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Biofilm Production and Porin Permeability Activity in Clinical Isolates of Ciprofloxacin-Resistant *Klebsiella pneumoniae* in the Tertiary Hospital in Purwokerto, Indonesia

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Abstract

World Health Organization reports that the current use of ciprofloxacin as a broad-spectrum antibiotic shows a pattern of high bacterial resistance in many countries. *Klebsiella pneumoniae* is known as a Gram-negative bacterium that most often causes infection and is resistant to antibiotics. This study aimed to find patterns of resistance mechanisms based on biofilm production and porin permeability activity in *K. pneumoniae* clinical specimens from patients at RSUD Prof. Dr Margono Soekardjo Purwokerto, Indonesia. Several isolates of ciprofloxacin-resistant *K. pneumoniae* were isolated from clinical specimens of blood, sputum, urine, pus, stool and pleural fluid from August to October 2022. Identification and sensitivity testing of *K. pneumoniae* to ciprofloxacin were performed using Vitek® 2 Compact. A test for biofilm production is carried out by measuring optical density (OD) with a microplate at a wavelength of 630 nm. The porin permeability activity was determined using the value of the minimum inhibitory concentration (MIC) by the LC-MS spectrophotometry method at a wavelength of 630 nm. The results showed that 72 (47 %) isolates were resistant to ciprofloxacin. Among them, 41.3 % (24/58) of ciprofloxacin-resistant *K. pneumoniae* were able to produce strong biofilm. Porin permeability is indicated by MIC values of 1, 2 and 4 mg/L with bacterial cell counts of 32×10^6, 19×10^6 and 34×10^6 CFU/mL, respectively. This number decreased from the initial control with a bacterial cell count of 10.15×10^7 CFU/mL. Analysis of the correlation between the significance of biofilm production and the number of bacterial cells indicated that biofilm formation was related to the number of bacterial cells ($R = 0.628$, $p \leq 0.05$). In conclusion, the resistance mechanism of ciprofloxacin-resistant *K. pneumoniae* involves biofilm production and decreased porin permeability activity.

Keywords: Antibiotic resistance, Biofilm, Ciprofloxacin, *Klebsiella pneumoniae*, Porin

Introduction

K. pneumoniae is a Gram-negative bacterium that has been the leading pathogen in human infections for several decades [1-3]. *K. pneumoniae* dominates (15.10 %) [4-6] among other Gram-negative bacterial strains during infections [7]. The presence of *K. pneumoniae* in hospitals is a major concern, as this bacterium has been linked to nosocomial infections [8]. The emergence of antibiotic-multiresistant strains has increased and spread widely in recent decades [9,10]. It makes antibiotic treatment in cases of *K. pneumoniae* infection even more difficult [11-13]. Patients in intensive care for a long time increase the risk factor for severe infection caused by *K. pneumoniae* [14]; therefore, the intensive care room can be a place for the persistence and proliferation of antibiotic-resistant microorganisms [2,4,15]. The patients used in this study met special categories by considering the characteristics of gender, age, treatment room in the hospital and type of patient specimen.

Over-prescribing and improper antibiotic use can trigger an increase in antibiotic resistance [16,17]. Antibiotic treatment for patients must pay attention to the sensitivity of bacterial isolates to various types of antibiotics [18,19]. Ciprofloxacin as a broad-spectrum antimicrobial agent [20] is one of the first-line

choices for Enterobacteriaceae bacterial infection therapy [21,22]. The degree of resistance to these antibiotics varies among bacteria caused by the use and dosage of antibiotics. World Health Organization reported a higher increase in the bacterial resistance of *K. pneumoniae* in 33 and 34 country regions, i.e., from 4.1 to 79.4 % [23]. Ciprofloxacin is the most effective antibacterial agent with a resistance rate of 27.8 % in Multi Drug Resistance (MDR) *Staphylococcus aureus* [24]. Major resistance mechanisms in *K. pneumoniae* decreased cell permeability from porin activity [25] and biofilm formation [26,27].

Bacteria produce biofilm to increase their resistance to antibiotics [28]. Biofilm cells will become 10 - 1,000 times more resistant to antimicrobial actions than planktonic cells [29]. *K. pneumoniae* possesses type 3 fimbriae, which are thought to help with biofilm development. It is the main determinant of specificity in fimbrial binding; therefore, this bacterium has a good ability to form biofilms of 98.8 % [30]. A study at Klaten Tertiary Hospital reported that a total of 64.7 % of *K. pneumoniae* clinical isolates produced weak, medium and strong biofilms [26]. *K. pneumoniae* isolates from sputum, blood and urine specimens were able to produce 37.6 % of the biofilms, while in MDR cases, it was found to produce 38 % of the biofilms [31]. The production of biofilm correlates with bacterial resistance to specific antibiotics. Most biofilm-producing strains exhibit higher resistance than non-biofilm producers.

