Association of drug resistant pattern with Biofilm production by using Microtitre plate method in clinical isolates of *Klebsiella pneumoniae*

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Abstract

Health care associated infections (HAIs) are frequently caused by *Klebsiella pneumoniae (K. pneumoniae)* which has a high level of antibiotic resistance pattern and the ability to form biofilms. In this research work, 105 *K. pneumoniae* was isolated from both inpatients and outpatients between January 2021 and January 2022 at the tertiary care hospital viz, Mahatma Gandhi Medical College and Research Institute (MGMCRI), India. These *K. pneumoniae* isolates were further analysed using morphological analysis of the colonies, microscopic examination, and biochemical testing. The Kirby Bauer disk diffusion method and adhesion quantitative assays were utilized for testing antibiotic susceptibility and biofilm- producing capacity. *K. pneumoniae* isolates were mostly derived from the urine specimens (42.9%) and pus (19.0%). Most of the K. *pneumoniae* were resistant to a wide range of antibiotics and were also well known to the ability to produce biofilm. The present study shows that the biofilm producing *K. pneumoniae* had been a good resistant to Ceftriaxone/Cefotaxime (80.9%), Amikacin (78.7%), Piperacillin+ Tazobactam (79.8%), and Meropenem (79.8%). On the contrary the present study also shows that among non-biofilm producing *K. pneumoniae also* showed good resistant to Gentamicin (87.5%), ciprofloxacin+ Norfloxacin (87.5%), Amikacin 15 (93.8%) and Cefoperazone+ sulbactam 12 (75.0%) of resistance. Thus, in this study, it is shown that among 105 *K. pneumoniae* isolates that were tested 89 (84.76%) isolates were found to be biofilm producer and 16 (15.2%) isolates were non-biofilm producers. Among biofilm producers, it is also shown that there were 36 (34.28%) isolates as strong, 42 (40%) isolates as moderate, and 11 (10.47%) isolates identified as weak biofilm producers. Furthermore, it is shown that the majority of the *K. pneumoniae* isolates showed resistance to a variety of antibiotics and were capable of producing biofilms.

Keywords: *Klebsiella pneumoniae,* Biofilm**,** Antibiotics**,** Clinical and Laboratory Standards Institute.

Introduction

Communities of bacteria are known as biofilms form when they stick to a surface and create an extracellular polymeric substance (EPS) matrix. Because the EPS matrix shields the microorganisms from environmental stressors like antibiotic therapy, biofilm associated illnesses

are more challenging to cure[1]. Biofilms prevent the entry of antibiotics, inhibit the growth of bacteria, promote the development of persisted cells, and allow for genetic exchange[2,3]. Bacterial resistance to antibiotics has been steadily rising as a result of the extensive usage of antibiotics worldwide. The frequency with which functional genes are acquired through mobile components has increased, leading to a rise in drug resistance and virulence in K. *pneumoniae*[4]. *K. pneumoniae* is a gram-negative bacillus that can cause urinary tract infections (UTIs), pneumonia, bacteremia, and liver abscesses in young people and other healthy individuals[5]. The two pathogenic varieties of K. *pneumoniae that* are now known to exist are classical *Klebsiella pneumoniae (*cKp) and hypervirulent *Klebsiella pneumoniae (h*vKp). Because of their genes for antibiotic resistance, ckp and hvKp are more challenging to treat. Furthermore, *K. pneumoniae has* a strong propensity to build biofilms, which exacerbates the already difficult illness[6]. *K. pneumoniae is* linked to a significant amount of ventilator-acquired pneumonia and hospital acquired pneumonia, which usually affects susceptible patients in intensive care units[7]. *K. pneumoniae strain* is frequently colonized in hospitalized patients and is more prevalent in immunocompromised people, such as diabetics, the elderly, and children[8]. Hospital infections are commonly associated with highly biofilm-forming bacteria, including *Escherichia coli, Pseudomonas aeruginosa, and* Klebsiella *pneumoniae*[9]. The misuse of antibiotics has led to treatment challenges for *K. pneumoniae* and reduced alternatives for the efficient control of this bacterial infection[10-11]. Treatment for infections resulting from K. *pneumoniae strains* that develop biofilms is more challenging than for other strains[12].

The present study was designed to evaluate biofilm development and drug resistant pattern among *K. pneumoniae* clinical isolates.

