MULTIDRUG-RESISTANT *Salmonella* sp. ISOLATED FROM SEVERAL CHICKEN FARMS IN WEST JAVA, INDONESIA

Aprilia Hardiati[1], Safika[1], Fachriyan Hasmi Pasaribu[1]*, and I Wayan Teguh Wibawan[1]

[1]Medical Microbiology Division, Department of Animal Disease and Veterinary Public Health, Faculty of Veterinary Medicine, IPB University, Bogor, Indonesia

*Corresponding author: fhpasaribu@gmail.com

ABSTRACT

This study was aimed at isolating and identifying *Salmonella* sp. and then conducting an antibiotics susceptibility test in order to detect resistant genes. One hundred and five chicken cloaca swab samples were used in this study. 30 samples were taken from a layer farm in Bogor, 45 from a broiler farm in Sukabumi and 30 from a broiler farm in Cianjur. In order to isolate and identify the bacteria, a tetrathionate broth was used, which was then cultured in a Salmonella-Shigella agar, and finally a Gram stain and biochemical test was conducted. To confirm the presence of *Salmonella* sp., a pair of primers were used for the polymerase chain reaction (PCR) method to determine the presence of the i*nvA* gene.. An antibiotics susceptibility test was used with the Kirby-Bauer disk diffusion method. Nine antibiotics were used in this study. Each primer pair was used for the detection of *tetA*, *blaTEM*, *aac(3)-IV*, *gyrA* and *ermB* genes, and for genes encoding antibiotic resistance a PCR test was used. Eight (7.6%) *Salmonella* sp. were isolated in this study. All isolates showed positive results with PCR confirmation. The results of the antibiotics susceptibility test showed that *Salmonella* sp. isolates were resistant to tetracycline (75%), oxytetracycline (75%), amphicillin (75%), gentamicin (12.5%), nalidixic acid (100%), ciprofloxacin (12.5%), enrofloxacin (0%), erythromycin (100%), and chloramphenicol (0%). The distribution of antibiotic resistance genes in *Salmonella* sp. were *tetA* (33.3%), *blaTEM* (100%), *aac(3)-IV* (0%), *gyrA* (100%) and *ermB* (0%) positive. In conclusion, *Salmonella* sp. was isolated. All isolates showed positive results in the PCR confirmation. *Salmonella* sp. isolates were resistant to tetracycline, oxytetracycline, amphicillin, gentamicin, nalidixic acid, ciprofloxacin, and erythromycin. Only the *tetA*, *blaTEM,* and *gyrA* genes were detected in *Salmonella* sp. isolates.
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Key words: antibiotics, chicken, resistance gene, *Salmonella* sp.

ABSTRAK

Penelitian ini bertujuan isolasi dan identifikasi Salmonella sp., melakukan uji kepekaan bakteri terhadap antibiotika dan deteksi gen resistensi beberapa antibiotika pada isolat Salmonella sp. Seratus lima sampel usap kloaka ayam digunakan dalam penelitian ini. Sampel diambil dari 30 peternakan layer Bogor, 45 peternakan broiler Sukabumi dan 30 peternakan broiler Cianjur. Isolasi dan identifikasi bakteri menggunakan kaldu tetrationat, kemudian dikultur pada agar Salmonella-Shigella, pewarnaan Gram dan pengujian biokimia. Konfirmasi isolat Salmonella sp. menggunakan satu pasang primer untuk metode polymerase chain reaction (PCR) bertujuan mengetahui keberadaan gen invA. Uji kepekaan bakteri terhadap antibiotika dilakukan dengan metode difusi cakram Kirby-Bauer. Sembilan antibiotika digunakan dalam penelitian ini. Masing-masing 1 pasang primer digunakan untuk deteksi gen tetA, blaTEM, aac(3)-IV, gyrA dan ermB, gen penyandi antibiotik resisten, menggunakan PCR. Delapan (7,6%) Salmonella sp. dapat diisolasi pada penelitian ini. Hasil uji kepekaan bakteri terhadap antibiotik menunjukkan bahwa Salmonella sp. resisten terhadap tetrasiklin (75%), oksitetrasiklin (75%), ampisilin (75%), gentamisin (12,5%), asam nalidiksat (100%), siprofloksasin (12.5%), enrofloksasin (0%), eritromisin (100%), dan kloramfenikol (0%). Distribusi gen resistensi antibiotika pada Salmonella sp. yaitu positif tetA (33,3%), blaTEM (100%), aac(3)-IV (0%), gyrA (100%), dan ermB (0%). Kesimpulannya, Salmonella sp. terisolasi. Semua isolat menunjukkan hasil positif pada konfirmasi PCR. Salmonella sp. isolat resisten terhadap tetrasiklin, oksitetrasiklin, ampisilin, gentamisin, asam nalidiksat, siprofloksasin, dan eritromisin. Hanya gen tetA, blaTEM, dan gyrA terdeteksi pada isolat Salmonella sp.
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Kata kunci: antibiotika, ayam, gen resistensi, Salmonella sp.

