Detection of antibiotic resistance coding gene in *Klebsiella pneumoniae* bacteria isolated from broiler chickens in West Java, Indonesia

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**ABSTRACT**

Antibiotic resistance is now a global health issue. Antibiotic abuse leads microorganisms to grow resistant to a variety of antibiotics. Antibiotic resistance and resistant genes in *Klebsiella pneumoniae* in broiler chickens in West Java Province were investigated. Broilers from Bogor and Sukabumi were used to obtain 200 cloacal swab samples. Cultures on MacConkey agar, Gram staining, biochemistry test, and polymerase chain reaction (PCR) were used to isolate and identify the samples. The Kirby–Bauer disk diffusion method was used to investigate antibiotic sensitivity, and PCR was used to detect resistance genes. A total of 20% of the samples tested positive for *K. pneumoniae*. Among other drugs, *K. pneumoniae* isolates demonstrated resistance to erythromycin (100%), oxytetracycline (97.5%), ampicillin (97.5%), tetracyclines (95%), nalidixic acid (95%), enrofloxacin (EN) (87.5%), EN (82.5%), ciprofloxacin (75.0%), gentamicin (45.0%), and chloramphenicol (25%). All isolates (100.0%) had *gyrA* and *bla* TEM genes, 85.0% had *tetA* genes, and 52.5% had *ermB* genes, according to the molecular test. *Klebsiella pneumoniae* may be isolated and identified from broiler chickens in West Java, Indonesia, according to this study. *Klebsiella pneumoniae* isolated possessed *gyrA*, *bla* TEM, *tetA*, and *ermB*-resistant genes and was multidrug-resistant.

**INTRODUCTION**

The need for animal protein in Indonesia has increased every year. One of the affordable sources of animal protein for all levels of society is poultry. The area with the largest broiler in Indonesia is West Java, namely in the districts of Bogor and Sukabumi (Hardiati et al., 2021; Livestock and Animal Health Statistics, 2020). Broilers have the advantage of having a high level of productivity, but the disadvantage is that they have a low level of disease immunity. Chickens’ immunity can be boosted in a variety of methods, including the administration of vitamins, immunizations, and antibiotics (Alagawan et al., 2020; Dibner and Richards, 2005). Antibiotics such as β-lactams, enramycin, bacitracin, oxytetracycline (OT), streptomycin, and chlorotetraacycline were often used as growth promoters before being banned by the Indonesian government (Department of Nutrition Science and Feed Technology, 2018).

The systematic and excessive use of antibiotics in the poultry farming sector, without regard for the guidelines for usage, has a negative impact. The growth of antibiotic resistance is one of the effects. Antibiotic use generates stress, which can trigger resistance in DNA, resulting in bacterial cell mutations and genetic alterations (Huddleston, 2014). According to the World Health Organization (WHO, 2014), microorganisms resistant to antibiotics cause infection in animals, resulting in higher rates of morbidity and mortality. Both harmful bacteria and normal flora can develop antibiotic resistance. *Klebsiella pneumoniae* is one of the bacteria that is at risk of antibiotic resistance.

*Bacterium K. pneumoniae* is Gram-negative and is found in the skin, mouth, and intestines as part of the normal flora. Inhalation transmission of *K. pneumoniae* can result in
pneumonia, bacteremia, and nosocomial infections. Chicken meat is a potential reservoir for the transmission of antibiotic-resistant *K. pneumoniae* from animals to humans (Davis et al., 2015). The CTXM gene is produced by *K. pneumoniae*, an extended-spectrum β-lactamase (ESBL) generating bacteria. This gene can be passed down directly and indirectly (Mahanti et al., 2018). Resistant Gram-negative and Gram-positive bacteria will be difficult to treat, if not impossible to treat, with antimicrobials (Akova, 2016).

