

Detection of *qnrS* and *tetA* resistance coding genes in *Pseudomonas aeruginosa* of egg-laying hens farming Cianjur Regency, West Java

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Abstract. The use of antibiotics is an alternative to reduce disease incidence and is used to increase egg-laying hens. This study aims to detect the presence of antibiotic resistance to resistance coding genes in *Pseudomonas aeruginosa* bacteria. The cloacal swab samples were collected randomly as many 66 samples from several egg-laying hens farms in Cianjur Regency, West Java. The collected isolates were examined bacteriologically. The results of the antibiotic sensitivity test of 8 samples (12.1%) of the *Pseudomonas aeruginosa* showed the resistance level of antibiotics class tetracycline (tetracycline 50%, oxytetracycline 75%, and doxycycline 50%), fluoroquinolone (enrofloxacin 12.5%), and phenicol (chloramphenicol 12.5%). *Pseudomonas aeruginosa* was resistant to tetracyclines, oxytetracyclines, doxycycline, enrofloxacin and is sensitive to chloramphenicol. The detection of resistance coding genes showed the genes amount of *tetA* (62.5%) and *qnrS* (75%). Efforts to determine the level of resistance and use of antibiotics in farms need regular surveillance and monitoring to ensure the wise use of antibiotics.

Keywords: antibiotics, egg-laying hens, *Pseudomonas aeruginosa*, resistance genes

INTRODUCTION

Community needs for livestock products are increasing every year. One of the livestock products that different social levels can consume is eggs. The consumption rate of eggs of 18.16 kg per capita/year [1]. In 2019, egg-laying hens in Indonesia reached 263,918,004 egg-laying hens, while the hen's population in West Java Province was 24,491,231 egg-laying hens [1]. Increasing the population of egg-laying hens aims to meet the consumption level of eggs (animal protein) of the community. In Indonesia, one of the areas that have an egg-laying hens farm is Cianjur Regency.

Hens immunity may have the potential to cause disease and require antimicrobials to control disease incidence. Krivonogova *et al.* [2] stated that using antimicrobials is the most effective way to fight pathogenic and opportunistic bacteria. The opportunistic microorganisms that

adapt most quickly to antimicrobials are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus* [2]. *Pseudomonas aeruginosa* in hens is an opportunistic pathogenic bacterium that can cause respiratory infections, diarrhea, and septicemia. It can cause diarrhea, dehydration, dyspnea, septicemia, and death of newly hatched chicks in malignant strains. Infection can occur through skin wounds or contaminated vaccines, or needle contamination used for injection [3].

In addition to suppressing disease incidence, antimicrobials are also used in production applications such as stimulating egg production, muscle growth, and others. However, it is feared that antimicrobial use that is not following the rules of use can cause resistant microbes through genetic and non-genetic mechanisms [4]. The high incidence of antibiotic resistance in egg-laying hens is due to the continuous use of antibiotics during the productivity period, the administration of antibiotics that are not right and the dosage is not correct. This use of antibiotics can lead to changes like the bacteria towards resistance, causing failure in treatment. Resistant bacteria

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in laying hens can reach humans through the food chain, environment (water, air, and soil), as well as direct contact between animals and humans [5].

The high incidence of antimicrobial resistance is of concern to international agencies such as the World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the World Organization for Animal Health (OIE) [6]. Incidence of antibiotic resistance can contaminate products from livestock and potentially be a source of microbial contamination to humans, which is very influential in increasing the economic value of overcoming microbial resistance. Testing for bacterial resistance, one of which *Pseudomonas* to antibiotics in farms, needs to be carried out regularly to ensure the wise use of antibiotics. The antibiotic resistance genes in *Pseudomonas aeruginosa* can cause failure in the treatment program, cause harm to humans, animals, and the environment.

METHODOLOGY

Samples Collection

The sample collection was conducted from November 2020 to March 2021. The samples of cloacal swabs were collected randomly as many 66 samples from several egg-laying hens farms in Cianjur Regency, West Java. Samples were collected in Phosphate Buffered Saline (PBS) as a transport medium and stored at 4°C.

