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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 chlortetracycline 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

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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 Surabaya, Indonesia, with 61.54% resistance to tetracycline (TET), 15.38% resistance to gentamicin (CN), 7.69% resistance to cefoxitin, 38.46% resistance to sulfametizole, 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 Surabaya, 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 CAG TTC CGC GTT GGC 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°Cand final extension at 72 n condition al ard 5'-AAC CAG TTC*K. 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), *bla*TEM (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 1. Constraint zone diameter standard (CLSI, 2018).

Antibiotic classes	Antibiotics	Doses (µg)	Inhibition zone diameter (mm)		
			S	*I*	*R*
TET	TET	30	≥15	12–14	≤11
	OT	30	≥19	15–18	≤14
Macrolide	Erythromycin (E)	15	≥23	14–22	≤13
Quinolone	CIP	5	≥21	16–20	≤15
	EN	5	≥23	14–22	≤13
	NA	30	≥19	14–18	≤13
Aminoglycoside	CN	10	≥15	13–14	≤12
Chloramphenicol	Chloramphenicol (C)	30	≥18	13–17	≤12
β-lactam	AMP	10	≥17	14–16	≤13

S: Susceptible, *I*: Intermediate, *R*: Resistant.

Table 2. Primer lists of antibiotic resistance coding genes.

Antibiotic group (Gen)	Base sequence (5'–3')	Amplicon (bp)	Annealing (°C)	Citation
Quinolone (*gyrA*)	(F) CGACCTTGCGAGAGAAAT (R) GTTCCATCAGCCCTTCAA	626	60	Nawaz *et al*. (2012)
TET (*tetA*)	(F) GTAATTCTGAGCACTGTCGC (R) CTGCCTGGACAACATTGCTT	965	62	Chuah *et al*. (2018)
β-lactam (*blaTEM*)	(F) ATCAGCAATAAACCAGC (R) CCCCGAAGAACGTTTTC	516	54	Hassan *et al*. (2018)
Macrolide (*ermB*)	(F) GAAAAGGTACTCAACCAAATA (R) GTAACGGTACTTAAATTGTTTAC	639	53	Hao *et al*. (2016)

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 *bla*TEM (AMP). Figures 5 and 6 show the electrophoresis of PCR amplification products using a UV transilluminator. The *gyrA* and *bla*TEM genes were found in all 40 samples analyzed (Table 5).

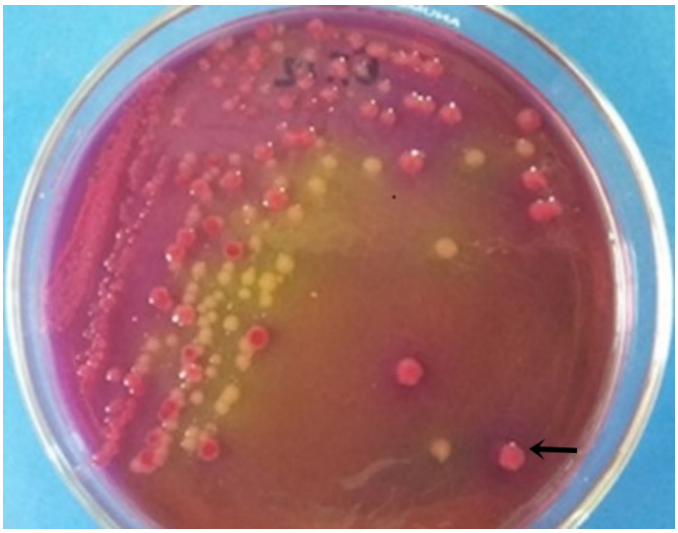

Figure 1. *Klebsiella* colonies on MCA medium.