According to some reports, *K. pneumoniae* has developed drug resistance. Antibiotic resistance was found in *K. pneumoniae* isolated from chicken meat in Surabay, Indonesia, with 61.54% resistance to tetracycline (TET), 15.38% resistance to gentamicin (CN), 7.69% resistance to cefoxitin, 38.46% resistance to sulfacetamide, 53.85% resistance to nalidixic acid (NA), and 15.38% resistance to chloramphenicol (Yulistiani et al., 2017). In a 2005 survey, at a hospital in Surabay, Indonesia, broad-spectrum β-lactamases were detected in 20% of *Escherichia coli* and 28% of *K. pneumoniae* (Lestari et al., 2008). Antibiotic resistance and the discovery of antibiotic resistance coding genes in *K. pneumoniae* bacteria from hens in Indonesia are currently understudied. As a result, antibiotic resistance and antibiotic resistance coding genes in *K. pneumoniae* bacteria isolated from broilers in Bogor should be researched.

### MATERIALS AND METHODS

#### Procedures

**Isolation and sample identification**

In this study, 200 cloaca swab samples from broiler farms in Bogor and Sukabumi were cultured on MacConkey agar (MCA) (Oxoid) in a selective medium for *K. pneumoniae* bacteria. For 18–24 hours, the culture was incubated at 37°C. Gram staining was used to identify *Klebsiella* colonies. The Indole, Methyl Red, Voges Proskauer and Citrate (IMViC) test, methyl red/Voges–Proskauer test (Oxoid), Simmons citrate test (Oxoid), triple sugar iron agar, urease test, motility test, and carbohydrate test in the form of fermentation of glucose, sucrose, lactose, maltose, and Mannitol were used to test positive isolates.

**Confirming *K. pneumoniae* sample using polymerase chain reaction (PCR)**

Identification of *K. pneumoniae* bacteria was carried out by detecting the RNA polymerase β subunit (*rpoB*) gene with an amplicon length of 1,090 bp. *Klebsiella* bacteria extraction carried out using the PrestoTM Mini gDNA Bacteria Kit protocol (Geneaid). The primers used in this study were forward 5'-AAC CAC TTC CGC GTG CTG G-3' and reverse 5'-CCT GAA CAA CAC GCT CGG A-3' (Alves et al., 2006). The reagent used for amplification was MyTaqTM HS Red Mix (Bioline) with a total PCR of 50 µl. The amplification condition began with predenaturation at 94°C for 2 minutes; then 30 cycles with denaturation at a temperature of 94°C for 30 seconds; annealing at 54°C for 1 minute; extension at 72°C for 4 minutes; and final extension at 72°C and final extension at 72 n condition al ard 5'-AAC CAG TTCK. *pneumoniae* ATCC 700603.

**Antibiotics susceptibility test**

Bacterial isolates positive for the *rpoB* gene were then tested for antibiotic resistance. The antibiotic resistance test followed the Kirby–Bauer disk diffusion method using Mueller–Hinton agar based on the guidelines (CLSI, 2018). The antibiotics used were TET 30 µg, OT 30 µg, erythromycin (E) 15 µg, ciprofloxacin (CIP) 5 µg, enrofloxacin (EN) 5 µg, NA 30 µg, CN 10 µg, chloramphenicol (C) 30 µg, and ampicillin (AMP) 10 µg (Table 1). The selection of antibiotics was based on the results of a questionnaire, namely the top antibiotics most often used in chicken farms in West Java (Nilasari et al., 2018).

**Molecular detection of antibiotic resistance gene by PCR amplification**

Antibiotic resistance coding genes were detected using primary target genes gyrA (quinolone), tetA (TET), blaTEM (β-lactam), and ermB (macrolide). MyTaqTM HS Red Mix (Bioline) was used in the application of this study. The total volume of the PCR was 50 µl containing forward primers (2 µl), reversed primers (2 µl), DNA templates (3 µl), MyTaqTM Red Mix (25 µl); then H2O was added so that the total volume would reach 50 µl. The following primers were used in the study (Table 2).