Materials and Methods

Bacterial Isolation and Identification

In the present study, 105 *K. pneumoniae* strains were isolated at Mahatma Gandhi Medical College & Hospital in Jan 2021 to Jan 2022. The *K. pneumoniae* strains were recovered from blood, sputum, surgical wound swabs, and urine samples of hospitalized patients (Outpatients & Inpatients). Definitive identification of isolates was confirmed using colony morphology, gram staining, manual biochemical testing[13]. After recognition, the *K. pneumoniae strains* were stored in tryptic soy broth (TSB) (Merck Co., Germany) with 10% glycerol at -80 degree Celsius. All isolates were freshly subculture on brain-heart infusion (BHI) agar prior to every analysis.

Antibiotic Susceptibility Tests of *K. pneumoniae*

Antibiotic susceptibility tests were conducted by using the Kirby Bauer disc diffusion method. According to the Clinical and Laboratory standards Institute 2020[14], *K. pneumoniae* were

categorised as resistant, intermediate, or susceptible. To this study, correlate biofilm production under resistant pattern [14].

Biofilm Formation Assay

The Biofilm formation test was conducted using a quantitative adherence assay. For every isolate, an overnight culture in Trypticase Soy Broth (TSB) at 37 degrees Celsius was carried out. Following this, 198 microlitre of TSB was enclosed in 96 well flat bottom polystyrene microtitre plates that were sterile and inoculated with 2 microliter of cell suspension. Each test also included negative control wells containing 200 microlitre of uninoculated TSB. For twenty-four hours, incubation was maintained at 37 degrees Celsius. The wells were gently rinsed three times with 200 microlitre of phosphate buffered saline (PBS). The wells were dried with the bottoms facing up. Mass of biofilm-stained utilising 50 microlitres of 0.1% crystal violet. The wells were gently cleaned three times using 200 microlitres of distilled water, and then they were dried inverted. Ultimately, the stained biofilm mass was solubilized by dissolving the wells in 200 microliters 5% isopropanol. An Optical Density (OD) measurement was performed at 570 nm using a microplate reader. By examining each isolate or negative control for eight to twelve wells, the mean OD was calculated. Optical Density Cut-off (ODC) was assigned as an average OD of negative controls + (3* Standard Deviation (SD) of negative controls). Isolate with OD ≤ ODC categorized as non-Biofilm producer. Meanwhile, the isolate was categorized as biofilm producer consisting of weak biofilm producer if 2*ODC < OD ≤ 4*ODC; moderate 2* ODC* OD ≤ 4*ODC; and strong biofilm producer if OD > 4* ODC[15].

Statistical analysis

The collected data from the *K. pneumoniae isolates* and their biofilm production and antibiotic drug resistant pattern were further analysed and compared using the chi-square test and Fischer's exact test. The statistical analyses had been performed using SPSS (Statistical Package for the Social Sciences) statistics software for Windows, version 15 (which is available from SPSS (statistical package for the social sciences) Inc., Chicago, USA). The p - value ≤0.05 had been taken as a statistically significant.

Ethics statement

The study was approved by the IHEC (MGMCRI/RAC/02/2020/XX/IHEC/137) of the MGMCRI of Sri Balaji Vidyapeeth University, Pondicherry, India.

Results

Demographics of patients from which *K. pneumoniae were* isolated is given in the following table: Table-1: Demographics

Table-1**: Demographics**

Sex	**No of Isolates**	**N in %**
Male	59	59 (56.2%)
Female	46	46 (43.8%)
Age Interval		
≤ 20	2	2 (1.9%)
21-40	39	39 (37.1%)
41-60	45	45 (42.9%)
61-80	18	18 (17.1%)
> 80	1	1 (1.0%)
Sample Types		
Ear swab	3	3 (2.9%)
ET aspirate	5	5 (4.8%)
Pleural fluid	4	4 (3.8%)
Pus	20	20 (19.0%)
Sputum	6	6 (5.7%)
Tissue	3	3 (2.9%)
Urine	45	45 (42.9%)
Vaginal swab	4	4 (3.8%)
Wound swab	15	15 (14.3%)

Table-2: **Antibiotic resistant pattern and biofilm producing capacity of *K. pneumoniae* clinical Isolates**

