

Prevalence of *Salmonella* spp. in fresh fish and shrimp in Semarang's Traditional Markets (Indonesia) through Polymerase Chain Reaction

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Abstract. Amalia U, Darmanto YS. 2020. Prevalence of *Salmonella* spp. in fresh fish and shrimp in Semarang's Traditional Markets (Indonesia) through Polymerase Chain Reaction. *Biodiversitas* 21: 2500-2505. Fresh fish products are rich in macronutrients for meeting the protein needs of the consumer's community. Semarang is a city located in the North Coast of Java, indirectly closely related to the production of catches and processed fish and shrimp, including several large markets in Semarang, e.g.: Rejomulyo, Jatingaleh, and Rasamala. The problem was that there are still many fresh fish and shrimp traders in traditional markets who did not understand how to handling of fresh fish and shrimp properly so that it triggers the growth of bacteria, one of which is *Salmonella* spp. Indonesian National Standard requires that *Salmonella* must be negative in food, because these bacteria are pathogenic. Generally, detection of *Salmonella* in food using conventional methods requires 5 days. This study aims to determine how far the prevalence of *Salmonella* in fresh fish and shrimps in three traditional markets in Semarang through Polymerase Chain Reaction, Real-Time PCR compare with conventional methods. The results showed that 4 out of 9 fresh fish samples (44%) and also 5 out of 9 fresh shrimp samples (55.6%) in three traditional markets in Semarang was contaminated by *Salmonella* spp through conventional method; while all of samples (100%) was detected *Salmonella* spp using PCR.

Keywords: Fish, PCR, prevalence, *Salmonella*, shrimp

INTRODUCTION

Fishery products have been widely used as protein sources and non-oil export commodities. CSDMFFI (2019) recorded that until the end of the year export of fishery products towards the end of 2018 reached US \$ 5 billion, up 10 from 2017 which amounted to US \$ 4.52 billion. Shrimp became the main commodity, which reached US \$ 1.8 billion, with a volume of 180,000 tons. In Central Java, shrimp's distribution has increased from January to August 2018. The export increased is also driven by the awareness of stakeholders, especially suppliers of fishery processing raw materials-in meeting the requirements for quality control certificates to support export transactions abroad. This indicates that the quality assurance of Central Java's exported fishery products is also increasingly gaining recognition in the international market. However, besides export orientation, the distribution of fishery products in the form of fresh fish and shrimp is also spread in several traditional markets in Semarang. For example, Rejomulyo Market or better known as Kobong Market, has a high activity of buying and selling fresh fishery products. Many fish traders from various cities in the province of Central Java such as Kendal, Salatiga buy fish in the market to be resold in their home city. Likewise, the presence of fresh fish and shrimp in Jatingaleh Market and Rasamala Market, Banyumanik to meet household needs in the upper and surrounding Semarang region. The quality and sanitation standards applied for fresh fishery products to be consumed are actually the same, but there are still many fish and

shrimp traders found in traditional markets that have not applied the basic principles of sanitation and proper handling of fishery products. This can accelerate the process of deterioration in the quality of fresh fish and shrimp that begins with the growth of bacteria, one of which is *Salmonella* spp.

Prevalence of *Salmonella* spp. on fresh fish and fisheries products from South East Asia's countries has been reported, such as in 2011 USFDA, Kumar et. al. (2015) and Maritsa et. al. (2018) found the prevalence of *Salmonella* spp on fish and fresh chicken sold in traditional market around Jambi University. Semarang as a big city also has high activity related fresh fisheries market, especially in 3 big traditional market, i.e: Rejomulyo, Jatingaleh, and Rasamala's markets. Generally, for verifying the quality, guarantee system has done by final product assays, so the product standardization has fulfilled. Even though safety couldn't be guaranteed, product assays could be convinced the consumers. *Salmonella* spp. 's assays by conventional method generally require long time. By technology, many methods have been developed. *Salmonella* spp.'s detection method based on PCR has been reported by Amalia et. al. (2014), Jeyasekaran et al. (2012), and Upadhyay et. al. (2010). This study aims to know the sensitivity of conventional method, PCR and Real-Time PCR related *Salmonella*'s prevalence detection in fresh fish and shrimp at 3 big markets in Semarang city, Central Java, Indonesia.

