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Antibiotic Resistant Pattern and Resistant Gene Identification of *Staphylococcus aureus* from Chicken Farm in Bogor

(POLA RESISTANSI ANTIBIOTIK DAN IDENTIFIKASI GEN RESISTANSI PADA STAPHYLOCOCCUS AUREUS DARI PETERNAKAN AYAM DI BOGOR)

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ABSTRACT

Chicken is one of the important protein source in Indonesia. Moreover, the largest population of chicken layer and poultry in Indonesia is known situated at West Java province with Bogor manicipality as the main producer. The aims of this study were to determine the antibiotic resistance pattern of *Staphylococcus aureus* isolated from poultry and layer farm in Bogor. The study also identified gene encoded the resistance. Cloacal swab samples were collected from chicken broiler and layer farm in Bogor manicipality. The samples were then cultured in Mannitol Salt Agar (MSA) medium to obtain *S. aureus*. Suspected colony was then confirmed by biochemical test. Positive strains were tested against several antibiotics and the diameter of clear zone arround of colony was interpreted based on Clinical and Laboratory Standard Institute. Furthermore, the DNA from resistant strains were then extracted, followed by detection of the resistance gene by using polymerase chain reaction (PCR) method. A total of 14 isolates of *S. aureus* were positive from poultry farm, and 15 isolates from layer farm. Most of all were resistant to tetracycline, ampicillin, oxytetracycline, erythromycin and nalidixic acid. On the other hands, several strains were sensitive to gentamycin and chloramphenicol. The study showed 28 isolates out of them were multi-drug resistant. Resistant gene such as blaTEM, gyrA and tetA were also identified in some isolates except for ErmB gene which was found in isolates originated from poultry farm. In conclussion, *S. aureus* in both farm showed mostly multi-drug resistant to several antibiotics which were supported by identification of resistant gene among isolates.

Keywords: antibiotic; chicken; resistant gene; *S. aureus*

ABSTRAK

Ayam merupakan salah satu sumber protein penting di Indonesia. Provinsi Jawa Barat memiliki populasi ayam pedaging dan petelur terbesar di Indonesia, dan Bogor sebagai daerah dengan populasi tertinggi. Tujuan penelitian ini adalah untuk menentukan pola resistensi antibiotik pada *Staphylococcus aureus* yang diisolasi dari peternakan ayam pedaging dan petelur serta mengidentifikasi gen resistansinya. Sampel usap kloaka dari peternakan ayam pedaging dan petelur dibiakkan pada medium *Mannitol Salt Agar* (MSA) untuk memperoleh *S. aureus* dan diteguhkan menggunakan uji biokimiawi. Isolat positif *S. aureus* diuji resistansinya terhadap beberapa antibiotik dan zona hambat yang terbentuk ditafsirkan berdasarkan *Clinical and Laboratory Standard Institute*. Senyawa DNA dari isolat resistan diekstraksi dan dilacak keberadaan gen resistansinya dengan *polymerase chain reaction* (PCR). Sebanyak 14 isolat *S. aureus* diperoleh dari peternakan ayam pedaging dan 15 isolat diperoleh dari peternakan ayam petelur. Sebagian besar isolat dari kedua peternakan resistan terhadap tetrasiklin, ampisilin, oksitetrasiklin, eritromisin dan asam nalidiksat. Isolat masih peka terhadap gentamisin dan kloramfenikol. Sebanyak 28 isolat dari kedua peternakan tahan terhadap beberapa antibiotik (*multidrug resistant*) karena

mengalami resistansi terhadap lebih dari tiga jenis antibiotik. Gen resistansi seperti blaTEM, gyrA, dan tetA ditemukan di kedua peternakan, sedangkan gen ermB hanya ditemukan di peternakan ayam pedaging. Bakteri *S. aureus* dari kedua peternakan tahan terhadap beberapa antibiotik dan memiliki gen resistansi terhadap antibiotik pada isolat resistan.

Kata-kata kunci: antibiotik; ayam; gen resistansi; *S. aureus*

INTRODUCTION

Chicken was one of favourite protein source in Indonesia since its easiness to find and cheap price. West Java province has known as the largest chicken population in Indonesia with total population 644,923,955 chicken in 2018 (Disnak Jabar, 2018). Moreover, Bogor regency is the central of poultry chicken production in West Java with poultry chicken population in Bogor regency was 19,062,875 heads in 2018. West Java province also has known as the largest layer population with 14,469,405 chicken in 2018. Largest population of layer chicken also found in Bogor regency with 4,826,000 heads in 2018 (Ditjen PKH, 2018).

