

Original Research Paper

# Detection of *tet* Gene in Multidrug-Resistant *Salmonella* spp. Isolates from Layers and Broiler Breeders

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**Abstract:** The present study was conducted with the following aims: (i) To detect the multidrug-resistant *Salmonella* spp. isolates recovered from faeces, litter and drinking water in layers and broiler breeders' farms and (ii) to carried out the detection of the *tet* gene. A total of 21 *Salmonella* isolates were subjected to Polymerase Chain Reaction (PCR) assay to determine the presence of *tet* gene. Out of 21 isolates, 14 (66.7%) and 7 (33.3%) were found positive for *tet(A)* and *tet(B)*, respectively. In antimicrobial susceptibility tests, the *Salmonella* isolates showed resistance to tetracycline, oxytetracycline, ampicillin, nalidixic acid, enrofloxacin, gentamicin and chloramphenicol. It can be concluded that the high prevalence of the *tet* gene indicates a high potential of *Salmonella* isolates for horizontal transmission of tetracycline resistance genes.

**Keywords:** Multidrug-Resistant, *Salmonella*, *tet(A)*, *tet(B)*

## Introduction

Resistance to tetracycline was widely observed in different niches of the environment (Huang *et al.*, 2016; Jiang *et al.*, 2018; Shi *et al.*, 2019). Tetracycline resistance (*tet*) genes are one of the important determinants which enabling bacteria to resist tetracycline and are frequently associated with the development of Multidrug Resistance (MDR) in bacteria (Hedayatianfard *et al.*, 2014). The *tet* genes such as *tet(A)* and *tet(B)* can promote drug efflux (Opal and Pop-Vicas, 2015). Thus, drug efflux as a major mechanism of resistance to tetracyclines which could be find in enteric Gram-negative organism is encoded by *tet* genes (Giovanetti *et al.*, 2003).

There are now over 40 recognized determinants of tetracycline resistance and *tet(A)* were frequently detected in different environments (Ling *et al.*, 2013). Among the various *tet* genes, the *tet(A)* and *tet(B)* are significantly found in the Gram-negative bacteria (Jones *et al.*, 2006). *Salmonella* played a big role in many outbreak investigations of poultry worldwide (Demirbilek, 2017). In Indonesia, control of *Salmonella* spp. in layers and breeders are difficult to perform since

these bacteria are resistant to some antimicrobials (Gupta *et al.*, 2019).

The prevalence of *tet* genes in *Salmonella* spp. of layers and broilers origin has not been previously reported from Indonesia. The present study was conducted with the following objectives: (i) To detect the multidrug-resistant *Salmonella* spp. isolates recovered from faeces, litter and drinking water in layers and broiler breeders' farms and (ii) to carry out the detection of the *tet* gene.

## Materials and Methods

### Bacterial Isolation and Identification

Cloacal swabs, litter and water samples were collected from layers and broiler breeder farms in Bandung and Purwakarta, West Java, Indonesia. A total of 70 these samples (cloacal swabs, litter and water) were prepared in Buffer Peptone Water (BWP). SNI (2008) for the identification procedure of *Salmonella* spp. was applied. Each sample (1 mL) was transferred into 50 mL of Tetrathionate Broth (TB) (Oxoid, UK) for the enrichment and incubated at 37°C for 18-24 h.

After the enrichment procedure, one loop of the broth was inoculated onto *Salmonella* Shigella Agar (SSA) (Oxoid, UK) for selective and differential procedures. After 18-24 h incubation at 37°C, up to black coloured colonies were transferred onto Tryptone Soy Agar (TSA) (Oxoid, UK) and incubated at 37°C for 24 h. The isolates were characterized by Triple Sugar Iron Agar (TSIA), urea, indol, Methyl-Red (MR), Voges-Proskauer (VP) and Citrate (IMViC). Confirmation test for *Salmonella* spp. was performed by Polymerase Chain Reaction (PCR).