MATERIALS AND METHODS

Study area

Sample used for this study were fresh fish and shrimp obtained from traditional market (Jatingaleh, Rasamala, and Rejomulyo) in Semarang, Central Java, Indonesia.

Procedures

Sample collection and bacterial enrichment

Positive control used *Salmonella* Typhimurium obtained from Medical Lab, Diponegoro National's Hospital and also media for conventional detection method such as Buffered Peptone Water (BPW) (Oxoid, England), Rappaport-Vassiliadis Broth (RVB), Muller-Kauffmann Tetrathionate-Novobiocin Broth (MKTtnB), *Brilliance Salmonella* Agar (BSA) and Xylose Lysine Deoxycholate Agar (XLDA), Triple Sugar Iron Agar (TSIA), Lysine Iron Agar (LIA); biochemical assays Microgen GN-ID Test Kit; rapid detection methods: PCR Master Mix (Fermentas), sterile distilled water, DNA template, and primer targeting *invA* of *Salmonella* obtained from AIT-Biotech (Singapore).

Salmonella spp. identification

Fresh fish and shrimp samples were taken from three traditional markets in Semarang. Pre enrichment of the sample was carried out by putting 25 g of each sample (minced fish meat and minced shrimp meat) into 225 ml of BPW and incubated at 37°C for 18 h. RVB is incubated at 41.5°C for 24 h and the MKTtn Broth at 37°C for 24 h. Respectively, followed by selective media of XLDA and BSA and incubated for 24 h at 37°C. For confirmation, colonies of presumptive *Salmonella* are subcultured and their identity is confirmed by TSIA and LIA.

DNA Isolation for PCR

DNA was isolated from 1 mL aliquots of overnight bacterial culture in Tryptone Soya (TSB) (LP0042, OXOID). In brief, one mL aliquot of bacterial culture is centrifugated at 4,000 x g for 10 min and the cell pellets were suspended in 190 µL TE Buffer (Invitrogen) and 10 µL Lysozyme (Invitrogen) and incubated at 30°C for 30 min. The cell suspension is centrifugated at 4,000 x g for 5 min and the cell pellets were resuspended in 400 µL of DNA *Lysis Buffer T* (Invitrogen), added with 20 µL Proteinase K (Invitrogen), 15 µL RNaseA (Promega) and mixed well by brief vortexing. The mixture was incubated at 70°C for 30 min and 200 µL of DNA *Binding Buffer* (Invitrogen) was added. Purification was conducted by adding the mixture into spin column and centrifugation at 10,000 x g for 1 min. The spin column containing solid phase was moved to new tubes, added with 650 µL DNA *Wash Buffer* (Invitrogen) contained 100% ethanol and centrifuged at 10,000 x g for 10 min. the spin column was placed in a new sterile 1.5 mL microcentrifuge tube and added with 50 µL DNA *Elution Buffer* (Invitrogen) incubated at room temperature for 3 min. DNA precipitation was carried out by centrifugation at 6,000 x g for 1 min. To recover more DNA, a second elution step was performed using the same volume of the same elution

buffer. The clear supernatant (DNA) was collected and stored at -20°C for PCR. The concentration of DNA was determined by optical density (OD) at the wavelength of 260 nm and its purity was evaluated by the ratio of OD at the wavelength of 260/280. The DNA were kept at -20°C until further use.

PCR assays

The specific primer for PCR assays used a forward primer F139 (5'- GTGAAATTATCGCCACGTTCCGGG CAA – 3') and a reverse primer R141 (5' – TCATCGCACCGYCAAAGGAACC – 3'). This primer amplify gene encoding for *invA* located within 287 to 312 and 571 to 550 bp regions (AIT-Biotech Singapore). PCR amplification was performed with PCR Applied Biosystem 2720 Cycler using PCR mixtures containing 1.5 µL of primers, 7.5 µL PCR Master Mix. The thermocycling condition were 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 64.5°C for 30 sec and elongation at 72°C for 30 sec, with final extension at 72°C for 4 min. The PCR products were visualized by electrophoresis on 1.5% agarose gel (Fermentas, USA) at 100 V for 30 min, stained with SYBR SAFE (0.6 µg/100 mL), visualized under UV transilluminator (Bio-Rad) and photographed. DNA of *S. Typhimurium* and sterile aquabidest were used as positive and negative control, respectively.

