Detection and prevalence of multidrug-resistant *Klebsiella pneumoniae* strains isolated from poultry farms in Blitar, Indonesia

DIAN AYU PERMATASARI[1], ADIANA MUTAMSARI WITANINGRUM[1], FRESHINTA JELLIA WIBISONO[2], MUSTOFA HELMI EFFENDI[1,3,♥]

[1]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Airlangga, Jl. Raya Mulyorejo, Surabaya 60115, East Java, Indonesia.
[2]Department of Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Wijaya Kusuma Surabaya. Jl. Dukuh Kupang XXV No. 54, Surabaya 60225, East Java, Indonesia
[3]Halal Research Center, Universitas Airlangga, Jl. Raya Mulyorejo, Surabaya 60115, East Java, Indonesia. Tel./fax.: +62-31-5915551, ♥email: mheffendi@yahoo.com

Abstract. *Permatasari DA, Witaningrum AM, Wibisono FJ, Effendi MH. 2020. Detection and prevalence of multidrug-resistant* Klebsiella pneumoniae *strain isolated from poultry farms in Blitar, Indonesia. Biodiversitas 21: 4642-4647.* Antibiotics are commonly used as therapy and disease control in humans and animals. However, the widespread use of antibiotics may also trigger the rise of antibiotic resistance. Therefore, the aim of this study was to study the occurrence of Multidrug-Resistant (MDR) of *Klebsiella pneumoniae* from poultry farms in Blitar. Chicken cloacal swabs from 63 poultry farms (32 broilers and 31 layers) were taken randomly, using total samples 160 broilers and 155 layers. The collected swab samples were inoculated on MacConkey agar medium for isolation and identification. Single colony was isolated after primary positive cultures and identified by using the IMViC test and TSIA. 28 (8.88%) out of samples were found positive for *Klebsiella pneumoniae*. The antimicrobial confirmation test showed that 53.57% of the *K. pneumoniae* isolates were Multi-Drug Resistance (MDR) bacteria. The percentage of MDR bacteria against different antibiotics included ampicillin 75%, Erythromycin 42.86%, Tetracycline 35.72%, Sulfamethoxazole 32.14% and Streptomycin 21.4%. This study confirmed that the percentage of resistant isolates of *Klebsiella pneumoniae* from 5 antimicrobial agents of broiler chicken are higher than layer chicken. The presence of multidrug-resistant bacteria is a threat to public health and livestock. The impact of these conditions leaves a limited treatment option as chicken farmers in Indonesia still using antibiotics without veterinarian supervision.

Keywords: Antibiotic resistance, *Klebsiella pneumoniae*, MDR, poultry farms, public health

INTRODUCTION

Antibiotics are commonly used as therapy and to control microbial infections in humans and animals. However, the widespread use of an antibiotic may trigger the rise of antibiotic resistance not only in humans but in animals also (Kempf et al. 2015; Hayati et al. 2019). The European Centre for Disease Prevention and Control/European Food Safety Authority/European Medicines Agency (ECDC/EFSA/EMA) joint report in 2014 stated that the average antibiotic consumption in animals (152 mg/kg) was higher than in humans (124 mg/kg). It is estimated that two-thirds of antimicrobials produced globally are consumed in the livestock and poultry (CDDEP 2015). The raising small-scale commercial poultry farms (PFs) demands low investment, it has been expanding at a high rate, mostly in the rural and semi-urban areas which contribute to national economic growth, considerably. These PFs are often run by unskilled, non-professional managers having poor knowledge of biosecurity alike other developing countries (Conan 2012). Since most of these poultry farms neither do have good surveillance systems nor well-documented monitoring mechanisms to record potential pathogenic microorganisms or other poultry-hazards claiming serious public health implications (Nahar et al. 2014). The increase in the prevalence of infections caused by multidrug-resistant (MDR) bacteria belonging to the Enterobacteriaceae group poses a great concern since these are common natural inhabitants of microbiome (Navon-Venezia et al. 2017).

