

ORIGINAL ARTICLE

## Detection of *Salmonella* pathogenicity island and *Salmonella* plasmid virulence genes in *Salmonella Enteritidis* originated from layer and broiler farms in Java Island

Ernes Andesfha<sup>1</sup>, Agustin Indrawati<sup>1</sup>, Ni Luh Putu Ika Mayasari<sup>1</sup>, Irma Rahayuningtyas<sup>2</sup>, Istiyaningsih Jusa<sup>2</sup>

<sup>1</sup>Department of Animal Infectious Diseases and Veterinary Public Health, Faculty of Veterinary Medicine, IPB University, Bogor, Indonesia

<sup>2</sup>National Veterinary Drug Assay Laboratory NVDAL, Bogor, Indonesia

### ABSTRACT

**Objective:** The incidence of salmonellosis in humans and animals is still high due to the occurrence of virulence factors in *Salmonella enterica* which play a role in the process of infection in the host and the spread of disease and most of the *S. enterica* can infect humans and animals. The present study was aimed to identify *Salmonella Enteritidis* and detect virulence genes related to *Salmonella* pathogenicity islands (SPIs) and *Salmonella* plasmid virulence (Spv).

**Materials and Methods:** A total of 27 *S. Enteritidis* archive isolates belonging to the National Veterinary Drug Assay Laboratory (NVDAL) were used in this study. The bacteria were collected in 2016 and 2017 from samples of the cloaca and fecal swabs from layer and broiler farms in five provinces of Java Island. Isolates were cultured in specific media, biochemical tests and Gram staining. Detection of *S. Enteritidis* and virulence genes was done by polymerase chain reaction (PCR) method.

**Results:** Identification of serovar showed 100% (27/27) isolates were positive for the *sdfl* gene (304 bp). The result confirmed that all strains were *S. Enteritidis*. PCR based detection of virulence genes showed that 100% of isolates had virulence genes in SPI-1 to SPI-5, namely, *invA*, *ssaQ*, *mgcC*, *spi4D*, and *pipA* genes. All the isolates (27/27) were also positive to *spvB* gene-based PCR.

**Conclusion:** All the isolates of *S. Enteritidis* in this study carry virulence genes related to SPI-1 to SPI-5 and plasmid virulence. The existence of virulent genes indicates that the *S. Enteritidis* strain examined in this study is highly virulent and poses a potential threat of worse disease outcome in humans and animals.

### ARTICLE HISTORY

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### KEYWORDS

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### Introduction

Java Island is the center of poultry farming in Indonesia. More than 66% of the national population of layer and broiler chickens [1] are on Java Island. The large population of chickens allows the transmission of animal diseases, one of which is *Salmonella* infection. Cases of *Salmonella* infection continue to occur with a high percentage in both animals and humans. The European Surveillance System reported a total of 31 829 cases in 2015 and 3 709 incidents in early 2016 caused by *S. Enteritidis* infection from 18 countries in Europe [2]. The report of *Salmonella enterica* contamination in poultry products in several countries tends to be high, including *S. Paratyphi B* 76% and *S. Heidelberg* 23% in Columbia

[3], *S. Enteritidis* 37.3% in Egypt [4], and *S. Enteritidis* 30% in Malaysia [5]. *Salmonella enterica* infections in humans mostly occur through foodborne transmissions. Humans can be infected by *S. Enteritidis* by consuming animal products (poultry, swine, fish, fish, crustaceans, and shellfish) and also indirectly related to the consumption of cheese, chocolate, vegetables, and juices [6]. Generally associated with gastrointestinal organs, *S. enterica* can cause invasive infections and bacteremia in young age and elderly people as well as in individuals with low immunity [7].

*Salmonella enterica* have many virulence factors that are necessary for the infection process to the host and the spread of the disease. The main virulence factor of *S. enterica* was determined in the chromosomal gene which was

**Correspondence** Agustin Indrawati ✉ [titin.seta@gmail.com](mailto:titin.seta@gmail.com) 📧 Department of Animal Infectious Diseases and Veterinary Public Health, Faculty of Veterinary Medicine, IPB University, Bogor, Indonesia.

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located in the *Salmonella* pathogenicity islands (SPI). SPI is needed for invasion and proliferation in host cells [8]. Some *S. enterica* serovars also have virulence plasmids called Salmonella plasmid virulence (Spv). This plasmid virulence has several virulence genes that cause suppression of host's innate immune response [9]. Horizontal transfer of virulence genes can cause bacterial pathogenesis evolution. This evolution is the force that initiates the emergence of new pathogenic species, which can adapt to new hosts and are specific sites in the host. Most virulence genes can move between bacteria called pathogenicity island and are known as a quantum leap in the process of bacterial pathogenesis evolution [10]. In *Salmonella* bacteria, virulence gene clusters are located in specific areas of the chromosome called SPI. SPI act as virulence factors in the pathogenicity of *Salmonella*. SPI is characterized by an elemental composition that is different from the core genome. SPI is often associated with mobile genetic elements and *tRNA* genes, such as insertion element, phage genes, or transposons [11]. This difference indicates that there are sequences of SPI compilers obtained through horizontal transfers, although the origin of the sequence and the process of transferring sequences is still unclear [12].