Real-time PCR assays

The PCR products (400 µL) were used in PCR assays was transferred to a 1.5 mL microtube containing 100 µL ddH₂O solution and 0.5% saponin solution in 1mL of 10-fold dilutions of PBS (Phosphate-Buffered Saline), then stored at 4°C for 24 hours. The stored sample was centrifuged at 12,000 rpm for 10 minutes, then discarded the supernatant. 1 mL of PBS solution was added to the microtube and centrifuged at 12,000 rpm for 5 minutes, then discarded the supernatant. 100 µL of ddH₂O solution and 50 µL of 20% Chelex were added to the microtube, then incubated at 90°C-100°C for 10 minutes and vortex. The sample was then centrifuged at 12,000 rpm for 10 minutes and a 1 mL supernatant was transferred to a new microtube and stored at -20°C before use.

RESULTS AND DISCUSSION

Salmonella spp.'s detection on fresh fish and shrimp by conventional methods

The results showed that from each of the 9 fish samples and 9 fresh shrimp samples grown on TSB media, all showed turbidity (positive). The next stage is selective enrichment by using two types of media namely Muller-Kauffmann Tetrathionate-Novobiocin Broth (ISO) (MKTtn) and Rappaport Vassiliadis (RV). Both of these media selectively enriched the amount of *Salmonella* found in the sample, all of them showed positive results, in the form of turbidity and precipitation on MKTtn media and turbidity on RV media. In MKTtn media, selective compounds in the form of bile salts inhibit the growth of

gram-positive bacteria. Novobiocin supplement contained in the media can increase *Salmonella* growth. In addition there are selective compounds such as sodium thiosulfate and tetrathionate to inhibit the growth of coliform bacteria. Tetrathionate is formed in the media due to the addition of iodine and potassium iodide (I₂-KI). On MKTtn media, *Salmonella* can grow because it has the enzyme tetrathionate reductase (Post 1997). The presence of the tetrathionate reductase enzyme in *Salmonella* causes *Salmonella* to be resistant to the toxic effects of tetrathionate (S₄O₆²⁻) during enrichment. On RV media selective compounds such as malachite green and magnesium chloride combined with a low pH (5.2 ± 2) inhibit the growth of natural microbes originating from the digestive tract other than *Salmonella* (D'Aoust 1989). In addition, the growth of *Salmonella* is also supported by the presence of soy peptone in the media. The soy peptone contained in the RV media serves as a source of nitrogen, carbon, and amino acids for *Salmonella*.

Salmonella's isolation was then performed using two specific media namely Xylose Deoxycholate Agar (XLDA), and Brilliance *Salmonella* Agar (BSA). Typical colonies on XLDA media were pink with or without black in the middle, some appeared as large, shiny black colonies in the center or appeared as colonies that were all black (BAM 2017). Typical colonies in BSA were purple. Around the colony usually be blue at first and became black with increasing incubation time, which is called the halo effect. Typical or non-typical *Salmonella* colonies isolated from XLDA and BSA media were then inoculated on agar media to tilt Triple Sugar Iron Agar (TSIA) and Lysine Iron Agar (LIA) for biochemical confirmation by means of scratches and pricks, then their growth was observed after incubation at 35 ± 2 ° C for 24 hours. The biochemical confirmation of TSIA was marked by the formation of red on the surface and yellow (producing acid) at the bottom of the tube, with or without gas forming on the agar. The red color occurred because *Salmonella* can ferment a limited amount of glucose in the media, so that if the glucose runs out this bacterium uses peptone as an energy source that occurs on the surface of the agar and produces a byproduct in the form of a base (red). The formation of H₂S was marked in black because the sodium

thiosulfate content in order to be reduced by H₂S which then reacts with iron salt produces a black color. Biochemical confirmation in LIA was marked by the formation of purple on the surface and black on the bottom of the tube (producing H₂S). The purple color occurred because *Salmonella* can decarboxylized lysine to produce cadaverine amine, which was indicated by changing the purple bromochresol pH indicator to purple color. Table 1 shows the results that the media that produced the most positive biochemical confirmation tests on fresh fish samples were typical and atypical colonies from BSA from both MKTtn and RV selective enrichment media, where 4 and 5 of 9 fresh shrimp fish samples respectively (44.4 %) originating from MKTtn and RV were suspected to be *Salmonella*. And for the results of the media that most produce positive biochemical confirmation tests on fresh shrimp samples are typical and atypical colonies from XLDA from MKTtn and RV selective enrichment media, where each of 5 out of 9 fresh shrimp samples (55.6%) originating from MKTtn and RV suspected *Salmonella*.