Klebsiella pneumoniae is a Gram-negative, rod-shaped, non-motile, encapsulated opportunistic pathogen. This bacterium is part of the Enterobacteriaceae family, facultative anaerobe with the mucoid colony (Fielding et al. 2012; Al-Ammiri et al. 2016). The increase of multidrug resistance in Gram-negative bacteria is now a serious challenge (Exner et al. 2017). Five factors have been proposed for *Klebsiella* pathogenesis, including capsular antigen (as the most important virulence factor in *K. pneumoniae*), adhesives, lipopolysaccharide, siderophore (Struve et al. 2009). Cases of multidrug resistance (MDR) have been reported in *K. pneumoniae* isolates against more than three types of antibiotics (Hayati et al. 2019). Notably, the prevalence of antibiotic resistance is increasing among Enterobacteriaceae, including *K. pneumoniae* strains isolated from animals (Davis and Price 2016). Due to the extensive use of antibiotics in humans, veterinary medicine, and agricultural practice during the last few decades, the emergence of *K. pneumoniae* strains that harbor various resistance genes has increased

considerably *K. pneumoniae* has acquired increasingly high levels of antimicrobial drug resistance (Wu et al. 2019).

Antimicrobial resistance is often caused by overuse of antibiotics in human and animal medicine and has now emerged as a global health problem (Pitout and Laupland 2008; Widodo et al. 2020). The resistance of microorganisms to antimicrobial drugs that are initially sensitive is called antimicrobial resistance (AMR). This is a natural process and is facilitated by excessive antimicrobial abuse (Riwu et al. 2020). Gram-negative bacteria have developed this resistance by developing enzymes that can destroy antibiotics, by having resistant metabolic pathways, and by changing receptors for antimicrobial agents (Okonko et al. 2009; Wibisono et al. 2020). *K. pneumoniae* has two types of antibiotic resistance mechanisms. One mechanism involves the expression of ESBL, which develops resistance in bacteria to cephalosporins and monobactams. The second mechanism involves carbapenemase expression, which helps develop resistance to all available β-lactams (Riwu et al. 2020). The presence of the Extended Spectrum Beta-Lactamase (ESBL) gene consisting of SHV, TEM, and CTX-M encoded by plasmids in *K. pneumoniae* has an impact on resistance to different antibiotics (Ahmed et al. 2014). *Klebsiella* spp has also developed antimicrobial resistance, which is an alarming situation in the field of human medicine (Lynch et al. 2013). This antimicrobial treatment failure is safe for humans and animals (Guardabassi et al. 2004).

Antibiotic sensitivity of *K. pneumoniae* has not been well studied, especially in poultry. The use of antimicrobials in broiler chickens was higher than that of laying hens, this was due to maintaining the speed of body weight growth of broilers. Whereas in laying hens there are fewer antimicrobials just to maintain egg production (Wibisono et al. 2020). Given the differences between the types, it is necessary to understand how the incidence of resistance varies in order to precisely provide the policies in which they will have the greatest impact (Brower et al. 2017). The aim of this study was to study the occurrence of Multidrug-Resistant (MDR) isolates of *Klebsiella pneumoniae* from poultry farms in Blitar.

MATERIALS AND METHODS

Research design, location, and sampling

This cross-sectional study was done in May-June 2019. Chicken cloacal swabs were collected randomly from 63 poultry farms in Blitar (31 layers and 32 broilers) with total of 160 cloacal swabs of broilers and 155 cloacal swabs of layers. The cloacal swabs were transferred by using transport swabs in Amies transport medium from Biomedics-Madrid, Spain (Vasilakopoulou et al. 2020), and carrying by the cooler box to the laboratory for bacterial isolation and identification.

Isolation and identification of *Klebsiella pneumoniae*

Samples were brought to the laboratory for the isolation and identification of *K. pneumoniae* bacteria. Isolated

colony of bacteria was identified as *Klebsiella pneumoniae* based on Gram's staining, colony character on selective MacConkey Agar media from Oxoid-England incubated at 35-37°C for 20-24 hrs (Effendi et al. 2018). Single colonies were isolated after primary positive cultures and identified by using the IMViC test (Indol-Motility, Methyl Red, Voges Proskauer, Citrate) and Triple Sugar Iron Agar (TSIA) (Arya et al. 2020).