INTRODUCTION

Antibiotics have saved humans against disease infections for almost seven decades. Animals that are used as food sources are also given antibiotics in an effort to fight harmful bacteria or to increase production. The use of antibiotics for increased production is considered to be a very controversial practice. The use of low-dose antibiotics has a positive effect according to farmers, namely by increasing 4-5% of the average growth of livestock. However, the use of antibiotics also carries with it a negative impact which is an increase in the incidences of bacterial resistance (Diarra *et al.* 2007).

The use of antibiotics on farms plays a major role in the development of commensal and pathogenic bacterial resistance to antibiotics. Risk to humans can also occur due to the developed resistant bacteria (WHO 2010). Deaths due to bacterial infection are estimated to increase every year from 700.000 to 10.000.000 by 2050. Not only do these resistant bacteria pose a threat to human health, but also the worldwide economic cost of fighting these bacteria is estimated to reach US$ 100 trillion (O'Neill 2014). The occurrence of resistant bacteria complicates the treatment process of bacterial infections due to the antibiotics being ineffective at treating the illness. Antibiotic resistant bacterial infections in humans increases material losses in the form of life quality loss, increased death, and reduced health improvement programs (WHO 2010).

Several studies on bacterial resistance to antibiotics have been carried out in Indonesia. Salmonella species have been found to be resistant to nalidixic acid (44.4%) and ampicillin sulbactam (11.1%) (Aprilian *et al.* 2015). According to Loisa *et al.* (2016), *Salmonella* sp. has been found to be resistant to erythromycin (66.7%), streptomycin (33.3%) and chloramphenicol (33.3%). *Salmonella* sp. is still relatively susceptible to enrofloxacin, tetracycline, and gentamicin.

The emergence of bacterial resistance to antibiotics is a major concern to the world. Programs to monitor

such resistance are mostly carried out in various countries to protect human and animal health (Li *et al.* 2012). Concerns about bacterial and multidrug-resistance is also being studied in Salmonella. Increased resistance in Salmonella not only poses a threat to animal life but also to human life as well because Salmonella is a zoonotic agent (Nahar and Ridwan 2018).

One characteristic of bacteria that makes them deadly is their natural resistantability to protect themselves from harmful agents. These resistant properties can be obtained from mutations, gene transfer by conjugation or transformation, transposon, integron and bacteriophage (Cogliani *et al.* 2011). At present the use of molecular detection, specifically polymerase chain reaction (PCR), is widely used to detect resistant genes.

This study was aimed at isolating and identifying *Salmonella* sp. and then conducting an antibiotics susceptibility test in order to detect resistant genes. The isolates used in this study were taken from Chicken Farms in West Java, Indonesia.

MATERIALS AND METHODS

Sample

Isolation and identification of bacteria was obtained from 105 poultry cloaca swab samples. Samples were collected randomly from three districts (Bogor, Sukabumi, and Cianjur) in the West Java region. Thirty samples were taken from a layer poultry farm in Bogor, 45 samples were taken from a broiler poultry farm Sukabumi and 30 samples were taken from a broiler poultry farm in Cianjur. Samples were put into a buffered peptone water media and stored in an icebox then transported to the Medical Microbiology Laboratory, Department of Animal Disease and Veterinary Public Health, Faculty of Veterinary Medicine, IPB University, Indonesia.

