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Detection of virulence and specific genes of *Salmonella* sp. indigenous from Jember, Indonesia

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Abstract. *Yulian R, Narulita E, Iqbal M, Rochmiyah D, Suryaningsih I, Ningrum DEAF. 2020. Detection of virulence and specific genes of* Salmonella *sp. indigenous from Jember, Indonesia. Biodiversitas 21: 2889-2892.* The bacterium *Salmonella sp.* is the most common cause of foodborne infection in humans. It has the ability to *inv*Ade the host, Salmonellosis is highly influenced by the combination of chromosome and plasmid in which *Salmonella* Pathogenesis Islands (SPIs) are the biggest gene in the chromosome that are responsible to build specific interaction between *Salmonella* and the host. This research performed detection on virulence and specific genes in the SPIs area using PCR method for two *Salmonella* spp. bacterial isolates originated from Jember which were KP2 and P21D. Seven pairs of primers including *stn, fimA, spvR* (virulence genes), *invA, ivaB, spvC,* and *fliC-d* (specific genes) were used. The result revealed that P21D bacteria containing 3 virulence genes i.e. *stn, fimA,* and *spvR*. Meanwhile, only two virulent genes *stn,* and *spvR* were present in KP2 bacteria. For the detection of specific genes, three genes *invA, ivaB,* and *spvC* were present while fliC-d genes were not detected. Only two, *invA,* and *ivaB* genes were detected in P21D bacteria, while *spvC,* and *fliC-d* were not detected.

Keywords: *Salmonella,* SPIs, virulence gene, specific gene

INTRODUCTION

All genes in the *Salmonella* chromosome are in the area that is called *Salmonella Pathogenesis Island* (SPIs) (Chaudhary et al. 2015; Andesfha, et al. 2019). SPIs in the *Salmonella* chromosome is responsible for building interaction between the *Salmonella* and the host (Sabbagh et al. 2010). The genes in the SPIs area can be treated as molecular marks to identify *Salmonella* and also the virulence type of those bacteria (Kumar et al. 2006). Specific genes that could be used to identify *Salmonella* bacteria had been widely reported. One of them was the detection of *S.typhi* using PCR with primer pairs such as *invA, ivaB, fliC-d, spv* genes (Kumar et al. 2006). *viaB, prt,* and *fliC-d* genes, each of them, was used to encode in synthesizing Vi (Capsule), O (LPS), and H antigen (flagellar) (Shanmugasamy et al. 2011). The virulence genes, i.e. *stn, fimA,* and *spvR* had been widely reported on many research especially for detection virulence genes in bacteria caused of foodborne disease (Naravaneni and Jamil 2005), for classification of *Salmonella* (Alphons and Jaap, 2005; Card et al. 2016), as well as for knowing nontyphoidal *Salmonella* in gastroenteritis (Araque 2009; Aoki et al. 2017).

The amplification of *invA* genes has been internationally acknowledged for the detection of *Salmonella* genus. *invA* gene is the primary gene in the operon *invABC* which encodes the protein in the inner membrane of *Salmonella* that is responsible for *inv*Ading intestinal epithelial cells of the host (Phumkhachorn and Rattanachaikunsopon 2017). *flic-D* gene-encoded flagellin protein genes in phase 1 (Shanmugasamy et al. 2011). Genes *ivaB* and *fliC-d* encodes the synthesis of Vi (capsule), O (LPS), and antigen H (flagellar). Each antigen becomes the basis of detection and classification for *Salmonella* by Kauffmann-White scheme (Kumar et al. 2006; Mthembu 2019; Figueira and David 2012). *spv* gene holds the role in systemic infection by infecting host in the macrophage. *spv* gene allows to increase the virulence of *S. enteritidis* in the internal organs because one main function of *spv* operon is to potentiate the systemic spread of pathogen (Chaudhary et al. 2015). This research used *spvC* which was virulence-related gene in plasmid that is needed to survive in the host cell (Tekale et al. 2015).

Regarding the existence of virulence and specific genes of *Salmonella*, this research used two Jember *Salmonella* indigenous bacteria namely P21D and KP2 which were isolated from Puger and Kencong. There were 255 cases of food poisoning recorded in 2016 in Kencong and Puger (Diah 2016). Even though the status was decreasing, the bacterial contamination in Kencong area and surrounding was still detected in the early of March 2017 from the sampling conducted by Jember Health Department in which, in the sampling process, *Salmonella sp.* bacteria were still found. The recent case of food poisoning in Jember was in early of January 2020 (Health Crisis Center). Thus, this study aimed to detect virulence and specific gene contained in Jember *Salmonella* indigenous bacteria.

