of 570 nm (OD 570 nm) and were graded as per Christensen et al. These OD values were considered as an index of bacteria adhering to the surface and forming biofilms. The experiment was performed in triplicate[19].

Statistical analysis

The statistical analysis was performed using SPSS software version 23.0. Data were presented as percentages and proportions. The Chi-square test was applied when two or more sets of variables were compared. The critical value of P indicating the probability of significant difference was taken as <0.05. Based on the available literature, we considered TCP to be gold standard method of biofilm detection (Fig.1) amongst the Tube methods for our study and calculated the other statistical parameters accordingly. (Fig 1 & 2)

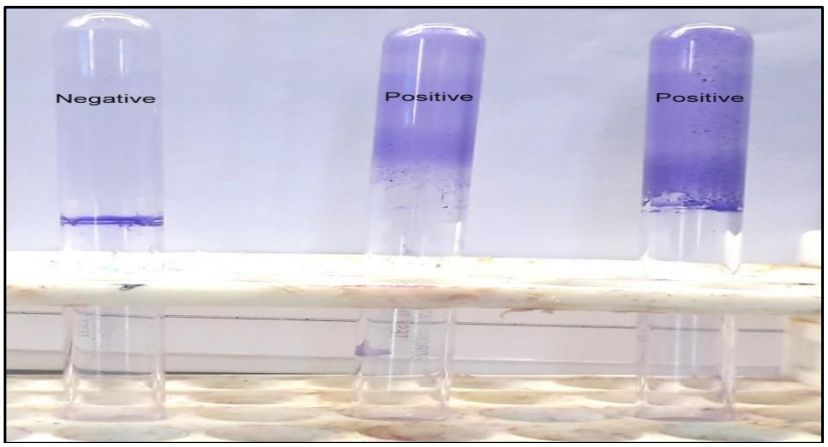

Fig. 1: Biofilm Detection by Tube Method

Table 1: Classification of bacterial adherence by TCP method Mean OD values Adherence Biofilm formation

Mean OD values	Adherence	Biofilm formation
<0.120	None	None/weak
0.120-0.240	Moderate	Moderate
≥0.240	Strong	High

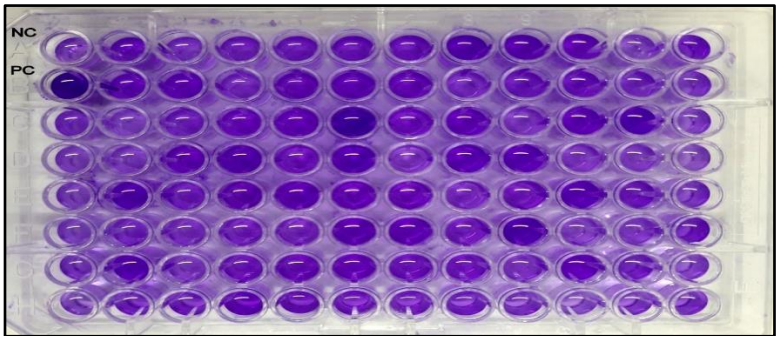

Fig. 2: Biofilm Detection by Tisue Culture Plate Method

Result

During the study period, out of total 83 isolates were identified as *Klebsiella pneumoniae*. Out of 83 isolates were 57 (68.6%) male patients and 26(31.2%) female patients. Most of *Klebsiella pneumoniae* were isolated from patients aged more than 46-60 years age group (31.6%) followed by more than 60 years old 24(28.6%).

Age group	Female		Male		Total	
<15	02	2.4%	03	3.6%	05	6.0%
16-30	05	6.0%	07	8.5%	12	14.5%
31-45	06	7.2%	10	12.1%	16	19.3%
46-60	08	9.6%	18	21.8%	26	31.4%
>60	05	6.0%	19	22.8%	24	28.8%
Total	26	31.2%	05	68.8%	83	100%

Table 2: Distribution of Age and Sex wise in *Klebsiella pneumoniae*

Out of 83 isolates were 47(56.6%) Biofilm Producer by tissue culture method and 36 (43.4%) were Non biofilm producer by Tissue Culture Plate Method, and by the Tube method were 62.7% Biofilm Producer and 37.3% Non-Biofilm Producer.