Genomic DNA was extracted using the boiling method (De Medici *et al.*, 2003). *Salmonella* spp. was identified by PCR using specific nucleotide primers for detection of the *invA* gene. The forward and reverse sequences of the primers were as follows 5'-ACAGTGCTCGTTTACGACCTGAAT-3' and 5'-AGACGACTGGTACTGATCGATAAT-3' (Chiu and Ou, 1996) respectively. Amplification of *InvA* was performed with (KAPA2G Fast Hotstart Readymix PCR kit, KAPA Biosystems, Cape Town, South Africa) in a total volume of 25 µL with 2 µL DNA template, 12.5 µL master mix, 1.6 µL primer forward 10 µM, 1.6 µL primer reverse 10 µM and 7.3 µL dH<sub>2</sub>O (DNase, RNase free). Temperature conditions consisted of an initial 95°C denaturation step for 3 min followed by 35 cycles of 95°C for 30 s, 57.5°C for 1 min and 72°C for 1 min. The final cycle was followed by one cycle at 72°C for 5 min in the thermal cycling system. The amplified fragments using standard PCR markers (KAPA™ Universal Ladder, KAPA Biosystems) were evaluated using agarose gel electrophoresis.

#### Antibiotic Susceptibility Testing

Antibiotic susceptibility was determined by disk diffusion method of Kirby Bauer using the following disks (Oxoid, UK): Tetracycline (30 µg/disk), oxytetracycline (30 µg/disk), ampicillin (10 µg/disk), nalidixic acid (30 µg/disk), erythromycin (15 µg/disk), enrofloxacin (15 µg/disk), gentamicin (10 µg/disk) and chloramphenicol (30 µg/disk). Isolates were categorized as susceptible, intermediate and resistant based upon interpretative criteria developed by Clinical and Laboratory Standards Institute (CLSI, 2017).

**Table 1:** Primer sequences used in the PCR assay and the expected sizes of the products

Target gene	Size (bp)	Primer	Sequence
<i>tet(A)</i>	577	TET(A)-F	5'-GGTTCACCTCGAACGACGTCA-3'
		TET(A)-R	5'-GGGCAGCAAATGTGGAGCAA-3'
<i>tet(B)</i>	634	TET(B)-F	5'-CCTCAGCTTCTCAACCGCGTG-3'
		TET(B)-R	5'-GCACCTTGCTCATGACTCTT-3'

#### Detection of *tet(A)* and *tet(B)* Genes

The presence of *tet(A)* and *tet(B)* gene was determined with PCR. Specific nucleotide primers are listed in Table 1, according to Randall *et al.* (2004). PCR was performed in a total volume of 10 µL with 1µL DNA template, 5µL master mix (KAPA2G Fast Hotstart Readymix PCR kit, KAPA Biosystems), 0.6 µL primer forward 10 µM, 0.6 µL primer reverse 10 µM and 3.8 µL dH<sub>2</sub>O (DNase, RNase free). A 1-µL DNA template was added to the PCR solution, which underwent an initial denaturation step of 95°C for 3 min before 35 cycles of 95°C for 30 s, 50-60°C for 30 s and 72°C for 1 min and then a final step of 72°C for 5 min for the last cycle. The amplified fragments using standard PCR markers (KAPA™ Universal Ladder, KAPA Biosystems) were evaluated using agarose gel electrophoresis.

### Results

#### Identification of *Salmonella* spp. Isolates by PCR for *InvA* Gene

The phenotyping was done for 33 *Salmonella* spp. isolates. Only 21 were identified as *Salmonella* spp. based on the presence of *InvA* gene by PCR. The detail of positive samples for *Salmonella* spp. is presented in Table 2.