Isolation and Identification of *Pseudomonas aeruginosa*

The collection was cultured in Mac Conkey agar (MCA) medium. The cultured samples were incubated aerobically at 37°C for 18-24 hours. Bacterial colonies growing on MCA medium were observed. Colonies of the genus *Pseudomonas* bacteria are large, irregular, translucent and do not ferment lactose [7]. A single colony suspected of being the genus *Pseudomonas* bacteria was then observed microscopically using Gram staining. The cell morphology of the bacteria was observed under a microscope at a magnification of 10 × 100. The cell morphology of *Pseudomonas* bacteria has a rod shape and is characteristic red of Gram-negative bacteria [8].

Furthermore, the positive samples were subcultured on Trypticase Soy Agar (TSA) slant medium and then incubated aerobically at 37°C for 18-24 hours. Isolates suspected as *Pseudomonas aeruginosa* colonies were identified with biochemical tests. The biochemical tests carried out were the Oxidase test, the Triple Sugar Iron Agar (TSIA) test, the Sulfide Indole Motility (SIM) test, the urea test,

the Simmon's Citrate test, then the fermentation test for glucose, lactose, sucrose, maltose and mannitol [8].

Confirmation of *Pseudomonas aeruginosa* with Polymerase Chain Reaction (PCR)

Pseudomonas aeruginosa, isolated and identified based on conventional tests, was extracted to obtain deoxyribonucleic acid (DNA). The method used in the extraction is heating (boiling) [9]. Furthermore, molecular confirmation was carried out with the PA-SS gene. PA-SS gene detection used primers; forward 5'- GGG ARF TCT TCG GAC CTC A -3 'and reverse 5'- TCC TTA GAG TGC CCA CCC G-3' amplicon length of 956 bp [10]. The DNA amplification of *Pseudomonas* sp. with a total reaction volume of 25 µl consisting of 4 µl template, 2 µl reverse primer (20 µM), 2 µl forward primer (20 µM), 12 µl MytaqTM HS Red Mix and adjusted with ddH₂O up to 25 µl. The PCR process was carried out with a *Thermocycler GeneAmp® PCR System 9700*. Predenaturation at 95°C for 5 minutes was used as the initial stage. DNA amplification stage with 30 cycles contained denaturation at 95°C for 1 minute, annealing 50°C for 30 seconds, extension at 72°C for 2 minutes, and final extension at 72°C for 8 minutes. Visualization of PCR results was performed by electrophoresis at 1% agarose in Tris-Acetate-EDTA (TAE) buffer and staining using 1 µl FloSafe DNA Stain (1st BASE). The DNA ladder (GeneRuler®) of 100 bp was used as the standard measure.

Antibiotic Sensitivity Test

The antibiotic sensitivity test followed the Disk Diffusion Kirby-Bauer method using Mueller-Hinton Agar based on the Clinical and Laboratory Standards Institute guidelines [10]. Antibiotics used were tetracycline 30 µg, oxytetracycline 30 µg, doxycycline 30 µg, chloramphenicol 30 µg and enrofloxacin 5 µg.

Table 1. Diameters of zone standard antibiotics [11]

| Antibiotics | Dose (µg) | Inhibition zone diameter (mm) | | |
|-------------------------|--------------|----------------------------------|-------|------|
| | | S | I | R |
| Tetracycline | | | | |
| Tetracycline (TET) | 30 | ≥ 15 | 12-14 | ≤ 11 |
| Doxycycline (DO) | 30 | ≥ 16 | 13-15 | ≤ 12 |
| Oxytetracycline(OT) | 30 | ≥ 19 | 15-18 | ≤ 14 |
| Fluoroquinolones | | | | |
| Enrofloxacin (EN) | 5 | ≥ 23 | 14-22 | ≤ 13 |
| Phenicol | | | | |
| Chloramphenicol (C) | 30 | ≥ 18 | 13-17 | ≤ 12 |

S: sensitive, I: intermediate, R: resistant

The purified isolates of *Pseudomonas aeruginosa* were subcultured on TSA medium and incubated at 37°C for 24 hours, and the pure growing colonies were suspended with physiological NaCl 0.9%, then homogenized to the turbidity level in a standard solution of 0.5 McFarland. 0.5 ml of the suspension was poured into a petri dish containing Mueller Hinton Agar (MHA) medium, then leveled using a cotton bud. Paper disks containing antibiotics were placed on the surface of the medium and incubated at 37°C for 18 hours, and the diameter of the antibiotic inhibition zone was measured. This antibiotic sensitivity test was repeated three times at the same time. The inhibition zone diameter in the antibiotic susceptibility test followed the standard of inhibition zone diameter by CLSI [11] in Table 1.