Antibiotics	**Weak**	**Moderate**	**Strong**	**Chi-square value**	**p-value**
Cotrimoxazole	9 (81.8%)	29 (69.0%)	28 (77.8%)	1.335	0.721
Ceftriaxone/cefotaxime	9 (81.8%)	33 (78.6%)	30 (83.3%)	4.928	0.177
Ciprofloxacin/ Norfloxacin	7 (63.6%)	32 (76.2%)	25 (69.4%)	2.637	0.451
Gentamicin	10 (90.9%)	32 (76.2%)	26 (72.2%)	2.698	0.441
Amikacin	8 (72.7%)	34 (81.0)	28 (77.8%)	2.417	0.490
Imipenem	8 (72.7%)	25 (59.5%)	33 (91.7%)	10.457	0.015*

Meropenem	10 (90. 9%)	32 (76.2%)	29 (80.6%)	2.089	0.554
Piperacillin+Tazobactam	7 (63.6%)	37 (88.1%)	27 (75.0%)	12.666	0.005*
Cefperazone+Sulbactam	8 (72.7%)	24 (57.1%)	23 (63.9%)	2.055	0.561
Nalidixic acid	6 (54.5%)	22 (52.4%)	23 (63.9%)	1.836	0.607
Nitrofurantoin	7 (63.6%)	30 (71.4%)	25 (69.4%)	0.256	0.967
Fosfomycin	8 (72.7%)	25 (59.5%)	20 (55.6%)	2.397	0.494
Type of patient (IP)	10 (90.9%)	36 (85.7%)	27(75.0%)	2.176	0.537
Out Patient Department (OPD)	1 (9.1%)	6 (14.3%)	9 (25.0%)		
Biochemical Characterization					
K. pneumoniae	11 (13.09%)	39 (46.42%)	34 (40.4%)		
K. ozaenae	0	3 (60%)	2 (40%)	1.925	0.588

* Indicates statistically significant

Table-3: **Antibiotic drug resistant pattern with biofilm and non- biofilm producers**

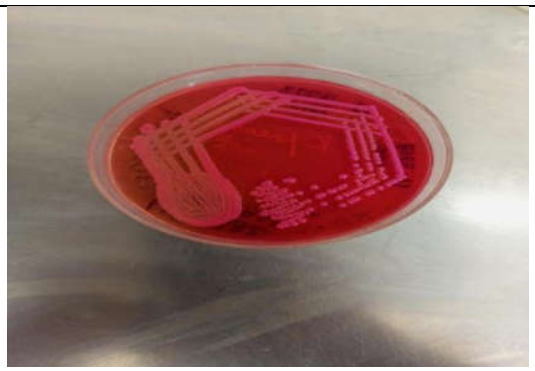
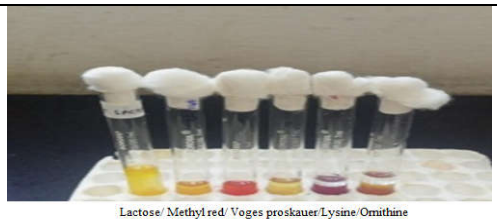
Antibiotics	Non-biofilm producers	Biofilm producers	Chi-square value	p-value	Fischer
Cotrimoxazole	11(68.8%)	66(74.2%)	0.203	0.652	
Ceftriaxone+cefotaxime	9(56.2%)	72(80.9%)	4.673	0.031*	0.029
Ciprofloxacin+ Norfloxacin	14(87.5%)	64(71.9%)	1.726	0.189	
Gentamicin	14(87.5%)	68(76.4%)	0.976	0.323	
Amikacin	15(93.8%)	70(78.7%)	2.005	0.157	
Imipenem	11(68.8%)	66(74.2%)	0.203	0.652	
Meropenem	11(68.8%)	71(79.8%)	0.964	0.326	
Piperacillin+Tazobactam	7(43.8%)	71(79.8%)	9.214	0.002*	0.004
Cefoperazone+sulbactam	12(75.0%)	55(61.8%)	1.024	0.312	
Nalidixic acid	11(68.8%)	51(57.3%)	0.735	0.391	
Nitrofurantoin	11(68.8%)	62(69.7%)	0.005	0.942	
Fosfomycin	7(43.8%)	53(59.6%)	1.383	0.24	

* Indicates statistically significant.