Gram-negative bacteria are wrapped by an outer membrane that acts as an antimicrobial permeability barrier. Bacterial outer membrane permeability to antimicrobial substances influences inherent resistance [32]. Porins are non-specific diffusion transport proteins that play a part in the antibiotic resistance process [33]. *K. pneumoniae* that lacks extended-spectrum-beta-lactamase (ESBL) can express OmpK35 and OmpK36 porins. Case studies of ciprofloxacin use were found to be a major factor in bacterial resistance to antibiotics and their relation to patterns of spread in hospitals. The increasing prevalence of resistant *K. pneumoniae* strains encourages the importance of studying their resistance to ciprofloxacin. The evolutionary development in the case of antibiotic resistance has an impact on the emergence of understanding in new perspective patterns on how to overcome health problems related to antibiotic resistance.

This study is expected to help with the management of antibiotic usage in hospitals and the general population, hence reducing the development of MDR strains. The study's uniqueness and urgency emerge from its simultaneous examination of the mechanisms of resistance of *K. pneumoniae* isolates to ciprofloxacin in patients at RSUD Prof. Dr. Margono Soekarjo Purwokerto, Indonesia.

Materials and methods

Ethical approval

This research received ethical approval (Number: 420/08640) from the Ethics Commission of the Regional General Hospital, Prof. Dr Margono Soekarjo Purwokerto, Indonesia.

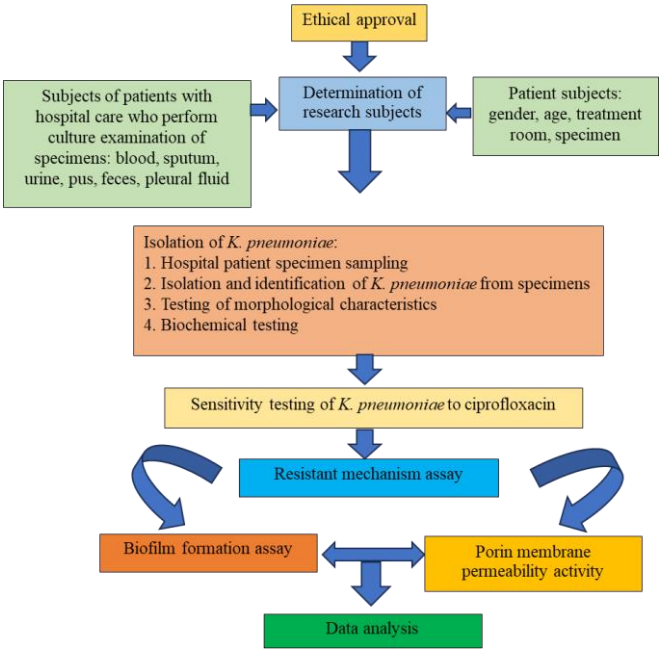

Figure 1 Research workflow.

Isolation and identification of *K. pneumoniae* clinical isolate

This study used clinical specimens, including blood, urine, sputum, pus, stool and pleural fluid, from inpatients at RSUD Prof. Dr Margono Soekarjo Purwokerto, Indonesia. Different isolation techniques were performed to isolate *K. pneumoniae* from different types of specimens. Samples were grown on MacConkey agar media. The growing bacteria were identified by Gram staining and biochemical assays using Vitek® 2 Compact.

Antibiotic sensitivity test

Testing of bacterial resistance to ciprofloxacin was carried out with the Vitek® 2 Compact tools and using the Gram-negative VITEK® 2 AST-GN93 bacterial reagent. The Clinical and Laboratory Standards Institute (2015) is used to differentiate between bacteria that are susceptible, intermediate, or resistant [34].

Biofilm formation

A total of 3 mL of bacterial culture was inoculated on 50 mL of Trypticase Soy Agar (TSB) media and incubated for 6 h at 37 °C using a shaking incubator. Bacterial culture diluted 1:100 (10 µL of bacterial culture: 1 mL of TSB media). A total of 100 µL of TSB was poured on a 96-well microtiter plate and 10 µL of diluted bacterial culture and incubated at 37 °C for 18 - 24 h. To eliminate non-adherent bacteria, the microtiter plate is washed with 300 µL of phosphate-buffered saline (PBS). For 30 min, 100 µL of 1 % crystal violet is applied to the microtiter plate. After removing the crystal violet, the plate is cleaned and dried. A 5 % of isopropanol acid is added to the stained plate. The 70 % ethanol is used as a blank sample. The OD value was measured at a wavelength of 630 nm. Interpretation of the OD value of biofilm-producing cultures was carried out based on average OD values (**Table 1**).