Based on Table 1, it appeared that the highest possibility of getting a colony suspected by *Salmonella* in fish is to isolate the colony from both BSA media and XLDA media, both from RV and MKTtn add-on media; whereas in fresh shrimp by isolating the colony from XLDA media. In this case, XLDA media was more sensitive to isolate *Salmonella* in shrimp compared with BSA media. Research related to the identification of *Salmonella* in several food and non-food objects in the past five years using XLDA media as a selective media, as conducted by (Albino et al. 2014; Yhils et al. 2015; Oni et al. 2016; Hasan et al. 2018 and Terfassa et al. 2018). XLDA media is used as a selective media, together with media such as Brain Heart Infusion Agar (BSIA), Brilliant Green Agar (BGA) and Brilliance *Salmonella* Agar (BSA). Typical colonies on XLDA media are pink with or without black in the middle, some will appear as large, shiny black colonies in the center or appear as colonies that are all black (BAM 2007). Typical colonies in BSA are purple. Around the colony will usually be blue at first and will become black with increasing incubation time, which is called the halo effect.

Table 1. Percentage of *Salmonella* presumptive colonies after TSIA LIA's confirmation

Information	Colonies	Selective agar media		Σ positive samples of TSIA LIA and it's sensitivity	
		BSA	XLDA	BSA	XLDA
Total samples of fresh fishes = 9; fresh shrimps = 9					
Total fresh fish samples of <i>Salmonella</i> spp presumptive on selective enrichment by RV media	Typical	6	5	4 (44.4%)	4 (44.4%)
	Atypical	3	4		
Total fresh shrimp samples of <i>Salmonella</i> spp presumptive on selective enrichment by RV media	Typical	5	6	2 (22.2%)	5 (55.6%)
	Atypical	4	3		
Total fresh fish samples of <i>Salmonella</i> spp presumptive on selective enrichment by MKTtn media	Typical	6	7	5 (55.6%)	5 (55.6%)
	Atypical	3	2		
Total fresh shrimp samples of <i>Salmonella</i> spp presumptive on selective enrichment by MKTtn media	Typical	5	6	4 (44.4%)	5 (55.6%)
	Atypical	4	3		

