

MULTIDRUG RESISTANCE OF *Klebsiella pneumoniae* IN CATS IN BOGOR, INDONESIA

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ABSTRACT

This study aims to measure the level of antibiotic resistance to *Klebsiella pneumoniae* isolated from clinical cats in Bogor. Samples were isolated and identified macroscopically, microscopically, and biochemically. Positive isolates were tested for antibiotic sensitivity using the Kirby-Bauer disk diffusion method. The results showed that *Klebsiella pneumoniae* isolated from sputum and laryngeal swabs of clinic cats in Bogor had experienced Multidrug Resistance (MDR). The highest level of resistance to *Klebsiella pneumoniae* occurred in the β -lactam group (ampicillin 76%) followed by the tetracycline group (oxytetracycline 72% and tetracycline 68%), then the quinolone group (enrofloxacin 52%), and finally the aminoglycoside group (gentamicin 44%). The results of this study are expected to be taken into consideration in the use of antibiotics for the treatment of cases related to the *Klebsiella pneumoniae* bacteria.

Key words: antibiotic resistance, cats, *Klebsiella pneumoniae*

ABSTRAK

Penelitian ini bertujuan mengukur tingkat resistensi antibiotika terhadap *Klebsiella pneumoniae* yang diisolasi dari kucing klinik hewan di kota Bogor. Sampel diisolasi dan diidentifikasi secara makroskopis, mikroskopis, dan biokimia. Isolat positif dilakukan uji sensitivitas terhadap antibiotik menggunakan metode Kirby-Bauer disk diffusion. Hasil penelitian menunjukkan bahwa isolat *Klebsiella pneumoniae* yang diisolasi dari sputum dan usap laring pada kucing klinik di kota Bogor telah mengalami Multi Drug Resistant (MDR). Tingkat resistensi *Klebsiella pneumoniae* tertinggi terjadi pada golongan β -laktam (ampisilin 76%) yang diikuti oleh golongan tetrasiklin (oksitetrasiklin 72% dan tetrasiklin 68%), golongan kuinolon (enrofloksasin 52%), serta golongan aminoglikosida (gentamisin 44%). Hasil dari penelitian ini diharapkan dapat menjadi bahan pertimbangan dalam pemakaian antibiotika untuk pengobatan kasus terkait dengan bakteri *Klebsiella pneumoniae*.

Kata kunci: resistensi antibiotika, *Klebsiella pneumoniae*, kucing

INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*) belongs to the Enterobacteriaceae family, which is a Gram negative bacterium in the form of bacilli (Podschun and Ullmann, 1998). *K. pneumoniae* has been described as an agent of Friedlander's pneumonia, which is a severe lobar pneumonia with a high mortality rate. *K. pneumoniae* is still one of the main causes of pneumonia in several countries (Brisse *et al.*, 2009). These bacteria can form a capsule (Podschun and Ullmann, 1998), and this capsule that surrounds *K. pneumoniae* serves to protect against phagocytosis activity and bactericidal serum. This is considered to be the most important determinant of *K. pneumoniae* virulence (Brisse *et al.*, 2009). Other factors involved in the virulence of the *K. pneumoniae* strain include capsular serotype, lipopolysaccharide, iron scavenging system, fimbrial and nonfimbrial adhesion. Historically, this species of bacteria, in addition to causing upper respiratory tract infections (Adler *et al.*, 2007), has also been shown to cause urinary tract infections (Ling *et al.*, 2001), pyometra (Stone *et al.*, 1988), and septicemia (Roberts *et al.*, 2000).