Table 1 Interpretation of OD values of biofilm production.

Biofilm production	OD value (mean)
Weak	ODc/ODc < ~ ≤ 2×ODc
Medium	2×ODc < ~ ≤ 4×ODc
Strong	> 4×ODc

Note: Meanwhile, the isolate was categorized as a biofilm producer consisting of weak biofilm producer if 2×ODc < OD ≤ 4×ODc, moderate biofilm 2×ODc < OD ≤ 4×ODc and strong biofilm producer if OD > 4×Odc [35]. Optical density cut-off value (ODc) = average negative control OD + 3×negative control SD. The absorbance is read at a wavelength of 630 nm.

Porin membrane permeability activity

The MIC value was determined by quantitative methods using Mueller-Hinton agar and broth media (Oxoid). The required solvent is adjusted for ciprofloxacin as an indicator antibiotic. In the quinolone group, water is used as a solvent. The antibiotic is dissolved in the solvent to a final volume of 1 mL and added to 19 mL of warm MHA medium (temperature 45 - 50 °C), then poured into a petri dish (9 cm in diameter). Inoculum preparation: 0.5 McFarland suspension is prepared (the McFarland bacterial suspension has a density of 5×10^5 CFU/mL). Preparation of broth medium: 0.5 McFarland suspension diluted 100×(9.9 mL broth media + 0.1 mL suspension) to a density of 10^6 CFU/mL. The suspension is poured into wells containing the appropriate concentration of antibiotics (50 µL bacterial inoculum plus 50 µL liquid medium with antibiotics or 10 µL inoculum to 100 µL diluted antibiotics). The bacterial inoculum must be adjusted to a 0.5 McFarland suspension as measured by a densitometer measurement test or spectrophotometer. The absorbance wavelength of 630 nm is in the range of 0.08 to 0.13. Inoculum was added to liquid or solid media with antibiotics to maintain adequate cell density (CFU/mL). During the test, the medium was aerobically incubated at 35 ± 1 °C for 18 - 24 h. The resultant MIC value is the lowest antibiotic concentration at which bacterial growth is inhibited. The concentrations of ciprofloxacin tested were 1, 2 and 4 mg/L. Bacterial growth was characterized by turbidity of the broth medium at the lowest concentration compared to control growth [36].

Statistical analysis

The variables in this current study were described using frequency and percentage. Correlation between biofilm formation variables in resistant *K. pneumoniae* isolates and the number of bacterial colonies based on MIC values describing porin permeability. The correlation between biofilm formation

variables in resistant *K. pneumoniae* isolates and the number of bacterial colonies based on MIC values was tested using Pearson's correlation. The correlation segregation analysis used is logarithmic with IMB SPSS Statistics 26 software. The data is presented as prevalence ratios with 95 % confidence intervals. The statistical significance level is set at < 0.05.

Results and discussion

Isolation and identification of clinical isolates

K. pneumoniae were isolated from 86 male patients (55.48 %) and 69 female patients (44.52 %) from ICU, PICU, ICCU, HCU, poly-surgery and inpatient installations. *K. pneumoniae* was a Gram-negative bacteria that was found to dominate in clinical specimens, including blood (6.45 %), sputum (65.16 %), pus (17.42 %), urine (8.39 %), stool (1.94 %) and pleural fluid (0.65 %) (**Table 2**). The age distribution of patients ranged from 9 days to 82 years, with the majority of patients over 60 years old (56.77 %).

Table 2 Patient characteristics of clinical isolate origin of *K. pneumoniae*.

Characteristics		Total	
		n	**%**
Sex	Male	86	55.48
	Female	69	44.52
Age (years)	< 15	17	10.97
	15 - 59	49	31.61
	> 60	88	56.77
Room	Non-ICU	97	62.58
	ICU/PICU/ICCU/HCU	58	37.42
Specimen	Blood	10	6.45
	Sputum	101	65.16
	Pus	27	17.42
	Urine	13	8.39
	Stool	3	1.94
	Pleural fluid	1	0.65
Total		**155**	**100**

The presence of *K. pneumoniae* in different patient specimens in the current study is comparable with the findings of [37], who discovered that 28 % of ICU patients tested positive for *K. pneumoniae*. In the cases of urinary tract infection, *K. pneumoniae* was found to be dominant (23 %), followed by *Escherichia coli* [38]. The majority of *K. pneumoniae* infections in this study were found in male patients. Separate studies in India and Nigeria found that males are more likely than females to be infected with *K. pneumoniae* [39], [40]. A similar study in Klaten Indonesia by Nirwati *et al.* [26] found the percentage of *K. pneumoniae* infection in men was 64.07 %. Male individuals are more likely to become infected with *K. pneumoniae* [41]. A poor lifestyle, smoking and alcoholism in men are often associated with the incidence of *K. pneumoniae* infection. However, no statistically significant differences between both genders of individuals were discovered [40].