Staphylococcus aureus is an opportunistic pathogen in human and animal. It considered as the third most common pathogen caused food poisoning in the world (Kadariya *et al*., 2014). The species caused several diseases in poultry it associated with several diseases, such as staphylococcosis, osteomyelitis, arthritis, and bumble foot disease (Giedraitiene *et al.,* 2011). The agents can be transmitted via feed, litter, water, and may also be transmitted transovarially (Hakkani *et al.,* 2016).

Antibiotic was widely used for fight the infection caused by *S. aureus*, such as penicillin, erythromycin, and tetracycline. Unfortunately, farmers also used antibiotic in poultry as a prophylaxis and growth promotor. However, the use of extensive antibiotic with improper procedure can promote the increasing of antibiotic resistant bacteria (Wongsuvan *et al.,* 2018). It is known most *S. aureus* strains are heterogeneously resistant to several antibiotic such as beta-lactams, aminoglycosides, macrolides, clindamycin and tetracycline. Erythromycin, tetracycline and penicillin-G were few antibiotic used to treat infection in poultry (Ali *et al.,* 2017). *Staphylococcus aureus* was resistant to most used antibiotic. In the last decade, public are concerning about antibiotic-resistance (Khodadadi *et al*., 2016).

The aim of this research was to identify the pattern of antibiotic resistance in chicken broiler and layer at Bogor regency. The research also detected the gene encoded the antibiotic resistance in *S. aureus*.

RESEARCH METHODS

Isolation and Identification of *S. aureus*

Cloacal swab samples were collected from chicken broiler and layer farm in Bogor regency and stored them in phosphate buffered saline (PBS) until processed in laboratorium. Samples were cultured in mannitol salt agar (MSA) and incubated at 37ºC for 24 hours. *Staphylococcus aureus* colony ferment mannitol and changed the medium color from red to yellow. The suspected colony was identified according to Indonesian National Standard for *S. aureus* identification number SNI 2332.9-2011. The test initiated by Gram staining, catalase test, coagulase test, and glucose fermentation microaerophilic (SNI, 2011). The deoxyribonucleic acid (DNA) material was extracted from positive isolate using PrestoTM Mini gDNA Bacterial kit (Geneaid) based on manufacturer guidance. *Staphylococcus aureus* specific *femA* was detected by polymerase chain reaction (PCR) using forward primer 5^1 – GCAAACTGTTGGCCACTATG – 3^1 and reverse primer 5^1 – TCATCACGATCA GCAAAAGC – 3^1 with its 594 bp amplicon (Riyaz-Ul-Hassan *et al*., 2008).

Antibiotic Susceptibility Test

Isolate of *S. aureus* was tested against several antibiotics based on Kirby-Bauer disk diffusion method. *Staphylococcus aureus* were suspended in NaCl solution until its density reached McFarland 0.5 and then cultured on Mueller-Hinton agar (MHA) plate. Antibiotic disks were placed on cultured MHA and incubated in 37ºC for 24 hours. Tetracycline (30 µg), oxytetracycline (30 µg), ampicillin (10 µg), gentamycin (30 µg), nalidixic acid (30 µg), enrofloxacin (5 µg), ciprofloxacin (5 µg), chloramphenicol (5 µg), and erythromycin (15 µg) were used for this test. The diameter of inhibition zone were measured and then

interpreted according to the Clinical and Laboratory Standard Institute (CLSI) 2018 (CLSI, 2018).

Identification of Resistance Gene

The DNA of resistant strain were extracted by PrestoTM Mini gDNA Bacterial Kit (Geneaid) and it were detected by single PCR method. Total volume of PCR reaction mix was 50 µL containing 3 µL of DNA template, 25 µL of PCR mix, 2 µL of 10 pm forward primer, 2 µL of 10 pm reverse primer, and 18 µL of ddH_2O (Table 1). Polymerase chain reaction was performed using a thermocycler (Biorad) with five minutes of initial denaturation at 95ºC, followed by 30 cycle of denaturation at 95ºC for 30 seconds, annealing for one minute, extension at 72ºC for one minutes, and final extension at 72ºC for 10 minutes. Polymerase chain reaction product were analysed on 1% agarose gel electrophoresis and were analysed under UV-transiluminator after florosafe gel staining was added to the gel.

