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DETECTION AND MOLECULAR IDENTIFICATION OF *Salmonella* spp. IN TRADITIONAL SHRIMP PASTE (*TERASI*): INSIGHTS FROM MULTIPLEX PCR AND 16S rDNA SEQUENCING

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Abstract

One of the key criteria for quality food products is the absence of pathogenic microbes. Shrimp paste (*terasi*), a traditionally fermented product, is prone to contamination by unexpected bacteria, including *Salmonella* spp. This study aimed to isolate and identify *Salmonella* spp. in traditional shrimp paste using a multiplex polymerase chain reaction (mPCR) approach. The analyzed samples consisted of shrimp paste formulated from shrimp (UA, UB, UC), from fish (IA, IB, IC), and from a combination of both raw materials (CA, CB, CC). The research involved isolating *Salmonella*, performing biochemical tests with triple sugar iron agar (TSIA) and lysine iron agar (LIA), and molecular detection using mPCR. Isolate identification based on 16S rDNA sequencing was conducted for samples where mPCR failed to detect *Salmonella* at either the serovar or genus level. The presence of presumptive *Salmonella* spp. was confirmed through isolation and biochemical characterization in five shrimp paste samples, namely UA, UB, UC, CA, and CC, with the UC sample exhibiting the highest bacterial density at 8.3×10^6 CFU/g. Further TSIA and LIA tests verified that *Salmonella* spp. was only present in UC. Seven UC isolates showed biochemical characteristics typical of *Salmonella* spp. (i.e., glucose fermentation, hydrogen sulfide production, and colony morphology on xylose lysine deoxycholate agar). mPCR confirmed these seven UC isolates as belonging to the *Salmonella* genus, and one of them (UC8) was successfully identified as *Salmonella* Typhimurium. The 16S rDNA sequencing results showed that six isolates were not identified at the serovar level were classified as *Salmonella enterica* subsp. *enterica* serovar Newlands, which is reported for the first time in Indonesian shrimp paste. Meanwhile, one isolate (UC4) that could not be identified at the genus level was confirmed as *Proteus mirabilis*, indicating non-*Salmonella* contamination. These findings highlight the importance of improving hygiene in traditional shrimp paste production to minimize the risk of pathogenic microbial contamination.

Keywords: fermented products, food safety, pathogen, *Proteus mirabilis*, shrimp

Deteksi dan Identifikasi Molekuler *Salmonella* spp. dalam Terasi Tradisional: Analisis Menggunakan Multiplex PCR dan Sekuensing 16S rDNA

Abstrak

Kriteria utama produk pangan berkualitas salah satunya adalah tidak adanya mikrob patogen. Terasi sebagai produk fermentasi tradisional rentan terhadap kontaminasi bakteri yang tidak diharapkan

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termasuk *Salmonella* spp. Penelitian ini bertujuan untuk mengisolasi dan mengidentifikasi *Salmonella* spp. dalam terasi tradisional menggunakan pendekatan *multiplex* PCR (mPCR). Sampel yang digunakan mencakup terasi berbahan dasar udang (UA, UB, UC), ikan (IA, IB, IC), serta campuran keduanya (CA, CB, CC). Penelitian ini meliputi isolasi *Salmonella*, uji biokimia dengan TSIA dan LIA, serta deteksi molekuler menggunakan mPCR. Identifikasi isolat berbasis sekuensing 16S rDNA dilakukan pada sampel yang tidak terdeteksi *Salmonella* melalui mPCR, baik pada tingkat serovar maupun genus. Hasil isolasi dan karakterisasi biokimia mengonfirmasi keberadaan *Salmonella* spp. dalam lima sampel terasi (UA, UB, UC, CA, dan CC), dengan densitas bakteri tertinggi ditemukan pada sampel UC ($8{,}3 \times 10^6$ CFU/g). Uji TSIA dan LIA lebih lanjut memverifikasi keberadaan isolat *Salmonella* spp. hanya pada sampel UC sejumlah tujuh isolat, menunjukkan fermentasi glukosa, produksi hidrogen sulfida, serta morfologi koloni khas pada media XLD. Tujuh isolat dalam sampel UC tersebut teridentifikasi melalui mPCR sebagai genus *Salmonella*, dan satu diantaranya berhasil teridentifikasi sebagai *Salmonella* Typhimurium (UC8). Hasil sekuensing 16S rDNA menunjukkan bahwa enam isolat yang tidak diketahui serovarnya diidentifikasi sebagai *Salmonella enterica* subsp. *enterica* serovar Newlands, yang untuk pertama kalinya dilaporkan pada terasi di Indonesia. Sementara itu, satu isolat (UC4) yang tidak teridentifikasi pada tingkat genus terkonfirmasi sebagai *Proteus mirabilis*, yang mengindikasikan adanya kontaminasi non-*Salmonella*. Temuan ini menyoroti pentingnya peningkatan kebersihan dalam produksi terasi tradisional guna mengurangi risiko kontaminasi mikroba patogen.