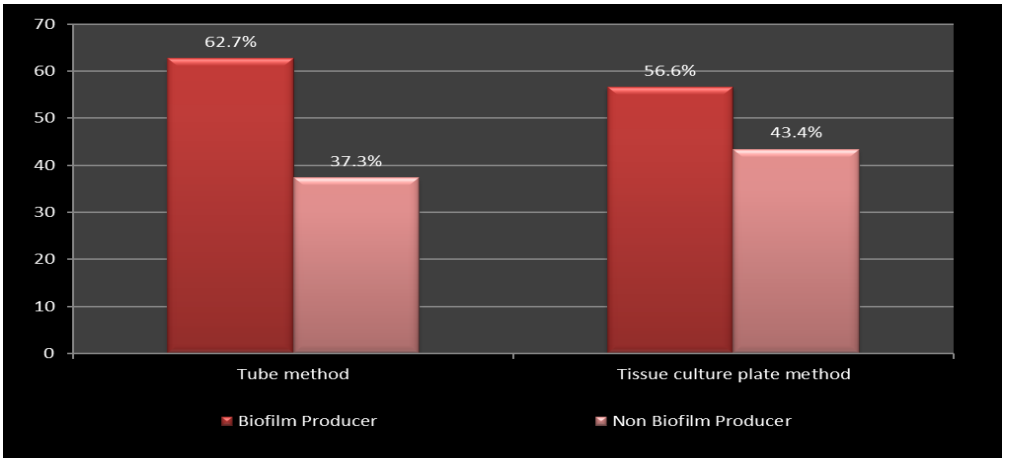

Fig. 3: Comparison between Tube Method and Tissue Culture Plate Method in *Klebsiella pneumoniae.*

In this study, among the 83 *Klebsiella pneumoniae* isolates tested by Tissue Culture Plate Method, there were 47(56.6%) isolates as biofilm producer and 36 (43.4%) isolates that were not biofilm producers. Among biofilm producers, there were 16 (19.3%) isolates as strong, and 31(37.3%) isolates as moderate biofilm producers.

Interpretation of Results

Mean OD values:- <0.120 = Negative, 0.120-0.240 = Moderate and >0.240= High Biofilm Producer.