In this study, most *K. pneumoniae* infections were found in patients over 60 years old. Cases of *K. pneumoniae* infection are highest in patients aged 20 - 29 years [41]. Patients over 60 years of age constitute the majority group primarily associated with pneumonia, chronic obstructive pulmonary disease infection, and other respiratory diseases, while the lowest age group ranges from 16 to 30 years [43]. Previous findings also revealed that patients over 70 years old had a significantly higher chance of becoming infected with *K. pneumoniae* than younger age groups [42]. The percentage of infection cases in various age groups is related to the human immune system [43]. Patients under the age of 40 tend to have stronger immunity and

put pressure on bacteria to fight the immunity of their host. The contrary happens as one gets older, which increases the risk of *K. pneumoniae* infection due to the prevalence of comorbidities [14].

This study succeeded in isolating *K. pneumoniae* from various sources, including sputum, blood, urine, pus, stool and pleural fluid. Sputum samples are the most common source of *K. pneumoniae*. A study reported 28 sputum specimens infected with *K. pneumoniae* [44]. Infection with *K. pneumoniae* in the lungs can cause destructive changes, necrosis, inflammation and bleeding within the lung tissue. Sometimes, it results in sputum becoming viscous, bloody and mucoid [45]. *K. pneumoniae* may be found in various locations across the human body, such as the skin, pharynx, digestive tract, wounds and urine. Wang *et al.* [46] reported that the percentage of *K. pneumoniae* infection in specimens of sputum, urine, blood and bronchoalveolar fluid was 68.48, 15.24, 5 and 3.34 %, respectively, in China teaching hospitals. Macromorphological observations of *K. pneumoniae* isolates grown on MacConkey media revealed characteristics, including large colony size, pink-brick red colour, smooth colony surface, convexity and sliminess (**Figure 2**).

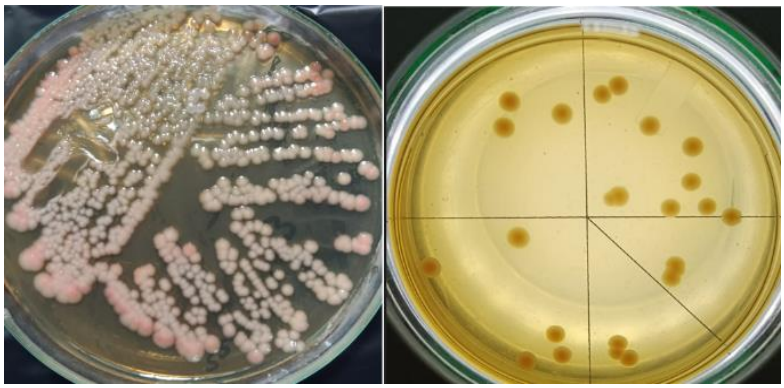

Figure 2 Macromorphological characteristics of *K. pneumoniae* isolates grown on MacConkey agar medium. (Note: Large colony size, pink-brick color, smooth colony surface, convex and slimy). The *K. pneumoniae* isolate was obtained from sputum specimens grown on MacConkey growth media for 1×24 h.

These results are consistent with the characteristics of *K. pneumoniae* isolates reported by previous research in clinical settings. *K. pneumoniae* can be isolated from various specimens, such as pus, tracheal aspirate, sputum, wound swabs and catheter tips [47]. Similarly, [48] isolated *K. pneumoniae* from urine, blood, stool, peritoneal and pleural fluid.

Resistance of *K. pneumoniae* to ciprofloxacin

Testing of bacterial resistance to ciprofloxacin was carried out by phenotypic observation. The results showed that 72 (46.5 %) cases were resistant, 73 (47 %) were sensitive and 10 (6.5 %) were intermediates. The percentage of resistant *K. pneumoniae* in this current study is similar to a reported case in the UK and Ireland. Phenotypic results showed that more than 65 % of the sample population was resistant to ciprofloxacin (resistant: 164, sensitive: 62 and intermediate: 24) [3]. Microbiological diagnosis reported 39 % of resistant cases, which increased to 60 % during the study [49]. The main agents of Enterobacteriaceae (32 %), non-fermented Gram-negative bacteria (27.6 %) and pathogenic microbes varied significantly.