Several studies on the isolation of *Salmonella* bacteria from animal products have been reported [13,14]. However, research on virulence genes in *S. Enteritidis* from samples of cloacal swabs and poultry feces is still lacking in Indonesia. This study aims to identify *S. Enteritidis* and detect virulence genes of *S. Enteritidis* originated from layer and broiler farms in Java Island.

## Materials and Methods

### Bacterial isolates and ethical approval of experimental animals

A total of 27 *S. Enteritidis* isolated from cloacal swabs and feces collected from the layer and broiler farms in the Province of West Java, Banten, Yogyakarta, Central Java, and East Java in 2016 and 2017 were used in the current study (Table 1). All isolates were belonging to NVDAL, West Java, Indonesia. No live animals were used in the present study. Therefore, no ethical approval was needed to conduct this study.

### Identification of *Salmonella Enteritidis* and biochemical tests

Freeze dry bacteria were revived in Heart Infusion Broth (HIB) medium at 37°C for 24 h. The bacteria were then cultured on Xylose Lysine Deoxycholate (XLD) and Rapid Salmonella Agar (RSA) media which were incubated at 37°C for 24 h. Specific colonies of *Salmonella* on RSA media were cultured on the Heart Infusion agar (HIA) media for 24 h at 37°C. Then, the bacterial colonies were

stained using Gram staining and were cultured on Triple Sugar Iron Agar (TSIA) media for 24 h at a temperature of 37°C. *Salmonella* bacteria cultured on TSIA media were subjected to be tested for biochemistry including indole test, Methyl Red-Voges Proskauer (MR-VP), lysine, and citrate. Indole test was done using Sulfide Indole Motility (SIM) media and incubated at 37°C for 24 h. After incubation, Kovac's reagent indicator was given. The MR-VP test used MR-VP broth media and incubated at 37°C for 48 h. After incubation, the media were added with the reagent indicators [methyl red, Alpha naphthol, and potassium hydroxide (KOH)]. Lysine test was carried out using Lysine Decarboxylase Broth (LDB) media and incubated at 37°C for 48 h. Citrate test was done using Simmons Citrate Agar media and incubated at 37°C for 24 h [15].

### Identification of *Salmonella Enteritidis*

Bacterial isolates positively as *Salmonella* in the biochemical test were enriched on HIB media for 24 h at 37°C. The isolates were purified by culturing on HIA media for 24 h at 37°C. Colonies grown on HIA media were taken 1–2 ose and dissolved in 100 µl PBS to extract DNA using the boiling method [16]. The samples were homogenized using vortex for 10–30 sec then heated at 100°C for 10 min. The samples were cooled for 2 min and centrifuged at 14000 rpm for 2 min, and the supernatant was taken as much as 50 µl as a DNA master stock.

*Salmonella Enteritidis* was identified by detecting *sdfl* gene on the amplicon length of 304 bp. The primary forward sequence of *sdfl* gene used was 5'-TGT GTT TTA TCT GAT GCA AGA GG-3' while the primary reverse sequence of *sdfl* gene used was 5'-TGA ACT ACG TTC GTT CTT CTG G-3' [17]. The amplification reagent of *S. Enteritidis* used in this study was the HotStarTaq® Master Mix Kit (Qiagen, Germany) with a total polymerase chain reaction (PCR) reagent volume of 25 µl consisting of 12.5 µl HotStarTaq master mix, 1 µl forward primer (20 µM), 1 µl reverse primer (20 µM), 5.5 µl dH<sub>2</sub>O, and 5µl DNA template. The PCR process begins with a pre-denaturation cycle of 95°C for 15 min, and lastly the next process was 30 cycles of denaturation at 94°C for 1 min, annealing at 57.5°C for 1 min, then 1 min of extension at 72°C, and a final extension at 72°C for 10 min. Visualization of PCR results was done using 1.5% agarose gel, SYBR safe staining, and 100-bp marker. The documentation was done using Gel Documentation Systems (Thermo Fisher Scientific, USA).

### Detection of Virulence Genes in *S. Enteritidis*

Simplex PCR was performed targeting *invA* (SPI-1), *ssaQ* (SPI-2), *mgtC* (SPI-3), *spi4D* (SPI-4), *pipA* (SPI-5), and *spvB* genes (plasmid virulence). The primer sequences, gene targets, amplicon sizes, and annealing temperatures are presented in Table 2.