#### Multidrug Resistance

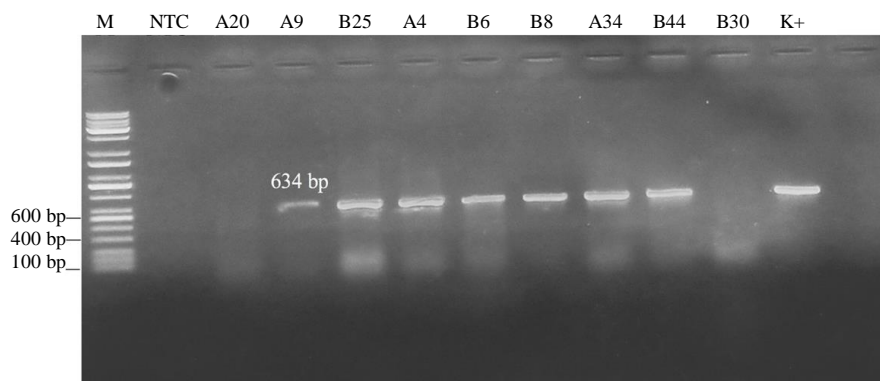
The antibiotic resistance profiles of the *Salmonella* spp. isolates against eight antimicrobial agents are shown in Table 3, respectively. The results indicated that *Salmonella* spp. in this study were resistant to more than two of the eight antimicrobials which are tetracycline (100%), ampicillin (100%), oxytetracycline (95%), nalidixic acid (95%), erythromycin (80%), enrofloxacin (71%), gentamicin (43%) and chloramphenicol (24%), in declining order.

#### Detection of *tet(A)* and *tet(B)* Genes

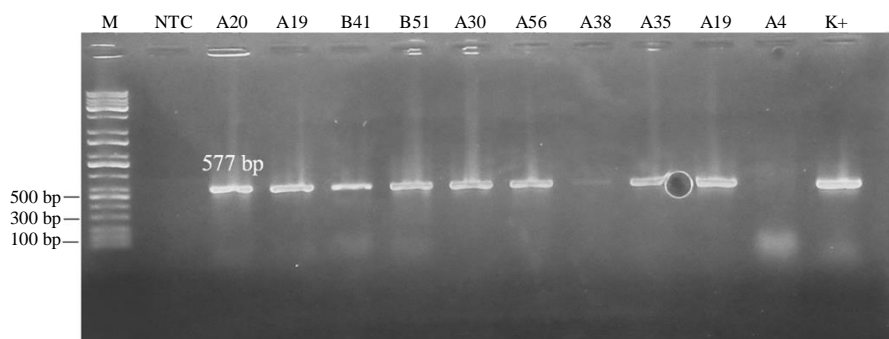
All isolates of *Salmonella* spp. in the present study were resistant to tet genes. The tet genes distribution is listed in Table 4. Based on the results, most isolates were positive for *tet(A)* (n = 14) (Fig. 2) compared to *tet(B)* (n = 7) (Fig. 1).

**Table 2:** Results of identification of *Salmonella* spp. isolates by PCR for *InvA* gene

Farm	Sample n (%)			<i>InvA</i> gene n (%)
	Faeces	Litter	Drinking water	
Layer	7 (10)	2 (2.9)	1 (1.4)	10 (14.3)
Broiler	4 (5.7)	3 (4.3)	4 (5.7)	11 (15.7)
Total n (%)	11 (15.7)	5 (7.1)	5 (7.1)	21 (30)



**Fig. 1:** Amplification of the *tet(B)* gene (634 bp) encoding tetracycline resistance in *Salmonella* spp.. A total of seven isolates showed positive results on *tet(B)*. M: 100 bp (marker); NTC: Non-template control



**Fig. 2:** Amplification of the *tet(A)* gene (577 bp) encoding tetracycline resistance in *Salmonella* spp.. A total of 14 isolates showed positive results on *tet(A)*. M: 100 bp (marker); NTC: Non-template control