Detection of Antibiotic Resistance Coding Genes

DNA templates from *Pseudomonas aeruginosa* isolate obtained from the extraction process were then carried out by the resistance gene detection process using primers target genes, namely, *qnrS* (enrofloxacin) and *tetA* (tetracycline, doxycycline and oxytetracycline). PCR reaction to detect the gene coding for antibiotic resistance using *Mytaq™ HS Red Mix* (Bioline). The total volume of the reaction was 25 µl consisting of 3 µl of template DNA, 2 µl of reverse primers (20 µM), 2 µl of forward primers (20 µM), 12 µl of Mytaq™ HS Red Mix and added with H₂O to 25µl.

The amplification process began with predenaturation at 95°C for 3 minutes, then 30 cycles of the amplification process with a denaturation temperature of 95°C for 30 seconds, annealing at 52–62°C according to the primer used (Table 2), extension at 72°C for 1 minute, and the end of amplification process was performed with a final extension at 72°C for 5 minutes. The amplified PCR product was maintained at 12°C. The amplified samples were then visualized by electrophoresis on 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer.

RESULTS AND DISCUSSION

Characteristics of *Pseudomonas aeruginosa* isolates

The Culture results on the MCA medium showed that 12 samples (18.1 %) were suspected of being *Pseudomonas aeruginosa* colonies. The colonies were large, irregular, and did not ferment the lactose (Figure 1a). Furthermore, Gram staining was carried out to see the morphological shape of

the bacteria, microscopically showing that it was rod-shaped and red as Gram-negative bacteria (Figure 1b). Rapi *et al.* [14] stated that Gram-negative bacteria would be red because the lipids in their cell walls will dissolve during the washing process with alcohol so that the pores and cell walls will enlarge and cause the release of the crystal violet dye which was previously absorbed. The bacteria will be brightly colored after treated with safranin dye. Colonies suspected of *Pseudomonas aeruginosa* were then reconfirmed using biochemical tests.

The biochemical test results showed the characteristics of biochemical properties; the oxidase test was positive, it could ferment carbohydrates but did not form H₂S and gas, was motile, the citrate test was positive, and the indole test was positive. According to Cowan and Steel [8], *Pseudomonas aeruginosa* showed the results of biochemical tests on citrate-positive, oxidase-positive, glucose-positive, sucrose and lactose positive/negative, and mannitol positive/negative. Based on these characteristics, the colony showed consistent results with the characteristics of *Pseudomonas aeruginosa*.

Further identification was made through confirmation using Polymerase Chain Reaction. The confirmed Isolates showed that 8 out of 12 isolates (66.7%) were positively detected as isolates having the *PA-SS* gene (Figure 2). *PA-SS* gene is a 16S rRNA subunit specially designed to identify *Pseudomonas aeruginosa* with an amplicon length of 956 bp. According to Spilker *et al.* [9] the *PA-SS* gene was designed to identify *Pseudomonas aeruginosa* with 100% specificity and sensitivity to targets. The 16s rRNA subunit is currently the "gold standard" in determining the bacteria phylogenetics [15].

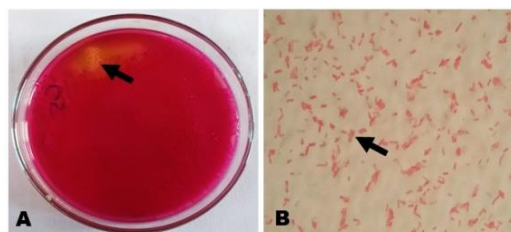


Figure 1. Arrows show *Pseudomonas aeruginosa* colonies on Mac Conkey Agar medium (A) and the morphology of *Pseudomonas aeruginosa* cells at 1000 × magnification (B).