Microbiology Analysis

The samples were inoculated in 10 mL of tetrathionate broth media aseptically and incubated at 37° C for 48 hours (Yue *et al.* 2014). Bacteria grown in the tetrathinate broth media were cultured on a Salmonella-Shigella agar media and then incubated at 37° C for 24 hours. Colonies referring to Salmonella were cultured on a tryptic soy agar and then incubated at 37° C for 24 hours. The test to distinguish Enterobacticeae groups from other Gram negative bacil bacteria, such as Pseudomonas, can be assessed with the use of a triple sugar iron agar. The biochemical test carried out in this study was urease and indole, methyl red, Voges Proskauer and citrate (IMViC). Bacterial incubation on the urea media was carried out at 37° C for 18-24 hours. Testing of indole, methyl red, Proskauer and citrate was conducted at 37° C for 48 hours. The 0.5 mL Erlich reagent was added to the culture in the media, and then the tube was shaken and left to sit for a while. A red indole ring showed positive results. One drop of methyl red was dropped on the culture in the media. Positive results were indicated by a change in color to red. A Voges Proskauer test was carried out by dripping 2 drops of α-naphthol 5% followed by KOH 40%. Positive results were indicated by a change in color to red (Markey *et al.* 2013).

Extraction of DNA *Salmonella* Isolates

The bacteria that had grown on the tryptic soy agar were then inoculated on a tryptic soy broth media and incubated at 37° C for 24 hours. Extraction of the bacterial DNA used the Presto™ Mini gDNA bacteria kit (Geneaid Biotech, Taiwan) according to manufacturing procedures. The extraction results were then amplified using the PCR method using a specific primer *Salmonella* sp. and gene-specific primers encoding antibiotic resistance (Table 1).

Confirmation of *Salmonella* sp. Isolates by PCR Method

The specific primer of *Salmonella* sp. used was the nucleotide base sequence forward 5´-TCA TCG CAC CGT CAA AGG AAC C -3´and reverse 5´ -GTG AAA TTA TCG CCA CGT TCG GGC AA-3´ in the *invA* gene with 284 bp amplification product (Li *et al.* 2012). The total volume of the PCR reaction used for amplification of the *invA* gene was 25 μL consisting of 12 μl MyTaq™ Red Mix (Bioline, Singapore), 2 μL primary (forward), 2 μL primer (reverse), 4 μL DNA templates and ddH$_2$O added to 25 μL. Amplification using T100™ Thermal Cycler (Bio-Rad, Singapore) with 30 cycles consisting of 5 minutes initial denaturation at 95° C, 30 seconds denaturation at

Table 1. Primer sequence used for detection antibiotics resistance genes of *Salmonella* sp.

Antibiotics	Resistance genes	Primer sequence	Size	Annealing temperature
Tetracycline Oxytetracycline	*tetA*[a]	(F)5´-GTA ATT CTG AGC ACT GTC GC-3´ (R)5´-CTG CCT GGA CAA CAT TGC TT-3´	965 bp	62° C
Ampicilin	*blaTEM*[b]	(F)5´-ATC AGC AAT AAA CCA GC -3´ (R)5´-CCC CGA AGA ACG TTT TC -3´	516 bp	54° C
Gentamicin	*aac(3)-IV*[c]	(F)5´-CTT CAG GAT GGC AAG TTG GT-3´ (R)5´-TCA TCT CGT TCT CCG CTC AT-3´	286 bp	55° C
Nalidixic Acid Ciprofloxacin Enrofloxacin	*gyrA*[d]	(F)5´-CGA CCT TGC GAG AGA AAT-3´ (R)5´-GTT CCA TCA GCC CTT CAA-3´	626 bp	62° C
Erythromycin	*ermB*[e]	(F)5´-GAA AAG GTAC TCA ACC AAA TA-3´ (R)5´-GTA ACG GTA CTT AAA TTG TTT AC-3´	639 bp	50° C

[a]= Chuah *et al.* (2018); [b]= Colom *et al.* (2008); [c]= Van *et al.* (2008); [d]= Nawaz *et al.* (2012); [e]= Song *et al.* (2004)

95° C, 1 minute annealing at 58° C, 5 minutes extension at 72° C and final extension 10 minutes at 4° C. Visualization of PCR results was carried out by electrophoresis on 1% agarose gel, Tris-Acetate-EDTA (TAE) buffer (1x), FloroSafe DNA staining, 1 mL (1st BASE, Singapore). The DNA ladder used was 100 bp as a standard size.