**Table 3:** The antibiotic resistance profiles of the *Salmonella* spp. isolates against eight antimicrobial agents

No	Salmonella spp.	TET	OT	AMP	NA	E	ENR	G	C
1	A34	R	R	R	R	R	R	R	R
2	A4	R	R	R	R	R	R	R	S
3	B8	R	R	R	R	R	R	R	S
4	B19	R	R	R	R	I	R	S	R
5	B38	R	R	R	R	I	R	S	S
6	A51	R	R	R	R	R	I	S	S
7	A41	R	R	R	R	R	S	S	S
8	A6	R	R	R	R	R	S	S	S
9	A9	R	R	R	R	R	R	R	R
10	A30	R	R	R	R	R	R	R	R
11	A19	R	R	R	R	R	R	R	R
12	B41	R	S	R	R	S	I	S	I
13	B6	R	R	R	R	R	I	R	I
14	A27	R	R	R	R	S	R	I	I
15	A25	R	R	R	R	R	R	S	I
16	B35	R	R	R	R	R	R	S	S
17	A56	R	R	R	R	R	R	S	I
18	B44	R	R	R	S	R	I	I	I
19	A20	R	R	R	R	R	R	R	I
20	B25	R	R	R	R	R	R	R	I
21	A21	R	R	R	R	R	R	I	I
S (%)		-	5	-	5	10	10	43	33
I (%)		-	-	-	-	10	19	14	43
R (%)		100	95	100	95	80	71	43	24

TET (tetracycline), OT (oxytetracycline), AMP (ampicillin), NA (nalidixic acid), E (erythromycin), ENR (enrofloxacin), G (gentamicin), C (chloramphenicol), S (sensitive), I (intermediate), R (resistant)

**Table 4:** The distribution of *tet(A)* and *tet(B)* genes

No.	Sample code	Poultry flock (layers/broiler breeder)	<i>tet(A)</i> (+/-)	<i>tet(B)</i> (+/-)
1	A34	Layer	-	+
2	A4	Broiler breeder	-	+
3	B8	Layer	-	+
4	B19	Broiler breeder	+	-
5	B38	Broiler breeder	+	-
6	A51	Layer	+	-
7	A41	Layer	+	-
8	A6	Broiler breeder	+	-
9	A9	Broiler breeder	-	+
10	A30	Layer	+	-
11	A19	Layer	+	-
12	B41	Broiler breeder	+	-
13	B6	Broiler breeder	-	+
14	A27	Layer	+	-
15	A25	Layer	+	-
16	B35	Broiler breeder	+	-
17	A56	Layer	+	-
18	B44	Broiler breeder	-	+
19	A20	Broiler breeder	+	-
20	B25	Broiler breeder	-	+
21	A21	Layer	+	-
		Total	14	7

## Discussion

*Salmonella* spp. has been recognized as a global threat and raises public health concerns since these bacteria have a number of virulence mechanism to interfere with host defence systems in different infection stages. In addition *Salmonella* may develop resistance to antibiotics (Moultotou *et al.*, 2017). Resistance to antibiotics in *Salmonella* spp. is significant in poultry isolates from Indonesia. In the present study the results of antibiotic sensitivity test (Table 3) indicated that all *Salmonella* spp. are resistant to tetracycline. Our results agreed with previous study (Yulistiani *et al.*, 2017). The high resistance to tetracycline in poultry farm caused by the abundant and quite long use of tetracycline on poultry farms. Although, Indonesia has banned the use of antibiotic growth promoter in farms, farmers are still using antibiotics to overcome the problem of Salmonellosis on their farms.

Because of the frequent use of antibiotics for the therapy, bacteria may develop multidrug resistant phenotype (Chatterjee *et al.*, 2019). The high number of *Salmonella* spp. infections despite antibiotic therapy raises several questions such as: Are available antibiotics efficient for the therapy of existing *Salmonella* spp.? Therefore, a few farmers start using vaccinations to treat *Salmonella* spp.. In this investigation *Salmonella* was identified in 30% of samples collected from several commercial layer and broiler breeder farms in Purwakarta, Indonesia. Another finding (Li *et al.*, 2017) from layer farms also detected *Salmonella* spp. using PCR targeting *InvA* gene with a low prevalence of 11.6%. In the present study, a total of 21 isolates of *Salmonella* spp. (30%) were