Bacterial resistance to ciprofloxacin can be caused by independent risk factors, for example, unsuitable use of ciprofloxacin therapy. In this present study, the prevalence of ciprofloxacin-resistant *K. pneumoniae* was relatively high, i.e., 46.5 % of cases. The precision level of *K. pneumoniae* against ciprofloxacin is 40 % [50]. The level of ciprofloxacin resistance shows that the infection prevention procedures and antimicrobial management adopted by RSUD Prof. Dr Margono Soekardjo Purwokerto have to be improved. However, they have reduced selective pressure and limited the spread of ciprofloxacin resistance. RSUD Prof. Dr Margono Soekardjo Purwokerto is a level 3 referral (tertiary) hospital that accepts patients from level 1 and 2 healthcare facilities. Thus, patients may have received antibiotic therapy at the previous healthcare facility.

Antibiotics of the fluoroquinolone group and ciprofloxacin derivatives are still commonly used. This antibiotic has a broad spectrum and is effective in the treatment of various infections in hospitals, including *K. pneumoniae* infection. Its virulence and broad pathogenicity resulted in a rise in ciprofloxacin resistance. Bacterial resistance can occur because bacteria have several traits that are intrinsically acquired, externally acquired and adaptive [51].

This study showed that *K. pneumoniae* from sputum specimens was more resistant to ciprofloxacin than in urine, blood and other specimens. We observed the highest rate in sputum samples, followed by samples of urine and blood, which is consistent with nationwide findings in China [52]. This suggests that *K. pneumoniae* is one of the main pathogenic bacteria of respiratory tract infections, which should be taken into account during diagnosis.

The resistance of *K. pneumoniae* to ciprofloxacin can be caused by several mechanisms, including the destruction or modification of ciprofloxacin and the ability of *K. pneumoniae* to survive by forming a biofilm. Changes in target sites with site mutations, enzymatic sites and protected sites, as well as changes in overproduction, can also increase antibiotic resistance. In addition, *K. pneumoniae* was able to reduce antibiotic accumulation by decreasing membrane permeability (porin activity) and/or increasing efflux pump activity [9,54,55].

Gram-negative bacteria acquire antibiotic resistance because the outer membrane functions as a permeability barrier for various substances. The natural resistance of some Gram-negative bacteria to antibiotics is due to the low permeability of the outer membrane to specific antibiotics [32]. Changes in the outer membrane's permeability might play a role in the development of acquired resistance.

Biofilm formation

The ability of ciprofloxacin-resistant *K. pneumoniae* to produce biofilm is 1 mechanism of their resistance to antibiotics. The discovery of biofilm-forming, ciprofloxacin-resistant *K. pneumoniae* could impact the administration and quantity of antibiotic prescriptions in hospitals. Saha *et al*. [56] stated that biofilm-forming resistant bacteria make antibiotic treatment inefficient and lead to chronic infections. This present study showed that a total of 58 isolates were able to produce biofilm (**Table 3**).

Table 3 Biofilm production capacity of ciprofloxacin-resistant *K. pneumoniae*.

Category	n (%)
Weak biofilm producer	12 (20.69)
Medium biofilm producer	22 (37.93)
Strong biofilm producer	24 (41.38)
Total	**58 (100)**

Biofilm testing with spectrophotometric methods is an indirect method to estimate the presence of bacteria in situ that quickly analyzes the adhesion of several strains of biofilm-forming bacteria [56]. The ability of isolates to produce biofilm can be seen from the OD value. The average OD value was calculated based on the cut-off value (ODc = 0.089459). A total of 41.4 % (24/58) isolates were classified as strong biofilm producers, with an average OD of 0.9401. The medium biofilm-producing group was 37.9 % (22/58) with an average OD of 0.2428. The weak biofilm-producing group of 20.7 % (12/58) produced an average OD of 0.1176. This OD value is much higher when compared to control isolates (sensitive isolates) that do not produce biofilm.

A total of 24 isolates of potent biofilm-producing *K. pneumoniae* were isolated from patients in intensive care (ICU, ICCU, PICU and HCU) and inpatients. Most *K. pneumoniae* were isolated from sputum specimens. However, isolated bacteria can also be found in urine, blood, pus, stool and pleural fluid. Patients infected by potent biofilm-producing *K. pneumoniae* were found mostly in patients over 40 years old. However, there was 1 case in a 55-day-old baby. Biofilm production by ciprofloxacin-resistant *K. pneumoniae* was performed in TSB media for 24 h using a microtiter plate. The growing biofilm is a mixture of bacterial colonies that are protected by a polysaccharide matrix, DNA and proteins. The biofilm produces extracellular polymeric substances (mucous membrane, slime). Biophilic observation showed the appearance of interlocking bonds and intertwining between colonies (**Figure 3**).

