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Identification of *Salmonella* sp. Using ISO 6579-1:2017 Method for Frozen Salmon (*Salmo salar*) Products

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Abstract

Salmon is a type of marine fish from the Salmonidae family that lives in the Atlantic Ocean, both on the northern coast of America and Europe, as well as the Pacific Ocean. Fish and fishery products are perishable foods because they contain high amounts of protein and water. The highwater content of fresh fish accelerates the breeding process of the spoilage microorganisms contained in it. One example of bacteria that contaminate fishery products is *Salmonella*. *Salmonella* sp. is a pathogenic microorganism that causes human disease through food and drink. Contamination can occur at any food production, distribution and consumption chain. This research aims to determine the microbiological quality of fishery products in the form of frozen salmon. Tests carried out to identify *Salmonella* use the ISO 6579-1:2017 test method. This test consists of pre-enrichment, enrichment, and biochemical tests. Identification of *Salmonella* sp. bacteria on frozen salmon products at the BUSKIPM Quality Microbiology Laboratory using the ISO 6579-1:2017 standard test method showing negative test results for *Salmonella sp.* The reading of the results of the confirmation test carried out did not meet the specific requirements for *Salmonella sp.* bacteria. Therefore, no *Salmonella* was detected in any of the tested samples, indicating that the frozen salmon products examined in this study were free from *Salmonella* contamination and did not pose a risk of salmonellosis foodborne illness.

Keywords: Frozen salmon; ISO; *Salmonella* sp.

INTRODUCTION

Imports of fishery commodity in Indonesia still have a higher value and volume compared to the size of fishery exports in Indonesia. Salmon is one of the main fishery import commodities in Indonesia with an import share reaching 41%, dominated by Norway (Arthatiani & Deswati, 2020; Khairunisaa *et al*., 2024). The success of mariculture for salmon has made Norway the largest producer of salmon-trout in the world. Several other countries that import salmon to Indonesia are Japan, Australia and Chile (Putri *et al.,* 2023). The main problem with this type of food is that it is highly perishable. In addition, fishing areas and the target markets are sometimes far apart. Freezing is an excellent alternative to extend the shelf-life of the fish, as well as an efficient means of storage and transportation (Irsyad *et al*., 2021; Mayangsari & Sipahutar, 2021). Salmon is a type of marine fish from the Salmonidae family that lives in the Atlantic Ocean, both on the northern coast of America and Europe, as well as the Pacific Ocean (Apriliani & Deswati, 2020). Salmon are an anodromous group, meaning they migrate to spawn. The

migration process in salmon will result in gradual changes in body composition (Lestari *et al*., 2017). Salmon is a rich source of animal protein containing essential amino acids, healthy fats rich in omega 3 fatty acids, like *Docosahexaenoic Acid* (DHA) and *Eicosapentaenoic Acid* (EPA), and various vitamins and minerals (Eng *et al*., 2015). Apart from containing many nutrients, salmon is usually consumed with a variety of dishes. One of the famous dishes is Sushi. Sushi is a traditional Japanese dish made from raw fish and rice. A small amount of rice is balled up, seasoned with a little sugar, salt and vinegar, then wrapped or topped with slices of raw fish, crustaceans, squid or fish egg (Trahutami, 2018).

Fish and fishery products are perishable foods because they contain high amounts of protein and water. The highwater content of fresh fish accelerates the breeding process of the spoilage microorganisms contained in it. One example of bacteria that contaminate fishery products is *Salmonella* bacteria (Aulia *et al*., 2015). *Salmonella* sp. it is one of the pathogenic bacteria that causes human disease through food and drink. Contamination can occur at any stage of the food production, distribution and consumption chain. Several forms of environmental pollution can originate from water, air, soil, and improper food storage and processing (Popa & Popa, 2021). *Salmonella* sp. are the most prevalent species of pathogens implicated in food-borne infections. The effect of frozen storage of pathogenic microorganism in salmon did not decrease the potential survival of food-borne pathogens over a period of ten months (Eng *et al*., 2015; Martanda, 2019). Research by Putri *et al*. (2023) shows that there was growth of *Salmonella typhi* bacteria in 7 sushi samples (87.50%), which did not meet the requirements for consumption. 1 sample (12.50%) did not show growth of *Salmonella typhi* bacteria on *Salmonella Shigella Agar* (SSA) media, suggesting that the sushi food served, using raw or semi-ripe did not meet consumption requirements. Based on the description of the background above, the aim of this study is to identify *Salmonella* sp. with the ISO 6579-1:2017 Method in Frozen Salmon (*Salmo sp*.) Products.