The existence of a bacterial infection can be overcome by administering antibacterial through antibiotics. Antibiotics are chemical substances produced by microbes with antimicrobial properties (Entjang, 2003). According to Boogard (2001), based on field observations, the antibiotics commonly used for the prevention and treatment of disease in animals

are penicillin, tetracycline, and sulfonamide antibiotics. Inaccurate selection of antibiotics, indications, dosage, administration method, frequency, and duration of administration are the causes of inaccurate treatment of infection with antibiotics (Nelson, 1995). A study conducted in Japan, reported that there was a positive relationship between antibiotic use and the level of resistance that occurred (Asai *et al.*, 2005). The careless or inappropriate use of antibiotics, in addition to failing therapy, can also cause other dangers such as resistance, Multidrug Resistance (MDR), supra infection, and other negative side effects (Sastramihardja and Hendry, 1997).

MDR is the bacterial resistance to three or more antibiotic groups (Magiorakos *et al.*, 2012). MDR in bacteria is caused by two mechanisms. First, the accumulation of several genes encoding a trait that is resistant to one antibiotic in a single cell. This accumulation occurs in the R (resistance) plasmid. Second, the increased of gene expression which responsible for coding the efflux pump against several antibiotics (Nikaido, 2009). Many cases of MDR *K. pneumoniae* have been found at several parts of this world, among others, in Tunis-Tunisia, Capetown-South Africa, and Shijiazhuang-China (Messaudi *et al.*, 2009; Fielding *et al.*, 2012; Guo *et al.*, 2016).

Antibiotic resistance to *Klebsiella* spp. has become a major concern around the world (Lynch *et al.*, 2013). In recent years, research about antibiotic resistance to *Klebsiella* spp. in pets has been published in several

countries in Europe, including Germany (Stolle *et al.*, 2013; Ewers *et al.*, 2014), Italy (Donati *et al.*, 2014), France (Haenni *et al.*, 2012; Poiriel *et al.*, 2013), Spain (Hidalgo *et al.*, 2013), and Switzerland (Wohlwend *et al.*, 2015). Brisse and Duijkeren (2005) stated that *K. pneumoniae* isolates collected from dogs and cats were resistant to ampicillin, cephalosporins, ceftazidime, enrofloxacin, gentamicin, and tetracyclines.

The incidence of bacterial resistance to antibiotics is very essential to note because it has been shown that bacteria that have developed resistance can cause serious illness in pets and can complicate treatment. Therefore, it is necessary to study the resistance of *K. pneumoniae* bacteria to several antibiotics in cats from animal clinics in Bogor.

MATERIALS AND METHODS

Isolation and Identification

The samples used in this study were sputum samples and laryngeal swabs from cats that were hospitalized at the veterinary clinic in Bogor. *K. pneumoniae* bacteria can cause upper respiratory tract infections (Adler *et al.*, 2007), thus bacteria isolation through sputum and laryngeal swabs is a proper method to be done. Samples were taken from 10 clinics with a total of 58 samples. Samples were put into 0.1% buffer peptone water (BPW) and stored at 4° C. The sputum samples and laryngeal swabs were cultured in MacConkey Agar (MCA) media by taking samples using one and cultured on MCA media then incubated at 37° C for 24 hours. Microscopic observation using Gram staining was done by taking one loop of bacteria swab with sterile physiological NaCl and fixed on microscope slides, followed by giving crystal violet solution for 3 minutes, lugol for 1 minute, 95% acetone alcohol for 30 seconds and safranin for 2 minutes. The bacteria morphology was observed under a microscope at 10×100 magnification. Gram positive bacteria will provide purple color while Gram negative bacteria will develop red colour (Willey *et al.*, 2008). The morphology of the genus *Klebsiella* bacteria has a stem, mucoid shape and is characteristic red of Gram negative bacteria.

Confirmation with Biochemistry Test

A single colony suspected of being genus *Klebsiella* bacteria was then subcultured on Tryptic Soy Agar (TSA) agar media by inoculating one bacterial colony with one derived from MCA media and cultured on TSA media then incubated at 37° C for 24 hours.

Bacteria suspected to be colonies of genus *Klebsiella* bacteria on macroscopic examination were then identified using biochemical tests. The biochemical tests carried out were the Triple Sugar Iron Agar (TSIA) test, Urease test, IMVIC test consisting of Sulfide Indole Motility (SIM) test, the Methyl Red-Voges Proskauer (MRVP) test, Simmon's Citrate test, and fermentation tests of glucose, lactose, sucrose, maltose, dulcitol, and mannitol.