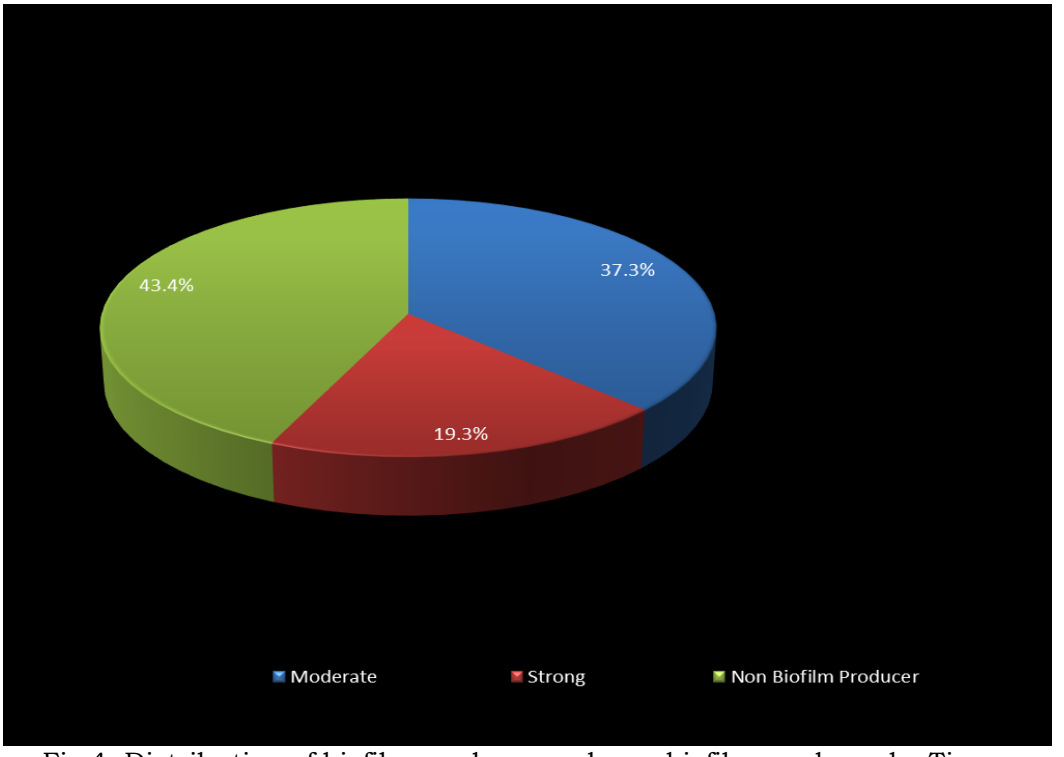

Fig 4: Distribution of biofilm producer and non-biofilm producer by Tissue Culture Plate Method Klebsiella pneumoniae

Out of 83 Klebsiella pneumoniae isolates 24 samples were isolated from urine sample followed by 18 isolates from endotracheal tube and 12 isolates from sputum samples. In this study biofilm producer (By TCP method) were 66.67%, 72.02% and 41.66% have in urine, endotracheal tube and sputum samples while non biofilm producer were 33.33%, 27.78% and 58.33% respectively.

Samples	Total		biofilm producer By TCP method		Non biofilm producer	
URINE	24		16	66.67%	08	33.33%
ET	18		13	72.02%	05	27.78%
SPUTUM	12		05	41.66%	07	58.33%
PUS	11		06	54.55%	05	45.45%

BLOOD	10		03	30.00%	07	70.00%
SWAB	08		04	50.00%	04	50.00%
Total	83		47	56.63%	36	43.37%

Table 3: Distribution and comparison of biofilm producer and non-biofilm producer by Tissue Culture Plate Method in clinical samples Klebsiella pneumoniae

Among biofilm producer and non-biofilm producer isolated, *Klebsiella pneumoniae* had most Resistant to Ceftazidime (100%) & (61.0%), followed by Ceftriaxone (56.0%) & (95.7%), Amoxyclav 95.7% & 47.0% and Ciprofloxacin (95.7%) & (44.0%) respectively. The association between biofilm and antibiotic resistance was noted to be statistically significant for aminoglycosides, fluoroquinolones, Cephalosporin's, Imipenem, Meropenem Amoxyclav and Piperacillin Tazobactam. No resistance was observed against Colistin and Polymyxin B.

Drugs	Biofilm Producer Resistant n= 47		Non Biofilm Producer Resistant n=36		P. value
Amoxyclav	45	95.7%	17	47.0%	.000
Piperacillin Tazobactam	36	76.6%	13	36.0%	.000
Ceftazidime	47	100%	22	61.0%	.000
Cefotaxime	44	93.6%	17	47.0%	.039
Ceftriaxone	45	95.7%	20	56.0%	.000
Imipenem	37	78.7%	02	5.6%	.001
Meropenem	33	70.2%	0	0.0%	.010
Gentamycin	40	85.1%	05	14.0%	.001
Ciprofloxacin	45	95.7%	16	44.0%	.021
Colistin	0	0.0%	0	0.0%	.000
Polymyxin B	0	0.0%	0	0.0%	.000

Table 4: Comparison of biofilm producer and non biofilm producer by Tissue Culture Plate Method in antibiotics resistant *Klebsiella pneumoniae*. *p-value was calculated by using the Chi-square test

Discussion

During the study period, *Klebsiella pneumoniae* isolates were collected from male patients. Out of total 83 isolates identified as *Klebsiella pneumoniae*, there were 57 (68.6%) isolates from male patients and 26 (31.2%) isolates from female patients, a similar study by Hera Nirwati *et al* [20] were isolated from the male (64.07%) and female (35.93%) patients. This result was in line with Osagie *et al.*[21] who collected the samples from 5 primary health care centers in Nigeria and reported that *Klebsiella pneumoniae* infection was higher in males than females. Akter *et al.*[22] also reported that male patients had a higher risk to get *Klebsiella pneumoniae* infection than females. The association between sex and the incident of *Klebsiella pneumoniae* was associated with poor lifestyle choices in the form of smoking and alcoholism[21]. However, no statistically significant differences between female and male subjects were reported in those studies. Most of *Klebsiella pneumoniae* in this study were obtained from patients of all age groups.

