Prevalence and diversity of ciprofloxacin-resistant *Klebsiella pneumoniae* clinical isolates

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Abstract. The persistence and proliferation of multidrug-resistant microorganisms have the potential to occur in health centers and patient care units. WHO reports that cases of *Klebsiella pneumoniae* infection treated with ciprofloxacin in 34 countries have resistance from 4.1% to 79.4%. This study aimed to analyze the prevalence and diversity of ciprofloxacin-resistant *K. pneumoniae* from patients at Margono Soekardjo Hospital, Purwokerto. This study used a purposive sampling technique from blood, sputum, urine, pus, feces, and pleural fluid. The research process includes isolates characterization and identification, ciprofloxacin resistance testing, and its prevalence. This study recorded 99 cases of *K. pneumoniae* infection, and 48.5% of isolates were resistant to ciprofloxacin from August-September 2022. Biochemical and morphological tests showed a characteristic diversity of isolated *K. pneumoniae* against *K. pneumoniae* subspecies *pneumoniae*. The prevalence of ciprofloxacin-resistant *K. pneumoniae* was from blood (7.1%), sputum (59.6%), pus (23.2%), urine (6.1%), feces (3.03%), and pleural fluid (1.01%).

Keywords: *Klebsiella pneumoniae*, ciprofloxacin-resistant, prevalence, diversity

1. Introduction

Klebsiella pneumoniae is the main human pathogen causing infectious diseases over the past few decades [1]–[3], including pneumonia, urinary tract infections, bacteremia, and liver abscess [4]. In the hospital, the presence of *K. pneumoniae* is a major concern. Cases of bloodstream infections in hospitals were acquired from *K. pneumoniae,* predominated (15.10%) Gram-negative bacteria which caused 12-80% mortality [5]–[8]. *K. pneumoniae* is known as pathogenic bacteria and is resistant to several antibiotics [9]. The emergence of multidrug-resistant bacterial strains has increased in recent decades [10],[11]. Antibiotic treatment of *K. pneumoniae* infection has become increasingly difficult as a result of the emergence of resistant strains [12]–[14].

K. pneumoniae caused more than 90,000 infection cases and 7,000 deaths annually in Europe [15]. The infections of multidrug resistance (MDR) bacteria are estimated to cause more than 23,000 in the United States and 33,000 in Europe [16]. The increase in cases of MDR bacterial infection tends to increase globally, e.g. more than 75% of bloodstream infections were caused by MDR *K. pneumoniae* [15]. Colonization of MDR pathogenic bacterial infection is influenced by many factors, including length of hospital stay, exposure to bacteria in the intensive care unit, use of mechanical ventilators,

invasive colonization, exposure to broad-spectrum antibiotic agents, postoperative and invasive procedures, and disease severity [17], [18]. Patients with intensive care for a long period increase the risk factors for severe infections caused by *K. pneumoniae* [19]. Therefore, the intensive care room may become a place for the persistence and proliferation of drug-resistant microorganisms [3], [7], [20].

Inappropriate use of antibiotics and over-prescribing are triggers for MDR problems [21], [22]. Treatment will be difficult with increasing antibiotic resistance, as well as geographic variations of isolates whose resistance becomes a reference for selecting antibiotics for certain species of bacterial isolates [23], [24]. Attention has been paid to cases of MDR since the 1980s in strains that produce broad-spectrum beta-lactamase (ESBL), and are resistant to broad-spectrum cephalosporins, including ceftazidime [25].

Awareness and concern in cases of MDR bacterial infection and its role in increasing patient morbidity and mortality, has increased over the last 10 years with concerns over Gram-negative bacterial infections. The presence of cases of resistant bacteria in the ICU requires appropriate treatment, and the administration of broad-spectrum antibiotics can have a negative impact when compared to cases of infection by susceptible strains [26]. Antibiotics from the broad-spectrum quinolone group are generally effective in cases of multidrug-resistant bacteria [27], [28].

Definitive combination therapy is recommended for carbapenemase-producing Enterobacteriaceae such as *K. pneumoniae*, considering the presence of severe infections from Pseudomonas and Acinetobacter spp., when -lactams cannot be used [29]. The limited selection of available treatments for treating infections and the lack of development of new antibiotics in overcoming resistance are serious problems. Antibiotic stewardship programs play an important role in the spread of pathogens, and as a development strategy for the management of infections caused by these bacteria.