Kata kunci: keamanan pangan, patogen, produk fermentasi, *Proteus mirabilis*, udang

INTRODUCTION

Traditional shrimp paste, or *terasi* is a solid, paste-like condiment commonly used as a cooking spice or flavor enhancer. It is typically dark brown to black in color and is characterized by a strong, distinctive aroma. Shrimp paste is produced through the spontaneous fermentation of shrimp, fish, or a mixture of both, with added salt and other ingredients (Prihanto & Muyasyaroh, 2021). The fermentation process involves the proteolytic activity, resulting in the development of desirable sensory properties (Karim *et al*., 2014). High-quality shrimp paste is characterized by its unique flavor, pleasant aroma, reddish-brown color, and the absence of physical contaminants such as insects, larvae, and maggots (Aristyan *et al*., 2014).

Traditional shrimp paste is usually packaged using banana leaves, plastic wrappers or paper. The occurrence of bacterial contamination in shrimp paste can occur when its processing or packaging is carried out traditionally (Linda *et al.,* 2017). Traditional shrimp paste processing, in general, has not been able to meet good food safety quality standards in terms of nutrition, durability, and packaging. This is due to the low knowledge of the traditional shrimp paste making community in handling food safety quality control. The presence of pathogenic bacteria is caused by unhygienic practice in traditional shrimp paste making. Less than perfect handling and processing can cause contamination of foodstuffs (Rosida & Faridayanti, 2013). Bacterial contamination in traditional shrimp paste can first occur from the time of its processing and storage. Additionally, at the time of preparation, traditional shrimp paste contamination can occur due to the use of wrappers or containers that have already been contaminated (Yanestria, 2020).

One of the pathogenic bacteria that can contaminate foodstuffs is *Salmonella* spp. The bacteria can cause salmonellosis, diarrhea, and typhoid fever. These diseases can be transmitted through food of animal origin contaminated with *Salmonella*. Improper food cooking can also be a source of *Salmonella* transmission (European Centre for Disease Prevention and Control, 2024). Salmonellosis is endemic in almost all major cities in Indonesia, with 33.1 cases of salmonellosis caused by *Salmonella* Typhi per 1,000 people at all age levels. Indonesia is third after China and India with the highest incidence of endemic salmonellosis, followed by Pakistan and Vietnam (Ochiai *et al*., 2008). Cases of non-typhoid *Salmonella* infections in humans in the world are estimated at 93.8 million annually, with a mortality rate of 155 thousand inhabitants (Majowicz *et al.,* 2010).

In addition to fresh and frozen shrimp, *Salmonella* has also been shown to be present in processed shrimp products, including shrimp paste (Gaffar *et al*., 2022). However, information regarding the presence of *Salmonella* spp. bacteria in traditional shrimp paste (*terasi*) is very limited. Although traditional shrimp paste is typically cooked before consumption, the risk of *Salmonella* transmission through cross-contamination remains a significant food safety concern. This is especially relevant in traditional household and market settings where hygienic practices are often lacking (Linda *et al*., 2017; Rosida & Faridayanti, 2013). Additionally, due to its strong odor and fermented nature, traditional shrimp paste is often perceived as safe, which may lead to negligence in handling and storage (Neyaz *et al*., 2024). To make matters worse, surveillance for foodborne pathogens in artisanal foods in developing countries remains limited, increasing the possibility of underreporting (European Centre for Disease Prevention and Control, 2024). Importantly, the SNI 7388:2009 standard requires that fermented fishery products, including shrimp paste, must be completely free from *Salmonella* in 25 grams of sample (Badan Standardisasi Nasional [BSN], 2009). Therefore, the early detection of *Salmonella* in traditional shrimp paste is crucial—not only to ensure food safety but also to fulfill regulatory compliance. This study was conducted to detect the presence of *Salmonella* spp., which was isolated from traditional shrimp paste made of various raw materials. It is hoped that this study will be able to provide information related to *Salmonella* spp. in processed fishery products using the Multiplex PCR (mPCR) approach.

mPCR is a variation of conventional PCR techniques that amplifies various gene sequences simultaneously. It contains a wide variety of primary sets with a mixture of single PCR reagents to produce amplicons of various sizes that are specific to different DNA sequences. The advantage of mPCR is its ability to simultaneously amplify two or more target sequences in a single reaction, thereby conserving both equipment and reagents. The specificity, efficiency, and sensitivity of mPCR amplification are similar to those of simplex PCR, which is influenced by several factors, including annealing temperature. Too high an annealing temperature prevents primers to attach to the target DNA, renderring the PCR process to be unsuccessful. On the other hand, too low an annealing temperature will cause false priming (Adikara *et al.,* 2016). Gaffar *et al.* (2022) using mPCR analysis, detected the presence of *Salmonella* Typhimurium and *Citrobacter freundii* in traditional shrimp paste. However, the mPCR primers used in their study differed from those employed in the present research. Therefore, this study aimed to isolate and identify *Salmonella* spp. from traditional shrimp paste (*terasi*) using an optimized mPCR approach.

MATERIALS AND METHODS

Isolation of *Salmonella* spp.

Shrimp paste samples were categorized into three groups: shrimp-based samples (UA, UB, and UC), fish-based samples (IA, IB, and IC), and samples based on a mixture of shrimp and fish (CA, CB, and CC). The samples were collected in March 2022 from traditional markets in Malang City, East Java, Indonesia. The isolation of *Salmonella* spp. began with a pre-enrichment stage, where 25 g of each sample was weighed and placed into an Erlenmeyer flask containing 225 mL of Buffered Peptone Water (BPW). The mixture was then homogenized using a vortex for 1 min and incubated at 37°C for 24 hours. Bacterial growth was indicated by the presence of turbidity and a distinct odor. The process continued with selective enrichment, in which 0.1 mL of the incubated BPW culture was transferred into 10 mL of Rappaport-Vassiliadis (RV) broth and incubated at 42°C for 24 hours. The presence of bacterial growth in the RV medium was indicated by a cloudy appearance (BSN, 2006).