**Table 1.** Data of archival isolates of *Salmonella Enteritidis* bacteria (n = 27).

No	Province	Districts	Farm	Isolate Code	Age	Year of sampling
1	Banten	Tangerang	Layer	SE/100/16L/Tangerang	24 weeks	2016
2		Serang	Layer	SE/101/16L/Serang	18 weeks	2016
3		Serang	Layer	SE/105/16L/Serang	18 weeks	2016
4	West Java	Bogor	Broiler	SE/211/17B/Bogor	13 days	2017
5		Bogor	Broiler	SE/236/17B/Bogor	29 days	2017
6		Bogor	Broiler	SE/237/17B/Bogor	29 days	2017
7		Ciamis	Broiler	SE/411/17B/Ciamis	19 days	2017
8		Ciamis	Broiler	SE/412/17B/Ciamis	19 days	2017
9		Ciamis	Broiler	SE/429/17B/Ciamis	7 days	2017
10		Ciamis	Broiler	SE/430/17B/Ciamis	7 days	2017
11		Ciamis	Broiler	SE/431/17B/Ciamis	34 days	2017
12		Ciamis	Broiler	SE/443/17B/Ciamis	12 days	2017
13	Central Java	Banyumas	Broiler	SE/157/17B/Banyumas	15 days	2017
14		Kendal	Layer	SE/069/17L/Kendal	28 weeks	2017
15		Kendal	Broiler	SE/119/17B/Kendal	35 days	2017
16		Kendal	Broiler	SE/120/17B/Kendal	32 days	2017
17		Semarang	Layer	SE/155/16L/Semarang	28 weeks	2016
18		Semarang	Layer	SE/158/16L/Semarang	28 weeks	2016
19	Special Region of Yogyakarta	Bantul	Layer	SE/115/16L/Bantul	1.5 years	2016
20		Bantul	Layer	SE/117/16L/Bantul	1.5 years	2016
21		Bantul	Layer	SE/118/16L/Bantul	1.5 years	2016
22	East Java	Kediri	Layer	SE/467/17L/Kediri	20 months	2017
23		Kediri	Layer	SE/496/17L/Kediri	12 months	2017
24		Kediri	Layer	SE/502/17L/Kediri	20 weeks	2017
25		Kediri	Broiler	SE/508/17B/Kediri	23 days	2017
26		Kediri	Broiler	SE/511/17B/Kediri	22 days	2017
27		Kediri	Broiler	SE/513/17B/Kediri	22 days	2017

**Table 2.** List of primer sequence virulence genes of *S. Enteritidis*.

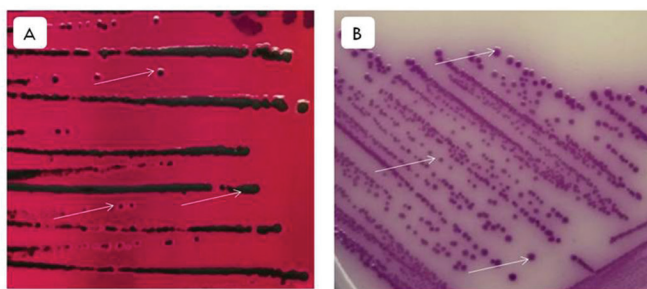
Gene target	Forward (F) and reverse (R) base pair sequence	Amplicon size (Annealing temperature)	Reference
<i>invA</i> (SPI-1)	(F) 5'-GTGAAATTATCGCCACGTTCCGGGCAA-3' (R) 5'-TCATCGCACCGTCAAAGGAACC-3'	284-bp (59°C)	[18]
<i>ssaQ</i> (SPI-2)	(F) 5'-GAATAGCGAATGAAGAGCGTCC-3' (R) 5'-CATCGTGTATCCTCTGTCAGC-3'	677-bp (60°C)	[19]
<i>mgtC</i> (SPI-3)	(F) 5'-TGACTATCAATGCTCCAGTGAAT-3' (R) 5'-ATTACTGGCCGCTATGCTGTTG-3'	655-bp (60°C)	[19]
<i>spi4D</i> (SPI-4)	(F) 5'-GAATAGAAGACAAAGCGATCATC-3' (R) 5'-GCTTTGTCCACGCCTTTCATC-3'	1231-bp (60°C)	[19]
<i>pipA</i> (SPI-5)	(F) 5'-CTCTTGATGATTTTCTTTTA-3' (R) 5'-CTTATCTCAGGCGGGTGG-3'	406-bp (58°C)	[19]
<i>spvB</i> (Plasmid virulen)	(F) 5'-CTATCAGCCCCGACGGAGAGCAGTTTTTA-3' (R) 5'-GGAGGAGCGGTGGCGTGGCATCATA-3'	717-bp (60.6°C)	[20]

PCR was performed in 25 µl reaction volume containing master mix reagents consisting of 12.5 µl HotStarTaq master mix (Qiagen, Germany), 1 µl forward primer (5 µM), 1 µl reverse primer (5 µM), 5.5µl dH<sub>2</sub>O, and 5µl DNA templates. The PCR process begins with a pre-denaturation cycle at 95°C for 15 min, followed by 30 cycles of 94°C for 1 min, annealing for 1 min (the primer and temperature used were shown in Table 2), and extension at 72°C for 1 min, and then a final extension at 72°C for 10 min. The PCR product was analyzed using 1.5% agarose (Thermo Fisher Scientific, USA) and visualized using Gel Documentation Systems (Thermo Fisher Scientific, USA) upon staining with SYBR safe (Thermo Fisher Scientific, USA).

## Results and Discussion

The results of the culture of *S. Enteritidis* isolates on XLD and RSA media showed that 100% (27/27) of isolates growing with typical colonies of *Salmonella spp.* Macroscopic observations of XLD showed pink colonies with a black center; there are also black colonies as a whole because H<sub>2</sub>S was produced (Fig. 1A). In RSA media, bacterial colonies are circular and the color is purple (Fig. 1B). Microscopic examination with Gram staining showed the stem morphology of *S. Enteritidis* bacteria and the color is red (Fig. 2).