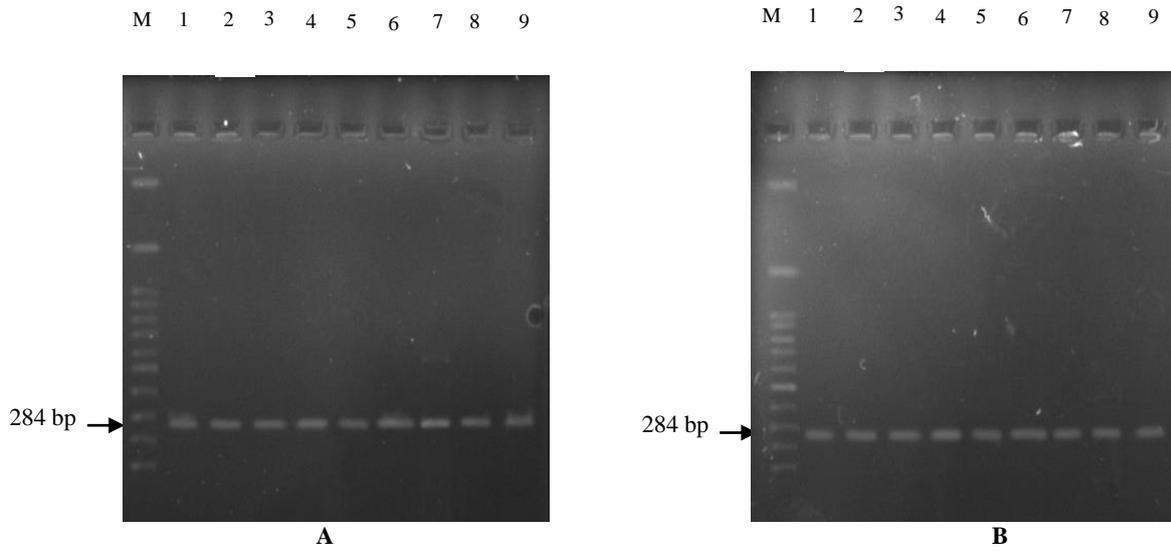


Figure 1. DNA Visualization of Amplification *Salmonella*'s *inv A* genes on 1.5% agarose. A. Line 1-9: fresh fish samples (all positive results); line M : marker DNA ladder 100 bp. B. Line 1-9: fresh shrimp samples (all positive results); line M : marker DNA ladder 100 bp

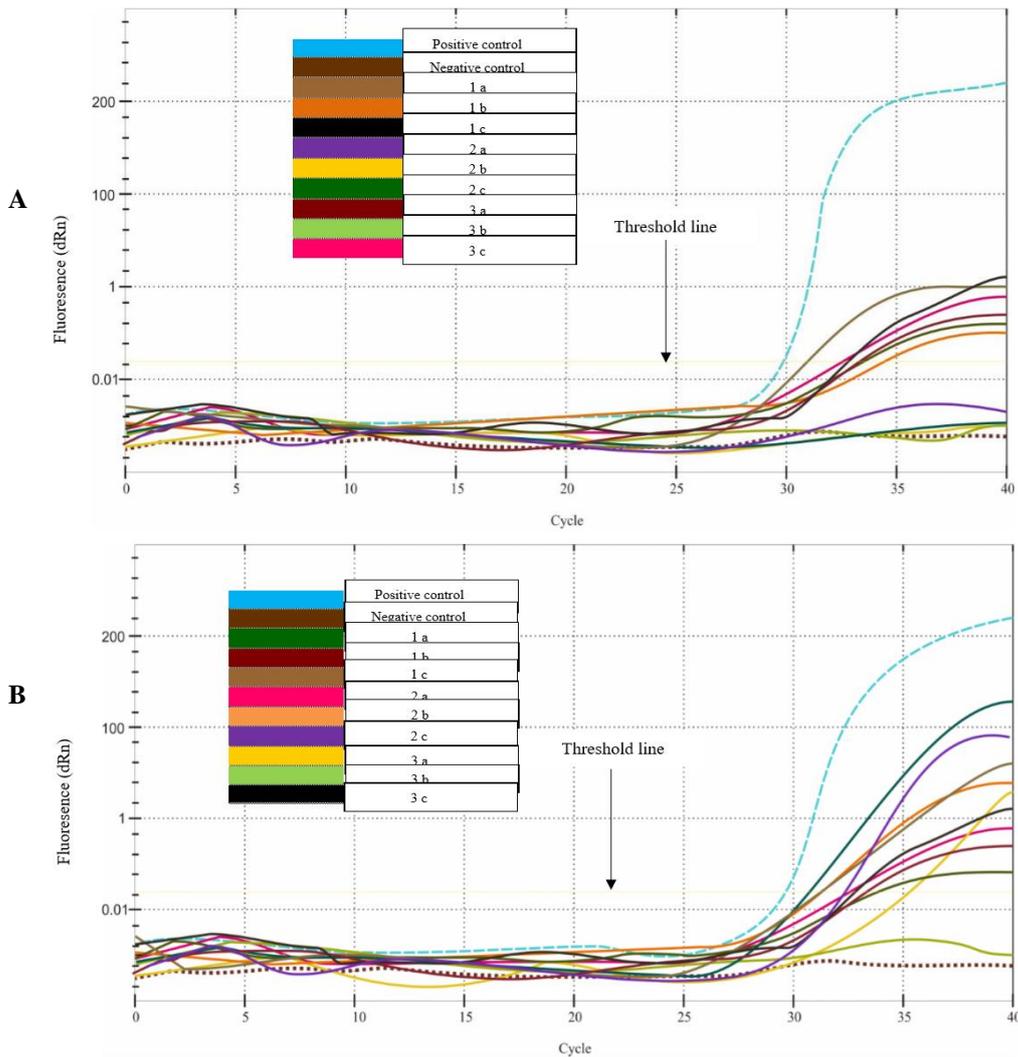


Figure 2. A. Amplification curve of fresh fish samples from 3 big traditional markets (each market consisted of 3 different seller: a, b, and c); B. Amplification curve of fresh shrimp samples from 3 big traditional markets (each market consisted of 3 different seller: a, b, and c)