MATERIALS AND METHODS

Procedures

Salmonella *sp. culture*

Two *Salmonella* isolates (KP2, and P21D) were obtained from fish and vegetable wastes from Puger and Kencong, respectively. Reactivation was done by inoculating the bacteria from SSA medium to *Luria-Bertani* medium (LB). The bacteria were cultured for 24 hours in a shaker.

Isolation and extraction DNA of Salmonella *sp.*

The bacteria were grown in LB medium for 24 hours which then 1000µl aliquot was retrieved and centrifuged at 4°C, 1000rpm for 10 minutes. The obtained supernatant contained DNA transferred to other Eppendorf tubes and stored at 4°C. DNA extraction by boiling was done with a modification. The collected material was placed into a tube containing 40 µL, then boiling for 10 minutes. The mixture was centrifuged at 12000 rpm for 10 minutes. The supernatant was collected and 70µL volumes of cold absolute ethanol were added, then centrifuged for 20 minutes. The pellet was washed in EtOH, dried the genome, and resuspended in TE buffer (Dashti et al. 2009).

DNA amplification

The first PCR amplification process was conducted using gradient temperature to obtain optimum temperature for each primer pairs (Table 1). The gradient temperature setting was based on melting temperature (T_m) of each primer at 5°C below of T_m with 35 cycles. The PCR condition was denaturated at 95°C for 2 minutes, followed by 35 cycles, denaturation at 95°C for 30 seconds, annealing temperature at 46.9°-50.8°C for 30 seconds, and extension at 72°C for 45 seconds, and final extension at 72°C for 7 minutes (Sunar et al. 2014). The amplification products were separated by electrophoresis in 1% agarose gel stained with 5µg-/-ml ethidium bromide using 1kb DNA ladders as molecular-weight size marker.

RESULTS AND DISCUSSION

The research step initiated by conducting genomic DNA extraction using colony and boiling PCR method which was then checked using electrophoresis in 1% agarose gel. The genomic visualization results of two isolates were seen for >1kbp band (Figure 1). Next, in PCR process, the amplification result of both *Salmonella* sp. samples which were KP2 and P21D showed difference in annealing temperature for each primer. The *stn* gene was amplified at annealing temperature of 46,9°C. Meanwhile, both *spvR* gene, and *fimA* gene were amplified at the annealing temperature of 50,8°C. For specific genes, the optimum annealing temperatures were *invA* gene (50,8°C), *ivaB* gene (47°C), *spvC* gene (57°C), while *fliC-d* gene did not show any amplified band at the optimum temperature gradient between 47°C - 54°C. The amplification result of *stn* gene showed there were 260 bp DNA bands on both samples (Figure 2). *Salmonella enterotoxin* (*stn*) is a gene encoding *stn* protein that causes gastroenteritis leading to nausea, vomiting, abdominal cramps, fever, and diarrhea. *stn* gene is detected in all *Salmonella* strains from broilers (Fekry et al. 2018). The virulence genes detected in this study were the *stn* gene, the *fimA* gene, and the *spvR* gene. The three genes in the *Salmonella* sp. are pathogenic. Virulence genes encode products that assist organisms in expressing virulence in host cells (Muthu et al. 2014). According to Thomas and Wigneshweraraj (2014) products of genes that facilitate successful colonization and bacterial survival or cause damage to the host are considered as determinants of virulence. The presence of virulence genes in *Salmonella* is related to the causes of salmonellosis and foodborne diseases in humans. *Salmonella* virulence is a combination of chromosome and plasmid factors (Chaudhary et al. 2015).

Table 1. Primers for detection of virulence and specific genes of *Salmonella* sp.