Antibiotics Susceptibility Test

The *Salmonella* sp. isolates were assessed against varying antibiotics, namely amphicillin (AMP) 10 µg, tetracycline (TE) 30 µg, oxytetracycline (OT) 30 µg, gentamicin (CN) 10 µg, chloramphenicol (C) 15 µg, nalidixic acid (NA) 30 µg, ciprofloxacin (CIP) 5 µg, enrofloxacin (ENR) 5 µg and erythromycin (E) 30 µg. This antibiotic resistance test used the Kirby-Bauer diffusion disk method on Mueller-Hinton agar media. The bacterial suspension of the tryptic soy agar was diluted with a sterile physiological NaCl until it matched the McFarland standard of 0.5 or equivalent to 1.5×10^8 CFU/mL. The suspension was cultured using sterile cotton buds on Mueller-Hinton agar, and then set to dry for around 10-15 minutes. It was then placed on antibiotic discs on a Mueller-Hinton agar using sterile tweezers. The maximal antibiotic discs used in one 100 mm diameter Petri dish were six pieces. Tests were carried out in triple replications, and incubation was carried out at 35° C for 16-18 hours (CLSI 2018).

Detection of Genes Encoding Antibiotic Resistance

Positive confirmation of *Salmonella* sp. from PCR was detected as well as its antibiotic resistance encoded gene. Detection of resistance encoded genes was only carried out on isolates belonging to the group resistant to the antibiotics tested. The bacteria were extracted using a Presto™ Mini gDNA bacteria kit (Geneaid, Taiwan) in the previous stage. A PCR reaction was used to detect antibiotic resistant gene coding using MyTaq™ HS Red Mix (Bioline, Singapore). The total volume of PCR reactions of 25 µL consisted 4 µL templates, 2 µL reverse primers (20 µM), 2 µL forward primers (20 µM), 12 µL MyTaq™ Red Mix, 2x and ddH₂O which were added to 25 µL. Primary targets for detection of the bacterial resistant genes against antibiotics are presented in Table 1. Amplification using Thermal Cycler T100™ (Bio-Rad, Singapore) with 30 cycles consisting of a 5 minute initial denaturation at 95° C, 30 second denaturation at 95° C, 1 minute annealing (temperature in Table 1), 5 minute extension at 72° C and final 10 minute extension at 4° C. Observation of the results is carried out by electrophoresis on 1% agarose gel, Tris-Acetate-EDTA (TAE) buffer (1x) and FloroSafe DNA Staining, 1 mL (1st BASE, Singapore). The DNA ladder used was 100 bp as a standard size.

RESULTS AND DISCUSSION

The use of unregulated antibiotics in chicken maintenance systems causes many incidences of resistant bacteria, one of which is *Salmonella s*p. These bacteria are enteric bacteria that can cause foodborne diseases (Newell *et al.* 2010). In this study, 7.6% (8 of 105 samples) of *Salmonella* sp. isolates were grown on a media so that the Salmonella-Shigella agar corresponded to a transparent colony with a black center in the middle. According to Leboffe and Burton (2011), these black dots are precipitates formed from the reaction of ferric citrate in the medium and hydrogen sulfide produced from the reduction of sulfur by the bacteria. Microscopically, *Salmonella* sp. cells were stem-shaped and had a pink color, thus it belongs to the category of Gram-negative bacteria. In the triple sugar iron, isolates presented a red (base) in the slant and yellow (acid) in the butt part of the media and produced hydrogen sulfide. Acidic conditions describe the ability of bacteria to ferment lactose and /or sucrose and glucose. Bacteria that cannot ferment sucrose or lactose under limited glucose conditions will use pepton in the media to produce energy. Pepton metabolites are alkaline and cause red slants (Markey *et al.* 2013). Urea tests showed negative results. *Salmonella* sp. do not use the urease enzyme to hydrolyze urea to ammonia and carbon dioxide (Leboffe and Burton 2011).