Triple Sugar Iron Agar (TSIA) test was initiated by taking a macroscopic loop of positive *K. pneumoniae*

bacteria and scratching it on the surface of the TSA agar slant. There are 3 indicators can be seen in the TSIA test: the formation of gas which is indicated by the formation of air cavities in the media; the ability to ferment carbohydrates which is marked by a change in color on the media to yellow; and the ability to produce H₂S which is indicated by the black color presence in the media. The TSIA media is an enriched medium used for bacterial differentiation based on its ability to ferment glucose, lactose, sucrose and to reduce sulfur (Laboffe and Burton, 2011).

The motility test was started with the inoculation of bacteria on the SIM medium by vertically stabbing on the media and incubating it at 37° C for 24 hours, a positive result for the motility test was indicated by the presence of bacterial growth in the puncture area and spread to the media surface. In indol test, 2-3 drops of Earlich's reagent was added to the same media as the motility test, then left it for 5 seconds. A positive result was indicated by the formation of a red ring.

The MR test was conducted by inoculating bacteria on the methyl red medium and then 3-5 drops of methyl red reagent were added. Positive results were indicated by color change of the media from yellow to red. The VP test was carried out by adding 5 drops of VP reagent (α naphthol) and 5 drops of 40% KOH to the media, homogenized and then let it for 5-10 minutes. A positive result was indicated by color change of media from yellow to red (Markey *et al.*, 2013).

Simmon's Citrate test using Simmon's Citrate Agar (SCA) media with the inoculation process was carried out on the media surface and incubated at 37° C for 24 hours. A positive result was indicated by color change of the media from green to blue, and the negative result shows no color change.

The fermentation test for the sugars was begun with the inoculation of bacteria in each medium consisting of glucose, lactose, sucrose, maltose, dulcitol, and mannitol then incubated at 37° C for 24 hours. Positive results for the sugar fermentation test in each medium were indicated by a change in color from red to yellow.

Antibiotic Resistance Test using Kirby-Bauer Disk Diffusion Method

The antibiotic sensitivity test followed the Kirby-Bauer disk diffusion method using Mueller-Hinton agar based on the Clinical and Laboratory Standards Institute Guidelines (CLSI, 2018) (Table 1). The sample tested was a sample that shows a positive result in the Biochemical test. The bacterial colonies obtained from TSA were diluted with physiological NaCl until they reached the McFarland standard 0.5 or equivalent to 1.5×10^8 CFU/mL, 1 mL suspension was poured on Muller-Hinton agar. Diffusion disks containing antibiotics were placed on the surface Muller-Hinton uses sterile tweezers then incubated at 35° C for 16-18 hours and the inhibition zone was measured by referring to the CLSI (2018). Antibiotics used were ampicillin 10 μ g, tetracycline 30 μ g, oxytetracycline 30 μ g, gentamicin 10 μ g, and enrofloxacin 5 μ g. This sensitivity test was replicated 3 times.

RESULT AND DISCUSSION

Isolation and Identification

Sputum and laryngeal swab samples were isolated and identified and 49 out of 58 samples (84.48%) which were cultured on MCA media showing the bacteria growth of genus *Klebsiella*. The quantity of the sample size was adjusted to the capacity of the inpatient room and the intensity of antibiotic use. In each animal clinic, from the isolated samples it was found bacteria that had pink, convex, and mucoid colony which are characteristic of the genus *Klebsiella* bacteria. When investigating the colony variant *K. pneumoniae*, Julianelle (1928) reported that the *K. pneumoniae* colony had a mucoid shape and was slightly slimy. The mucoid colony form of *K. pneumoniae* was also reported by Hadley (1925) and O'Neal (1933). Burke (2009) stated that the presence of hypermucoviscous and lactose fermentation also occurred in the *K. pneumoniae* colonies, resulting in a color change in the color of the MCA media to pink. Mucoid colonies of the genus *Klebsiella* bacteria are pictured in Figure 1a. Gram staining was conducted in order to see the shape of the bacteria. *Klebsiella* has a rod shape with red color which is characteristic of Gram negative bacteria (Figure 1b).