The results of culture on TSIA media showed that 100% (27/27) of the isolates reacted with the transformation to red in the TSIA slant media and yellow in the bottom part, H<sub>2</sub>S and gas bubbles were formed in the bottom part. *Salmonella* bacteria cannot ferment lactose and sucrose, so the TSIA media in the slant remains red. However, *Salmonella* bacteria can ferment glucose which produces pyruvic acid that causes the media on the bottom to become acidic and yellow because the red phenol indicator



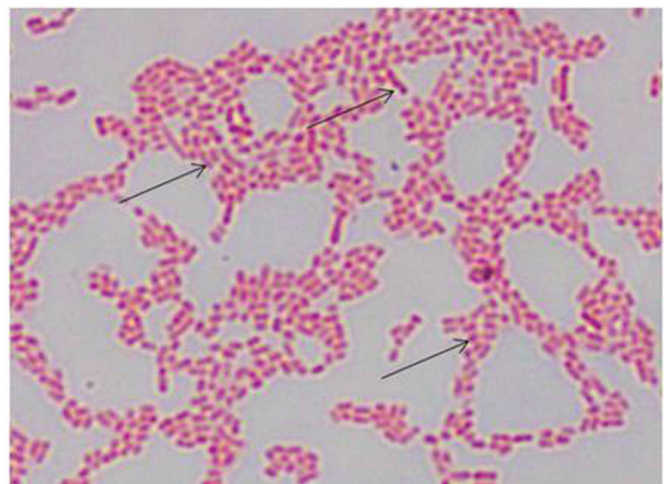
**Figure 1.** Macroscopic observation of *S. Enteritidis* in XLD and RSA media. (A) *S. Enteritidis* colonies on XLD media. The arrows indicate *S. Enteritidis* colonies are pink with the black center because H<sub>2</sub>S was produced, there are also colonies that are full black because more H<sub>2</sub>S was produced. (B) *S. Enteritidis* colonies in RSA media. The arrows indicate the circular *S. Enteritidis* colonies and the color is purple.

turns yellow [21]. The biochemical test showed that 100% (27/27) of *S. Enteritidis* isolates have a positive reaction in the MR, lysine, and citrate test. All isolates showed a negative reaction in the indole and VP test (Table 3).

The results of detection of *S. Enteritidis* with the *SdfI* gene target showed all isolates and positive control of *S. Enteritidis* ATCC 13076 were positive for the *sdfI* gene with an amplicon length of 304 bp (Fig. 3). These results confirm that all isolates (27/27) of the archives were *Salmonella Enteritidis*.

*S. Enteritidis* is the only serovar that has the unique fragment of *sdfI* gene. The unique fragments were isolated using the Suppression Subtractive Hybridization (SSH) method by multiplying fragments through matrix [17]. This unique fragment is not found in other bacteria so that its existence can be used as a diagnostic marker. The *sdfI* primer sequence specificity has been tested to 73 *S. enterica* non-*Enteritidis* isolates. The results showed *sdfI* primer did not amplify all *S. enterica* non-*Enteritidis* isolates. Other tests used 33 *S. Enteritidis* isolates from humans, pigs, cattle, turkeys, chickens, and poultry environmental samples. The result showed positive for *sdfI* primer in all 33 isolates [17].

Identification based on the results of bacterial culture on specific media, biochemical tests, Gram staining, and molecular identification showed that all isolates were *S. Enteritidis*. Rapid test and detection methods for the identification of *S. enterica* until the level of serovar are very important and fundamental to detect *S. Enteritidis* because of the high prevalence of salmonellosis and many serovars of *S. enterica*. The identification of *S. Enteritidis* with the *sdfI* gene target can be used because it provides a reliability benefit of an important test method with rapid, sensitive, and specific test results only for *S. Enteritidis*.



**Figure 2.** Microscopic observation of *S. Enteritidis* with Gram staining. The arrow indicates the morphology of the *S. Enteritidis* in the form of a stem and the color is red (1000×).

PCR targeting the virulence genes in SPI and SPV showed 100% isolates (27/27) of *S. Enteritidis* were positive to *invA*, *ssaQ*, *mgtC*, *spi4D*, *pipA*, and *spvB* genes (Figs. 4–9).

There are a total of 17 SPI described in *Salmonella* [22]. SPI-1 to SPI-5 have been widely characterized and can be found in most *S. enterica*. Other SPIs were less widely distributed [12]. While the genes that make up each SPI

are not known whether all serovars have the same SPI constituent gene. In *S. Typhimurium* [23] and *S. Typhi* [22], the SPI-1 to SPI-5 genes have been wholly identified. Amavisit et al. [24] reported that there were genetic variations between SPI-1, SPI-3, and SPI-5, while the genetic variation of SPI-4 and SPI-2 was not easily changed among 13 *Salmonella* serovars isolated from cattle, pigs, poultry, horses, environment, and humans [24].