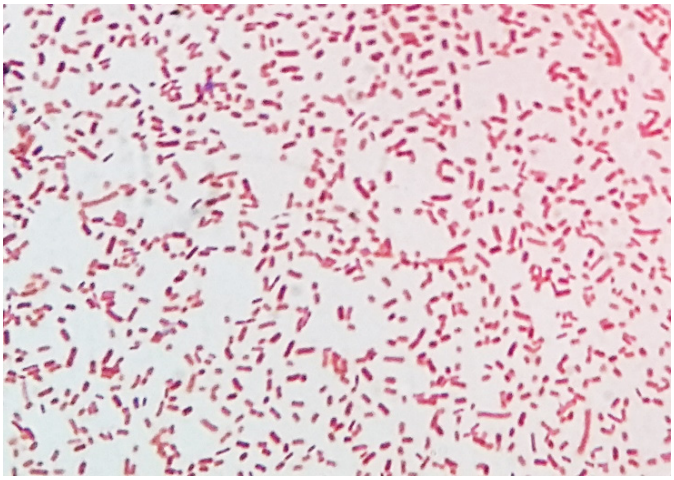

Figure 2. Gram stain of *Klebsiella* bacteria.

Table 3. Results of isolation and identification *K. pneumoniae.*

Poultry group	Samples' origin	Samples tested	Positive samples		
			Biochemical test	Molecular test	Percentage (%)
Broiler	Sukabumi	100	23	19	
	Bogor	100	22	21	
Total Samples		**200**	**45**	**40**	**20.00**

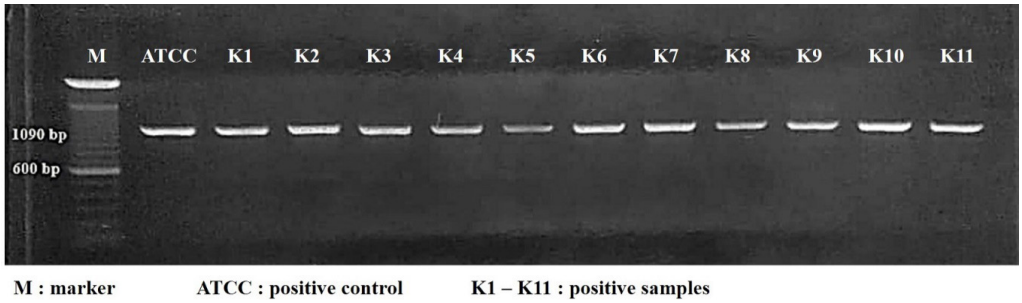

Figure 3. Amplification of the *rpoB* gene (1,090 bp) in *K. pneumoniae* isolates isolated from broilers in West Java. M: marker, ATCC: *K. pneumoniae* positive control, K1–K12: *K. pneumoniae* positive isolates.

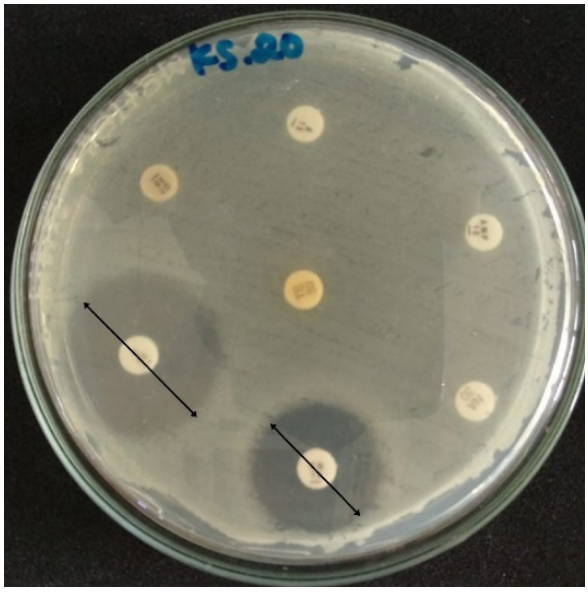

Figure 4. Measurement of the inhibition zone diameter on the diffusion disk. The black arrow indicates the diameter of the inhibition zone that is formed.

DISCUSSION

The bacteria *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 *Klebsiella* bacteria (Hayati *et al*., 2019), while in Nigeria 60% of poultry cloacal swab samples were positive for *Klebsiella* bacteria (Chika *et al*., 2017).

According to the findings, *K. pneumoniae* was found in 40 out of the 45 samples. Because the isolates were not *K. pneumoniae* species, but other *Klebsiella* genus species, such as *K. oxytoca* or *K. variicola*, five negative samples were suspected. The difference in positive biochemical and molecular test results is thought to be due to the fact that biochemical testing did not employ all criteria; hence, some isolates were positive in biochemical tests but not in *K. pneumoniae* species tests.