Gene target	Primer sequence (5-3')	Target size (bp)	Reference
fimA	F: CCT TTC TCC ATC GTC CTG AA R: TGG TGT TAT CTG CCT GAC CA	85	Chaudhary et al. (2015)
Stn	F: CTT TGG TCG TAA AAT AAG GCG R: TGC CCA AAG CAG AGA GAT TC	260	Chaudhary et al. (2015)
spvR	F: CAG GTT CCT TCA GTA TCG CA R: TTT GGC CGG AAA TGG TCA GT	310	Chaudhary et al. (2015)
invA	F: GTG AAA TTA TCG CCA CGT TCG GGC AA R: TCA TCG CAC CGT CAA AGG AAC C	284	Chaudhary et al. (2015)
ivaB	F: GTT ATT TCA GCA TAA GGA G R: ACT TGT CCG TGT TTT ACT C	599	Phumkhachorn and Rattanachaikunsopon (2017)
fliC-d	F: ACT CAG GCT TCC CGT AAC GC R: GGC TAG TAT TGT CCT TAT CG G	763	Phumkhachorn and Rattanachaikunsopon (2017)
spvC	F: ACT CCT TGC ACA ACC AAA TGC GGA R: TGT CTT CTG CAT TTC GCC ACC ATC A	571	Chaudhary et al. (2015)

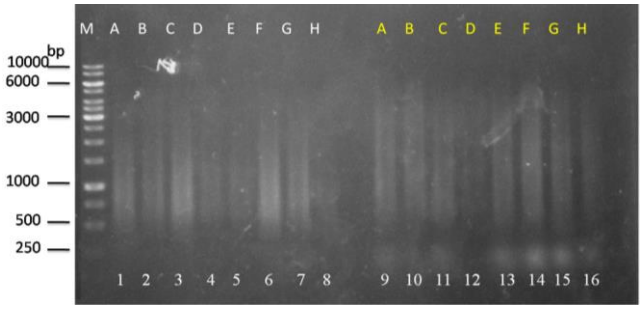

Figure 1. Electroforegram of *flicd* amplification showed no band at all. M: marker; line 1-8: *Salmonella* sp. P21D; and line 9-16: *Salmonella* sp. KP2

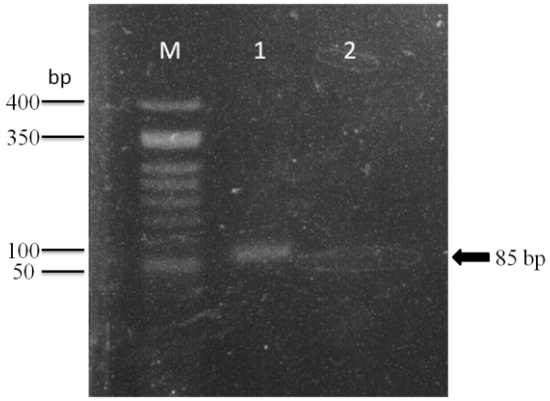

Figure 3. *fimA* gene with size of 85 bp. Line M: marker; line 1: *Salmonella* sp. P21D; and line 2: *Salmonella* sp. KP2

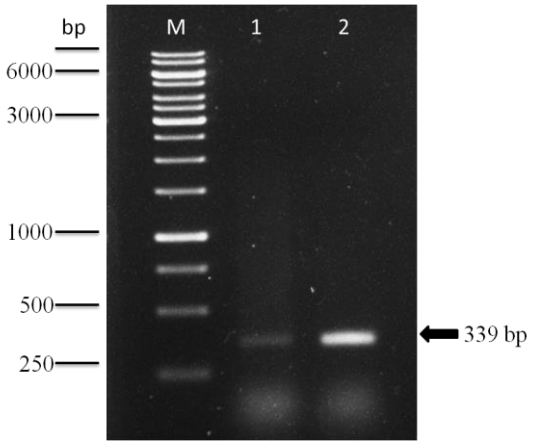

Figure 2. *stn* gene with size of 339 bp. Line M: marker; line 1: *Salmonella* sp. P21D; and line 2: *Salmonella* sp. KP2

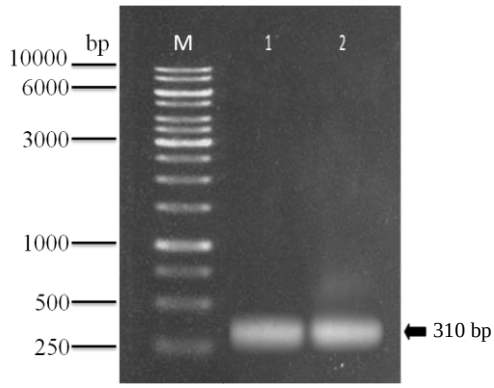

Figure 4. *spvR* gene with size of 310 bp. Line M: marker; line 1: *Salmonella* sp. P21D; and line 2: *Salmonella* sp. KP2

Salmonella sp. is a member of Enterobacteriaceae, Gram-negative, rod-shaped, non-spherical bacteria, and generally moves using peritrich flagellum (Araque 2009). *Salmonella* bacteria have facultative anaerobic properties to grow at a temperature range of 5-45°C with the optimum at 35-37°C. Other characteristics of *Salmonella* are that they multiply by dividing, easy to grow on simple mediums, and resistant to certain chemicals like. The DNA band of *fimA* gene only appeared on P21D while KP2 produced no bands (Figure 3).