Table 2 List of primers used for detection of antibiotic resistance coding genes

| Antibiotic (Resistance genes) | Base sequence | Amplicon (bp) | Annealing (°C) | Refere nce |
|--|--|------------------|-------------------|---------------|
| Enrofloxacin (<i>qnrS</i> ^a) | (F) 5'-ACG ACA TTC GTC AAC TGCAA-3' (R) 5'-TAA ATT GGC ACC CTG TAGGC-3' | 417 | 55 | [12] |
| Tetracyclines, doxycycline and Oxytetracycline(<i>tetA</i> ^b) | (F) 5'-GGT TCA CTC GAA CGA CGT CA-3' (R) 5'-CTG TCC GAC AAG TTG CAT GA-3' | 577 | 57 | [13] |

Table 3 Percentage of antibiotic resistance test results on *Pseudomonas aeruginosa* (n = 8)

| Antibiotic classes | Number and percentage of samples | | |
|-------------------------|----------------------------------|--------------|-------------|
| | Sensitive | Intermediate | Resistant |
| Tetracycline | | | |
| Tetracycline (TET) | 3/8 (37.5%) | 1/8 (12.5%) | 4/8 (50%) |
| doxycycline (DO) | 2/8 (25%) | 2/8 (25%) | 4/8 (50%) |
| oxytetracycline (OT) | 2/8 (25%) | 0/8 (0%) | 6/8 (75%) |
| Fluoroquinolones | | | |
| Enrofloxacin (EN) | 1/8 (12.5%) | 6/8 (75%) | 1/8 (12.5%) |
| Phenicol | | | |
| Chloramphenicol (C) | 7/8 (87.5%) | 0/8 (0%) | 1/8 (12.5%) |

Table 4 Pattern of multi-drug resistance in *Pseudomonas aeruginosa* isolates.

| number of antibiotics resistant | Number of isolates | Resistance pattern | Percenta ge (%) |
|------------------------------------|-----------------------|--------------------|--------------------|
| 5 | 1 | TE-OT-DO-EN-C | 12.5 |
| 3 | 2 | TE-OT-DO | 25 |

TE: tetracycline, OT: oxytetracycline, DO: doxycycline, EN: enrofloxacin, C: chloramphenicol

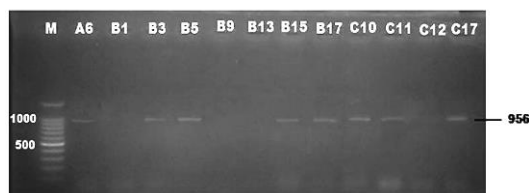


Figure 2. The results of PA-SS gene amplification (956 bp) in *Pseudomonas aeruginosa* isolates from laying hen farms in Cianjur Regency. M: marker; A6, B3, B5, B15, B17, C10, C11, C17: PA-SS gene-positive; B1, B9, B13, C12: PA-SS gene negative.

Antibiotic Sensitivity of *Pseudomonas aeruginosa* isolates

The resistance test of *Pseudomonas aeruginosa* to antibiotics used the Kirby-Bauer Disk Diffusion method. This method is a further test of 8 samples that have been molecularly confirmed. Determination of the resistance level based on the antibiotic inhibition zone on Mueller-Hinton Agar medium with CLSI as the standard reference [11]. In this research, the selection of antibiotics was made through interviews with breeders. Although there are antibiotics that are not commonly used in laying hens. Testing on these antibiotics was carried out to see the diversity of resistance levels to each class of antibiotics tested.

Antibiotic resistance test on eight isolates of *Pseudomonas aeruginosa* showed resistance levels to the antibiotics; tetracycline 50% (4/8),

doxycycline 50% (4/8), oxytetracycline 75% (6/8), enrofloxacin 12.5% (1/8) and chloramphenicol 12.5% (1/8) (Table 3). Research in Italy also reported that the isolate *Pseudomonas aeruginosa* from the cloacal swab had resistance to amoxicillin and sulfamethoxazole-trimethoprim (76.3%), doxycycline (71.2%), enrofloxacin (78%), gentamicin (28.9%). and oxytetracycline (81.3%) [16].