The isolation of *Salmonella* spp. resumed with the transfer of 1 mL of the incubated sample from a selective enrichment medium into 9 mL of saline solution (0.85% NaCl), followed by serial dilution from 10^{-1} to 10^{-5}. A 0.1 mL aliquot from each dilution was inoculated onto Xylose Lysine Deoxycholate (XLD) agar using the pour plate method and incubated at 37°C for 24

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hours. This serial dilution and pour plate technique facilitated the isolation of bacterial colonies. Suspected *Salmonella* spp. colonies on XLD agar appeared pink with a black center or entirely black (BSN, 2006). Colony counts were recorded, and morphological observations were conducted to confirm presumptive *Salmonella* spp. characteristics. Typically, *Salmonella* spp. cultures formed large, glossy colonies with a black center or completely black pigmentation. For further purification, selected colonies were transferred to Nutrient Agar (NA) slants, incubated at 37°C for 24 hours. The purified isolates were then stored as stock cultures in glycerol at -80°C for long-term preservation (Ikawikanti, 2013).

Gram Staining and Biochemical Tests

After obtaining bacterial isolates, Gram staining and biochemical tests were performed. Gram staining of *Salmonella* spp. was conducted on all samples. Biochemical tests included Triple Sugar Iron Agar (TSIA) and Lysine Iron Agar (LIA) tests. The TSIA and LIA tests were carried out by inoculating the isolate into the bottom of a tube (butt) and streaking it onto a slant surface. The inoculated tubes were then incubated at 37°C for 18-24 hours (Midorikawa *et al.,* 2014). A positive TSIA reaction for *Salmonella* spp. was indicated by a yellow butt, showing acid production, and a red slant, indicating that *Salmonella* spp. only fermented glucose, but not sucrose or lactose. The presence of black precipitate at the bottom confirmed H_2S production (Indriyani *et al.,* 2019). A positive LIA reaction was observed as a purple color change, which became more intense with increasing positivity, indicating lysine decarboxylation activity (Nisa *et al.,* 2018). Isolates that tested positive in biochemical assays were further identified using specific primers in the mPCR method.

Molecular Detection using mPCR

The selected bacterial isolate underwent DNA extraction using the heat treatment method according to the procedure used by Mulyawati *et al.* (2019). A growing bacterial colony was transferred into a microcentrifuge tube containing 200 µL of ddH_2O and heated in a water bath at 95°C for 20 mins. The sample was then centrifuged at 10,000 rpm for 5 mins at 4°C, and the supernatant containing the extracted DNA was collected. The extracted DNA was subsequently analyzed using multiplex PCR (mPCR) with specific primers (Table 1). To ensure primer specificity and evaluate amplification performance, mPCR was tested using DNA templates, along with positive control strain (Paião *et al.,* 2013). *Salmonella* Typhimurium was used as the positive control and sterile distilled water was used as the negative control.

The mPCR reaction mixture was prepared with a total volume of 25 µL, consisting of 12.5 µL of MyTaq HS Red Mix (containing dNTPs, Taq DNA polymerase, $MgCl_2$, and buffer solution), 0.5 µL of each forward and reverse primer (Table 1), 5 µL of DNA template, and 3.5 µL of ddH_2O. The mPCR protocol used by Paião *et al.* (2013) included InvA, IEI, and Flic-C primers with an annealing temperature of 58°C, while the ViaB primer for *Salmonella* Typhi had an optimum annealing temperature of 55°C (de Freitas *et al.,* 2010).

To determine the optimal annealing temperature for this study, a gradient PCR program was conducted with temperatures ranging from 55°C to 58°C. The results indicated that 57°C was the optimal annealing temperature. The PCR amplification process involved an initial denaturation process at 95°C for 3 mins, followed by 30 cycles of denaturation at 95°C for 2 mins, annealing at 57°C for 2.5 mins, and extension at 72°C for 2.5 mins, with a final extension at 72°C for 2.5 mins. The PCR products were separated using electrophoresis on 1.5% agarose gel, stained with GelRed, and visualized under a UV transilluminator. In cases where mPCR failed to identify isolates, 16S rDNA sequencing was performed.

Molecular Identification using 16S rDNA Sequencing

Molecular identification using 16S rDNA sequencing was performed when mPCR failed to detect *Salmonella* at both the

genus and serovar levels. Bacterial DNA was extracted using the Zymo-Spin™ Kit, following the manufacturer's protocol. The 16S rDNA sequence was amplified via Polymerase Chain Reaction (PCR) using the 27f (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-CTACGGCTACCTTGTTACGA-3') primers. The PCR reaction mixture was prepared in a total volume of 50 µL, consisting of 25 µL of MyTaq HS Red Mix, 2 µL of forward primer, 2 µL of reverse primer, 2 µL of DNA template, and 19 µL of ddH_2O. The PCR amplification process included an initial denaturation process at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1.5 minutes, with a final extension at 72°C for 5 minutes. The PCR products were separated using electrophoresis on 1.5% agarose gel, stained with GelRed, and visualized under a UV transilluminator (Arfani *et al.,* 2018).