The appearance of DNA band on the P21D indicated the presence of *fimA* gene while bacteria KP2 have no *fimA* gene. *fimA* is a fimbriae encoding gene in *Salmonella* (Zeiner et al. 2019). Fimbriae are found a lot on the enteric bacteria surface and mediating the attachment to eukaryotic cells. Type-1 fimbriae protein of *Salmonella typhi* was encoded by *fimA* gene group, with *fimA* assigned as single transcription unit. Based on amplification result of sample KP2, these bacteria have no type 1 fimbriae protein which contain *fimA* gene. Sample KP2 might have another fimbriae gene from *fimbrial gene cluster*. *FimA* is a fimbria coding gene in *Salmonella*. Fimbria is found in many surfaces of enteric bacteria and functions for attachment to eukaryotic cells.

Bacteria can be classified based on phylogeny (to see the relationship between *Salmonella* bacteria). A phylogenetic tree can be made by looking at the genome sequence of the bacteria using 16S rRNA analysis. Around 2,463 *Salmonella* serotypes were placed under 2 species due to differences in the genome sequence of the 16S rRNA analysis results, including *Salmonella enterica* (2443 serotypes) and *Salmonella bongori* (20 serotypes).

spvR gene could be amplified on the two samples (Figure 4). The size of amplified DNA was 310 bp. The appearance of single band on the amplification result revealed that both samples possessed gen *spvR* with thick band displaying a high DNA concentration. *spv* gene (*Salmonella plasmid virulence*) is a 7.8 kb locus consisting of 5 genes, *spvR*, located in a plasmid which is generally associated with some serotypes. *spv* gene possesses the ability to increase the severity of enteritis, infection, and persistence at extra-intestinal sites (Oliveira et al. 2003; Alphons and Jaap 2005; Card et al. 2016). The spv gene has the ability to increase the severity of enteritis and

infection and persistence at the extraintestinal location (Oliviera et al. 2003).

invA gene was amplified on the two *Salmonella* samples revealed a 244 bp band (Figure 5A). On the P21D bacteria sample, the band was slightly thinner than the band on KP2. The thickness of the bands may be due to the concentration of DNA in the bacteria. *invA* gene is the specific primer that owns solely by genus *Salmonella* which means that the two bacterial samples used in this research belong to *Salmonella* genus (Phumkhachorn and Rattanachaikunsopon 2017). *invA* gene makes the epithelial cell *invA*sion process, so when s*almonellosis* occurs, this gene will appear. Forty isolates of *Salmonella* had been studied for detection of *invA* gene using PCR, and *invA* appeared in all of the isolates (Sharma and Das 2016). The amplification result of *ivaB* primer gene on the two samples could be seen from the 599 bp band (Phumkhachorn and Rattanachaikunsopon 2017). Sample P21D has a thinner band than sample KP2 (Figure 5B). *ivaB* gene is especially owned by *Salmonella typhi* dan *Salmonella paratyphi.* The function of this gene is to operate in *viaB* area where locus on chromosome contains specific structural genes to express antigen Vi (Virulence) (Xiong et al. 2017).

From the result of the amplification, *spvC* primer gene only appeared in sample 2 (KP2) at 571 bp (Figure 6), while sample 1 (P21D) did not contain *spvC* gene. Gene *spv* (*Salmonella plasmid virulence*) is a 7.8 kb locus consisting of 5 genes namely *spvRAB* (Alphons and Jaap 2005; Card et al. 2016). P21D bacteria did not contain *spvC* gene, so they do not possess the ability in partial virulence. However, it is possible for P21D sample to have the other 5 *spv* genes. According to the research, *spv* primer gene, *spvR, and psvC*, on 102 *Salmonella enteritidis* samples from poultry, human feces and foodborne disease plague were successfully amplified (Oliveira et al. 2003). The outcome was detection of 91.2% *spvR* and 90.2% *spvC* virulence genes from all *Salmonella enteritidis* samples. *spvR* gene has a role to regulate LysR protein to express other *spv* genes (Alphons and Jaap 2005; Card et al. 2016). The absence of *spvR gene* might result in avirulence while the absence of *spvC* gene could show partial virulence. *Salmonella Enterica Serovar Typhimurium* strain, found on the rat, was inoculated subcutaneously, and contained *spvB* gene (Matsui et al. 2001). *spvB* gene expressed ADP-ribosylation enzyme which inhibited the process of polymerization actin in host cell, so it could inhibit the fusion between phagocyte and lysosome (Chu et al. 2016). *spvD* gene reduced the pro-inflammation regulation by inhibiting promotor activation which was controlled by NF-kB (Silva et al. 2017; Liu et al. 2017). The *spvR* gene functions to regulate the LysR essential protein to express other spv genes. Without the spvR gene, it may be avirulent while the absence of the *spvC* gene can show partial virulence.