Table-4**: Types of patients**

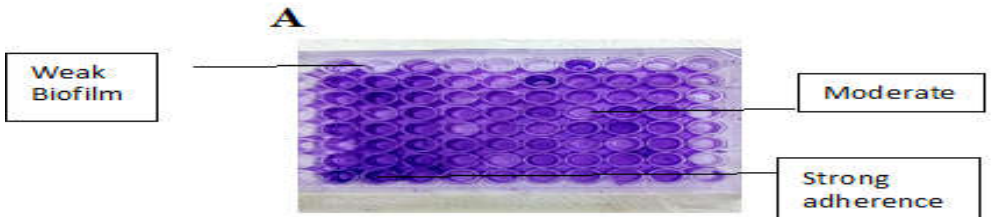
	Non-biofilm producer	Biofilm producer	Chi-square value	p-value
Inpatient	13(81.2%)	73(82.0%)	0.005	0.941
Outpatient	3(18.8%)	16(18.0%)		
K. pneumoniae	16(100.0%)	84(94.4%)	0.944	0.331
Ozaenae	0(0%)	5(5.6%)		

Figure-1: **Biofilm Production and Biochemical Tests**

<table>
<tr><td>A. MacConkey Agar</td><td>B. Mueller Hinton Agar</td></tr>
<tr><td>  </td><td>  </td></tr>
<tr><td colspan="2">Biofilm production - Microtitre plate Method</td></tr>
<tr><td>C. Biochemical Tests for K. pneumoniae</td><td>D. Biochemical Tests for the subspecies K. pneumoniae.</td></tr>
<tr><td>  </td><td> 
Lactose/ Methyl red/ Voges proskauer/Lysine/Ornithine</td></tr>
<tr><td></td><td></td></tr>
</table>

In the Figure-1 the following are displayed as:
Box A shows MacConkey Agar produces dome shaped, smooth, Lactose fermenting colonies
Box B shows Mueller Hinton Agar produces the antibiotic Susceptibility pattern
Box C shows the biochemical tests for *K. pneumoniae*
Box D shows the biochemical tests for subspecies *K. pneumoniae.*

Figure-2: **Biofilm characterization formation from weak to moderate and to strong adherences**



Characteristics of Clinical Samples
From Jan 2021 to Jan 2022, 105 *K. pneumoniae isolates* were examined from total clinical bacterial isolates at Mahatma Gandhi Medical College & hospital, India. *K. pneumoniae* isolates were isolated from 59 male (56.2%) and 46 (43.8%) female patients (Table-1). Most of K. *pneumoniae* were isolated from patients aged 41- 60 years old. *K. pneumoniae samples* were mostly isolated from urine specimens (42.9%) and pus (19.0%).

Antibiotic resistant Pattern
Most of *K. pneumoniae* were resistant to a wide range of antibiotics. Among biofilm producer isolates*, K. pneumoniae* had only a good resistant to ceftriaxone/cefotaxime (80.9%), Amikacin (78.7%), and Piperacillin+ Tazobactam (79.8%), Meropenem (79.8%). In contrast, among non-biofilm producer isolates, *K. pneumoniae showed* good resistant to Gentamicin (87.5%), ciprofloxacin+ Norfloxacin (87.5%), Amikacin 15 (93.8%) and Cefoperazone+ sulbactam 12 (75.0%) of resistance respectively. (Table-2)

Biofilm Formation Detection
In this study, among the 105 *K. pneumoniae* isolates tested, there were 89 (84.76%) isolates as biofilm producer and 16 (15.2%) isolates that were not biofilm producers. Among biofilm

producers, there were 36 (34.28%) isolates as strong, 42 (40%) isolates as moderate, and 11 (10.47%) isolates identified as weak biofilm producers (Table-2).

Association between type of patients and biofilm producing capacity

In this study, inpatients show more biofilm production than outpatients, because of immunocompromised and long term of hospitalization. In subspecies, *K. pneumoniae subspecies pneumoniae shows* more biofilm production than *K. pneumoniae* subspecies *ozaenae.*

Discussion

The percentage of *K. pneumoniae* isolates in the present study was 17.36% of all clinical bacterial isolates from January 2021 to January 2022. Considering that *K. pneumoniae* is one of the major global sources of MDR infections, this proportion be cause for significant anxiety. These bacteria are frequently linked to HAIs and extremely contagious outbreaks that have longer hospital stays and high fatality rates, all of which drive up healthcare expenses.[16]. Male patients provided the majority of the *K. pneumoniae isolates* used in this investigation. This outcome was consistent with the findings of Osagie et al.[17] who obtained samples from five primary healthcare facilities in Nigeria and said that males were more likely than females to be infected with *K. pneumoniae.* Additionally, Akter et al. [18] noted that male patients were more likely than female patients to be infected with *Klebsiella.* Gender and the occurrence of *K. pneumoniae* were linked to unhealthy lifestyle choices, such as alcohol consumption and smoking. Nevertheless, those investigations did not disclose any statistically significant differences between male and female subjects [17].