The amplified products were then sequenced at First BASE Sequencing Services, Malaysia. The obtained sequences were compared with available databases in NCBI using the Basic Local Alignment Search Tool (BLAST). Multiple sequence alignment was performed using ClustalW, and a phylogenetic tree was constructed using MEGA version 10.0 with the Neighbor-Joining method under the Tamura-Nei model with 1,000 bootstraps.

Data Analysis

The data obtained, including data on cell density, phenotypic characterization and molecular identification results, were analyzed descriptively.

RESULTS AND DISCUSSION

Salmonella spp. in Traditional Shrimp Paste

The isolation results of *Salmonella* spp. from the traditional shrimp paste samples revealed that not all samples were contaminated. Among the nine samples analyzed—three traditional shrimp-based samples, three fish-based samples, and three mixed samples—only five were suspected to contain *Salmonella* spp. These included all shrimp-based samples (UA, UB, and UC) and two mixed samples (CA and CC). According to SNI 7388:2009 (BSN, 2009), the standard for fermented fishery food products requires that *Salmonella* be absent in 25 g of sample. Based on the isolation results, only four samples—IA, IB, IC, and CB—met this standard. The five samples suspected of *Salmonella* contamination were characterized by a mushy texture, loosely sealed packaging, and moist, unhygienic storage conditions, which may have contributed to bacterial growth.

The isolation results from the five suspected samples revealed the growth of colonies resembling *Salmonella* spp. on XLD agar. Twenty-six isolates (Table 2) suspected to be *Salmonella* spp. were selected for further confirmation. The characteristic appearance of these colonies varied, displaying round, yellow, and pink coloration, with some colonies exhibiting a black center or entirely black pigmentation (Figure 1). Colonies of

Table 1 List of mPCR primer sequences
Tabel 1 Daftar sekuen primer mPCR

Primer	Sequences	Bacteria	Size (bp)	References
InvA forward	CGG TGG TTT TAA GCG TAC TCT T	*Salmonella* spp.	796	Paião *et al.* (2013)
InvA reverse	CGA ATA TGC TCC ACA AGG TTA			
IEI forward	AGT GCC ATA CTT TTA ATG AC	*Salmonella* Enteritidis	316	Paião *et al.* (2013)
IEI reverse	ACT ATG TCG ATA CGG TGG G			
Flic-C forward	CCCGCTTACAGGTGGACTAC	*Salmonella* Typhimurium	432	Paião *et al.* (2013)
Flic-C reverse	AGCGGGTTTTCGGTGGTTGT			
ViAB forward	CAC GCA CCA TCA TTT CAC CG	*Salmonella* Typhimurium	401	de Freitas *et al.* (2010)
ViAB reverse	AAC AGG CTG TAK CGA TTT AGG			

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Table 2 Number of *Salmonella* spp. cells in traditional shrimp paste from different raw materials
Tabel 2 Jumlah sel *Salmonella* spp. pada terasi tradisional dengan bahan baku berbeda

Samples	Sample code	Number of cells (CFU/g)	Number of isolates
Shrimp paste A	UA	3.4×10^{3}	4
Shrimp paste B	UB	3.9×10^{6}	6
Shrimp paste C	UC	8.3×10^{6}	8
Fish paste A	IA	n.d	-
Fish paste B	IB	n.d	-
Fish paste C	IC	n.d	-
Mixed shrimp-fish paste A	CA	3.2×10^{3}	4
Mixed shrimp-fish paste B	CB	n.d	-
Mixed shrimp-fish paste C	CC	9.8×10^{5}	4

n.d: not detected

Salmonella spp. on XLD agar typically appear red with a black center, indicating hydrogen sulfide (H_2S) production (Neyaz *et al.*, 2024).

XLD agar serves as a selective and differential medium for isolating *Salmonella* spp. It contains xylose, which allows *Salmonella* spp. to be differentiated from other enteric bacteria based on their fermentation ability. Additionally, the medium includes lysine, which can be decarboxylated by *Salmonella* spp., maintaining a neutral or slightly alkaline pH. Under these conditions, *Salmonella* spp. reduce sodium thiosulfate, producing hydrogen sulfide (H_2S), which reacts with iron salts to form black precipitates, distinguishing them from other coliforms. Furthermore,