Table 1. Primers used in this study

Gene	Sequence base	Amplicon (bp)	Annealing (ºC)	Reference
GyrA	(F) 5¹ ATGAGCGAATTAGCCAAAGA 3¹	582	62	Wang *et al*., 2010
	(R) 5¹ GCAACCGTCCAACACTTCAT 3¹			
tetA	(F) 5¹ CGACCTTGCGAGAGAAAT 3¹	965	62	Chuah *et al*., 2018
	(R) 5¹ GTTCCATCAGCCCTTCAA 3¹			
blaTEM	(F) 5¹ ATCAGCAATAAACCAGC 3¹	516	54	Colom *et al*., 2003
	(R) 5¹ CCCCGAAGAACGTTTTC 3¹			
ermB	(F) 5¹ GAAAAGGTACTCAACCAAATA 3¹	639	54	Song *et al*., 2004
	(R) 5¹ GTAACGGTACTTAAATTGTTTAC 3¹			

RESULTS AND DISCUSSION

A total of 79 samples were collected from chicken broiler and layer farm, which are 49 samples were collected from chicken broiler farm and 30 samples were obtained from layer farm. Fourteen (28%) isolates from chicken broiler farm and 15 (50%) from layer farm were identified as *S. aureus* using biochemical test and molecular examination of *femA*. *FemA* gene is a specific gene in *S. aureus* which encoded a 48 kDa protein on cell wall synthesis. The presented *femA* gene was detected by PCR (Figure 1) (Khodadadi *et al.,* 2016).

The presence of *S. aureus* in poultry is important in food poultry industry since it may affect to human health, and leads to food borne diseases (Ribeiro *et al.,* 2018). *Staphylococcus aureus* was often colonized on skin and mucous membrane in human and animals (Khodadadi *et al.,* 2016). In chicken, it caused several diseases such as staphylococcosis, osteomyelitis, arthritis, and bumble foot disease (Giedraitiene *et al.,* 2011). Infection which caused by *S. aureus* usually treated by antibiotics. The frequently using antibiotic to treat *S. aureus* infection is penicillin, erythromycin, and tetracycline (Ali *et al.,* 2017). Increasing of antibiotic use in chicken broiler and layer farm, increasing number of resistant *S. aureus* strain (Netsvyetayeva *et al.,* 2014). *Staphylococcus aureus* has an outstanding ability to acquire resistance to antibiotics, which is considered a major public health concern (Fernandez *et al.,* 2012; Meemken *et al.,* 2013).

The antibiotic susceptibility test result presented in Table 2. According to Table 2, *S. aureus* were isolated from chicken broiler farm were resistant to erythromycin and ampicillin

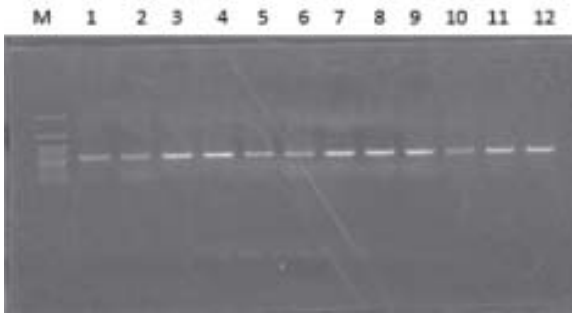

Figure 1. Polymerase Chain Reaction/PCR result of *femA* gene after electrophoresis (594bp). Lane M: marker (100bp); lane 1 to 6: samples from poultry farm; lane 7 to 10: samples from layer farm in Bogor

chloramphenicol (13%). No isolate were resistant to gentamycin.