<table>
<thead>
<tr>
<th>Antibiotic classes</th>
<th>Antibiotics</th>
<th>Doses (µg)</th>
<th>Inhibition zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td>TET</td>
<td>TET</td>
<td>30</td>
<td>≥15</td>
</tr>
<tr>
<td></td>
<td>OT</td>
<td>30</td>
<td>≥19</td>
</tr>
<tr>
<td>Macrolide</td>
<td>Erythromycin (E)</td>
<td>15</td>
<td>≥23</td>
</tr>
<tr>
<td>Quinolone</td>
<td>CIP</td>
<td>5</td>
<td>≥21</td>
</tr>
<tr>
<td></td>
<td>EN</td>
<td>5</td>
<td>≥23</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>30</td>
<td>≥19</td>
</tr>
<tr>
<td>Aminoglycoside</td>
<td>CN</td>
<td>10</td>
<td>≥15</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Chloramphenicol (C)</td>
<td>30</td>
<td>≥18</td>
</tr>
<tr>
<td>β-lactam</td>
<td>AMP</td>
<td>10</td>
<td>≥17</td>
</tr>
</tbody>
</table>

S: Susceptible, I: Intermediate, R: Resistant.
The amplification process began with predenaturation at 95°C for 3 minutes, then 95°C for 30 seconds, annealing at 53°C–62°C (Table 2), extension at 72°C for 1 minute, and final extension at 72°C for 5-minute amplification cycles of 30 times. The amplified samples were then visualized by electrophoresis on 1.0% agarose gel in a 1× Tris acetate-ethylenediaminetetraacetic acid buffer.

RESULTS

Isolation and identification of samples

Klebsiella pneumoniae colonies in MCA media were pink, round, convex, and mucoid (Fig. 1). Klebsiella pneumoniae bacteria have a rod-shaped, pink Gram stain, which is typical of Gram-negative bacteria (Fig. 2). The findings of the sample identification biochemical testing revealed 45 positive isolates (22.5%) as Klebsiella colonies (Table 3).

Confirmation of K. pneumoniae isolates using PCR

A total of 45 isolates were molecularly examined, with 40 of them having the rpoB gene (Table 3). This gene plays an essential role in transcribing DNA into RNA (Mosaei and Harbottle, 2019). The isolates showed a band with a length of 1,090 bp, according to the results of molecular identification (Fig. 3).

Sensitivity test

The sensitivity test was carried out by calculating the diameter of the antibiotic inhibition zone formed on the Mueller–Hinton agar. The area appears clear because it is not overgrown by bacteria (Fig. 4). Klebsiella pneumoniae bacterial resistance to antibiotics is extremely high in 40 samples. Among other antibiotics, erythromycin resistance is the highest (100%), followed by OT (97.5%), AMP (97.5%), and TETs (95%) (Table 4).

Detection of antibiotic resistance coding genes

Resistance genes were discovered in K. pneumoniae isolates with phenotypic intermediates and resistance (40 samples) utilizing the genes tetA (TETs and OTs), ermB (erythromycin), gyrA (CIP, NA, and EN), and blaTEM (AMP). Figures 5 and 6 show the electrophoresis of PCR amplification products using a UV transilluminator. The gyrA and blaTEM genes were found in all 40 samples analyzed (Table 5).
DISCUSSION
The bacteria \textit{K. pneumoniae} were isolated from the cloaca of West Javan broilers, in accordance with the findings of this research. In East Java, Indonesia, 7.8\% of cloacal swab samples from broiler chickens were positive for \textit{Klebsiella} bacteria (Hayati et al., 2019). \textit{Klebsiella pneumoniae} was also found in poultry drinking water and the feces of veterinarians and farm employees (Hamza et al., 2016). \textit{Klebsiella} can also be found in livestock waste, hospital waste, hospital worker gloves and clothing, and contaminated river water (Muraleedharana et al., 2019; Rock et al., 2014; Runcharoen et al., 2017). As a result, more research with a different sample source from this study is required. Although \textit{Klebsiella} bacteria are still considered low pathogenic microorganisms, they can induce primary infections in poultry that are immunosuppressed. \textit{Klebsiella} in chicken can contaminate the carcass, and if not properly treated, it can cause human infection. Because the bacteria are resistant to antibiotics, this bacterial infection becomes increasingly difficult to cure (Kowalczyk et al., 2017).