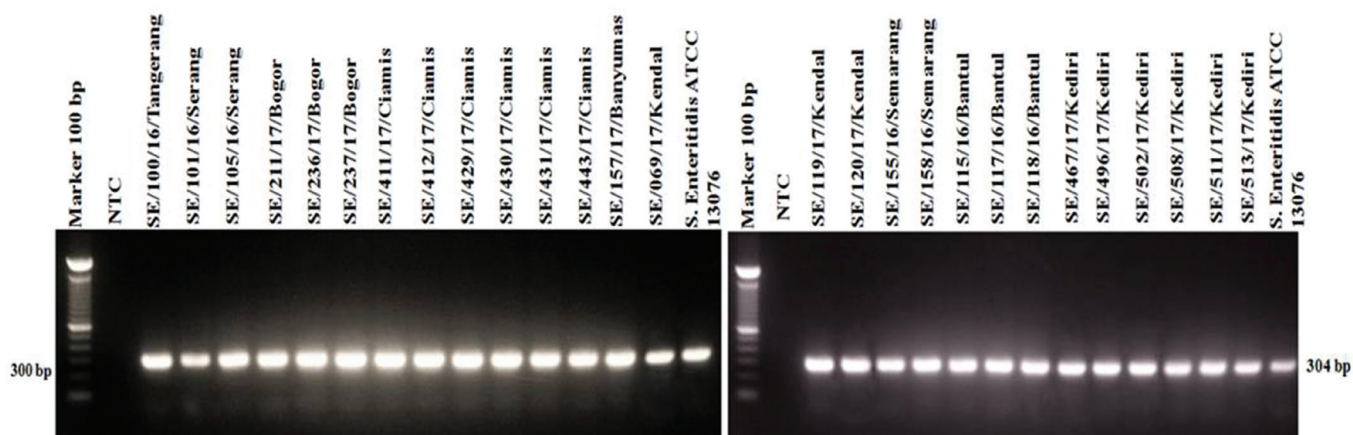
*Salmonella* bacteria can be found in the digestive organs of both animals and humans. Besides being found in feces, eggs, and meat, *Salmonella* also can be found at any tools and part of the rooms in the abattoirs, such as knife, transport crate, chopping board, or even in the drain water and wash water, as well as in defeathering machines, drain swabs, and apron [25]. *Salmonella* can also be found in animal feed ingredients, soil, bedding, litter, and feces which are generally a source of *Salmonella* contamination on farms. Chickens can be exposed to and infected with *Salmonella* bacteria through vertical transmission. In addition, horizontal transmission from the environment of the genes can occur through equipment, transportation, feed, and vectors (insects, rodents, as well as humans) [26].

The mechanism of *Salmonella* infection and the role of SPI-2 and SPI-1 in bacterial infiltration and invasion of host epithelial cells, proliferation in mononuclear to systemic infections are as follows. *Salmonella* contained in the feed will enter the digestive tract, after passing gastric acid will go to the small intestine, large intestine, and cecum which has a low pH, and *Salmonella* can survive in a low pH environment. In the small intestine, *Salmonella* will attach to host epithelial cells, then SPI-1 will express the first Type 3 Secretion Systems (T3SS) or T3SS-1 which are multiprotein complexes that facilitate bacteria in the process of invasion and absorption through endothelial [22]. The T3SS-1 acts as a syringe molecule in the form of a channel

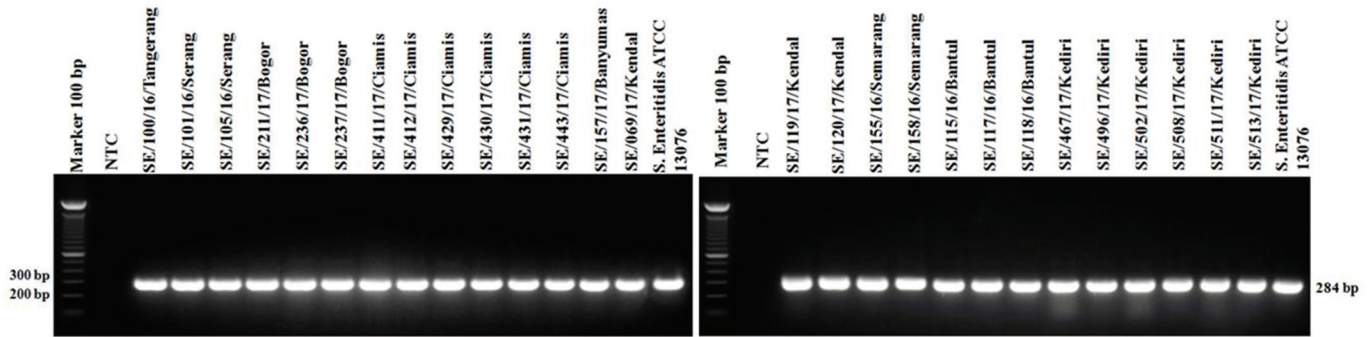
**Table 3.** Results of biochemical tests on *Salmonella Enteritidis* ( $n = 27$ ).

Type of test	Result		Information <sup>c</sup>
	Positive (+) <sup>a</sup>	Negative (-) <sup>b</sup>	
Indole	0	27	The surface of the media is yellow; no pink ring is formed after the Kovac's reagent was added
MR <sup>d</sup>	27	0	The methyl red indicator diffuses with the MR-VP media so that the color of the media is red
VP <sup>e</sup>	0	27	There is no red color on MR-VP media after alpha naphthol, and KOH were added
Lysine	27	0	The LDB <sup>f</sup> media returns to purple
Citrate	27	0	The development of <i>Salmonella</i> colonies shown with changes in the color of the media from green to blue