The majority of the *K. pneumoniae* used in this investigation came from patients between the ages of 41-60 years of age. This conclusion deviates from a prior study that found the majority of *K. pneumoniae* isolates were from patients who were older than 70. However, a different recent study revealed that individuals between the ages of 40 and 65 accounted for a higher proportion of *K. pneumoniae* isolates[19]. The variations in the age distribution of patients may be associated with the immune system response strength, which is predicted to decrease with ageing. Since patients under 40 often have stronger immune systems, K. *pneumoniae is* under more pressure to combat the host's immunity. On the other hand, because concurrent illnesses become increasingly common as people age, they are more likely to contract *K. pneumoniae*. *K. pneumoniae is* linked to a significant percentage of ventilator-acquired pneumonia and hospital-acquired pneumonia, which usually affects susceptible patients in intensive care units.

Urine specimens were the primary source of *K. pneumoniae* isolates. Ashurst and Dawson[15] highlighted that *K. pneumoniae* usually colonized the gastrointestinal tract and oropharynx mucosal surfaces in humans. Because of this, *K. pneumoniae* is thought to be the most frequent cause of hospital acquired pneumonia in the US. In contrast, wang et al.[20] found that the Republic of China's dominant site of K. *pneumoniae infection* was the respiratory system. Similar findings were made by Seifi et al.[21]who obtained samples from two hospitals in Tehran. They found that

the proportion of *K. pneumoniae* in the urine, surgical wounds, sputum and blood were 61.7%, 18.1%, 11.7% and 8.5% respectively.

The majority of *K. pneumoniae* was resistant to several antibiotics; the most effective combination against *K. pneumoniae* was Cefoperazone+ sulbactam, followed by Fosfomycin. The least combination was cotrimoxazole, ceftriaxone+ cefotaxime. The research done by Madahiah et al.[22] which indicated that the isolates of *K. pneumoniae were* 100% responsive to amikacin and 100% resistant to ampicillin, lends evidence to this account. The resistant percentages for ciprofloxacin and amoxicillin - clavulanic acid were 38.75% and 36.69%, respectively. This result is comparable to that of Cepas et al.[23] who found that 40% of *K. pneumoniae strains* were resistant to amoxicillin - clavulanic acid, as well as Ciprofloxacin.

The most important determinant in the development of antibiotic resistance is antibiotic exposure. Numerous causes contribute to the rise in antibiotic resistance, including the use of antibiotics in the population, in hospitals, in agriculture, and in the environment. Because they are frequently purchased, antibiotics are over prescribed. The key underlying cause responsible for the widespread transmission of nosocomial infections that are resistant to antibiotics and are difficult to cure in the health service context is most likely the extensive and continuous use of antibiotics[24].

In this study, among the 105 *K. pneumoniae* isolates tested, there were 89 (84.76%) isolates as biofilm producer and 16 (15.2%) isolates that were not biofilm producers. Among biofilm producers, there were 36 (34.28%) isolates as strong, 42 (40%) isolates as moderate, and 11 (10.47%) isolates identified as weak biofilm producers. Out of 110 *K. pneumoniae* studied, 70 isolates were found to be strong or moderate biofilm producers and 40 isolates to be weak biofilm producers, according to a similar study performed by Hassan et al.[25]. A different study by Cepas et al.[23] found that 37.6% of *K. pneumoniae* strains were capable of producing biofilm. According to Yang and Zhang, 62.5 % of the *K. pneumoniae isolated* from blood, sputum, urine, and wound swabs were biofilm producers. According to Seifi et al.[21] 93.6% of *K. pneumoniae were* biofilm producers, while the remaining 6.4% were not. Of the strains that produced biofilm, 33% were classified as strong producers, 52.1% as moderate producers, and 8.5% as weak biofilm producers. In a related investigation, Nirwati et al.[26] reported that biofilm production accounted for 85.63% of the K. *pneumoniae strains* identified from an Indonesian hospital[26]. Every isolate had a different capacity to form biofilms because, in simple terms, a number of factors influence this capacity, including the physicochemical characteristics of *K. pneumoniae,* the physical interactions between constituents, the type of surface to which the biofilm adheres, temperature, pH, and so on. The ability of *K. pneumoniae to* form biofilms and exhibit extensive drug resistance (XDR) has been shown by Vuotto et al.[13] to be correlated with a profile of antibiotic resistance.