Antibiotic sensitivity confirmation test

All Isolated *K. pneumoniae* bacteria were tested for their sensitivity to antibiotics with the disc diffusion test by Kirby-Bauer method on Mueller-Hinton Agar Merck-Germany (Effendi et al. 2019). All disks used in the disc diffusion test were obtained from Oxoid, England, in the following concentrations: Ampicillin (Amp 10 μg), Streptomycin (S 10 μg), Tetracycline (TE 30 μg), Erythromycin (E 2 μg), and Sulfamethoxazole (SXT 25 μg). The culture turbidity was adjusted to 0.5 McFarland standard. The sterile cotton swab was dipped into the suspension and spread evenly over the entire Mueller-Hinton Agar surface (Geta et al. 2019). The antibiotics discs were placed onto the surface of the inoculated plates and incubated at 37°C for 16-18 hrs. The zone diameters of inhibition were measured in millimeters and interpreted as susceptible, intermediate, and resistant (CLSI 2017). The evaluation of specimens for antibiotic resistance was conducted according to the clinical and laboratory standards institute (CLSI) guidelines (Watts 2013).

RESULTS AND DISCUSSION

Isolation and identification of *Klebsiella pneumoniae*

The result of isolation and identification of 155 samples of cloacal swabs on layer chicken farms showed that 6 (3.87%); and 160 samples of cloacal swabs on broiler chicken farms showed that 22 (13.75%) isolates were positive of *K. pneumoni*ae. The distribution of samples showing positive growth on MacConkey Agar was as follows: 5 samples in Srengat, 2 in Undanawu, 8 in Talun, 3 in Kademangan, 4 in Bakung, 3 in Ponggok, and 3 in Garum. Positive samples of *K. pneumoniae* on MacConkey Agar looks pink with colonies culture looks very mucoid (Masruroh et al. 2016). *K. pneumoniae* identified with lactose fermentation pink and dome-shaped mucoid colonies (Figure 2) and also confirmed by a biochemical test using IMViC and Triple Sugar Iron Agar. The biochemical test identified lactose fermenter, non-motile, Indole production negative, Voges-Proskauer test positive, Methyl Red negative, Citrate utilization test positive, TSIA acid/ acid with gas (Figure 3). In the antibiotic sensitivity test, the positive organism was sensitive to Ampicillin and Streptomycin, while it was resistant to Tetracycline, Erythromycin, and Sulfamethoxazole (Figure 4). The presence of multidrug-resistant (MDR) producing *K. pneumoniae* in layer and broiler chicken farms, shown in Table 1.

Table 1. Data of multidrug-resistant *Klebsiella pneumoniae* in poultry farms

Type of livestock	Location	Farms	Number of samples	*Klebsiella pneumoniae*		MDR	
				Positive	Percentage	Positive	Percentage
Layer	Srengat	17	85	1	1.17%	0	0%
	Udanawu	6	30	2	6.67%	1	50%
	Talun	4	20	2	10%	2	100%
	Kademangan	4	20	1	5%	0	0%
	Sub-total	**31**	**155**	**6**	**3.87%**	**3**	**50%**
Broiler	Srengat	6	30	4	13%	1	25%
	Kademangan	4	20	2	10%	1	50%
	Bakung	4	20	4	20%	3	75%
	Ponggok	10	50	3	6%	2	66.67%
	Talun	4	20	6	30%	3	50%
	Garum	4	20	3	15%	2	66.67%
	Sub-total	**32**	**160**	**22**	**13.75%**	**11**	**50%**
Total		**63**	**315**	**28**	**8.88%**	**15**	**53.57%**

Table 2. Antimicrobial resistance pattern of *Klebsiella pneumoniae* isolates

Antimicrobial agent	Number percentage of sensitive isolates		Number percentage of intermediate isolates		Number percentage of resistant isolates	
	Layer	Broiler	Layer	Broiler	Layer	Broiler
Ampicillin	2 (7.14%)	0 (0%)	0 (0%)	1 (3.57%)	4 (14.29%)	21 (75%)
Streptomycin	4 (14.29%)	14 (50%)	0 (0%)	2 (7.14%)	2 (7.14%)	6 (21.43)
Erythromycin	0 (0%)	6 (21.43)	3 (10.71%)	4 (14.29%)	3 (10.71%)	12 (42.86%)
Tetracycline	3 (10.71%)	11 (39.29%)	0 (0%)	1 (3.57%)	3 (10.71%)	10 (35.71%)
Sulfamethoxazole	3 (10.71%)	8 (28.57%)	0 (0%)	5 (17.86%)	3 (10.71%)	9 (32.14%)