IMViC test results were negative indole, positive methyl red, negative Voges Proskauer and citrate. Corresponding to the indole test, *Salmonella* sp. did not use the enzyme tryptophanase to produce indole. All *Salmonella* sp. isolates were motile. Based on the literature, Leboffe and Burton (2011), *Salmonella* sp. isolates were able to produce mixed acids known from the positive methyl red test results. The Voges Proskauer test shows that *Salmonella* sp. was unable to produce acetoin from glucose. *Salmonella* sp. can use citrate as the only source of carbon. Citrate (citric acid) is produced as acetyl coenzyme A (from pyruvate oxidation or β-oxidation fatty acids) which reacts with oxaloacetate when entering the Krebs cycle. The 8 isolates detected as *Salmonella* sp. were code C7.2 and G7.5 (from Bogor); A.56 and A.72 (from Sukabumi); and K4.13, K4.16, K4.18, K4.26 (from Cianjur).

The *invA* gene in 8 *Salmonella* sp. isolates was successfully amplified by the PCR method. Visualization of the *invA* gene PCR products showed an amplicon length of 284 bp (Figure 1). The *invA* gene works to encode membrane proteins in bacteria that are responsible for invading the host intestine cells (Sharma and Khasmiri 2016). The *invA* gene in Salmonella is located in Salmonella Pathogenicity Island-1 (SPI-1) which has a very important role in invading the epithelial cells of the host. This gene is very specific in most Salmonella serotypes (Li *et al.* 2012).

The results of the antibiotic susceptibility test were shown in Table 2. Most of *Salmonella* sp. isolates were resistant against several antibiotics. Only 3 isoates showed intermediate results. All isolates were susceptible to enrofloxacin. Based on the bacterial resistance test for antibiotics, all isolates showed multidrug-resistant (MDR) properties. Multidrug-

8
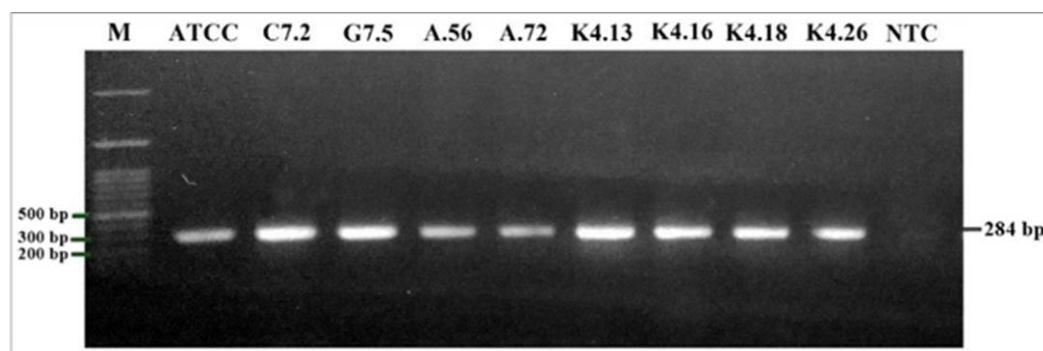
Figure 1. Amplification *invA* gene (284 bp) in *Salmonella* sp. M= Marker 100 bp; ATCC= *Salmonella* enteritidis ATCC 13076; C7.2, G7.5, A.56, A.72, K4.13, K4.16, K4.18, K4.26= Isolate code; NTC= Non template control

Table 2. Result of antibiotic susceptibility test against of *Salmonella* sp.

No.	Code	TE	OT	AMP	CN	NA	ENR	CIP	E	C
					Bogor					
1.	C7.2	R	R	R	S	R	S	R	R	S
2.	G7.5	R	R	R	R	R	S	I	R	I
					Sukabumi					
3.	A.56	R	R	S	S	R	S	I	R	S
4.	A.72	R	R	S	S	R	S	I	R	S
					Cianjur					
5.	K4.13	S	S	R	S	R	S	S	R	S
6.	K4.16	S	S	R	S	R	S	S	R	S
7.	K4.18	R	R	R	S	R	S	S	R	S
8.	K4.26	R	R	R	S	R	S	S	R	S
	Number of resistant	6	6	6	1	8	0	1	8	0
	%	75	75	75	12.5	100	0	1.25	100	9

TE= Tetracycline; OT= Oxytetracycline; AMP= Ampicillin; CN= Gentamicin; NA= Nalidixic acid; ENR= Enrofloxacin; CIP= Ciprofloxacin; E= Erithromycin; C= Chloramphenicol; S=Susceptible; I= Intermediate; R= Resistant

resistant properties are bacterial that are resistant to three or more antibiotic groups (Magiorakos *et al.* 2012). Multidrug resistance in bacteria is caused by two mechanisms: first, the accumulation of several genes, each of which encodes the resistance to one type of antibiotic in a single cell. This accumulation occurs on plasmid R (resistance). The second mechanism is due to the increase in gene expression that encodes the efflux pump for several antibiotics (Nikaido 2009).