Resistance to erythromycin, AMP, OT, TET, NA, EN, CIP, and CN was found in more than 50\% of the \textit{K. pneumoniae} strains studied (Table 4). \textit{Klebsiella} spp. isolated from broilers in Shandong province, China, was resistant to AMP 98.9\%, CIP 80.0\%, TET 78.9\%, and chloramphenicol 92.2\%, according to a similar study (Wu et al., 2016).

Antibiotic resistance can develop for a variety of reasons, including the length of antibiotic use (Moini et al., 2015). Antibiotic misuse has resulted in a global antibiotic resistance epidemic (Bartlett et al., 2013). The overuse of antibiotics in chickens is linked to the high rate of antibiotic resistance, increasing the danger of normal gut flora becoming resistant to antibiotics (Chishimba et al., 2016). Antibiotics like those employed in this study are often utilized in farms in West Java.
As a result, *K. pneumoniae* has a high level of resistance and is resistant to multiple antibiotic classes.

*Klebsiella pneumoniae* isolate was resistant to drugs from three different classes (multidrug-resistant). Bacteria are not sensitive to the majority of TET, AMP, and erythromycin classes. *Klebsiella* was found on a turkey farm in Oklahoma, and it possesses multidrug resistance to medicines such as AMP, TET, streptomycin, CN, and kanamycin (Kim et al., 2005). This has a devastating effect on the health of both animals and humans. WHO (2014) has identified bacteria that are the main focus of antimicrobial resistance research. *Klebsiella pneumoniae* is one of the bacteria that is the main focus in the international community. These bacteria are the source of the spread of resistance to antibiotics is shown in Figure 7 (Venezia et al., 2017).

Bacterial resistance to chloramphenicol antibiotics was only 25% in this study (Table 4), which could be because chloramphenicol in chicken has been banned in Indonesia since 1994. Chloramphenicol is included in the list of harsh drugs that are not authorized to be used for animals, according to Ditjen PP KemenKumHAM (1994) Decree of the Minister of Agriculture Number: 806/KFpts/TN.260/12/94 concerning veterinary pharmaceuticals classification regulation. Furthermore, the government notes that chloramphenicol is one of Indonesia’s nine illegal food additives via Permenkes Number: 1168/Menkes/

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Sensitivity</th>
<th>Intermediate</th>
<th>Resistance</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>TETs</td>
<td>2</td>
<td>0</td>
<td>38</td>
<td>95.0</td>
</tr>
<tr>
<td>OT</td>
<td>0</td>
<td>1</td>
<td>39</td>
<td>97.5</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>100.0</td>
</tr>
<tr>
<td>CIP</td>
<td>6</td>
<td>4</td>
<td>30</td>
<td>75.0</td>
</tr>
<tr>
<td>EN</td>
<td>0</td>
<td>7</td>
<td>33</td>
<td>82.5</td>
</tr>
<tr>
<td>NA</td>
<td>1</td>
<td>4</td>
<td>35</td>
<td>87.5</td>
</tr>
<tr>
<td>CN</td>
<td>21</td>
<td>1</td>
<td>18</td>
<td>45.0</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>24</td>
<td>6</td>
<td>10</td>
<td>25.0</td>
</tr>
<tr>
<td>AMP</td>
<td>0</td>
<td>1</td>
<td>39</td>
<td>97.5</td>
</tr>
</tbody>
</table>

Grey colors showed the highest antibiotic resistance, namely Erythromycin, followed by Tetracyclines, Oxytetracycline, Ampicillin, Nalidixic acid, Enrofloxacin and Ciprofloxacin. Gentamicin and Chloramphenicol were not detected for resistance genes.
Figure 7. The mechanism of emergence and spread of *K. pneumoniae* is MDR and extensively drug-resistant (XDR) and the accumulation of Antibiotic resistance gene (ARG) (Venezia et al., 2017).