Disc diffusion test for antibiotic sensitivity confirmation test

The number of positive samples from broilers exceeded the one obtained from Layers (11 and 3, respectively). Isolated strains (315) originated from 31 out of the 32 visited farms, i.e., 53.57% of the farms were positive for multidrug-resistant *K. pneumoniae*. As shown in table 1, the highest prevalence multidrug-resistant *K. pneumoniae* was detected in Talun whereas, the lowest was detected in Srengat and Kademangan. Percentages of isolates classified as sensitive, intermediate, or resistant were used to summarize resistance prevalence overall and disaggregated by farm (Table 2, Figure 1). A high percentage of *K. pneumoniae* resistant to AMP (75%), E (42.86%), TE (35.72%), SXT (32.14%), and S (21.4%) was observed. The degree of resistance to Ampicillin, Erythromycin, Tetracycline, Sulfamethoxazole, and Streptomycin was consistently high levels only in broiler farms.

Discussion

In this research, the results of biochemical test identified lactose fermenter, non-motile, Indole production negative, Voges-Proskauer test positive, Methyl Red negative, Citrate utilization test positive, TSIA Acid/ Acid with gas. The isolation and identification results were confirmed by *K. pneumoniae* character according to El-Mansi et al. (2000) in order to verify that the cured strain was indeed a derivative of *K. pneumoniae*, the IMViC tests were carried out routinely.

The antimicrobial confirmation test showed that 53.57% of the *K. pneumoniae* isolates were MDR bacteria. These results corroborate similar studies in broiler productions, but the prevalence reported here is similar or higher (Dierikx et al. 2013; Kar et al. 2015; Laube et al. 2013). Multidrug-resistant cases in *K. pneumoniae* caused longer treatment and were difficult to cure. The pattern of antibiotic resistance is expected to be a guide in selecting the right antibiotic for treatment. The widespread use of antibiotics without close supervision can lead to antibiotic resistance (Hayati 2019). Until recently, antibiotics were easy to obtain and could be used without veterinary supervision. As many as 72.3% of chicken farmers in Indonesia still using antibiotics without veterinarian supervision (Arief et al. 2016).

The resistance against ampicillin was 75%, erythromycin 42.86%, tetracycline 35.72%, sulfamethoxazole 32.14%, streptomycin 21.4%. Rashed et al. (2013) and Effendi et al. 2018 reported that *Klebsiella* spp. showed higher resistance against ampicillin (74%) and amoxicillin (90%). This resistance of *Klebsiella* spp. against amoxicillin was not in accordance with this result. *Klebsiella* spp. were naturally sensitive or intermediate to penicillin, all tested aminoglycosides, quinolones, tetracyclines, trimethoprim, cotrimoxazole, chloramphenicol, and nitrofurantoin (Stock and Wiedemann 2001). These are used singly or in combination with 2 or more ABs, which has been reported to contribute significantly to the emergence of multidrug-resistant in chicken isolates (McEwen and Fedorka-Cray 2002).
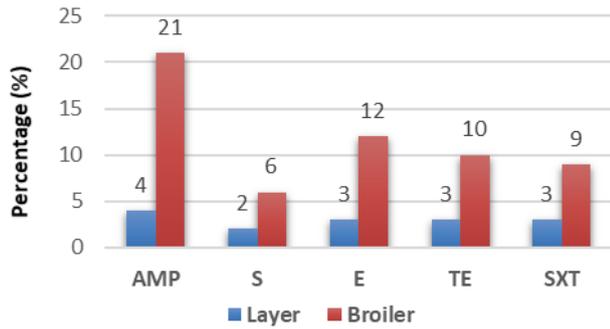
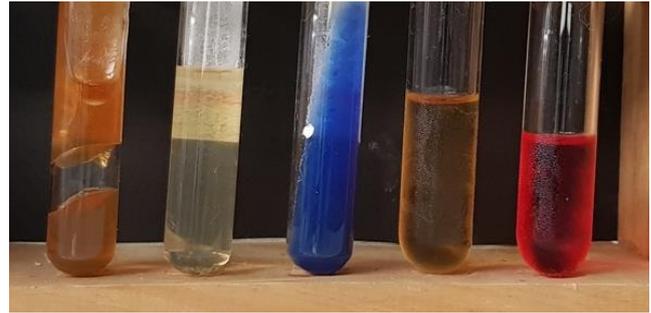**Figure 1.** Percentage of antimicrobial resistance *Klebsiella pneumoniae*