found in faeces (15.7%), litter (7.1%) and drinking water (7.1%). The distribution of *Salmonella* spp. in this study is higher compare to previous results (Abunna *et al.*, 2016) that found 15.12% of *Salmonella* spp. isolates from cloacal swabs, fresh faeces, litter and poultry drinking water samples. The results in this study also showed that cases of salmonellosis were higher in commercial layer chickens than in broiler breeders. This distribution agreed with previous studies (Shoaib *et al.*, 2019). This is not surprising because sanitation in the environment of broiler breeder farms is better than commercial layer farms in Indonesia, generally.

Also, the discovery of *Salmonella* spp. in drinking water indicates the possibility of contamination from the environment. Most likely, these chickens are infected from their drinking water contaminated by *Salmonella* spp.. The *Salmonella* contamination at this site probably caused by a biofilm formation by *Salmonella* spp. (Merino *et al.*, 2019) on the waterline or the water source are not free from *Salmonella* spp.. To figure it out, more investigation is needed.

The investigation was continued to address the question of unsuccessful handf of Salmonellosis in these farms. Based on the results of the investigation, all *Salmonella* spp. isolates were resistant to at least three of the antibiotics tested. These results show that the isolates are multidrug resistant. Besides, all *Salmonella* isolates in this study showed 100% resistance to tetracycline and ampicillin. In Indonesia, the types of antibiotics that are still used for *Salmonella* control in poultry include enrofloxacin, a combination of ampicillin and colistin

sulphate, a combination of ciprofloxacin and tylosin tartrate, floxamycine and a combination of amoxicillin and colistin sulphate. Therefore, the prevalence of *Salmonella* resistant to tetracycline in these farms was surprisingly high, considering that this antibiotic is not used anymore in Indonesia. This observation could address that the high prevalence of *Salmonella* spp. in these farms are not linked to the antibiotic usage. The previously finding by Liljebjelke *et al.* (2017) also showed that the therapeutic use of tetracycline in *Salmonella* spp. was not efficient. Therefore, detection of *tet(A)* (Fig. 2) and *tet(B)* genes (Fig. 1) was applied in this study. Based on the results, these genes are distributed in both types of farms with a greater distribution is *tet(A)* gene. The results in this study about the distribution of *tet(A)* and *tet(B)* genes were in confirmation with Waghmare *et al.* (2018) who reported that all *Salmonella* spp. were found to carry *tet(A)* while none of their isolates carried *tet(B)*. The *tet(A)* and *tet(B)* are the genes of the tetracycline resistance related to an efflux mechanism (Roberts and Schwarz, 2017) and it could be present in mobile elements and acquired by horizontal transfer by *Salmonella* spp.. It is not surprising since efflux pumps are the most abundant in Gram-negative bacteria (Gharajalar and Sofiani, 2017). Our result confirms the spread of multidrug-resistant *Salmonella* spp. from commercial layer and breeder broiler farms. The occurrence of multidrug resistant *Salmonella* spp. in poultry farms in Indonesia present a significant risk to human health.

## Conclusion

The prevalence of tetracycline and ampicillin in commercial layer and broiler breeder farms was high. This study revealed a significant rise in tetracycline resistance with presence of *tet(A)* and *tet(B)* genes in *Salmonella* spp. Dissemination of multidrug-resistant *Salmonella* spp. observed in commercial layer and broiler breeder farms may pose a serious risk to human health.

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## Author's Contributions

**Leila Nur Azizah, Maya Shofa and Usamah Afiff:** Prepared the protocol.

**Agustin Indrawati, I Wayan Teguh Wibawan and Safika:** Developed the study.

**Siti Gusti Ningrum:** Drafted and corrected the manuscript.

All authors read and approved the final manuscript.

## Ethics

The authors declare that there are no conflicts of interest or any ethical issues.

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