The presence of *K. pneumoniae* in chicken cloacal swabs suggests that this bacterium can be found in the digestive tract of chickens, as well as in the feces and environment of chicken farms. *Klebsiella pneumoniae* was also found in poultry drinking water and the feces of veterinarians and farm employees (Hamza *et al*., 2016). *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 *Klebsiella* bacteria are still considered low pathogenic microorganisms, they can induce primary infections in poultry that are immunosuppressed. *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 *K. pneumoniae* strains studied (Table 4). *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.

Table 4. Percentage of antibiotic resistance in *K. pneumoniae* bacteria (n = 40).

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

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.

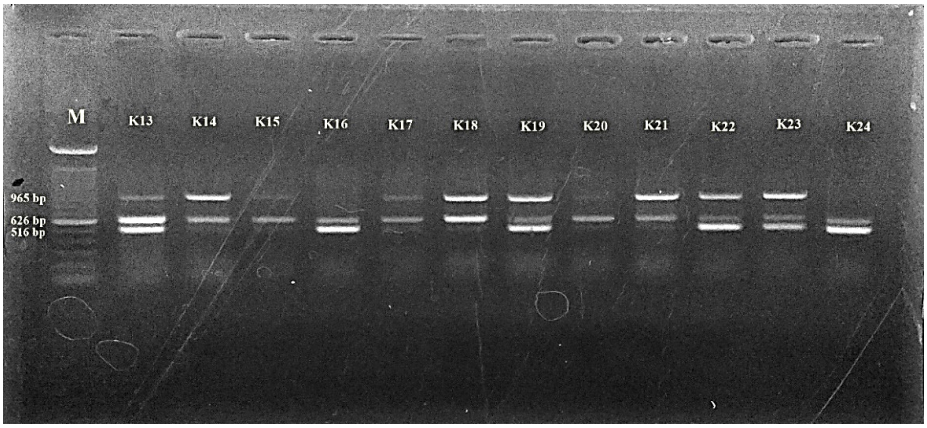

Figure 5. Amplification of the *gyrA* (626 bp), *blaTEM* (516 bp), and *tetA* (965 bp) genes. M: marker, K13–K24: *K. pneumoniae* isolates.

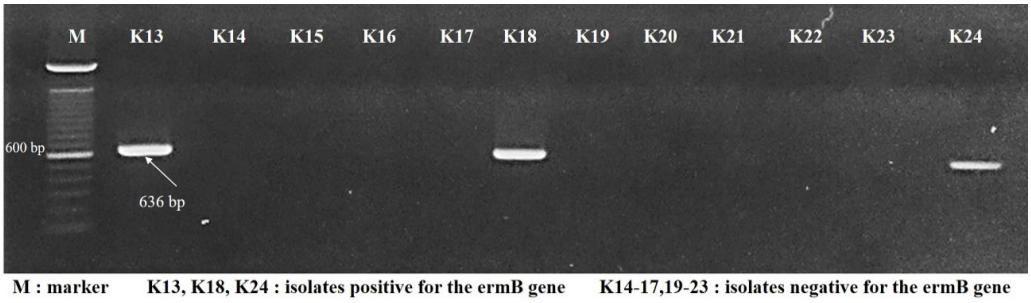

Figure 6. Amplification of gene *ermB* (636 bp). M: *marker*, K13–K24: *K. pneumoniae* isolate.

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

Samples' origin	Isolates (n)	Positive isolates have a resistance coding gene (%)			
		gyrA	***tetA***	***ermB***	***blaTEM***
Isolates	40	40 (100.00%)	34 (85.00%)	21 (52.50%)	40 (100.00%)