METHOD

This research was conducted from March 19 to 27, 2024 at the Quality Microbiology Laboratory, Balai Uji Standarisasi Karantina Ikan, Pengendalian Mutu, dan Keamanan Hasil Perikanan (BUSKIPM). The tools used for this test are laminar air flow, incubator, water bath, stomacher, plastic stomacher, scales, aluminum foil, scissors, tweezers, micropipette, microtips, test tube rack, pen, inoculation needle, bunsen, lighter. The materials employed in this test consisted of frozen Salmon samples in Jakarta, Salmonella sp (As a control), Buffered Peptone Water (BPW), Muller-Kauffmann Tetrathionate Novobiocin (MKTTn), Rappaport-Vassiliadis Soya (RVS), Xylose Lysine Deoxycholate (XLD), Hektoen Enteric (HE), Triple Sugar Iron Agar (TSIA), Lysine Iron Agar (LIA), Sulphide Indole Motility (SIM), Kovac Reagent, Urea Agar, positive control and alcohol. Tests carried out to identify *Salmonella* bacterial contamination use the ISO 6579-1:2017 test method. The International Organization for Standardization (ISO) is an international organization responsible for developing international standard documents including specifications, requirements, characteristics or guidelines aimed at ensuring that manufacturing processes, materials and products and even services meet certain objectives (Fauzi, 2019; Cahyaningsih *et al*., 2023; Yusmila *et al*., 2025).

The detection procedure for Salmonella followed a systematic workflow, which is illustrated in Figure 1. This workflow comprised four successive stages: The initial stage is pre-enrichment in non-selective liquid medium. A buffered peptone water (BPW) was inoculated with the test sample. This step was essential because Salmonella is often present in low numbers and might be outnumbered by other bacteria. The inoculated BPW was then incubated between 34 °C and 38 °C for 18 hours to allow any injured or low-level Salmonella cells to recover and multiply. After pre-enrichment, the culture was divided for further enrichment using selective media. Two enrichment routes were followed: One portion was inoculated into Rappaport-Vassiliadis medium with soya (RVS broth) or Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar. Another portion was inoculated into Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broth. The RVS broth or MSRV agar was incubated at 41.5 °C for 24 hours, while the MKTTn broth was incubated at 37 °C for 24 hours. (In some cases, the incubation period was extended by an additional 24 hours, depending on the nature of the product.) It is important to note

that MSRV agar is specifically designed for detecting motile Salmonella strains and is not suitable for non-motile ones.

The next stage is plating out on selective solid media. From the enriched cultures, two different selective solid media were used for isolation: that is Xylose Lysine Deoxycholate (XLD) agar, and A second selective medium that is complementary to XLD. The inoculated XLD agar was incubated at 37 °C and examined after 24 hours for the presence of colonies with typical Salmonella characteristics. The last stage is confirmation. Presumptive Salmonella colonies, which either displayed typical or atypical appearances on the selective solid media, were then subcultured. Their identity was confirmed using a series of appropriate biochemical and serological tests, ensuring that only Salmonella was ultimately identified. This multi-step process, from initial recovery through to final confirmation, ensured that even small numbers of Salmonella in the presence of other bacteria were reliably detected.