Figure 3. Identification *Klebsiella pneumoniae* by IMViC and TSIA

Figure 2. *Klebsiella pneumoniae* on MacConkey agar; grey arrow is dome-shaped mucoid colony
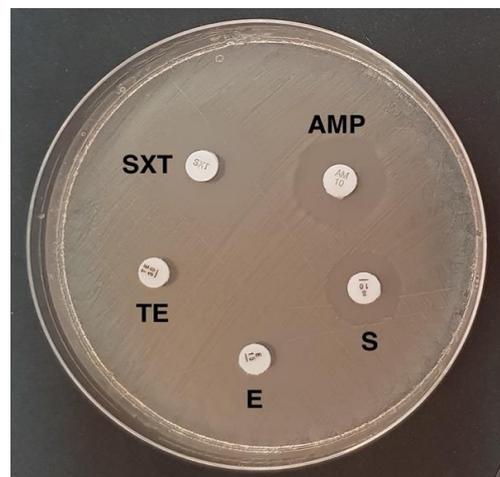

Figure 4. Antibiotic sensitivity confirmation test by disc diffusion by Kirby-Bauer method on Mueller-Hinton agar. Information: ampicillin (AMP), streptomycin (S), tetracycline (TE), erythromycin (E), and sulfamethoxazole (SXT).

The presence of multidrug-resistant bacteria is a threat to public health and livestock. These conditions could result in limited treatment options. The only laws on antimicrobial use in food animal production for domestic consumption mandate withdrawal of antimicrobials before processing of food animal products (Brahmachari et al. 2013). Furthermore, multidrug-resistant bacteria triggered the use of an antibiotic that has no longer been used for toxicity, such as colistin (Fard 2004). Measures that could be undertaken were building surveillance programs, conducting surveillance on feed and livestock. Breeders also need to improve biosecurity practice. Evidence indicates that antimicrobial residues in manure might also be responsible for the contamination of soil, surface water, and groundwater resources close to farms involved in intensive broilers rearing activities (FAO 2008). Policy actions should be implemented immediately in order to safeguard the effectiveness of antimicrobials since antimicrobial effectiveness is a globally shared resource and responsibility (Ganguly et al. 2011). Litter and manure waste must be properly managed in intensive production systems to prevent the contamination of air, soil, and water,

as well as negative consequences on human health (Thyagarajan 2014).

The use of antimicrobials in poultry is a well-known catalyst for developing resistance in bacteria (Wibisono et al. 2020), but unfortunately, no reliable data and information are available regarding the use of antimicrobials in poultry (Rahmahani et al. 2020). There is excessive use of antimicrobials in the livestock sector compared to pet animals. The risk of AMR is due to the close association between pet animals and humans and this association provides opportunities for two ways of commensal and pathogenic transfer (Pomba et al. 2017). ESBL is a β-lactamase that can hydrolyze oxyimino-based β-lactams, such as cefotaxime, ceftazidime, and aztreonam (Putra et al. 2019). Most *K. pneumoniae* produce SH-based non-ESBL β-lactamases such as SHV, and some *Escherichia coli* produce TEM, which is also a non-ESBL β-lactamase. Enzymes such as SHV-1 and TEM-1 can hydrolyze ampicillin, but oxyiminocephalosporins including ceftriaxone, cefotaxime, and ceftazidime cannot be hydrolyzed. These antibiotics are well designed to

counter hydrolysis by these bacterial enzymes (Karanika et al. 2016; Putra et al. 2020).

This study examined the prevalence of resistance in farms suspected of using antimicrobials. Given these limitations, our study mainly compared the resistance profile among different farms (broiler and layer) affecting resistance development. Moreover, it is possible to reduce the prevalence of antimicrobial resistance by placing restrictions on the use of antimicrobials in food animal production without negative impacts on productivity, as evidenced by the experience of both the poultry and pork industries in Denmark (Levy 2014).

In conclusion, this study confirms that the percentage of resistant isolates of *K. pneumoniae* from 5 antimicrobial agents of broiler chicken samples was higher than layer chicken sample. This study also showed highest levels of antibiotic resistance to broiler chicken samples to Ampicillin (AMP) antibiotic. Total of 75% *K. pneumoniae* from broiler chicken samples was resistant to Ampicillin (AMP) antibiotic. The presence of multidrug-resistant bacteria is a threat to public health and animals. The impact of these conditions limited and harder the treatment option as chicken farmers in Indonesia still using antibiotics without veterinarian supervision. Policy and veterinarian supervision are important to implement for disease treatment and animal husbandry in Indonesia.

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