Table 2. Identification results of 11 non-*Salmonella* bacteria on fresh fishes and shrimps

Total samples	Fish/shrimp	Bacteria identified	Probability percentage (%)
5	3 in fish and 2 in shrimp	<i>Proteus mirabilis</i>	96-98.9
3	Fish	<i>Enterobacter aerogenes</i>	35.57
1	Shrimp	<i>Providencia rettgeri</i>	75-99.9
1	Shrimp	<i>Serratia rubidea</i>	70-82
1	Shrimp	<i>Proteus vulgaris</i>	80.85

Positive results on TSIA and LIA media were subsequently confirmed by the Microgen GN-ID System device with GNA microwell test strips to confirm it as *Salmonella*. The GN-ID Microgen Device System microwell test strips GNA is a rapid test kit to identify bacteria in the family of Enterobacteriaceae and certain Gram-negative bacteria by making it easy to inoculate and read relevant test results. Test results with Microgen GN-ID System showed that 4 out of 9 fresh fish samples (44.4%) were *Salmonella* spp., with a very good final identification (80.89%). In fresh shrimp found 5 out of 9 samples (55.6%) *Salmonella* identified with very good final identification (81.95%). Each of the remaining samples (6 fish and 5 shrimp) were confirmed to be *Salmonella* spp. Identification of 11 types of bacteria based on these advanced biochemical tests is presented in Table 2.

***Salmonella* spp. 's detection on fresh fish and shrimp by PCR methods**

Detection results of the prevalence of *Salmonella* spp. in each of the 9 fresh fish and shrimp samples by PCR method is shown in Figure 1.

From the 18 fresh fish and shrimp samples tested by PCR, the results were obtained that all were contaminated by pathogenic *Salmonella* bacteria (100%). In this study, this indicated that PCR was more sensitive to detecting the presence of *Salmonella* through the *invA* gene amplification at 284 bp compared to conventional methods (50%). These results are similar to the research conducted by Upadhyay et al. (2010), who compared several enrichment media used before the PCR test, and concluded that the enrichment stage was very important and potential to obtain a higher detection rate, because it increased the number of cells, including DNA and mRNA. The results showed that 11 fresh fish and shrimp samples were negative *Salmonella* when tested by conventional methods, but positive with PCR indicated that *Salmonella* might be viable but not cultured (El-Safey 2013). This is because conventional methods are able to detect *Salmonella* only through living cells, which should be quantitatively calculated, can be resuscitated in pre-enrichment and selective enrichment. The amount of DNA needed to detect *Salmonella* spp. in fresh shrimp using more conventional methods, compared with the PCR method. However, the sensitivity of the PCR method is higher compared to conventional methods. The existence of *Salmonella* spp.

fresh fish and shrimp samples in traditional markets could be due to poor traditional market sanitation and hygiene practices. The main factor that allows *Salmonella* spp. in fish, fresh shrimp and other seafood found in traditional markets is *Salmonella* spp. from the digestive tract of humans or animals in the traditional market, because *Salmonella*'s main habitat is the intestinal tract of animals and humans (Hasan et al. 2018). Moreover, it can also be caused by water used to wash shrimp, equipment used such as knives, cutting boards, containers and contamination from workers and cross contamination from other foodstuffs during storage.

***Salmonella* spp. 's detection on fresh fish and shrimp by RT-PCR methods**

The results of detection of *Salmonella* spp prevalence in fresh fish and shrimp using the Real Time (RT) -PCR method were presented in Figures 2a and 2b.

Figures 2a and 2b show that with RT PCR, *Salmonella* spp's DNA in fresh fish and shrimp, the average threshold can be detected between 30 - 35 annealing cycles. This suggests that the primers used to detect the presence of the *Salmonella* spp gene are appropriate, whereas in this study the primary pair F 139 - R 140 encoding the *Salmonella* spp *invA* gene was used. These results are in line with research conducted by Nurjanah et al. (2018) and Amalia et al. (2014), in which both studies also used the same primers to detect the presence of *Salmonella* spp's DNA.

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