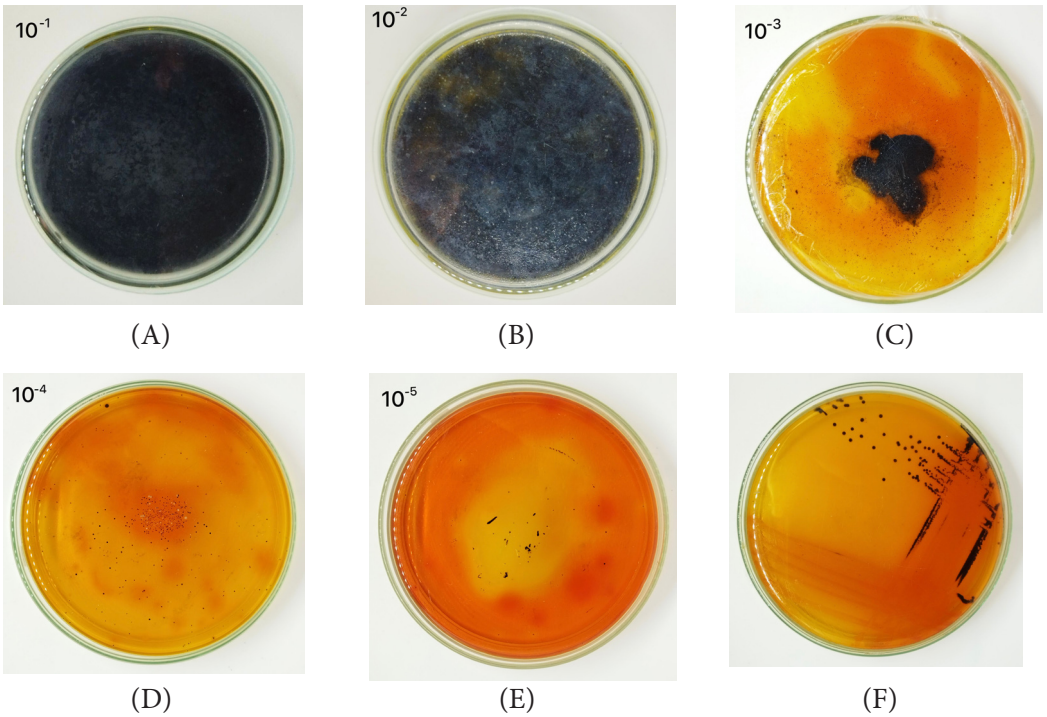

Figure 1 *Salmonella* spp. colonies on XLD agar; (A) 1st dilution, (B) 2nd dilution, (C) 3rd dilution, (D) 4th dilution, (E) 5th dilution, (F) pure isolate
Gambar 1 Koloni *Salmonella* spp. pada media XLD; (A) pengenceran ke-1, (B) pengenceran ke-2, (C) pengenceran ke-3, (D) pengenceran ke-4, (5) pengenceran ke-5, (F) isolat murni

sodium deoxycholate in XLD agar acts as a selective agent, inhibiting the growth of Gram-positive bacteria (Park *et al.,* 2012).

The total number of bacteria isolated may not fully represent the actual microbial load, as an enrichment process was performed before isolation. The highest bacterial cell density was observed in traditional shrimp-based sample C, reaching 8.3×10^6 CFU/g (Table 2). This high bacterial count was likely attributed to unhygienic processing methods, where traditional shrimp paste was produced under suboptimal sanitary conditions. Additionally, the mixing of raw materials during storage, lack of refrigeration, and exposure to open-air environments may have further facilitated microbial growth. These factors contributed to the increased bacterial contamination in traditional shrimp paste, highlighting the need for improved hygiene and storage practices.

This study found that the type of shrimp paste packaging did not significantly influence the shrimp paste quality. However, the quality of shrimp paste could be assessed based on the color and texture of the shrimp paste (Table 3). In this study, the shrimp paste samples suspected to be contaminated with *Salmonella* spp. exhibited a dark brown to blackish color, whereas high-quality shrimp paste samples were typically red in color, influenced by the astaxanthin pigment. The intensity of the red color was also affected by the salt concentration used during processing. In terms of texture, the shrimp paste samples suspected to be contaminated with *Salmonella* spp. tended to be slightly mushy to mushy, deviating from the solid and compact texture specified under SNI 2761:2016 (BSN, 2016). The texture of shrimp paste is largely determined by its moisture content, where higher water content may contribute to a softer consistency and increased susceptibility to microbial contamination (Hudayati *et al.,* 2021).

The results of this study indicated that shrimp-based shrimp paste was more susceptible to *Salmonella* spp. contamination than fish-based paste. This may be attributed to the chitin content in shrimp body parts, such as the shell and head, which contain 15–20% chitin. Chitin is a naturally occurring polysaccharide found in the exoskeleton of crustaceans and serves as a substrate for chitin-degrading pathogenic bacteria, including *Salmonella* and *Vibrio*. *Salmonella enterica* has been identified as a chitinolytic bacterial species isolated from shrimp shells (Halim *et al.,* 2022).

The primary raw material used in shrimp paste production is *rebon* shrimp (*Acetes* sp.), a small-sized species typically processed whole, including the shell and head (Karim *et al.,* 2014). The potential for *Salmonella* contamination arises from inadequate cleaning processes before shrimp paste production. Furthermore, unhygienic drying and grinding practices contribute to

Table 3 Characteristics and origin of shrimp paste samples
Tabel 3 Karakteristik dan asal sampel terasi

Shrimp paste samples	Packaging	Colors	Texture	Origins
Shrimp paste A	Plastic	Brown	Slightly mushy	Tuban
Shrimp paste B	Plastic	Dark brown	Mushy	Tuban
Shrimp paste C	Plastic	Dark brown	Mushy	Tuban
Fish paste A	Paper and plastic	Reddish brown	Congested	Tuban
Fish paste B	Leaf	Reddish brown	Congested	Pacitan
Fish paste C	Plastic	Reddish brown	Congested	Tuban
Mixed shrimp-fish paste A	Plastic	Brown	Slightly mushy	Tuban
Mixed shrimp-fish paste B	Leaf	Reddish brown	Congested	Lamongan
Mixed shrimp-fish paste C	Paper and plastic	Brown	Slightly mushy	Tuban

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an increased risk of microbial contamination. These factors highlight that shrimp-based paste is particularly vulnerable to *Salmonella* spp. contamination, emphasizing the need for improved sanitation measures during processing and handling.