WHO categorized the quinolone group of ciprofloxacin in 2018, as one of the most important class criteria antibiotics given as first-line therapy in humans [30]. Ciprofloxacin is one of the therapeutic options for infections caused by bacteria from the Enterobacteriaceae family [31]. This antibiotic works by inhibiting the bacterial DNA gyrase and topoisomerase IV enzymes which are required in the replication process. Quinolones and fluoroquinolones can inhibit nucleic acid synthesis [32]. Its antimicrobial activity disrupts the activity of topoisomerase type IIA, DNA gyrase, and topoisomerase IV enzymes [33].

Resistance to ciprofloxacin has been observed in several countries in Southeast Asia, WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS) data for 2020 in the Philippines contained 70.3% and Thailand 91.9% isolates resistant to ciprofloxacin. Treatment of cases with pathogenic *K. pneumoniae* infection using fluoroquinolones as the main class of antibiotics with the agent ciprofloxacin. Based on the Decree of the Minister of Health of the Republic of Indonesia Number HK.01.07/MENKES/342/2017 concerning the national guidelines for medical services for the management of sepsis, it is stated that the administration of antibiotics from the quinolone class for fluoroquinolones with the broad-spectrum category of ciprofloxacin [34].

The rate of resistance to ciprofloxacin as an antimicrobial drug commonly used to treat urinary tract infections varied, from 8.4% - 92.9% for Escherichia coli and increased higher in 4.1% to 79.4% for *K. pneumoniae* in 33 and 34 country regions reported in the WHO GLASS [35]. Ciprofloxacin from the fluoroquinolone group is a broad-spectrum antimicrobial agent [32] which is effective in the treatment of various infections including those caused by Klebsiella. Studies related to cases of resistance of *K. pneumoniae* to several antibiotics, including fluoroquinolones [36]–[39] have been carried out to overcome the problem of MDR, but the development of *K. pneumoniae* bacteria has increased mechanisms against these antibiotics.

The study aimed to analyze the prevalence and diversity of ciprofloxacin-resistant strains of *K. pneumoniae* from a sample of patients at the Prof. Regional General Hospital. Dr. Margono Soekarjo, Purwokerto, Banyumas, Central Java.

2. Methods

The research conducted includes; isolation and identification of bacteria, resistance testing, a confirmation test of *K. pneumoniae* referring to Bergey's Manual of Systematic Bacteriology, and measuring the prevalence of resistance of *K. pneumoniae* to ciprofloxacin antibiotics.

2.1 Isolation and identification of bacteria

Specimen samples in the form of blood, sputum, urine, pus, feces, and pleural fluid, were isolated using several different techniques depending on the type of specimen. (1) The patient's blood specimen sample is first cultured on BD Bactec fertilizing media, with a blood volume of 1-3 ml (infants), 8-10 ml (adults), inserted into the BACTEC 9050 device to be read within 6 hours-5 days, then inoculated on Mac Conkey and Blood Agar media, incubated for 1x24 hours at 37oC. (2) Specimen samples in the form of urine, pus, and pleural fluid can be directly streaked on Mac Conkey and Blood Agar media, then incubated 1x24 hours at 37oC. (3) Sputum specimens were first grown on TSB fertilizing media for 1x24 hours, then the culture was inoculated on Mac Conkey and Blood Agar solid media, then incubated for 1x24 hours at 37oC.

The next culture was identified as phenotypic by looking at the morphological characters of the bacteria by observing the colonies and staining with Gram stain. Gram staining is done by making preparations for bacterial smears that are given coloring reagents gradually; namely purple crystals for 20 seconds, iodine for 60 seconds, then decolorized with alcohol and giving safranin color for 30 seconds. Microscopic observations will show Gram-positive purple and Gram-negative red [40].

Preparation of bacterial suspension by inoculating isolates in 0.45% NaCl media up to standard turbidity of 0.5-0.63 Mc Farland measured with DensiChek plus Biomerieux and then inserted into the Vitek ® 2 Compact.

Biochemical testing of bacteria in the Gram-negative category using the Vitek ® 2 Compact includes; utilization of citrate (Simon Citrate), glucose fermentation, production of H2S, lysine decarboxylase, utilization of malonate, ornithine decarboxylase, utilization of sucrose, urease.