Table 5. The result of amplification of resistance coding genes in *K. pneumoniae* isolates (*n* = 40).

<table>
<thead>
<tr>
<th>Samples' origin</th>
<th>Isolates (<em>n</em>)</th>
<th>Positive isolates have a resistance coding gene (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>gyrA</strong></td>
</tr>
<tr>
<td>Isolates</td>
<td>40</td>
<td>40 (100.00%)</td>
</tr>
</tbody>
</table>
Per/X/1999 about food additives (Ditjenphk, 2018). Despite the fact that chloramphenicol was outlawed 27 years ago, this study indicates that bacterial isolates resistant to it still exist. Until now, it was thought that the gene coding for antibiotic resistance of the chloramphenicol type was still present in *K. pneumoniae*.

Quinolone phenotypic resistance is extremely common. Bacterial resistance to quinolones is induced by mutations in the genes gyrA and parC, which alter the interaction between quinolones and enzymes, causing quinolones to become toxic and destroy bacterial chromosomes (Aldred et al., 2014). A gene mutation in the quinolone resistance determining regions—gyrA and parC, gyrA or parC only, or both genes—is responsible for the high level of resistance to the quinolone group and its derivatives (Hooper and Jacoby, 2015). In the molecular test, all of the isolates tested positive for the gyrA gene. In Enterobacteriaceae, the gyrA gene has a significant impact on the high level of resistance. Resistance is caused by mutations in DNA gyrA at amino acid positions 83 (serine tyrosine/leucine/isoleucine/treonin) or 87 (aspartate asparagine) (Nawaz et al., 2012). According to another study, the gyrA mutation causes alterations at positions 83 (serine), 84 (alanine), and 87 (aspartate) in the protein (Pieksarska et al., 2015).

In this investigation, the TETs’ sensitivity was quite poor. Efflux pumps, ribosomal protection, degradation, and rRNA mutation are the four basic methods by which bacteria become resistant to TETs. The efflux pump is the most common source of TET resistance; so far, 28 genes have been identified as the efflux pump’s cause. And tetA is one of them (Nguyen et al., 2014). In this study, the tetA gene was found in 83.33% of the participants. Previous research has found that 100% of clinical *K. pneumoniae* isolates carry the tetA gene (Bokaesian et al., 2014).

Because it may be spread via plasmids, the tetA gene is frequently found in bacteria. The tetA coding gene in *Klebsiella* bacteria can be used to transfer TET resistance to other bacteria such as *E. coli* via plasmid mediation (Wang et al., 2014). The tetA gene in bacteria obtained from layer chickens can be transmitted to other bacteria, and the tetA gene can spread faster in the environment than the tetB gene (Balasubramaniam et al., 2014). The efflux pump is encoded by the tetA gene (Akiyama et al., 2013). TET efflux pumps are regulated by the TET repressor “tetR,” which tightly controls TET mRNA expression (Moller et al., 2016). TET-resistant *Klebsiella pneumoniae* isolates from Kenya had 18% of the tetA gene; tetD, tetB, tetG, and maybe more coding genes made up the rest. So it is possible that the isolates that were not detected by the tetA gene have alternative resistance coding genes or resistance mechanisms in addition to efflux pumps in this investigation (Taft et al., 2017).