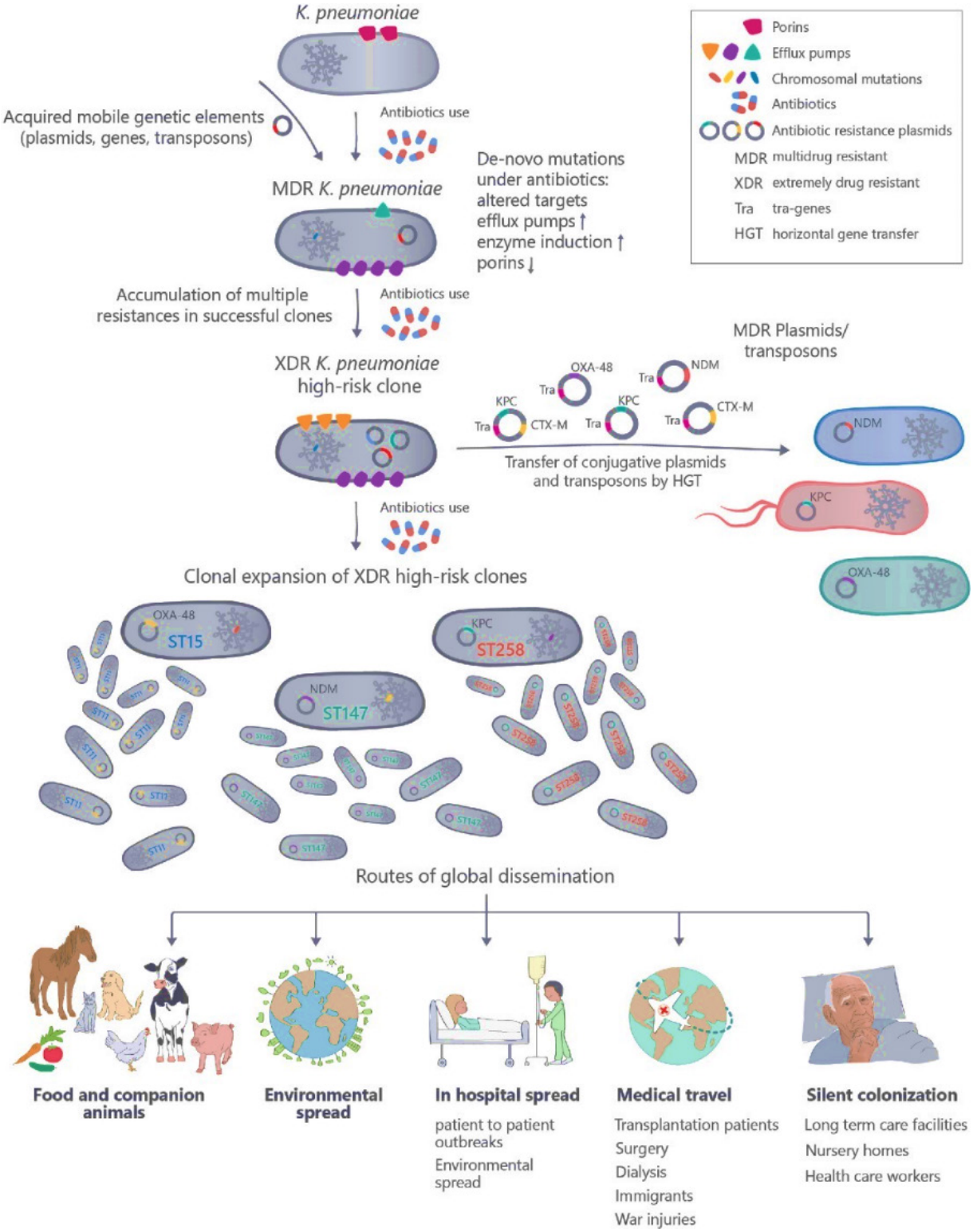

Figure 7. The mechanism of emergence and spread of *K. pneumoniae* is MDR and extensively drug-resistant (XDR) and the accumulation of Antibiotic resistance gene (ARG) (Venezia *et al*., 2017).

PER/X/1999 about food additives (Ditjenpkh, 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 gyrase and topoisomerase IV, which impair 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 (Piekarska *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 (Bokaeian *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 (Møller *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 (Taitt *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 temoniera (TEM) gene was identified as an ESBL gene in this investigation. When compared to other forms of ESBL, the presence of the *bla*TEM gene was the highest (Pishtiwan and Khadija, 2019). The *bla*TEM 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 *bla*TEM gene 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 *bla*TEM 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.

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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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