2.2 Confirmatory test for K. pneumoniae refers to Bergey's Manual of Systematic Bacteriology

Confirmation tests include colony morphology and bacterial isolates, as well as biochemical tests [41]. Confirmatory morphological testing was carried out by observing the character profile of Gram-negative bacteria, with straight rod morphology, 0.3-1.0 x 0.6-6.0 m arranged singly/pairs-lined/short-long chains, no spores, no mobile/non-motile, and encapsulated. Observation of colony growth culture on Blood Agar media, namely large, gray, smooth, convex, mucoid or not, a hemolytic, on Mac Conkey media; colony large, smooth, mucoid, convex, pink-brick red.

2.3 Antibiotic resistance test

The test for ciprofloxacin antibiotic resistance was carried out on a Vitek ® 2 Compact using VITEK ® 2 AST-GN93 Gram-negative bacteria reagent. System for testing clinical susceptibility of aerobic Gram-negative bacilli bacteria to certain antibiotics. Vitek ® 2 Compact will read a maximum of 6 hours and the results issued are already in the form of antibiotic resistance test results with a MIC value (minimum inhibitory concentration)

2.4 Measuring the prevalence of resistance of K. pneumoniae to antibiotics

The prevalence used to measure the proportion of ciprofloxacin-resistant *K. pneumoniae* in the population with the formula [42]

$$Prevalence = \frac{\sum \ Ciprofloxacin\ resistant\ Klebsiella\ pneumoniae}{\sum \ Total\ of\ Klebsiella\ pneumoniae\ infection\ cases} x100\%$$

3 Result and Discussion

A total of 99 samples of isolated and identified specimens showed infection with *K. pneumoniae*. The sample was further tested to determine the antibiotic resistance of ciprofloxacin. Sample specimens were obtained from patients treated at RSUD Prof. Margono Soekardjo from ICU, HCU, Surgery Poly, and Inpatient care. Qualification samples come from blood, sputum, urine, pus, feces, and pleural fluid. Patients who contributed to this study had an age distribution of 12 days-79 years. Based on the qualifications of the specimen samples collected from August-September 2022, the data obtained were blood samples (7/7.1%), sputum (59/59.6%), pus (23/23.2%), urine (6/6.1%), feces (3 / 3.03%), and pleural fluid (1/ 1.01%) Figure 1.

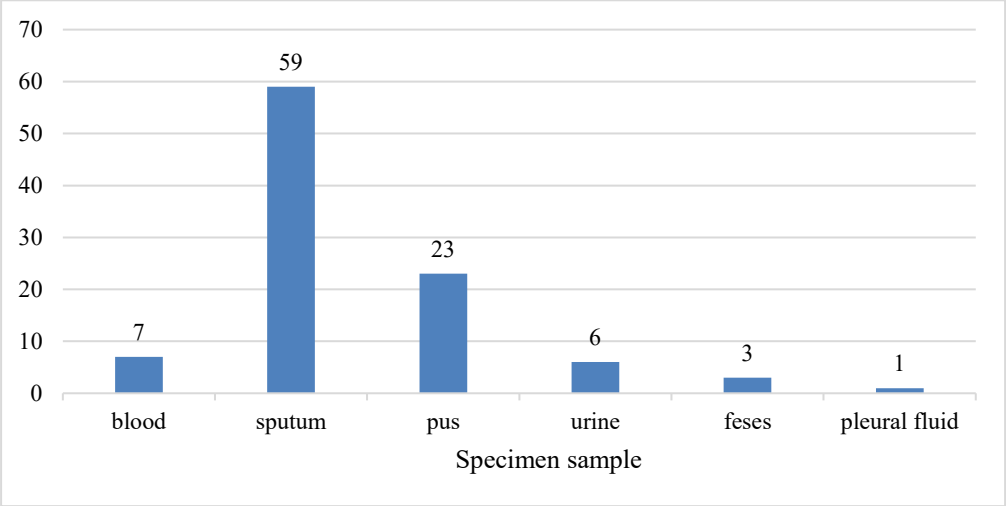

Figure 1. Graph of specimens obtained from patient samples of 99 specimens collected from blood (7), sputum (59), pus (23), urine (6), feces (3), and pleural fluid (1) during August-September 2022.