The tetracycline group has been marketed under various trade names. Adesiji *et al.* (2014) investigated *Salmonella* sp. gathered from human, poultry and seafood samples found that resistance to tertracyclin was 66.7%. In this study, 6 (75%) of the isolates were resistant to tetracycline and oxytetracycline. The resistance of Gram-negative bacteria, one of which being *Salmonella* sp., to the tetracycline group occurs mostly due to the efflux pumps. The genes for efflux pumps are related to mobile elements, one of which is *tetA* (Poole 2005). As many as 2 (33.3%) of the isolates are positive for the *tetA* gene, meaning efflux pump activity secretes tetracyclines from cells using protons as a successful energy source. According to Nguyen *et al.* (2014) tetracycline will exit to the bacterial surface periplasmic space at the time of proton exchange (H$^+$) during active transport so that it cannot bind to the

target ribosome subunit of 30s bacteria. This study also found 2 isolates which were negative to *tetA*. It is possible that other tet genes are involved in tetracycline group resistance in these isolates.

In general, resistance to β-lactam groups is caused by β-lactamase enzymes (Munita and Caesar 2016). Resistance to β-lactam class antibiotics in Gram-negative bacteria occurs mostly due to extended spectrum β-lactamase (ESBLs) (Rawat and Deepthi 2010). Extended spectrum β-lactamase encoded by the *blaTEM* gene will hydrolyze the β-lactam ring in the periplasmic space. Breaking bonds causes antibiotics not to function so there is no antibiotic reaction with penicillin-binding proteins (PBPs) (Munita and Caesar 2016). The *blaTEM* gene was detected in all *Salmonella* sp. isolates which is resistant to ampicillin.

The *gyrA* gene was detected in 8 (100%) isolates, which is resistant to quinolone and fluoroquinolone. Amino acid substitution that occurs in quinolon resistance determining region (QRDR) of the *gyrA* gene can cause resistance (Munita and Caesar 2016). Ogbolu *et al.* (2012) reported that mutation of the *gyrA* protein at codon position 83 (Serin → Leusin) in Gram-negative bacteria causes resistance to nalidixic acid and ciprofloxacin. Gram-negative-resistant enrofloxacin had amino acid substitution at codon position 83 (Serin → Leusin) and 87 (Aspartat → Glisin) (Jurado *et al.*

2008). Possibly the *Salmonella* sp. isolates in this study have been mutated in the *gyrA* protein.

The *ermB* and *aac(3)-IV* genes were not detected in the 8 *Salmonella* sp. isolates. According to Munita and Caesar (2016) more than 30 different *ermB* genes have been found in nature. Erythromicin-resistance is caused by the impermeable characteristics of Gram-negative bacteria against macrolide (Cesur and Demiroz 2013). Sandra *et al.* (2018) revealed that there are several genes encoding the aminoglycoside N-8-acetyltrasferase enzyme besides *aac(3)-IV* which can cause bacterial resistance to gentamicin is *aac(6)-I(a–d, e, f–z), aac(6)-II, aac(3)-I(a–b), aac (3)-II(a–c), aac(3)-III(a–c)* and *aac(3)-VII*. It is possible that other genes are involved in the resistance to gentamicin.

CONCLUSION

In conclusion, *Salmonella* sp. was isolated. All isolates showed positive results in the PCR confirmation. *Salmonella* sp. isolates were resistant to tetracycline, oxytetracycline, amphicillin, gentamicin, nalidixic acid, ciprofloxacin and erythromycin. Only the *tetA*, *blaTEM* and *gyrA* genes were detected in *Salmonella* sp. isolates.

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