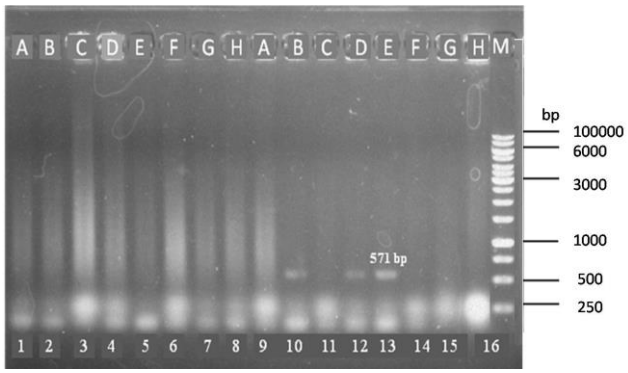

Figure 6. Electroforegram of *spvC* gene amplification of 571 bp. Line M: marker; line 1-8: *Salmonella* sp. P21D; and line 9-16: *Salmonella* sp. KP2

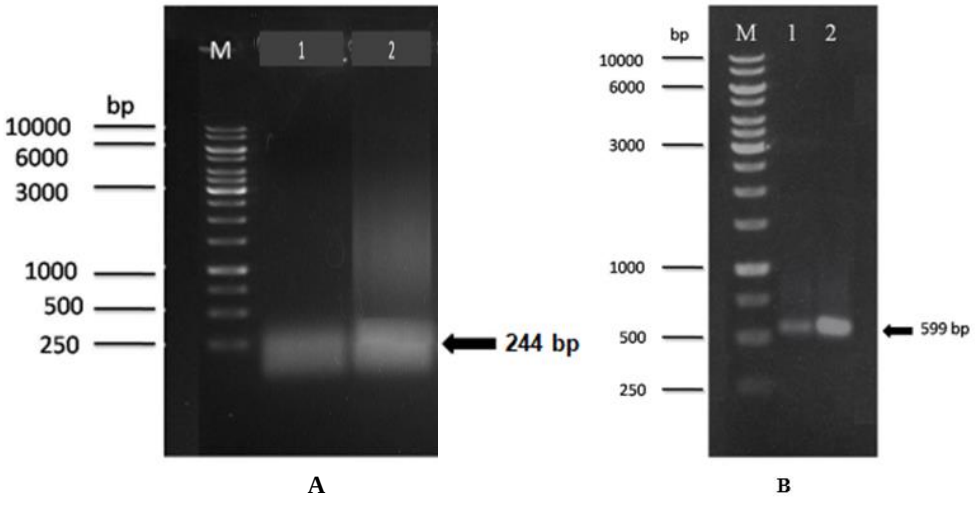

Figure 5. Electroforegram of *invA* (A) volume 25 µL and i*vaB* (B) volume 3 µL genes with size of 599 and 244 bp, respectively. Line M: marker; line 1: *Salmonella* sp. P21D; and line 2: *Salmonella* sp. KP2

In summarize, the detection result of virulence and specific genes on two *Salmonella* sp. bacterial isolates from Jember showed the presence of virulence genes namely *stn*, *fimA*, and *spvR* in P21D isolate. Meanwhile, the specific genes were *invA*, dan *ivaB*. In the KP2 isolate, virulence genes were *stn*, dan *spvR* while the detected specific genes were *invA*, *ivaB*, dan *spvC*. Many virulence factors have been shown to play various roles in the pathogenesis of *Salmonella* infections. These factors include flagella, capsules, plasmids, adhesion systems, and type 3 secretion systems (T3SS) encoded on the pathogenic islands of *Salmonella* (SPI)-1 and SPI-2 and other SPI.

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