Erythromycin resistance was phenotypically present in all isolates. However, not all isolates possessed the ermB gene following molecular testing. Plasmids are used to carry the ermB gene (Dong et al., 2018). By altering 23S rRNA from bacterial ribosomes and establishing cross-resistance to macrolides, the erm gene acts as a methyltransferase (Dzyubakand Yap, 2016). Like cross-resistance, cross-sensitivity to antibiotics is another major problem where antibiotics of same class or antibiotics having similar structure become reactive/allergic (Chaudhary et al., 2021). The erm gene has around 30 variants, with ermA, ermB, ermC, and ermF being the 4 primary classes found in pathogenic microbes. In *Staphylococcus*, the ermA and erm genes are commonly detected. The ermB class genes are mostly found in *Streptococcus* and *Enterococcus* bacteria (Hoek et al., 2011). The ermB gene responds to erythromycin resistance in *Enterococcus*. Isolates that are negative for the ermB gene may carry additional genes, according to research findings (Iweriebor et al., 2016). Furthermore, because the permeability of the outer membrane of Enterobacteriaceae bacteria is poor, it is innately resistant to the macrolide group (Gomes et al., 2016).

This study also detected the ESBL coding gene. Detection of the ESBL coding gene needs to be carried out because *Klebsiella* is a bacterium that produces ESBL (Muraleedharana et al., 2019). In India, 60.3% of *K. pneumoniae* isolates produced ESBL (Kaur et al., 2013). ESBL is an enzyme produced by Gram-negative bacteria which can cause β-lactam antibiotics. Since 2018, the bla gene has been identified in the description, and 223 TEM types, 193 SHV types, and 172 CTX-M types have been listed in the database (https://www.lahey.org/Studies) (Ramadan et al., 2018).

The ESBL coding gene was also discovered in this investigation. Because *Klebsiella* is a bacterium that produces ESBL, detection of the ESBL coding gene is required (Muraleedharana et al., 2019). 60.3% of *K. pneumoniae* isolates in India developed ESBL (Kaur et al., 2013). Gram-negative bacteria develop an enzyme called ESBL, which can make β-lactamase antibiotics ineffective. The bla gene has been found in 223 TEM types, 193 SHV types, and 172 CTX-M types since 2018 (Ramadan et al., 2018). The tetmoniera (TEM) gene was identified as an ESBL gene in this investigation. When compared to other forms of ESBL, the presence of the blaTEM gene was the highest (Pishtian and Khadija, 2019). The blaTEM gene is a β-lactamase (bla) gene that was found in *Klebsiella* isolates for the first time. This gene is a forerunner in the evolution of the bla gene, paving the way for the appearance of other ESBL coding genes, which later underwent changes. The bla gene can help antibiotics spread horizontally between strains via plasmids and transposons (Barguigua et al., 2011). The blaTEM is an antibiotic resistance gene found in plasmids that is most commonly found in Gram-negative bacteria in clinical settings (Wilopo et al., 2015).

Clinical disease is more usually linked to research on the bacterium *K. pneumoniae*. Environmental isolates are still rarely explored. Both phenotypically and genotypically, these isolates are remarkably similar to clinical isolates (Runcharoen et al., 2017). However, the two groups differed in terms of virulence characteristics (Davis et al., 2015). Only cloacal swab samples were used in this investigation, and only a few genes coding for antibiotic resistance were discovered. As a result, more research into *K. pneumoniae* samples from the environment and other chicken organs is required, as well as the utilization of more resistance coding genes for identification.

**CONCLUSION**

*Klebsiella pneumoniae* can be isolated and identified from broilers in West Java, Indonesia, according to this study. Resistance to more than three antibiotic classes indicates that the isolates are multidrug-resistant. In West Java, resistance coding genes such gyrA, tetA, ermB, and blaTEM were found in *K. pneumoniae* from broiler cloaca swabs.
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CONFLICT OF INTEREST

The authors declare no conflict of interest for this manuscript.

FUNDING


AUTHORS’ CONTRIBUTIONS

All the authors have equally contributed to study design, collection, analysis, and interpretation of the data, writing, and manuscript drafting. All the authors have read and approve the final version.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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REFERENCES


isolated from farm environments and retail products. stool isolates
species isolated from broiler chickens in Sukabumi and Bogor.

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