Pseudomonas aeruginosa isolates were intermediate to antibiotic classes, respectively; fluoroquinolone (enrofloxacin 75%) and tetracycline (tetracycline 12.5% and doxycycline 25%). The use of chloramphenicol in poultry farming has been prohibited since 1994 by the Indonesian government. The Decree of the Minister of Agriculture Number: 806/Kpts/TN.260/12/94 regarding the classification of veterinary drugs stated that chloramphenicol was included in the list of hard drugs that were not permitted used for animals. In addition, *Permenkes* (Minister of Health regulations) Number: 1168/Menkes/PER/X/1999, regarding food additives, explained that chloramphenicol was one of the nine types of food additives prohibited in Indonesia. This study shows that there are still isolates that experience resistance. This resistance is thought to impact the inappropriate use of chloramphenicol antibiotics in the past, so chloramphenicol resistance is still visible today.

Increasing antibiotic resistance in various livestock, especially poultry, poses a more significant challenge to public health than transferring antibiotic resistance genes from animals to animals and from animals to humans or vice versa [18]. Due to the limitations of the types of antibiotics used for veterinary treatment and the ease of access of antibiotics by farmers and the low price of antibiotics, it is not easy to control antibiotics in livestock in Indonesia [19].

The antibiotic resistance pattern in *Pseudomonas aeruginosa* isolates indicated that one isolate was resistant to five types of antibiotics and two isolates were resistant to three types of antibiotics (Table 4). Gill *et al.* [20] stated that bacteria with resistance to three or more types of antibiotics are categorized as multi-drug resistant (MDR). The pattern of antibiotic use in poultry farms is generally carried out for therapy, control and prevention of disease. Nearly 80% of protein-producing animals use antibiotics [21]. The use of antibiotics is increasing rapidly on livestock such as Bangladesh, Bhutan, India, Indonesia, Myanmar, Nepal, Sri Lanka, and Thailand [22]. The Directorate General of PKH [23] reported the use of antibiotics as disease prevention (81.4%) and the use of antibiotics as a growth promoter (0.3%). Improper use of antibiotics can adversely affect human and veterinary medications.

Antibiotic Resistance Coding Genes of *Pseudomonas aeruginosa* isolates

The detection of resistance coding genes on *Pseudomonas aeruginosa* isolates was carried out molecularly, which showed intermediate (enrofloxacin) and resistance (tetracycline and enrofloxacin) results to antibiotic resistance tests. The genes detected were the *tetA* gene (tetracycline, oxytetracycline and doxycycline) and the *qnrS* gene (enrofloxacin). However, in chloramphenicol, gene detection was not carried out because the incidence of resistance was relatively low.

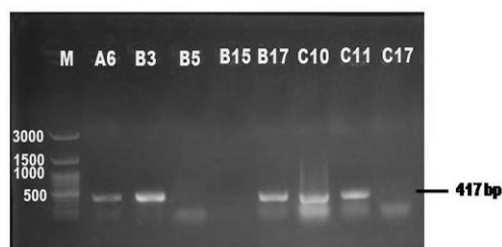


Figure 3 Amplification of the *qnrS* gene (417 bp) encoding resistance to enrofloxacin. M: marker; A6, B3, B17, C10, C11: *qnrS* positive, B5, B15, C17: *qnrS* negative.

Pseudomonas aeruginosa isolates that the *qnrS* resistance coding gene then detected experienced intermediate and resistance in the enrofloxacin. The detection results of the *qnrS* gene in *Pseudomonas aeruginosa* isolates showed that it was 6/8 (75%) detected with an amplicon length of 417 bp (Figure 3). This study shows that *Pseudomonas aeruginosa* isolates detected by *qnrS* resistance genes can transfer these genes horizontally or vertically, marked by detection of genes in isolates that are intermediate in the antibiotic resistance test. El-Badawy *et al.* [24] reported that antibiotic resistance in the fluoroquinolone class was increased, marked by the *qnrS* resistance coding gene (79.5%) isolated from Saudi Arabia. Another study also reported that 66% of the *qnrS* gene was detected from isolates from Kerman, Iran [25]. In 2007, the *qnr* gene was detected at 10 Gram-negative bacteria isolates isolated from zoo animals in Asa Zoological Park, Hiroshima prefecture, Japan [26].