Specimens analyzed for identification were found to dominate *Klebsiella pneumoniae* subs pneumoniae species with special characteristics observed morphologically the results were grown in Blood agar media showing large, gray, smooth, convex, mucoid, and Mac Conkey colonies with colony shape morphology, namely large colonies. -large, smooth, mucoid, convex, pink-brick in color shown in Figure 2. The results of microscopic observations by SEM this isolate belonged to the group of Gram-negative bacteria, long-short rod-shaped, found in pairs or rows, not spore-forming, non-motile, and encapsulated are shown in Figure 3, the results of observations with an SEM microscope. From the results of this study, it can be seen that the SEM size of *K. pneumoniae* was found to be 0.3-1.0-0.6-6.0 m arranged singly or in pairs and often surrounded by capsules.

The presence of capsules in *K. pneumoniae* is a polysaccharide capsule that acts as an important virulence factor because of its ability to block phagocytosis and can have an impact on increasing the severity of a disease. In general, the function of the capsule in bacteria is to protect, and its main function in bacterial pathogens is to protect the host immune system [43]. Production of large capsules gives rise

to large mucoid colonies with a viscous consistency. The capsular material also diffuses freely into the surrounding liquid medium as extracellular capsule material.

(b)

Figure 2. Morphological characteristics of *K. pneumoniae* isolates grown on (a) Mac Conkey media with the appearance of large, smooth, mucoid, convex, pink-brick-red colony morphology, and (b) Blood to appear large, gray colonies -gray, smooth, mucoid, convex.

K. pneumoniae isolates continued with a ciprofloxacin antibiotic resistance test. Table 1 shows ciprofloxacin-resistant *K. pneumoniae* isolates that were tested biochemically. The results of the biochemical tests carried out using Vitek ® 2 Compact are in Table 1, with 7 biochemical parameters used in the test generally producing a test picture with positive citrate, positive glucose, positive H2S production, negative lysine decarboxylase, positive malonate, negative ornithine decarboxylase, sucrose positive and urease positive. *K. pneumoniae* belongs to the Enterobacteriaceae group which is a facultative anaerobe and has a respiratory and fermentative metabolism. *K. pneumoniae* is capable of fermenting glucose by producing acid and gas (more CO2 is produced than H2), and most Klebsiella strains produce 2,3-butanediol as the main end product of glucose fermentation.

The discovery of *K. pneumoniae* in this study from several patient specimens, following data from the Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report of the World Health Organization in 2020, that the bacterium *K. pneumoniae* has a resistance frequency among high-priority pathogens that cause infection in humans. The microbiological diagnosis resulted in 39% of cases and increased to 60% during the study with the main agents being Enterobacteriaceae (32.%), non-fermenting Gram-negative bacteria (27.6%), and the causative microbes varied significantly [44].

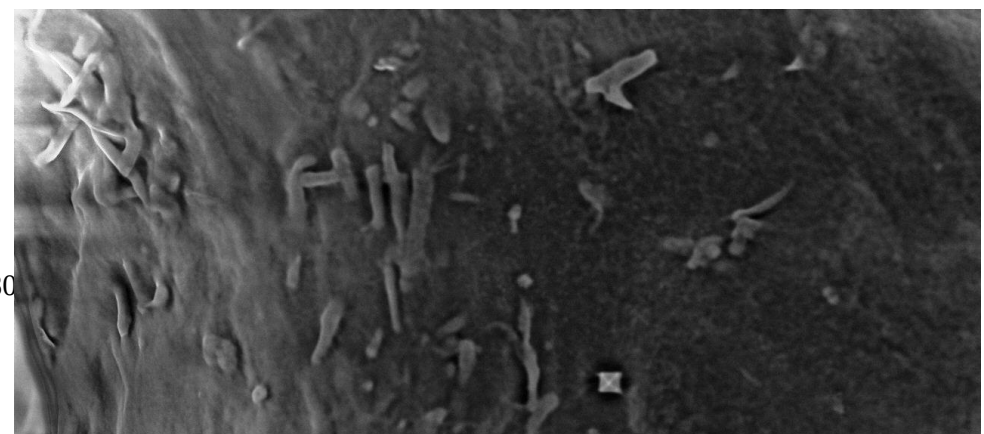

Figure 3. Observation of colony morphology of *Klebsiella pneumoniae* ss pneumoniae isolates with an SEM microscope with a magnification of x500, size 200 m, rod-shaped, long-short, can be paired and lined, non-motile, and encapsulated.