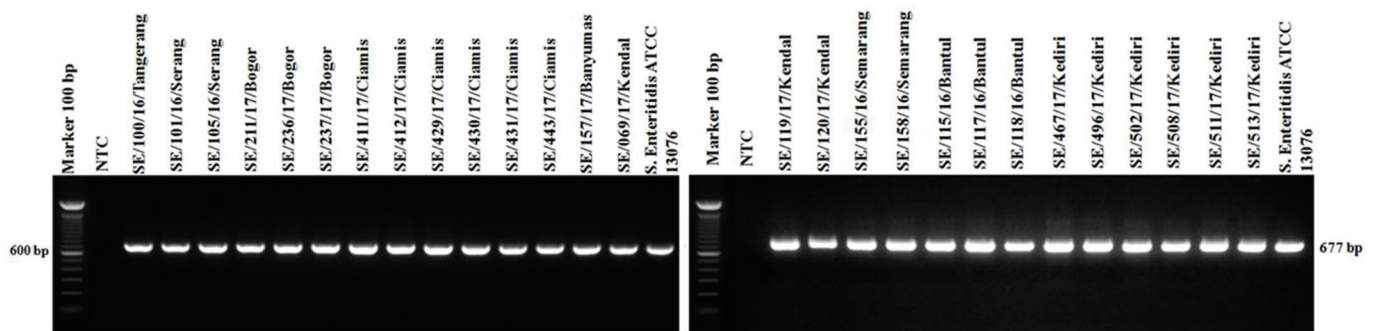
<sup>a</sup>The number of isolates was positive for biochemical tests, <sup>b</sup>the number of negative isolates against biochemical tests, <sup>c</sup>biochemical reactions that occurred, <sup>d</sup>MR = methyl red, <sup>e</sup>VP = Voges Proskauer, <sup>f</sup>LDB = Lysine Decarboxylase Broth.



**Figure 3.** Amplification of *sdfI* (304-bp) gene encoding *S. Enteritidis*. All or 100% (27/27) of isolates showed positive results against *sdfI* gene. NTC: non template control; *S. Enteritidis* ATCC 13076 as a positive control shows positive results against *sdfI* gene.



**Figure 4.** Amplification of *invA* gene on SPI-1 of *S. Enteritidis*. All isolates (27/27) showed positive *invA* results (284-bp). NTC: non-template control; positive control: *S. Enteritidis* ATCC 13076 showed positive *invA* results (284-bp).



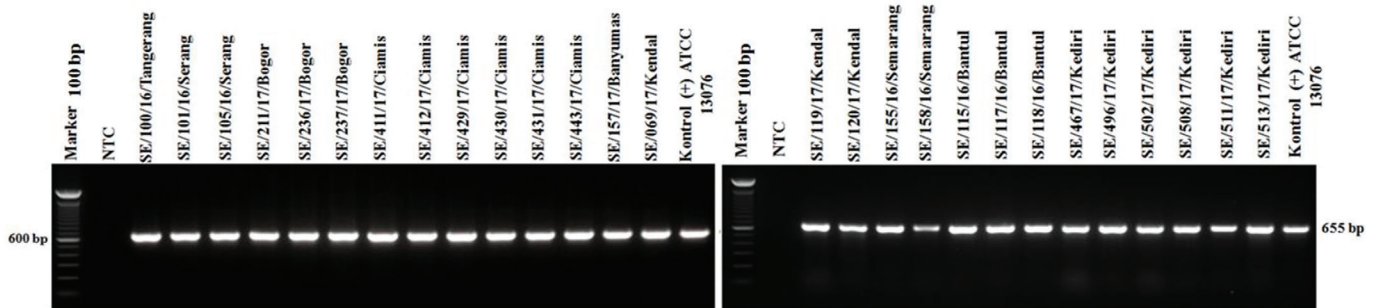
**Figure 5.** Amplification of *ssaQ* gene on SPI-2 of *S. Enteritidis*. All isolates (27/27) showed positive results *ssaQ* (677-bp). NTC: non-template control; positive control: *S. Enteritidis* ATCC 13076 showed positive results of *ssaQ* gene (677-bp).

to connect the bacterial cytoplasm with the membrane of the host cell. The T3SS-1 functions to transfer toxins and effector proteins of *Salmonella* encoded by SPI-1 into host intestinal cells. The number of effector proteins transferred is more than 20 structural proteins and regulating proteins [27]. One function of the effector protein makes the ruffling process on the host cell surface. Due to the wrinkled, supple, and curved surface of the host cell, *Salmonella* can easily enter the host cell membrane. *Salmonella* that have passed through the host cell membrane will bind to the host cell membrane to form a vacuole so that it is localized and known as *Salmonella* containing vacuole (SCV) [28].

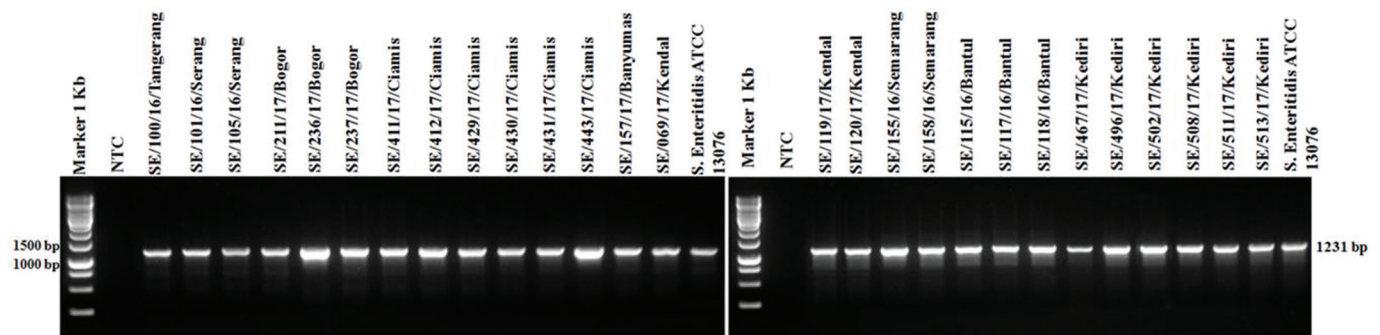
SPI-2 of *Salmonella* found in the SCV will express the second T3SS (T3SS-2). Through T2SS-2, the secretion of effector proteins has the function in the process of systemic infection and pathogenesis of infection inside the cell [26]. Other function of the effector proteins is their interaction with the motor protein and cytoskeleton in the formation of *Salmonella* induced filaments. T3SS-2 can modulate SCV movements to avoid fusion with lysosomes. SCV has a vital role in the process of survival as well as the proliferation of *Salmonella* bacteria in the macrophages and enterocytes [29].