Similar result showed that *S. aureus,* were isolated from chicken in Nigeria and Bangladesh were 100% resistant to erythromycin and tetracycline (Ali *et al.,* 2017; Onaolapo *et al.,* 2017). Antibiotic used in this study were commonly used by farmer in Indonesia (Zalizar *et al.,* 2015). Most common antibiotic used in chicken farm were erythromycin, tetracycline, penicillin, and doxycycline. *Staphylococcus aureus* in this study was least resistant to gentamycin and chloramphenicol, which is least used antibiotics in poultry. Similar result also occurred in Yogyakarta Indonesia and Kaduna, Nigeria where 26,1% and 3,1% of *S. aureus* were be resistant to gentamycin respectively (Khusnan *et al.,* 2016; Onaolapo *et al.,* 2017).

Antibiotics are being used in poultry industry in order to prevent bacterial infection on poultry, along with strict biosecurity and hygiene (Bermudez, 2003). Intensive chicken broiler farming in North America often use antibiotics such as tetracycline, bacitracin, tylosin, salinomycin, virginiamycin and bambermycin (Diarra and Malouin, 2014). Tetracyclines is used more than two-thirds of antimicrobials administered to animals (Ronquillo and Hernandez, 2017) in North America and only 37% in European Union (Carvalho and Santos, 2016). Using antibiotics as growth promotors have been banned by Indonesian Goverment since 2018 in order to minimize negative impact of antibiotics (Ditjen PKH, 2018).

Staphylococcus aureus, were isolated from both chicken broiler and layer farm, were be resistant to more than three antibiotics so it considered as multi-drug resistant. One isolate, was isolated from poultry farm was be resistant to all antibiotic tested, while the highest number of resistant pattern was TE-OT-AMP-NA-ENR-CIP-E where four isolate were be resistant. One isolate, isolated from layer farm was be resistant to eight antibiotics. Highest number of resistant pattern was TE-OT-AMP-NA-ENR-CIP-E where five isolate were be resistant (Table 3).

Multi-drug resistant species is species that is resistant to three, or more than three antibiotics (Bianchi *et al.,* 2014). The research result showed only one of 29 isolate was not as a multi-drug resistant species. The similar result was showed with study in Zagazig, Egypt and Karaj, Iran, that stated *S. aureus* from meat product poultry were multi-drug resistant (Morshdy *et al.,* 2018; Nouri-Gharajalar *et al.,* 2019).

Multi-drug resistance bacteria is important issue in public health because it exist in animals, humans, and environment. The antibiotic resistance caused by excesive use of antibiotics in animals and humans. Another factors such as antibiotics trading, increased international travels, poor sanitation and hygine, and antibiotics residue released into the environment contributes to the antibiotic resistance. Those factors also contribute to existence of multidrug resistant strain (Aslam *et al.,* 2018).

Ten isolates (six samples from chicken broiler and four samples from layer farm) were analysed by PCR amplification method using primer to detect resistance gene (Table 1). *GyrA, tetA, blaTEM,* and *ermB* genes were detected in six isolates of broiler farm (Table 4). *GyrA* and *blaTEM* gene were detected in four isolates of layer farm, *tetA* gene was detected in three isolates, meanwhile *ermB* was not found in layer farm sample (Figure 2-5).

All samples from poultry and layer farm have *blaTEM* gene. The similar result showed in Iran and Taiwan where 100% beta lactamase resistant strain have a *blaTEM* gene (Yang *et al.,* 2017; Nouri-Gharajalar *et al.,* 2019). *BlaTEM* gene encodes most encountered bacteria â-lactamase (Xu *et al.,* 2014). Most strains, which have *blaTEM* gene, exhibited a high degree of resistance to â-lactam antibiotics in China and Taiwan (Yang *et al.,* 2017). Modifcation of penicillin-binding protein (PLP2a) induces inactivity of penicillin and other beta lactam antibiotics; and it will develop cross-resistance of multiple antibiotics (Bounar-Kechih *et al.,* 2018).

GyrA gene also found in ten *S. aureus* from both farm. The similar gene has been detected in *S.aureus* isolated from Spanish hospital (Lozano *et al.,* 2018). The other research reported that quinolones-resistant *S. aureus* isolates, were isolated from clinical and soil sources, had independent alleles of *gyrA* (de Vries *et al.,* 2009). Mutation in *gyrA* is responsible for mutation that occurred in quinolone-resistance *S. aureus*, where the mutation occurred at codon 83 or 87 (Zhao *et al.,* 2018). Further sequencing needed to determine the mutational site in this study.