Table 1. Results of biochemical tests on ciprofloxacin-resistant *K. pneumoniae* isolates from patient specimens (blood, sputum, pus, urine, feces, and pleural fluid) using the Vitek ® 2 Compact

Isolate Code	Specimen	Isolates	Biochemical test							
			Citrate	Glucose	H2S production	Lysine decarboxylase	Malonate	Ornithin decarboxylase	Sucrose	Urease
22100233	pus	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	-	+	+
22100875	blood	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	-	+	+
22101254	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	-	-	+
22099046	pleura fluid	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	-	+	+
22100703	pus	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	-	+	+
22100432	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	-	-	+	+
22101801	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	+	+	+
22101737	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	+	+	+
22102390	sputum	*Klebsiella pneumoniae*	+	+	+	-	+	-	+	+
22102392	sputum	*Klebsiella pneumoniae*	+	+	+	-	+	-	+	+

22103094	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	-	-	+	+
22102848	urine	*Klebsiella pneumoniae*	+	+	+	-	+	-	+	+
22103825	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	-	+	+
22104146	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	-	-	+	+
22104500	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	-	-	+	+
22104147	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	-	-	+	-
22109032	sputum	*Klebsiella pneumoniae*	+	-	+	-	-	-	+	+
22109887	feses	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	-	+	+	+
22108695	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	-	-	+	+
22110261	pus	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	-	+	+
22109648	blood	*Klebsiella pneumoniae*	+	+	+	-	+	-	+	+
22111364	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	-	-	+
22111816	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	-	-	+	+
22112396	pus	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	-	-	+	+
22112938	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	-	+	+
22113516	sputum	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	+	-	+	+
22113986	pus	*Klebsiella pneumoniae* ss *pneumoniae*	+	+	+	-	-	-	+	+

The sensitivity test of *K. pneumoniae* isolates with the antibiotic ciprofloxacin obtained 48 (49%) resistant isolates, 46 (46%) sensitive, and 5 (5%) intermediate isolates. The prevalence value included in the calculation formula for cases of ciprofloxacin resistance during August-September was 48.5%. Demographic data showed that the distribution of cases of resistance in male (23) and female (25) patients was balanced. The use of antibiotics from the fluoroquinolone group with ciprofloxacin

derivatives is still used today, considering the ability of this antibiotic agent to be broad-spectrum which is effective in the treatment of various infections in hospitals, including bacterial infections due to *K. pneumoniae*. The development of virulence and the widespread of pathogenic *K. pneumoniae* led to an increase in its resistance to ciprofloxacin. Bacterial resistance can occur because bacteria have several intrinsic, acquired, and adaptive properties [45].

In this study, *K. pneumoniae* was resistant to ciprofloxacin due to the ability of its resistance mechanisms, including; the presence of destruction or modification of the antibiotic ciprofloxacin and its ability to survive by forming a biofilm; other mechanisms are indicated by changes in the target site with site mutations, site enzymatic, site protection, and changes in overproduction; the ability of *K. pneumoniae* to reduce antibiotic accumulation in the presence of decreased membrane permeability (porin activity), and/or increased efflux pump [10], [46], [47].

The mechanism of resistance in Gram-negative bacteria can occur due to the outer membrane as a permeability barrier for many substances including antibiotics. The low permeability of the bacterial outer membrane to certain antibiotic agents is responsible for the intrinsic resistance of some Gram-negative bacteria to antibiotics [48]. Changes in the permeability of the outer membrane may contribute to the development of acquired resistance. This research can be used as a basis for the development of further research related to the mechanism of resistance of gram-negative bacteria *K. pneumoniae*, thereby increasing the knowledge base and as a preventive effort to prevent Multi Drugs Resistant (MDR) cases.

4 Conclusion

This study is a study to know the prevalence and diversity of *K. pneumoniae* to ciprofloxacin antibiotics from patient specimens treated at Prof. Hospital. Margono Soekardjo Purwokerto. From this study, the prevalence of resistance of *K. pneumoniae* to the antibiotic ciprofloxacin increased during August-September 2022 by 48.5%. Ciprofloxacin resistance was also observed in several strains of *K. pneumoniae* ss pneumoniae isolates from patient specimens. The results of this study are expected to be a reference for policy and supervision in the appropriate administration of ciprofloxacin antibiotics, with better knowledge, thereby minimizing the spread of *K. pneumoniae* MDR.

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