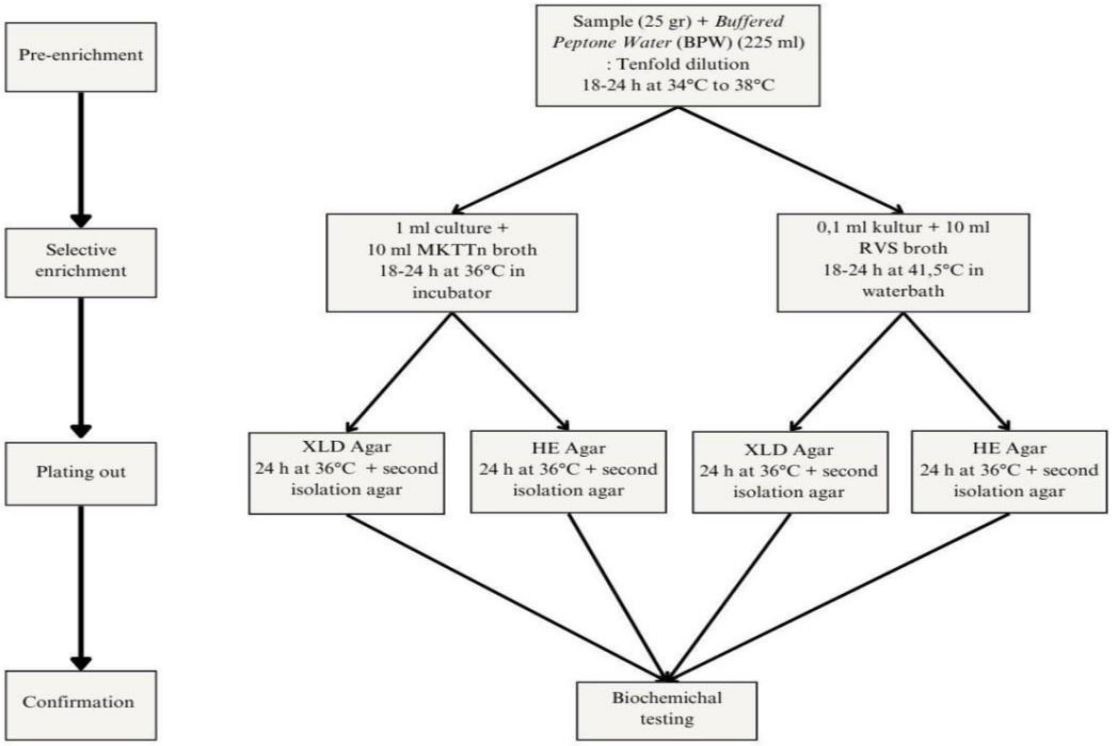
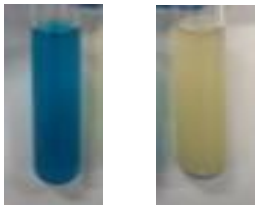


Figure 1. *Salmonella* test scheme ISO 6579-1:2017

RESULT AND DISCUSSION

In the pre-enrichment stage, testing was conducted using a non-selective medium, namely Buffered Peptone Water (BPW). BPW medium had a composition of peptone, sodium chloride, disodium phosphate, and potassium dihydrogen phosphate. This pre-enrichment stage test aimed to activate the growth of target bacteria if they were inactive or to increase the number of bacteria in the sample. The pre-enrichment stage test was considered successful or positive if it was marked by a color change in the BPW medium, becoming more cloudy (Sophian & Muindar, 2021). The enrichment stage of the test was carried out using MKTTn (Muller-Kauffmann Tetrathione Novobiocin) and RVS (Rapapport-Vasisiliadis Soya) media. Based on the test results on MKTTn and RVS, it shows that the color changes to cloudy on MKTTn and RVS (Figure 2).

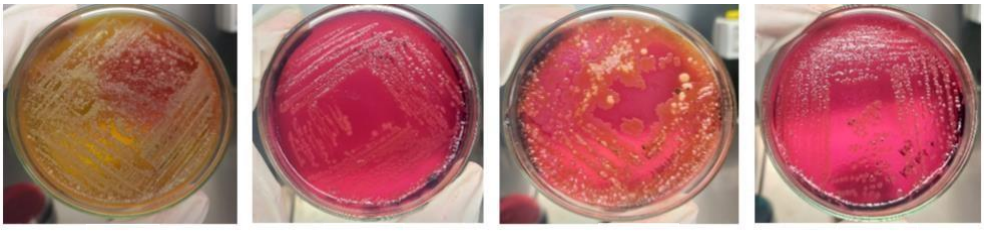


(a) (b)

Figure 2. Selective enrichment frozen salmon in media (a) RVS, (b) MKTTn.