Although *tetM and tetK* were common genes conferring tetracycline resistance in *S. aureus* (de Vries *et al.,* 2009), the research showed *tetA* gene was found in nine isolates. The previous

Table 2. Antibiotic resistant pattern of *Staphylococcus aureus* isolate from broiler and layer farm in Bogor

Antibiotic	Sample source	Interpretation of clear zone		
		Susceptible *n* (%)	Intermediate *n* (%)	Resistance *n* (%)
Tetracycline (30 µg)	PF	1 (7)	0 (0)	13 (93)
	LF	0 (0)	0 (0)	15 (100)
Oxytetracycline (30 µg)	PF	1 (7)	0 (0)	13 (93)
	LF	0 (0)	0 (0)	15 (100)
Ampicillin (10 µg)	PF	0 (0)	0 (0)	14 (100)
	LF	0 (0)	0 (0)	15 (100)
Gentamycin (30 µg)	PF	11 (79)	0 (0)	3 (21)
	LF	15 (100)	0 (0)	0 (0)
Nalidixic acid (30 µg)	PF	0 (0)	1 (7)	13 (93)
	LF	0 (0)	0 (0)	15 (100)
Enrofloxacin (5 µg)	PF	1 (7)	5 (36)	8 (57)
	LF	4 (27)	0 (0)	11 (73)
Ciprofloxacin (5 µg)	PF	4 (29)	2 (14)	8 (57)
	LF	4 (27)	5 (33)	6 (40)
Erythromycin (15 µg)	PF	0 (0)	0 (0)	14 (100)
	LF	0 (0)	0 (0)	15 (100)
Chloramphenicol (5 µg)	PF	7 (50)	2 (14)	5 (36)
	LF	13 (87)	0 (0)	2 (13)

Note: PF= Poultry farm; LF= Layer farm

Table 3. Multi-drug resistant pattern of *Staphylococcus aureus* isolate from poultry and layer in Bogor

Origin of samples	No of antibiotic resistant	Antibiotics	No of resistant isolate (%)
Poultry	9	TE-OT-AMP-CN-NA-ENR-CIP-E-C	1 (7,14)
	8	TE-OT-AMP-NA-ENR-CIP-E-C	2 (14,28)
	7	TE-OT-AMP-CN-NA-CIP-E	1 (7,14)
	7	TE-OT-AMP-NA-ENR-CIP-E	4 (28,57)
	7	TE-OT-AMP-NA-ENR-E-C	1 (7,14)
	6	TE-OT-AMP-CN-NA-E	1 (7,14)
	5	TE-OT-AMP-E-C	1 (7,14)
	5	TE-OT-AMP-NA-E	2 (14,28)
	3	AMP-NA-E	1 (7,14)
Layer	8	TE-OT-AMP-NA-ENR-CIP-E-C	1 (6,67)
	7	TE-OT-AMP-NA-ENR-CIP-E	5 (33,33)
	7	TE-OT-AMP-NA-ENR-E-C	1 (6,67)
	6	TE-OT-AMP-NA-ENR-E	4 (26,67)
	5	TE-OT-AMP-NA-E	4 (26,67)

(100%); tetracycline, oxytetracycline, and nalidixic acid (93%); enrofloxacin and ciprofloxacin (57%); chloramphenicol (36%); and gentamycin (21%). Meanwhile, *S. aureus* were isolated from layer farm were resistant to tetracycline, oxytetracycline, ampicillin, nalidixic acid, and erythromycin (100%); enrofloxacin (73%); ciprofloxacin (40%); and

Table 4. Number of resistant gene found in *Staphylococcus aureus* isolate [n (%)]

Resistant gene	Poultry farm	Layer farm
*Gyr*A	6 (100)	4 (100)
*tet*A	6 (100)	3 (75)
*Bla*TEM	6 (100)	4 (100)
*erm*B	6 (100)	0 (0)

resistance genes are often associated with conjugative and mobile genetic elements enabling horizontal transfer (Dayao *et al.,* 2016)

ErmB gene was only detected in *S. aureus* isolates that were isolated form chicken broiler farm. Study in Kerman, Iran showed that 20.5% of *S. aureus* isolates that were isolated from clinical sample has *ermB* gene (Fasihi *et al.,* 2016). *ErmB* is one of common gene encoded resistant to erythromycin and responsible for