In this study, most of *Klebsiella pneumoniae* were isolated from patients aged more than 46-60 years age group (31.6%) followed by more than 60 years old 24 (28.6%). Meanwhile, another recent study suggested that a greater number of *Klebsiella pneumoniae* isolates were obtained from patients aged between 40 to 60 years old[23]. The differences in terms of patient's age distribution could be related to the strength of the immune system response, which is expected to decline in senescence. Patients aged under 40, years tend to have stronger immune systems, thus giving more pressure to *Klebsiella pneumoniae* to fight the immunity of the host[24]. On the contrary, an increased age leads to a higher risk of *Klebsiella pneumoniae* infection because of the increased incidence of comorbid illness[25].

In this present study, 83 isolates were 47(56.6%), Biofilm Producer, by tissue culture method and 36 (43.4%) were non-biofilm producers by tissue culture method. And by tube method was 62.7% Biofilm Producer and 37.3% Non-Biofilm Producer. Compared to another study Gyaneshwar Tiwari *et al* [26] results were found between the tube method and tissue culture method 85.1% and 82.9% in those studies. In this study, among the 83 *Klebsiella pneumoniae* isolates tested by Tissue Culture Plate Method, there were 47(56.6%) isolates as biofilm producer and 36 (43.4%) isolates that were not biofilm producers. Among biofilm producers, there were16 (19.3%) isolates as strong, and 31(37.3%) isolates as moderate biofilm producers. A similar result in another study *Klebsiella pneumoniae* Gyaneshwar Tiwari *et al* [26] those results were by TCP method strong and moderate 11.53%, 33.33%, and Hera Nirwati *et al* [27] Moderate biofilm producer 48 (28.74) Strong biofilm producer 45 (26.95).

Antibiotic susceptibility pattern, Most of *Klebsiella pneumoniae* was resistant to a wide range of antibiotics. Among biofilm producer and non-biofilm producer isolates, *Klebsiella pneumoniae* had most Resistant to Ceftazidime (100%) & (61.0%), followed by Ceftriaxone (56.0%) & (95.7%), Amoxyclav 95.7% & 47.0% and Ciprofloxacin (95.7%) & (44.0%) respectively and our is compared to another study but not similar result found in other studies like; Hera Nirwati *et al* [27] Ceftriaxone 56.64 and 37.50, Piperacillin-Tazobactam 10.00% and 12.50%, Amoxicillin-Clavulanic acid 38.02 and 27.78, Ciprofloxacin 44.62 and 13.33 and another one Rabina Dumaru *et al* [28] Piperacillin–Tazobactam 40.82%, Ceftazidime 75.51%, in biofilm formation and statistically significant according to Rabina Dumaru *et al*[28].

The association between biofilm and antibiotic resistance was noted to be statistically significant for aminoglycosides, fluoroquinolones, cephalosporins, Imipenem, and Piperacillin Tazobactam. A higher proportion of antibiotic resistance in biofilm producers in comparison to non-producers has been documented in many studies[29-34]. No resistance was observed against Colistin and Polymyxin B. It's the last viable option for multi-drug resistant strains either being non-producers or producers of biofilm[35, 36].

Conclusion

In the present study, K. Pneumoniae isolated showed Multidrug resistance to a wide range of broad-spectrum antibiotics. This present study reported that

antibiotics resistance is found in K. pneumoniae infection due to biofilm formation of various capacities. Antibiotic resistance infection can be a burden on health care system and economy of the country. Global efforts should be intensified to prevent the spread of multi-drug resistant bacteria and eliminate the hospital-born microbes that are causing a dramatic rise in mortality. Our finding revealed that Polymyxin B and Colistin antibiotics are highly effective against bacterial infection as empirical treatment.

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