According to this study, *K. pneumoniae,* a biofilm - producing bacteria, had higher levels of antibiotic resistance than non- biofilm producing bacteria. Numerous studies have reported on

this conclusion. According to a study by Saha et al.[27] all of the isolates that produced biofilms showed more resistant pattern than isolates that produced not an isolated event; however, the defensive mechanisms found in biofilms are distinct from those that cause conventional antibiotic resistance. The protective layer of the sticky biomaterial in biofilms, which prevents antibiotics from penetrating, the adaptive reactions to stress, and the development of persistent cells are thought to form a multi-layered defence that makes eradication more difficult, particularly when paired with the bacteria's resistance. Although it seems that bacterial biofilm formation and antibiotic resistance are major contributing factors to the worldwide spread of K. *pneumoniae,* a different study by Alcantar Curie et al.[28] suggested that the precise nature of this association remains unclear. De Campos et al.[29] corroborate this conclusion by stating that there was no discernible correlation between the ability to create biofilms and clonal types of MDR bacteria.

Numerous studies have demonstrated that, in the majority of circumstances, a single antibiotic treatment is insufficient to eradicate biofilm forming infections. Consequently, for the successful treatment of infections linked to biofilms, controlling infections with currently available antibiotics and assessing the results have become crucial and necessary tasks. Due to their strong antibiofilm activity both inside and outside of living organisms, a number of studies suggest combining antibiotic therapy with macrolides like azithromycin, clarithromycin, and erythromycin as the primary antibiotics for biofilm associated infections caused by gram negative bacteria· Wu et al.[30] proposed that, in addition to the administration of combined antibiotics, removal of infected foreign bodies and the source of infection as well as the quorum sensing inhibitors or biofilm dispersal agents would result in a more effective management for biofilm infections, taking into account the currently known environmental and bioecological aspects.

Our investigation demonstrated the issues with antibiotic - resistant bacteria in hospital settings, which have previously been shown in another investigations. Taking into account the quantity and quality of antibiotic prescriptions expressed in the majority of hospitals, this scenario is concerning. In 2012, hospital surveillance in Surabaya revealed that 30.6% of antibiotics were given without indications validated by susceptibility testing. As a result, prescribing antibiotics continues to be a difficult task everywhere, even in Indonesia. According to Vander Meer, prescription antibiotic guidelines, are not the best in Netherlands, a country with low rates of antibiotic resistance in bacteria and utilisation of antibiotics. According to their research, 15% of antibiotic therapy in wards dedicated to internal medicine and surgery was deemed adequate.

Penicillin, Cephalosporins (including third generation cephalosporins), and aztreonam are among the drugs whose resistance is mediated by ESBLs. In order to determine which gram-negative bacteria, create biofilms, Dumaru et al. Conducted a study in which they also determined the antibiograms of these bacteria and detected the development of metallo-beta-lactamases (MBLs) and EBLs, A statistically significant correlation has been detected between

the formation of biofilm and MBL. But there was no significant association between ESBL and biofilm formation.

Lack of knowledge about infections and the administration of antibiotics is the main factor contributing to the incorrect prescription of antibiotics. A crucial phase in the prescription of antibiotics is modifying the first course of treatment on the basis of the clinical microbiology findings. Testing for antibiotic susceptibility is therefore necessary. Another crucial step is gathering clinical samples prior to giving antibiotics. Numerous medical professionals who provide antibiotic prescriptions are unaware of the potential effects of their improper recommendations on the emergence of bacterial resistance. The selection pressure on the pathogenic bacteria entrusted with hospital-based infections will be reduced by modifying the first antimicrobial therapy in accordance with the clinical microbiology results. Therefore, based on the most recent microbiological data, it is crucial that every hospital implement an antibiotic guideline or stewardship programme for all pharmacists and clinicians. To combat the fast spread of antibiotic resistant bacteria, ongoing efforts in hospital surveillance, infection control, and clinical audits are required in addition to these recommendations.

Conclusions

The majority of the *K. pneumoniae* isolates have shown resistance to a variety of antibiotics and are capable of producing biofilms. In our study, we compared the biofilm producing capacity with subspecies of *K. pneumoniae.* The development of biofilms is a crucial step in the cause and effect of Klebsiella *pneumoniae* disease, as it increases resistance to an environmental stresses and acts as a reservoir for the spread of the bacteria and further gene exchange with antibiotic drugs. The production of biofilms by bacterial competitors in their colonizing environment is facilitated by many virulence factors. Numerous of these compounds have been investigated as potential vaccination candidates or as targets for novel antibacterial medicines.

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