Confirmation of bacteria using the IMViC test showed that 51% (25/49) of the samples illustrated the biochemical properties of *K. pneumoniae*, namely positive for Indole, negative for bacterial motility in the SIM test, positive for the Methyl Red test, negative for the Voges-Proskauer test, positive for the Simmon's Citrate test, positive for the Urease test, negative for H₂S, positive for gas and sugar fermentation in the TSIA test, and positive for the fermentation test for glucose, lactose, sucrose, maltose, dulcitol, and mannitol (Holt and Krieg, 1994). Brisse et al. (2009) stated that *K. pneumoniae* is nonmotile bacteria, does not produce H₂S, and shows positive result in the

Simmon's citrate test. Brown and Ramon (1973) also stated that these bacteria are not motile, and rapidly ferment maltose and mannitol.

K. pneumoniae is a Gram negative bacterium with size around 2.0-3.0×0.6 μm, and one of facultative anaerobes common flora found in the intestinal and respiratory tract. *K. pneumoniae* has a large capsule, thus the colony culture looks very mucoid. *K. pneumoniae* is unable to move because it does not have a flagellum, but is able to ferment carbohydrates to form acids and gases. Based on their need for oxygen, *K. pneumoniae* is a facultative anaerobic bacterium. *K. pneumoniae* species showed growth of mucoid, large, nonmotile capsule polysaccharide (Anderson et al., 2007).

Factors involved in the virulence of the *K. pneumoniae* strain include capsular serotype, lipopolysaccharide, iron scavenging system, fimbrial and nonfimbrial adhesion. The polysaccharide capsules surrounding *K. pneumoniae* protect against phagocytosis activity and bactericidal serum considered as the most important virulence determinant of *K. pneumoniae* (Brisse et al., 2009).

These bacteria cause infections in the lungs, such as pneumonia, urinary tract infections, and sepsis in patients with weak immune systems (Brooks et al., 2005). *K. pneumoniae* causes extensive consolidation accompanied by hemorrhagic necrosis of the lung. These organisms sometimes cause urinary tract infections and bacteremia accompanied by focal infections in severe debilitated patients (Carpenter, 1990).

Resistance Test of *Klebsiella pneumoniae* against Antibiotic

The resistance test was carried out by calculating the diameter of the antibiotic inhibition zone formed on the Mueller-Hinton agar medium, then the results of the antibiotic resistance test were correlated with CLSI (2018) guidelines (Table 1). The results showed that

Table 1. Standard diameter of the inhibition zone (CLSI, 2018)

Type of antibiotic	Dose (μg)	Inhibition zone (mm)		
		S	I	R
Ampicillin (AMP)	10	≥17	14-16	≤13
Gentamicin (CN)	10	≥15	13-14	≤12
Tetrasiklin (TET)	30	≥15	12-14	≤11
Oxytetracycline (OT)	30	≥19	15-18	≤14
Enrofloxacin (EN)	5	≥23	14-22	≤13

S= Susceptible, I= Intermediate, R= Resistant

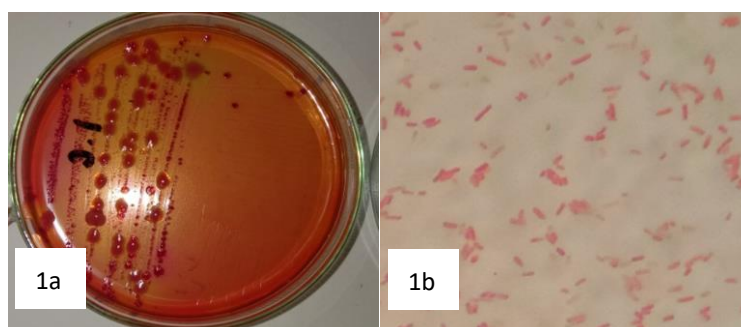


Figure 1. *Klebsiella* bacteria isolation of sputum samples and laryngeal swabs. 1a= Colonies form on MCA, 1b= Bacteria shape after Gram staining

K. pneumoniae against 5 antibiotics showed a high level of resistance. Overall, the isolates used in this study were resistant to at least three types of antibiotics.