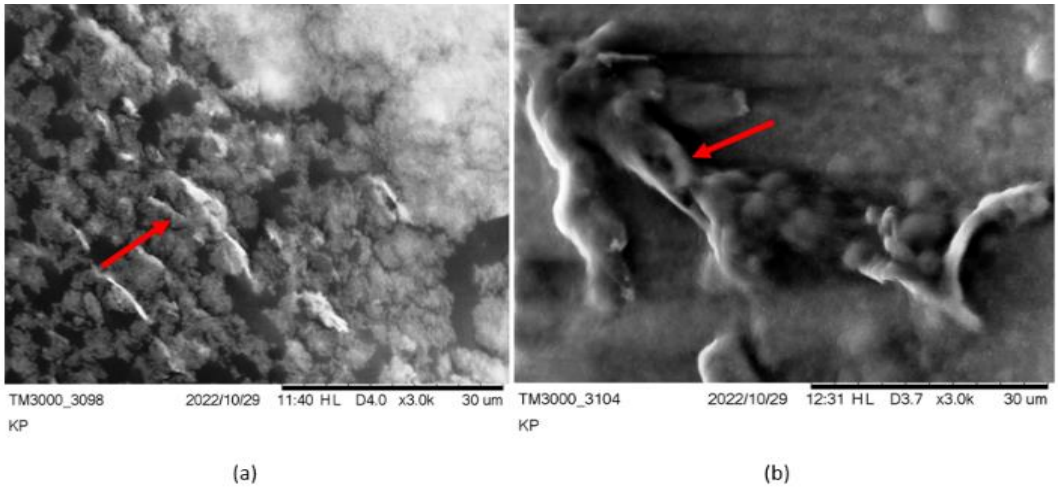

Figure 3 (a) Appearance of biofilm-producing ciprofloxacin-resistant *K. pneumoniae* colonies (aged 24 h) in SEM observations and (b) biofilms are protected by matrix extra polysaccharides (EPS) and extracellular polymers (mucus).

Biofilm-forming bacteria have been thought to have a major impact on antibiotic resistance through the release of planktonic bacteria into surrounding tissues. Biofilms become a potential source of infection and may cause chronic, persistent, or recurrent infections [57]. This current study showed that ciprofloxacin-resistant *K. pneumoniae* isolates produce biofilm (**Table 3**). Another study found that 37.6 % of *K. pneumoniae* strains are biofilm producers [31]. These bacteria are included in MDR cases; among them, 38 % of cases were resistant to 3 or more antibiotic groups, and most cases were resistant to ciprofloxacin. In another study, [26] isolated 143 (86.63 %) biofilm-producing *K. pneumoniae* isolates in the strong (26.95 %), medium (28.74 %) and weak (29.95 %) categories.

K. pneumoniae were isolated from various specimens, including sputum and urine, which were capable of producing biofilm [42]. *K. pneumoniae* isolates from sputum had differences in biofilm formation rates ($p \leq 0.01$) between older patients (more than 70 years) and younger patients (less than 70 years). Biofilm is more often formed in older patients. Biofilm formation is an important stage in bacterial virulence and resistance. In the logarithmic phase, biofilm formation provides a stronger resistance to antibiotics. Initial adhesion, microcolony growth, mature biofilm development and the discharge of planktonic bacteria from the biofilm define early-stage biofilm formation. It has been proposed that *K. pneumoniae* biofilm development contributes to bacterial resistance. It decreases bacterial susceptibility to antibiotics [58]. Biofilm-forming resistant bacteria will continuously develop their resistance to various antibiotics. The biofilm matrix prevents the efficiency of antibiotic diffusion, thus causing exposure to bacteria in the biofilm to decrease significantly.

The present study suggests that ciprofloxacin-resistant *K. pneumoniae* is a biofilm producer. Each isolate has a varying capacity to produce biofilms. It is influenced by factors such as the physicochemical characteristics of *K. pneumoniae*, physical interactions between constituents, the type of surface to which the biofilm attaches, environmental temperature and pH and others [59]. It has also been reported in many studies. In addition, biofilm-producing *K. pneumoniae* displays a more resistant pattern compared to non-biofilm producers [55].

The activity of porin permeability

Porin permeability is a mechanism of bacterial resistance. The porin permeability is analyzed by determining the MIC value using the microdilution method. This detection shows the ability of bacterial permeability to absorb minimal concentrations of ciprofloxacin, which can inhibit bacterial growth. The absorption analysis of the ciprofloxacin concentration was measured by the OD value as an indicator of turbidity.