According to Norhana *et al*. (2010), *Salmonella* spp. is not only present in fresh and frozen shrimp but is also found in preserved shrimp products, including dried shrimp and shrimp paste. Additionally, *Salmonella* spp. has been detected in cooked shrimp-based foods, such as shrimp sushi. Among the various *Salmonella* serotypes identified in shrimp and shrimp-derived products, *Salmonella* Weltevreden and *Salmonella* Typhimurium are the most frequently isolated.

Table 4 Cell characteristics and biochemistry of suspected isolates of *Salmonella* spp.
Tabel 4 Karakter sel dan biokimia isolat yang diduga *Salmonella* spp.

Isolates	Gram staining		Shape		Biochemical characters		
	Gram +	Gram -	Rod	Coccus	LIA test	TSIA test	H_2S
UA1	-	+	+	-	-	+	-
UA2	-	+	+	-	-	+	-
UA3	-	+	+	-	-	+	-
UA4	-	+	-	+	-	-	-
UB1	-	+	+	-	-	+	+
UB2	-	+	+	-	-	+	+
UB3	-	+	+	-	-	+	+
UB4	-	+	+	-	-	+	+
UB5	-	+	+	-	-	+	+
UB6	-	+	+	-	-	+	+
UC1	**-**	**+**	**+**	**-**	**+**	**+**	**+**
UC2	**-**	**+**	**+**	**-**	**+**	**+**	**+**
UC3	**-**	**+**	**+**	**-**	**+**	**+**	**+**
UC4	-	+	+	-	-	+	+
UC5	**-**	**+**	**+**	**-**	**+**	**+**	**+**
UC6	**-**	**+**	**+**	**-**	**+**	**+**	**+**
UC7	**-**	**+**	**+**	**-**	**+**	**+**	**+**
UC8	**-**	**+**	**+**	**-**	**+**	**+**	**+**
CA1	-	+	+	-	+	-	-
CA2	-	+	+	-	+	-	-
CA3	-	+	+	-	+	-	-
CA4	-	+	+	-	+	-	-
CC1	-	+	+	-	+	-	-
CC2	-	+	+	-	+	-	-
CC3	-	+	+	-	+	-	-
CC4	-	+	+	-	+	-	-

+ (positive reaction), - (negative reaction), row in bold and grey showed presumptive *Salmonella*

Cell Characteristics and Biochemical Test Results

Of the 26 isolates, one isolate, UA4, did not match the characteristics of *Salmonella* spp. (Table 4). This was determined based on Gram staining results, which showed a Gram-positive reaction and a coccus-shaped morphology, differing from the typical *Salmonella* spp. characteristics. Consequently, UA4 was excluded from further testing. Meanwhile, the remaining 25 isolates exhibited a Gram-negative reaction and a short rod-shaped morphology, consistent with the characteristics of *Salmonella* spp. *Salmonella* spp. is a Gram-negative, facultative anaerobic bacteria that possesses flagella and a capsule and does not form spores. The bacterial cells are rod-shaped, measuring approximately 1–3.5 µm in length and 0.5–0.8 µm in width, with colony diameters ranging from 2 to 4 mm (Gray & Fedorka, 2012).

Based on biochemical test results, seven isolates presumptively identified as *Salmonella* spp. yielded positive reactions in both Triple Sugar Iron Agar (TSIA) and Lysine Iron Agar (LIA) tests. Additionally, the presence of hydrogen sulfide (H_2S) deposits was observed at the bottom of the TSIA and LIA media (Table 4). These seven isolates were subsequently analyzed using mPCR.

In TSIA tests, a positive result for *Salmonella* spp. was indicated by a yellow butt, signifying acid production, and a red slant, indicating that the bacteria fermented only glucose, while lactose and sucrose were not utilized (Indriyani *et al.,* 2019). TSIA medium contains three sugars (glucose, lactose, and sucrose), along with phenol red as a pH indicator and $FeSO_4$ (iron sulfate) to detect H_2S production. When the pH drops below 6.8, the indicator turns yellow, whereas a pH above 6.8 results in a red color. As glucose is present in low concentration, bacteria rapidly utilize it and shift to amino acid oxidation, producing ammonia that increases the pH and turns the slant red. In the presence of H_2S production, *Salmonella* spp. reacts with iron sulfate, forming black precipitates at the bottom of the tube (Midorikawa *et al.,* 2014). Other H_2S-producing bacteria, such as *Proteus* spp., also generate black deposits, which may obscure the yellow butt.

In LIA tests, a positive result for *Salmonella* spp. was indicated by a color change to purple, confirming lysine decarboxylation activity. LIA is specifically designed to assess a bacterium's ability to decarboxylate lysine and produce H_2S. The deamination process occurs aerobically in the slant, producing ammonia, which reacts with ammonium citrate iron, turning the slant purple. Meanwhile, the lysine decarboxylase process takes place anaerobically in the butt, producing amine compounds, which react with the pH indicator, also resulting in a purple color (Nisa *et al.,* 2018).