This study was conducted on 10 veterinary clinics in Bogor with a different number of samples from each clinic. The most positive samples of *K. pneumoniae* came from clinic 7 (7 samples), while the smallest number of samples came from clinics 4, 8, and 10, which were 1 sample, but for clinics 5 and 9 there were no typical *K. pneumoniae* colonies found on MCA media. Thus, these colonies were not included in the resistance test.

Table 3 shows the different levels of antibiotic resistance to *K. pneumoniae* isolates used in this study. The resistance incidence for the β -lactam (ampicillin) group and the tetracycline (tetracycline) group was found in 7 out of 10 clinics (70%), while for the tetracycline (oxytetracycline) group was found in 6 out of 10 clinics (60%). Quinolone (enrofloxacin) group resistance was found in 5 out of 10 clinics (50%), but for the aminoglycoside (gentamicin) group it was found only in 4 out of 10 clinics (40%). This study results similar to Haenni et al. (2012) as *K. pneumoniae* isolated from dogs and cats were resistant to antibiotics of tetracycline, quinolone, and aminoglycosides.

The resistant incidence in *K. pneumoniae* isolates to antibiotics can occur due to various factors, including the use of antibiotics for a long time, excessive use and inappropriate dosage. Bartlett et al. (2013) explained that the excessive use of antibiotics has led to a global crisis of antibiotic resistance. Venezia et al. (2017) explained that *K. pneumoniae* bacteria are a source of the spread of resistance to antibiotics. *K. pneumoniae* continuously accumulates antibiotic resistance genes (ARGs) through denovo mutations, plasmid acquisition, and transferable genetic elements. These bacteria that produce extended spectrum beta lactamase (ESBL) have a higher level of risk and malignancy, and result in longer time of recovery for sick people (Tumbarello et al., 2006). The presence of antibiotic resistance genes causes bacteria to have resistant properties. The nature of antibiotic resistance in bacteria serves to protect itself from harmful agents (Cogliani et al., 2011).

The highest pattern of *K. pneumoniae* resistance occurred in the β -lactam group of antibiotics (ampicillin), while the lowest was the aminoglycoside group of antibiotics (gentamicin), for the quinolone group (enrofloxacin), while for the tetracycline group (tetracycline and oxytetracycline) (Table 4). The

Table 2. Isolation and identification of *Klebsiella* spp. from cats in Bogor

Sample source	Number of samples	Positive	Negative
Clinic 1	10	90% (9)	10% (1)
Clinic 2	6	100% (6)	0% (0)
Clinic 3	8	75% (6)	25% (2)
Clinic 4	2	100% (2)	0% (0)
Clinic 5	3	0% (0)	100% (3)
Clinic 6	5	80% (4)	20% (1)
Clinic 7	9	100% (9)	0% (0)
Clinic 8	2	100% (2)	0% (0)
Clinic 9	5	20% (1)	80% (4)
Clinic 10	8	12,5% (1)	87,5% (7)
Total	58	84.48% (49)	15.51% (9)

Table 3. Percentage of antibiotic resistance pattern in *Klebsiella pneumoniae* bacteria (n= 25)