After the *Salmonella* move through the intestinal epithelial cells, then enter the Peyer's patches that will be presented as antigen-presenting cells as phagocytic and neutralization efforts [30]. *Salmonella* that can survive in mononuclear cells of Peyer's patches will continue to grow and through the reticuloendothelial system will reach the liver and spleen to enter the bloodstream [31]. *Salmonella* will be excreted through feces which will contaminate water and become a source of transmission of *Salmonella* that can be spread through insects and other animals so that the bacterial life cycle will continue and increasingly spread [32].

All isolates *S. Enteritidis* (27 isolates) in this study carried virulence genes *invA*, *ssaQ*, and *mgtC* on the amplicon length 284, 677, and 655 bp, respectively. (Figs. 4–6). The central role of SPI-1 is to express the structure of T3SS and encode many effector proteins that are very important in the process of epithelial cell invasion and the *invA* virulence genes detected in the present study was reported to play a role in the process of invasion [22]. SPI-1 also plays a role in inducing inflammatory responses in the intestine [33]. Many genes are encoded by SPI-2 to form the secretion systems apparatus (*ssa*) is T3SS structure,



**Figure 6.** Amplification of *mgtC* gene on SPI-3 of *S. Enteritidis*. All isolates (27/27) showed *mgtC* positive results (655-bp). NTC: non-template control; positive control: *S. Enteritidis* ATCC 13076 showed positive of *mgtC* gene (655-bp).



**Figure 7.** Amplification of *spi4D* gene on SPI-4 of *S. Enteritidis*. All isolates (27/27) showed positive results of *spi4D* (1231-bp). NTC: non-template control; positive control: *S. Enteritidis* ATCC 13076 showed positive results *spi4D* (1231-bp).

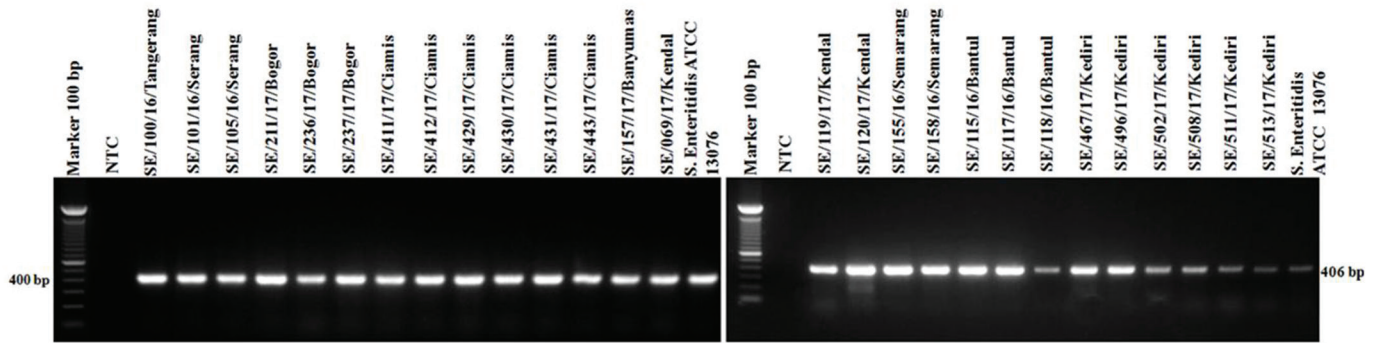
the SPI-2 virulence gene detected in this study is the *ssaQ* gene which plays a role in the formation of the T3SS structure [25]. SPI-2 also has a gene that encodes effector proteins, namely, secretion system effector (Sse) and encodes specific chaperones of effector proteins called secretion system chaperone (Ssc). These three systems make *Salmonella* survive, multiply in phagocytic cells, and facilitate the spread of systemic infections in organs [27,34]. SPI-3 encoded the magnesium transport system that is regulated by the *mgtB* and *mgtC* genes. Availability of magnesium plays an essential role as a key signal for *Salmonella* inside the host cell [35]. The *mgtC* virulence gene detected in this study also plays a role in activating  $\text{Na}^+$ ,  $\text{K}^+$ , and ATPase that regulates the potential membrane of *Salmonella* [36].

Detection of virulence genes of *spi4D* on SPI-4 and *pipA* on SPI-5 in this study showed that 100% isolates were positive (Figs. 7 and 8). The role of the *spi4D* virulence gene in SPI-4 is to encode Type 1 Secretion Systems (T1SS), cognate substrate protein, and mediate adhesion [37], whereas the virulent gene of *pipA* encodes effector proteins for T3SS in SPI-2 [38].