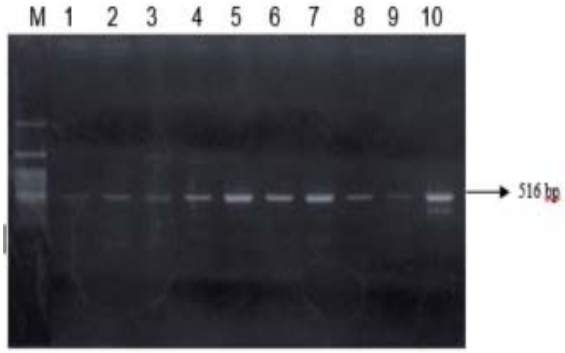

Figure 2. Polymerase Chain Reaction/PCR result of *blaTEM* gene after electrophoresis. Lane M: marker (100bp); lane 1 to 6: samples from poultry farm; lane 7 to 10: samples from layer farm in Bogor

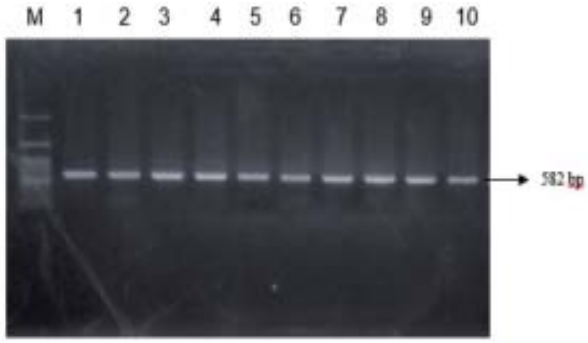

Figure 3. PCR result of *gyrA* gene after electrophoresis. Lane M: marker (100bp); lane 1 to 6: samples from poultry farm; lane 7 to 10: samples from layer farm in Bogor

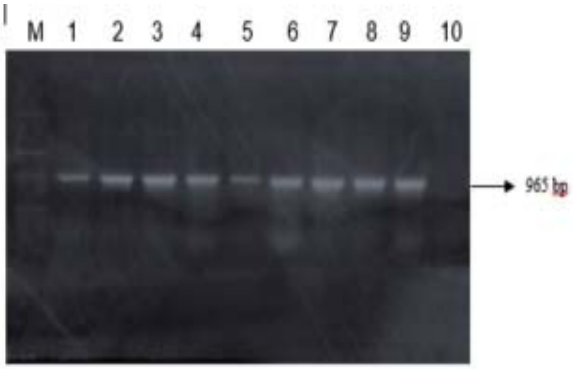

Figure 4. Polymerase Chain Reaction/PCR result of *tetA* gene after electrophoresis. Lane M: marker (100bp); lane 1 to 6: samples from poultry farm; lane 7 to 10: samples from layer farm in Bogor

Figure 5. Polymerase Chain Reaction/PCR result of *ermB* gene after electrophoresis. Lane M: marker (100bp); lane 1 to 6: samples from poultry farm; lane 7 to 10: samples from layer farm in Bogor

studies showed that 21-96% of tetracycline resistant bacteria possessed the *tetM* and *tetA* genes, suggesting that both genes are a significant contribution to the tetracycline resistant (Liyanage *et al.,* 2018). Tetracycline

ribosomal binding site modifications that are the most important macrolide resistance mechanisms (Takaya *et al.,* 2010, Zmantar *et al.,* 2011).

CONCLUSION

Staphylococcus aureus isolates were isolated from chicken broiler and layer in Bogor Regency have resistant to erythromycin, ampicillin, tetracycline, oxytetracycline, nalidixic acid, enrofloxacin and ciprofloxacin, and sensitive to chloramphenicol gentamycin. Antibiotic resistance gene such as *gyrA, tetA, blaTEM,* and *ermB* were detected in *S. aureus* isolates which isolated from chicken broiler and layer in Bogor Regency

SUGGESTION

There was still lack of information about antibiotic resistant from *S. aureus* isolated from poultry and layer in Indonesia. Findings in this study indicate that the rate of antibiotic resistant are alarming and increasing. Resistant gene in several isolate in this study are still unclear, therefore similar study can be developed with more additional resistant gene. This study also can be applied in other region in Indonesia to give a national data about antibiotic resistant in Indonesia. Further study may also be done to determine the best solution to manage and reduce antibiotic resistant.

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