No	Clinic	Number of samples	Antibiotic resistance pattern (%)														
			Amphicillin			Enrofloxacin			Gentamicin			Tetracyclines			Oxytetracycline		
			S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
1	K1	3	0	0	100	33,3	0	66,7	0	0	100	0	0	100	0	0	100
2	K2	5	0	20	80	40	0	60	40	0	60	20	20	60	20	0	80
3	K3	4	0	25	75	75	0	25	50	0	50	25	25	50	25	0	75
4	K4	1	0	0	100	100	0	0	100	0	0	0	0	100	100	0	0
5	K5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	K6	3	33,3	0	66,7	66,7	0	33,3	100	0	0	0	0	100	33,3	0	66,7
7	K7	7	14,3	14,3	71,4	28,6	0	71,4	42,9	14,2	42,9	14,2	28,6	57,2	28,5	0	71,5
8	K8	1	0	0	100	100	0	0	100	0	0	100	0	0	100	0	0
9	K9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	K10	1	0	100	0	100	0	0	100	0	0	0	0	100	0	0	100

R= Resistant, I= Intermediate, S= Sensitive, K1-K10= Clinical code

Table 4. Percentage of antibiotic resistance in *Klebsiella pneumoniae* bacteria (n= 25)

Type of antibiotic	S	I	R	Percentage (%)
Amphicillin	1	4	19	76
Gentamicin	13	1	11	44
Tetracyclines	4	4	17	68
Oxytetracycline	7	0	18	72
Enrofloxacin	12	0	13	52

incidence of *K. pneumoniae* resistance in pets (dogs and cats) is closely related to the prolonged use of antibiotics and treatment of nonbacterial causal diseases to avoid secondary infections (Rzewuska *et al.*, 2015). The β -lactam group and the tetracycline group are still the main choices and are most often used for therapy in cats because of their effectiveness against Gram positive and Gram negative bacteria. Carvalho *et al.* (2020) stated that *K. pneumoniae* isolates from domestic animals were resistant to the β -lactam group. Haenni *et al.* (2012) stated that in addition to β -lactam resistance, it turns out that *K. pneumoniae* isolates from dogs and cats are also resistant to several other antibiotic groups such as aminoglycosides, tetracyclines, and quinolones. Jassim *et al.* (2019) stated that one of the antibiotics with the highest level of resistance in veterinary clinics was the tetracycline group.

K. pneumoniae isolate in this study was resistant to more than one group of antibiotics, which is commonly known as MDR. Oliva *et al.* (2015) noted the widespread use and abuse of antimicrobial agents to treat disease in animals, *K. pneumoniae* has become highly resistant to most antibiotics. Ripabelli *et al.* (2018) stated that the emergence of MDR-resistant strains poses major challenges for the prevention and treatment of infections caused by *K. pneumoniae*. *K. pneumoniae* is an important opportunistic pathogen responsible for human and animal infections, and the emergence of MDR *K. pneumoniae* has made controlling this pathogen difficult worldwide.

Antibiotic resistance to *K. pneumoniae* isolates isolated from pets has been published in several countries in Europe, including Germany (Stolle *et al.*, 2013; Ewers *et al.*, 2014), Italy (Donati *et al.*, 2014), France (Haenni *et al.*, 2012; Poirel *et al.*, 2013), Spain (Hidalgo *et al.*, 2013), and Switzerland (Wohlwend *et al.*, 2015). Brisse and Duijkeren (2005) stated that *K. pneumoniae* isolates collected from dogs and cats were resistant to ampicillin, cepalexin, ceftacidime, enrofloxacin, gentamicin, and tetracycline. Hong *et al.* (2019) stated that *K. pneumoniae* isolated from pets such as dogs and cats has a resistance gene to the cephalosporin group of antibiotics.

CONCLUSION

Klebsiella pneumoniae isolate from sputum and laryngeal swabs of cats in veterinary clinics in Bogor, Indonesia has developed Multidrug Resistance (MDR). *Klebsiella pneumoniae* bacteria have been resistant to tetracycline, oxytetracycline, gentamicin, enrofloxacin, and ampicillin. Further studies are needed to determine the activity of genes resistant to each antibiotic. Thus, it can be taken into consideration for the use and treatment of cases associated with *Klebsiella pneumoniae* bacteria.

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