In MKTTn media containing tetrathionate, tetrathionate served as the key selective agent. It can inhibit the growth of most enterobacteria, except *Salmonella sp.* which could convert tetrathionate into thiosulfate. Novobiocin in medium further inhibited the growth of gram-positive bacteria (Torrico *et al*., 2022). With a low pH value of around 5.2 ± 2 and various specific compounds such as magnesium chloride and malachite green in RVS media can inhibit the growth of microbes other than *Salmonella sp*. originating from the digestive tract. *Salmonella sp* bacteria. can grow because of the soy peptone content in RVS media, which provides amino acids, carbon and nitrogen. The indicator of a positive test result on RVS media is characterized by a color change from blue to light blue, cloudy, and sometimes the formation of white precipitate at the bottom of the medium (Afrida *et al*., 2020).

At this stage, bacteria were inoculated from MKTTn and RVS media into Xylose Lysine Deoxycholate (XLD) media. XLD Agar media was a differential and selective medium with a sodium deoxycholate composition, which inhibited the growth of Gram-positive bacteria (Hutasoit *et al*., 2017). This medium contained lysine compounds, which caused colonies of *Salmonella* sp. bacteria to be differentiated because the bacteria decarboxylated lysine, making the pH alkaline. XLD media contained an H_2S indicator with a composition consisting of sodium thiosulfate and ferric ammonium citrate, so when the hydrogen sulfide compound was formed, it produced a black bacterial colony in the middle (Afrida *et al*., 2020). Bacterial culture was carried out using the streak method on the surface of XLD agar, and then the samples were incubated for 24 hours at a temperature of 36°C in the incubator (Figure 3).



(a) (b) (c) (d)

Figure 3**.** Plating-out (a) bacteria from frozen salmon, (b) positive control *Salmonella* sp. in media XLD from selective enrichment MKKTn ; and plating-out (c) bacteria from frozen salmon, (d) positive control *Salmonella* sp. in media XLD from selective enrichment RVS

Based on the isolation results using XLD media on salmon samples grown on MKTTn and RVS media, the results showed negative because there were no pink colonies.The positive control results (+) on the media MKTTn and RVS shows positive results which is characterized by the presence of pink colonies (Figure 4). This was in accordance with (Putri *et al*., 2021). On XLD media bacterial colonies will appear pink, with or without shiny dots or almost all colonies will be black. This was caused by the bacteria *Salmonella sp.* can ferment xylose, decarboxylate lysine compounds and produce hydrogen sulfide (H2S) from sodium thiosulfate. The results of this fermentation can change the pH of the XLD media to alkaline so that it could changed the color of the media to pink and produced black bacterial

colonies from the process of forming hydrogen sulfide (H_2S) (Samie *et al*., 2018). At this stage the second media used was Hektoen Enteric Agar (HE) media. Bacterial culture was performed using the streak method on the surface of HE agar, and the samples were incubated for 24 hours at temperature 36°C in the incubator.

(a) (b) (c) (d)

Figure 4. Plating-out (a) bacteria from frozen salmon, (b) positive control *Salmonella* sp. in HE medium from selective enrichment MKKTn ; and plating-out (c) bacteria from frozen salmon, (d) positive control *Salmonella* sp. in media HE from selective enrichment RVS

Based on the results of isolation using HE media, the salmon samples that had grown on MKTTn and RVS media showed positive results for *Salmonella* sp., characterized by the presence of bluish-green colonies with black spots. Similarly, the positive control results (+) on MKTTn and RVS media showed positive results, also characterized by bluish-green colonies with black spots. This was in accordance with Umidayati *et al*. (2020) who stated that suspected *Salmonella* sp. colonies on HE media exhibit bluish-green coloration with black spots in the center. The first stage of the biochemical test began with bacteria being isolated using Triple Sugar Iron Agar (TSIA) media. Bacteria are isolated on a streaked on an agar slant. After the isolate was transferred, it was incubated for 24 hours in an incubator at 36°C.