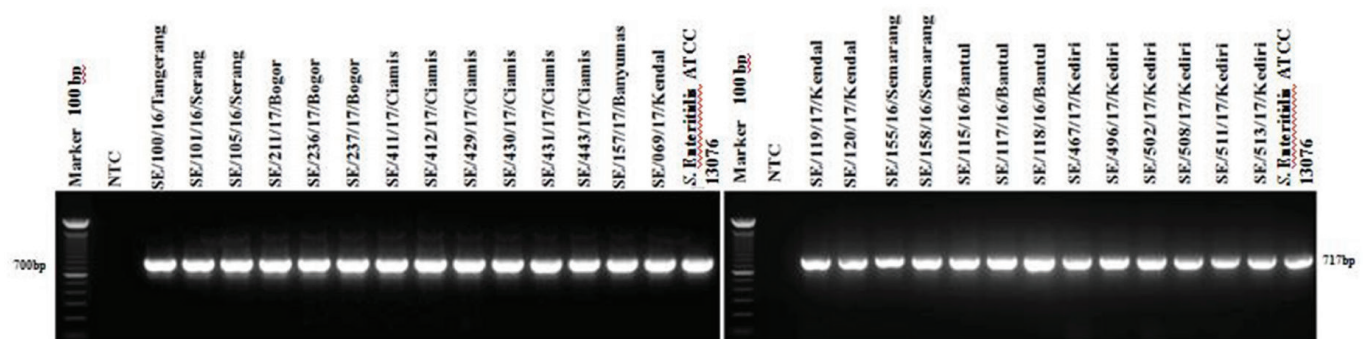
Plasmid containing the *Salmonella* plasmid virulence (*spv*) locus has an essential role in the bacterial

multiplication in organs of the reticuloendothelial system, such as the spleen and the liver [39]. Virulent plasmids play a crucial role in septicemia caused by non-typhoid *Salmonella* serovar infections. In this study, all *S. Enteritidis* isolates (27 isolates) were positive for *spvB* (Fig. 9), and common results were found in study by Amini et al. [40] that the *spv* genes were prevalent in 90% (humans), 88.6% (poultry), and 100% (bovine) [40]. In contrast, Smith et al. [41] reported lower prevalence (5%) of *spvB* gene in *Salmonella* spp. from meat samples [41]. However, the distribution of virulence genes among *Salmonella* has been confirmed by other researchers irrespective of the hosts [42].

Detection of virulence genes of *S. Enteritidis* has been studied widely. Mezal et al. [43] conducted a study on 60 isolates of *S. Enteritidis* from animals and humans targeting virulence genes including *invA* and *spvB* [43]. The result showed that the *invA* gene was found to be 100% positive, but two isolates were negative for the *spvB* gene, whereas in this study, *spvB* genes were found in all *S. Enteritidis* isolates. In the study of Osman et al. [44] which detected several virulence genes including *invA*, *ssaQ*, and *mgtC* in three isolates of *S. Enteritidis* from imported ducks and two isolates of *S. Enteritidis* from domestic ducks. As a



**Figure 8.** Amplification of *pipA* gene on SPI-5 of *S. Enteritidis*. All isolates (27/27) showed positive *pipA* results (406-bp). NTC: non-template control; positive control: *S. Enteritidis* ATCC 13076 showed positive of *pipA* gene results (406-bp).



**Figure 9.** Amplification of *spvB* gene on Spv of *S. Enteritidis*. All isolates (27/27) showed positive *spvB* results (717-bp). NTC: non-template control; positive control: *S. Enteritidis* ATCC 13076 proved positive of the *spvB* gene (717-bp).

result, all isolates (5/5) were positive for the *invA* gene, but in the imported duck, there was one isolate negative for the *mgtC* gene and three isolates were negative of the *ssaQ* gene [44]. These results indicate that although in the same serovar, *S. Enteritidis*, the virulence genes they possess may differ because in this study all *S. Enteritidis* isolates had virulence genes (*ssaQ* and *mgtC*), whereas in the study of Osman et al. [44], there are several *S. Enteritidis* that do not have the *ssaQ* and *mgtC* genes. The differences in the occurrence of virulence genes may be due to the transfer of virulence genes obtained through horizontal transfer mechanisms.

Two key virulence properties possessed by *S. enterica* species including *S. Enteritidis* are the capability of attacking nonphagocyte cells such as enterocytes and phagocytic cells such as macrophages. *Salmonella* in phagocytic cells is facultative intracellular pathogens so that they can replicate in eukaryotic host cells [8]. The results of the study found that all *S. Enteritidis* isolates had six virulent genes, indicating they are capable of infecting and survive in the host's body.

## Conclusion

Isolates of *S. Enteritidis* from layer and broiler farms in Java Island carried virulence genes related to SPI-1 to SPI-5 and plasmids virulence. The existence of virulent *S. Enteritidis* in both layer and broiler farms can be a source of human and animal infection through contaminated eggs, meat, and the environment.

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## Conflict of interests

The authors declare that they have no conflict of interest.



## Authors' contribution

EA designed the study and the manuscript drafted under the supervision of AI and NLPIM. IR and I collected samples and compiled the resource materials. EA conducted the experimental and data analysis under the supervision of AI. EA performed the molecular test detection of virulence genes under the supervision of NLPIM. The final manuscript has been read and approved by all authors.

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