The process of resistance to quinolone antibiotics can occur via chromosomes or be mediated by plasmids in *Pseudomonas aeruginosa* [25]. Chromosomal resistance occurs due to changes in enzyme targets (DNA gyrase or DNA topoisomerase IV). The *qnr* gene is known to be present in plasmids, called Plasmid-Mediated Quinolone Resistance (PMQR). Transforming plasmids through genetic elements in *Pseudomonas aeruginosa* is carried out horizontally, especially genes encoding resistance to quinolones. The presence of the *qnr*-mediated quinolone gene is often associated with multi resistant [27]. The mechanism for PMQR is the change in target by the *qnr* gene product, drug modification by aminoglycoside acetyltransferase (AAC (6')-Ib-cr), which can reduce ciprofloxacin activity as well as the pump efflux mechanism, which is connected by two pump effluxes known as olaquinox (OqxAB) and quinolone efflux plasmid (QepA) [28,29].

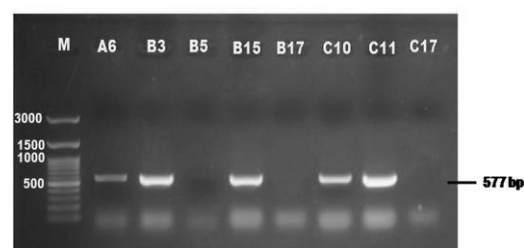


Figure 4 Amplification of the *tetA* (577 bp) encoding resistance to tetracycline-class M: marker; A6, B3, B15, C10, C11: *tetA* positive; B5, B17, C17: *tetA* negative.

The distribution of the resistance genes from these data shows that the distribution of the *qnrS* resistance gene is found throughout the world, although the percentage level varies from region to region. This resistance may harm both veterinary and human medicine.

The detection of the *tetA* resistance coding gene for the tetracycline antibiotic class that experienced intermediate and resistance in *Pseudomonas aeruginosa* isolates was detected by the *tetA* gene 5/8 (62.5%) with an amplicon length of 577 bp (Figure 4). Research in Egypt said that *Pseudomonas aeruginosa* isolates had 75.6% of the gene coding for *tetA* resistance [30]. Grossman [31] stated that one class of antibiotics with high resistance to bacteria is tetracyclines. The increased resistance to antibiotics is associated with antibiotics that are not following the rules of use to cause antibiotic resistance and the emergence of resistant coding genes. Obrequeet *al.* [32] stated that more than 40 genes cause resistance to tetracycline (*tet*), including the *tetA* and *tetB* genes that encode the efflux protein the role of pumping the tetracyclines out of *Pseudomonas aeruginosa*. The efflux pump activity removes tetracyclines from the bacteria using protons as an energy source [33]. This efflux process is a single transporter in the form of a membrane protein that can transfer several antibiotics from inside the cell to the substrate, causing resistance of these *Pseudomonas aeruginosa* to tetracyclines [34]. Therefore, it is essential to do antibiotic sensitivity testing to select an antibiotic specifically effective in overcoming antibiotic resistance.

CONCLUSION

Eight *Pseudomonas aeruginosa* isolate obtained through the cloaca swab of egg-laying hens farm in Cianjur Regency were resistant to antibiotics and tetracyclines oxytetracyclines, doxycycline, enrofloxacin. Intermediate to enrofloxacin, tetracycline, and doxycycline; and was sensitive to chloramphenicol. The resistance coding genes (*tetA* and *qnrS*) can be detected. Efforts to determine the level of resistance and use of antibiotics in farms need regular surveillance and monitoring to ensure the wise use of antibiotics. Further research is needed to detect samples from the environment, drinking water, feed, and staff on the farm.

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