Testing the activity of porin permeability was performed by using 1 mg/L of ciprofloxacin as the minimum MIC value and a dose of 4 mg/L as the maximum MIC value. The results showed that 34 isolates had a MIC value of 4 mg/L, 6 isolates had a MIC value of 2 mg/L and 18 isolates had a MIC value of 1 mg/L (**Table 4**). The ciprofloxacin absorption by *K. pneumoniae* isolates was indicated by a decrease in OD values. The resistant isolates (with 4 mg/L of ciprofloxacin) resulted in an OD value ranging from

0.033 to 1.005. This result was followed by a decrease in bacterial cells to 1.79×10^6 CFU/mL from the initial cell count of 10.15×10^7 CFU/mL.

Table 4 Ciprofloxacin MIC value with resistance variable parameter.

		MIC (mg/L)		
		1	**2**	**≥ 4**
Male	35			
Female	23			
Sample's number		18	6	34
Absorbance value (OD)	Minimum	0.55	0.065	0.033
	Maximum	1.253	0.927	1.005
	Mean (average)	0.584	0.4895	0.284
Bacterial cell count	Minimum	3	1	1
	Maximum	75	72	79
	Mean (average)	32	19	34
Inhibition (%)	Minimum	3.509	7.232	2.956
	Maximum	91.311	90.566	93.924
	Mean (average)	31.506	34.719	47.22
Biofilm producer (isolate)	Strong	6	1	17
	Medium	10	2	10
	Weak	2	3	7

This study showed that 6 isolates responded to the MIC value with a dose of 2 mg/L and an OD value of 0.065 - 0.927. The absorbance value shows a range of decreased OD of 0.064 - 0.624, compared to the control OD of 0.435 - 1.051. The number of bacterial cells decreased by 1.72×10^6 CFU/mL compared to the control value. The treatment of 1 mg/L ciprofloxacin in 18 isolates resulted in an OD value ranging from 0.055 - 1.253 with a decrease in OD of 0.002 - 0.934. The number of bacterial cells was 01.72×10^6 CFU/mL, lower than the initial bacterial cell count of 10.15×10^7 CFU/mL. The weak biofilm-producing isolates had a MIC value of 4 mg/L in 7 isolates, a MIC value of 2 mg/L in 3 isolates and a MIC value of 1 mg/L in 2 isolates. This condition shows that the level of resistance is related to biofilm formation.

This study tested porin permeability as an indicator of a resistance mechanism. The results showed that ciprofloxacin-resistant *K. pneumoniae* varied in MIC values ranging from 1, 2 and ≥ 4 mg/L. Decreased absorbance and reduced number of colonies in MIC treatment ≥ 4 mg/L indicated inhibition of bacterial growth by ciprofloxacin. Resistant *K. pneumoniae* isolates have a minimum limit to be inhibited at MIC concentrations ≥ 4 mg/L. This concentration can inhibit bacterial growth due to the malfunction of bacterial cell wall permeability in limiting the entry of antibiotics. The results of the MIC test showed that the isolates are categorized as highly resistant isolates. According to the standards of the Clinical and Laboratory Standards Institute (CLSI), bacteria resistant to ciprofloxacin have a MIC value of ≥ 4 mg/L [60], even if, this value is different from MIC in the case of susceptible isolates which have a lower MIC value, i.e., 0.016 to 0.75 mg/L.

The results confirmed, at each bacteriostatic and linearly significant concentration, a decrease in the number of colonies (CFU/mL) and suspension turbidity. In the resistant isolates, the antibiotic had bactericidal activity within 24 h of exposure. In susceptible isolates, the antibiotic has bactericidal to bacteriostatic activity within a 24-hour exposure period. The high MIC value in resistant isolates proves that the bacteria survive antibiotic exposure. They use membrane permeabilization to defend against the antibiotic; therefore, bacterial growth is increasing.

The distribution of biofilm production in ciprofloxacin-resistant *K. pneumoniae* is associated with bacterial growth. In weak biofilm producers, the average number of bacterial cells was 4×10^6 CFU/mL. It

is much less than the average number of bacterial cells in strong biofilm producers, i.e., 44×10^6 CFU/mL (**Table 5**).

Table 5 The statistical analysis of Pearson's correlation between biofilm production, the number of colonies and the MIC values.

Biofilm production		Cell count (10^6 CFU/mL)	R (correlation regression)	*p*-value
			0.628**	**0.000**
Weak	Minimum	1		
	Maximum	14		
	Mean (average)	4		
Medium	Minimum	2		
	Maximum	72		
	Mean (average)	34		
Strong	Minimum	13		
	Maximum	79		
	Mean (average)	44		

Note: **Statistical significance, $p \leq 0.005$.

The statistical analysis of Pearson's correlation between biofilm production, the number of colonies and the MIC values showed a significant, strong-positive relationship (R = 0.628, $p \leq 0.05$). The correlation regression test on the ANOVA showed statistical significance ($p \leq 0.05$). Strong biofilm production indicates a high number of *K. pneumoniae* cells (**Figure 4**).