(a) (b) (c)

Figure 5. Biochemical test TSIA test results (a) media TSIA, (b) bacteria from frozen salmon, (c) positive control *Salmonella* sp. in media TSIA

XLD salmon samples on MKTTn and RVS media exhibited an acid yellow color on the slant and a black-yellow base (H_2S). HE salmon samples on MKTTn and RVS media showed a basic red color on the slant and a red base without H_2S production (Figure 5). TSIA contained sodium thiosulfate, a substrate that produced H_2S, which appeared black to differentiate H_2S-producing bacteria from non-H_2S-producing bacteria. TSIA also contained 1% lactose and sucrose, 0.1% glucose, and phenol red, an indicator that changed color from purple-red to yellow (Zakaria *et al*., 2016). In the positive control (+), the slant was red, and the base was black and yellow. Because TSIA media contained sodium thiosulfate, which functioned as a substrate for the formation of hydrogen sulfide (H_2S), *Salmonella* sp. demonstrated the ability to reduce hydrogen sulfide (H_2S), producing a dark black color at the base. The deposition process of insoluble ferrous sulfide (FeS) caused the bottom to turn black (Krisnawati *et al*., 2022). The slant media on TSIA was red because *Salmonella* sp. could not ferment lactose and sucrose, whereas the

upright media was yellow due to the ability of *Salmonella* sp. to ferment glucose for growth. The second stage of the biochemical test involved isolating bacteria using Lysine Iron Agar (LIA) media. The bacterial isolate was first pricked and then streaked onto an agar slant. After the isolate was transferred, it was incubated at 36°C for 24 hours in an incubator.

(a) (b) (c)

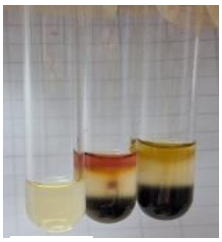
Figure 6. Biochemical test LIA test results (a) media LIA, (b) bacteria from frozen salmon, (c) Positive control *Salmonella* sp. in media LIA

The LIA test results showed that the XLD-MKTTn and HE-MKKTn salmon samples showed positive results, while the XLD-RVS and HE-RVS salmon samples showed negative results (Figure 6). On the other hand, positive control samples (+) show positive results. If the Lysine Iron Agar (LIA) test is positive, the color does not change or remains purple with or without H2S. The blue bromocresol pH indicator turns yellow at pH 5.2 or lower and purple at pH 6.8 or higher (Afrida *et al.*, 2020). *Salmonella sp*. bacteria. which has the ability to decarboxylate lysine and produce cadaverine, produces a purple color on the pH indicator of the neutral reaction LIA medium, while those which do not produce decarboxylation will be yellow. Sodium thiosulfate, the substrate that produces H2S in LIA, precipitates Ferrous sulfide (FeS) and makes the LIA base black (Christanti & Azhar, 2019). The third biochemical test isolates bacteria using urea agar media. The urease test is used to determine the ability of microbes to hydrolyze urea into ammonia. Bacterial isolates were streaked onto slanted agar, then incubated at 36°C for 24 hours in an incubator.

(a) (b) (c)

Figure 7. Biochemical testing urea test results (a) urea agar media, (b) bacteria from frozen salmon in urea agar, (c) positive control *Salmonella* sp.

According to the urea test results, samples (XLD) and (HE) overall showed negative results. This is caused by the urease enzyme not being able to break down carbon and nitrogen bonds, which produces ammonia and changes the pH of the medium (Mahmudah *et al.*, 2016). The urea test is carried out to test the ability of bacteria to produce urease. *Salmonella sp*. does not have the ability to produce the urease enzyme, but *Salmonella sp*. has the ability to ferment glucose, maltose and dulcitol (Hasanah *et al.*, 2023). The fourth biochemical test, the indole production test, aims to determine the ability of microbes to destroy the amino acid tryptophan. The results showed that the isolates obtained were unable to hydrolyze tryptophan (Gergonius & Yuni, 2016). Using a puncture, the bacterial isolate is transferred to Sulphide Indole Motility (SIM) media. To determine the reactions produced by bacteria, bacterial isolates were incubated for 24 hours in an incubator at 36°C. Then, the SIM media resulting from incubation was dripped with Kovac reagent.