Detection and Identification of *Salmonella* spp. using mPCR

Biochemical tests confirmed that seven isolates exhibited characteristics of *Salmonella* spp. (Table 4). These isolates were further analyzed using mPCR. The mPCR results showed that all seven isolates from UC sample belonged to the genus *Salmonella* spp., as indicated by the formation of a 796 bp DNA band (Figure 2). This band resulted from the amplification of the InvA primer pair, which targets the InvA gene, a unique genetic marker present in all *Salmonella* species. The InvA gene is located within the *Salmonella* Pathogenicity Island (SPI-1) and encodes proteins responsible for the bacterium's invasive properties in intestinal epithelial cells. Due to its conserved nature across all *Salmonella* species, InvA is widely used as a reliable genetic marker for detecting the presence of *Salmonella* (Muhsinin *et al.,* 2019).

Among the seven mPCR-confirmed *Salmonella* spp. isolates, only one isolate, UC8, was identified as *Salmonella* Typhimurium (Figure 2). This was characterized by the presence of two distinct DNA bands, a 796 bp band (InvA gene) and an additional 432 bp band, which resulted from the amplification of the Flic-C gene. The Flic-C gene is involved in the synthesis of flagellin, a key component of the flagella in *Salmonella* Typhimurium (Hatta *et al.,* 2011). The flagellum, a rope-like

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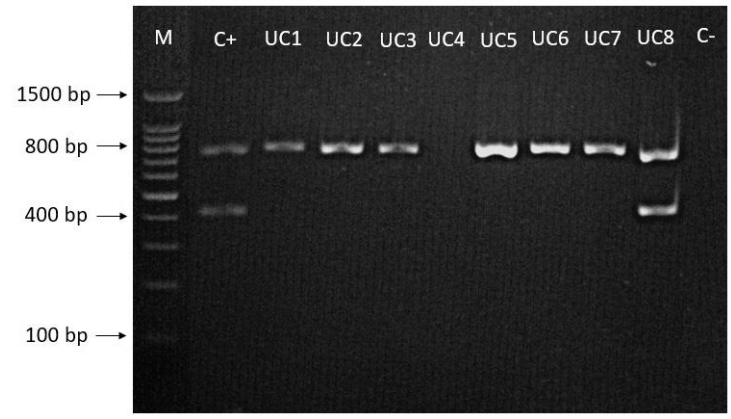

Figure 2 mPCR results for the identification of *Salmonella* spp. in traditional shrimp paste; (M) DNA Marker, (C+) Positive control (*Salmonella* Typhimurium), (UC1, UC2, UC3, UC4, UC5, UC6, UC7, UC8) Isolates suspected of being *Salmonella* spp., (C-) Negative control (sterile distilled water)

Gambar 2 Hasil mPCR untuk identifikasi *Salmonella* spp. pada terasi tradisional; (M) *DNA marker*, (C+) Kontrol positif (*S.* Typhimurium), (UC1, UC2, UC3, UC4, UC5, UC6, UC7, UC8) Isolat diduga *Salmonella* spp., (C-) Kontrol negatif (akuades steril)

structure protruding from the bacterial cell surface, enables bacterial motility. At its core is a filament composed of flagellin protein, which plays a crucial role in *Salmonella* movement and pathogenesis (Noviyanthi, 2019).

Several researchers have developed mPCR methods to detect various pathogenic bacteria, including *Salmonella* spp. Paião *et al.* (2013) successfully applied the mPCR method for detecting *Salmonella* spp. in broiler chickens, where all *Salmonella* spp. isolates from the broiler chicken samples produced a 796 bp DNA band, amplified using the InvA primer pair, confirming the presence of *Salmonella* spp. at the genus level. Further differentiation at the serovar level was achieved using specific primers. *Salmonella* Enteritidis was identified by the presence of two DNA bands: a 796 bp band (InvA gene) and a 316 bp band (IE1 gene). Similarly, *Salmonella* Typhimurium was confirmed by the presence of a 796 bp band (InvA gene) along with a 432 bp band (Flic-C gene).

These findings indicate that the InvA gene is a reliable marker for detecting *Salmonella* spp. at the genus level, while the IE1 and Flic-C genes serve as specific markers for identifying *Salmonella* Enteritidis and *Salmonella* Typhimurium, respectively. Based on these results, the InvA, IE1, and Flic-C genes are appropriate molecular targets for the mPCR-based detection and differentiation of *Salmonella* spp. and its serovars. The application of these primers enhances the accuracy and specificity of *Salmonella* detection in foodborne pathogens, supporting its critical role in food safety monitoring and bacterial surveillance.

The mPCR results for six isolates (UC1, UC2, UC3, UC5, UC6, and UC7) that were not identified at the serovar level showed the presence of *Salmonella* spp., as indicated by the formation of a 796 bp DNA band, confirming detection at the genus level. Since these isolates could not be further identified at the serovar level, UC5 isolate was selected as a representative isolate for further analysis using 16S rDNA sequencing.