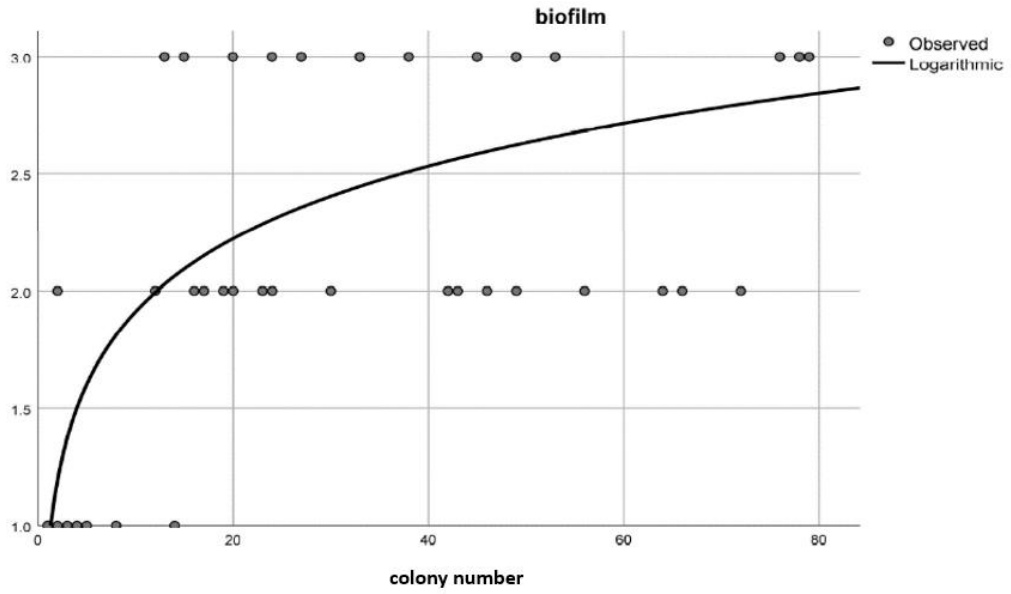

Figure 4 Logarithmic graph of the regression correlation of biofilm formation with colony number (note: MIC of 1, 2 and 4 mg/L).

Ciprofloxacin is a class of antimicrobial compounds that affect cell membranes and biofilm matrices. By functioning as a diffusion barrier, the biofilm matrix contributes to antibiotic resistance. Antibiotic susceptibility can be affected by changes in the biofilm matrix. Exopolysaccharides (EPS) influence material diffusion into and out of the biofilm, thereby creating microenvironmental diversity in the biofilm [61]. Microdilution analysis is used to determine the volume of living cells in biofilm below the MIC value.

Ciprofloxacin-resistant *K. pneumoniae* isolates resulted in strong, medium and weak (thin) biofilm formations of 41.38, 37.93 and 20.69 %, respectively. The final average number of bacterial cells was 44×10^6, 34×10^6 and 4×10^6 CFU/mL. This value is lower than the control, which is 10.15×10^7 CFU/mL. Tiwari *et al*. [61] used quaternary ammonium methacrylate as an antimicrobial compound, resulting in resistant isolates. The results showed that biofilm formation below the MIC value would be significantly thinner, indicating its formation was inhibited by antimicrobial compounds.

This present study showed that the bacteria that survived the treatment were considered to have intrinsic resistance factors. Bacterial resistance is affected by walls and membranes of cells [62]. For example, antimicrobials cannot easily access the working system of bacteria due to the lower permeability of the outer membrane as well as the ability of the efflux pump to cause an increase in antimicrobial efflux. Bacteria with an active pump will push ciprofloxacin out of the cell before entering the cell wall. The maturity of biofilms is also inseparable from their susceptibility to antimicrobial compounds. When the biofilm has formed, the antimicrobial compounds will diffuse into the biofilm at a lower concentration. It makes bacteria deactivate the efflux pump and susceptible to the compounds. Similarly, this study showed that during the formation of potent biofilms under MIC, the number of bacterial colonies was significantly lower than control values without MIC treatment.

Conclusions

K. pneumoniae subspecies *pneumoniae* was found to dominate in the clinical specimens (sputum, pus, urine, stool and pleural fluid) from patients with treatment at RSUD Prof. Dr Margono Soekardjo Purwokerto, Indonesia. The presence of ciprofloxacin-resistant *K. pneumoniae* increased from August to October 2022, which was 46.5 %. Ciprofloxacin-resistant *K. pneumoniae* produces biofilm as their resistance mechanism. They have low activity of porin permeability at MIC values of 1, 2 and 4 mg/L. The biofilm production has a strong positive significant correlation with the number of growing colonies at MIC values.

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