(a) (b) (c)

Figure 8. Biochemical testing Indol test results (a) SIM media, (b) bacteria from frozen salmon in SIM media, (c) positive control *Salmonella* sp.

The indole test showed that the salmon sample (HE) in the media showed a negative reaction (Figure 8). The indole test on the control showed that there was no color on the surface of the culture, while the sample test showed that there was a red layer on the surface. Because *Salmonella sp*. unable to hydrolyze tryptophan, the indole test results of the sample showed that there was a red layer on the surface (Hasanah *et al*., 2023). The principle of the indole test is that the hydrolysis process of tryptophan as a carbon source is catalyzed by the enzyme tryptophanase, producing metabolic products such as indole, pyruvic acid and ammonia. The surface is marked with a red layer (Rahayu & Gumilar, 2017). Using Kovac's reagent, indole can be identified by forming a red layer or ring on the surface of the medium. This occurs because the indole in the medium is extracted into the reagent layer by the butanol acid part, which then forms a complex with p-dimethylaminobenzaldehyde. Although *Salmonella sp.* can convert glucose into acid, it cannot convert the amino acid tryptophan into indole.

The indole test can only be made by certain types of bacteria, and *Salmonella sp.* usually gives negative results (no red ring formation on the media surface). The Genus *Micrococcus, Staphylococcus, Bacillus,* and the *Hafnia alvei* species are bacteria that give negative results in the indole test (Ihsan *et al*., 2018). One of the bacteria that forms indole is *Escherichia coli.* Although the biochemical test results on XLD media showed that the microorganisms isolated from MKKTn were positive, the results on other media were negative (Table 1). This suggests that the microorganisms present in frozen salmon could be other *Enterobacteriaceae* such as *Escherichia coli.* Lactose fermentation is a biochemical test differentiating Salmonella from other Enterobacteriaceae. *E. coli* has been recognized as a lactose fermenter and *Salmonella* as a non-lactose fermenter. Therefore, this information was used for the development of the differential agar media XLD, SS, and BG (Evangelopoulou *et al.*, 2024).

Table 1. The results of biochemical testing from frozen salmon

Sample	**Enrichment media**	**Plating Media**	**Biochemical test**									
			TSIA test					**LIA test**		**Urea test**	**Indol test**	**Conclusion**
			Glucose	**Lactose**	**Sucrose**	**Air**	**H_2S**	**Butt**	**H_2S**			
Frozen salmon	MKKTn	XLD	+	+	+	+	+	+	+	+	+	Negative *Salmonella* sp.
		HE	-	-	-			+	+	-	-	
	RVS	XLD	+	+	+	+	+	-	+	-	-	
		HE	-	-	-	-	-	-	+	-	-	
Positive control (*Salmonella* sp.)	MKKTn	XLD	+	-	-	-	+	+	+	-	-	Positive *Salmonella* sp.
		HE	+	-	-	-	+	+	+	-	-	
	RVS	XLD	+	-	-	-	+	+	+	-	-	
		HE	+	-	-	-	+	+	+	-	-	

CONCLUSION

Identification of *Salmonella sp.* bacteria. on frozen salmon products at the BUSKIPM Quality Microbiology Laboratory using the ISO 6579-1:2017 standard test method showing negative test results for *Salmonella sp*. The reading of the results of the confirmation test carried out did not meet the specific requirements for *Salmonella sp.* bacteria. Therefore, no Salmonella was detected in any of the tested samples, indicating that the frozen salmon products examined in this study were free from Salmonella contamination and did not pose a risk of Salmonellosis foodborne illness. This research is in line with global efforts to achieve sustainable development goals related to health and well-being and economic growth through safe trade. Assurance of frozen salmon products safety from *Salmonella* sp. contributes to a more resilient and responsible food system globally.

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