The 16S rDNA sequence analysis confirmed that the UC5 isolate belonged to *Salmonella enterica* subsp. *enterica* serovar Newlands (*Salmonella* Newlands), exhibiting 99.9% similarity to the SeqSC0082 strain (Figure 3). *Salmonella* Newlands SeqSC0082 was initially isolated from the distal ileum of pigs in China (Yin, 2019). In this study, *Salmonella* Newlands is reported for the first time in an Indonesian food product,

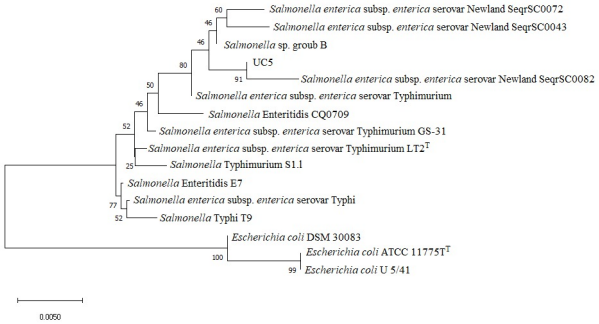

Figure 3 Phylogenetic tree of UC5 isolate and reference strains based on Neighbor-Joining construction with the Tamura-Nei model and 1,000 bootstraps

Gambar 3 Pohon filogeni isolat UC5 dan strain acuan berdasarkan konstruksi Neighbor-Joining dengan model Tamura-Nei dengan *bootstrap* 1.000 kali

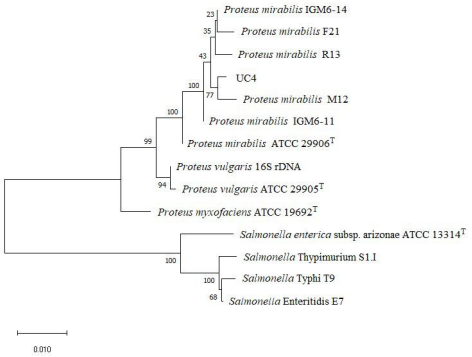

Figure 4 Phylogenetic tree of UC4 isolate and reference strains based on Neighbor-Joining construction with the Tamura-Nei model and 1,000 bootstraps

Gambar 4 Pohon filogeni isolat UC4 dan strain acuan berdasarkan konstruksi Neighbor-Joining dengan model Tamura-Nei dengan *bootstrap* 1.000 kali

specifically in shrimp paste, highlighting its presence as a previously unreported serovar in local fermented seafood products.

This serovar belongs to the non-typhoidal *Salmonella* (NTS) group, which is known to cause salmonellosis in humans. Globally, non-typhoidal *Salmonella* infections are estimated to result in 93.8 million cases per annum, with a mortality rate of approximately 155,000 deaths per year (Majowicz *et al.*, 2010). The detection of *Salmonella* Newlands in shrimp paste underscores the potential risk of foodborne transmission and the importance of stringent hygiene practices in traditional seafood processing.

UC4 isolate, which was not amplified by mPCR, was further analyzed using 16S rDNA sequencing. The sequence analysis identified UC4 isolate as *Proteus mirabilis* with 99.4% similarity to the M12 strain (Figure 4). *Proteus mirabilis* belongs to the genus *Proteus*, a group of pathogenic bacteria found in both humans and animals, primarily inhabiting the large intestine (Putri, 2014). This bacterium is known to cause urinary tract infections (UTIs) and can further lead to bacteremia and urosepsis, conditions that may become life-threatening. Most cases of *P. mirabilis*-induced UTIs originate from the gastrointestinal tract, where the bacterium can spread and colonize the urinary system (Schaffer & Pearson, 2017). The presence of *P. mirabilis* in shrimp paste highlights the potential risk of contamination by enteric pathogens, emphasizing the need for strict hygiene practices in traditional seafood processing to minimize bacterial

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transmission. Since UC4 isolate was presumed to be *Salmonella* based on a biochemical test, this discrepancy suggests that the biochemical test may have produced a false positive result. Therefore, it can be concluded that the primers used in the mPCR method were specific to *Salmonella*, and that molecular detection offers greater accuracy in confirming the identity of suspected isolates.

This study confirmed the presence of *Salmonella* spp. contamination in traditional shrimp paste, underscoring its potential risk to food safety. Although no official records reported illnesses directly linked to traditional shrimp paste consumption, the risk remains, especially since most shrimp paste products in Indonesia are produced by small-scale home producers under with minimal regulatory oversight (Prihanto & Muyasyaroh, 2021). Limited foodborne disease surveillance systems and underdiagnosis of cases further contribute to the potential underreporting of related illnesses (European Centre for Disease Prevention and Control, 2024). Additionally, *Salmonella* spp. can survive in low-moisture, high-salt, and acidic environments typical of fermented seafood products (Neyaz *et al*., 2024). To address this, strict application of Good Manufacturing Practices, proper packaging, and dry storage conditions are essential during processing and distribution (Yanestria, 2020). In addition to these preventive measures, the use of starter cultures from bacteriocin-producing lactic acid bacteria, as identified by Romadhon *et al*. (2018), may offer a promising biocontrol strategy to inhibit *Salmonella* growth in traditional shrimp paste. Routine microbial monitoring of traditional shrimp paste is, therefore, vital to ensure public health and safety.

CONCLUSION

This study successfully detected and identified *Salmonella* spp. in traditional shrimp paste (*terasi*) using biochemical tests, mPCR, and 16S rDNA sequencing. *Salmonella* Typhimurium and *Salmonella enterica* serovar Newlands were confirmed in the samples, marking the first report of the latter in Indonesian shrimp paste. The findings demonstrate the reliability of mPCR for rapid detection and highlight the need for improved hygiene practices in traditional seafood production to ensure food safety.

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