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Original Research

Identification of Salmonella typhi contamination by amplification fliC gene in grass-jelly from traditional markets and minimarket in Semarang city

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HIGHLIGHTS

We found positive samples with *S. typhi* in the markets by amplification of *fliC* gene but amplification of only *fliC* gene cannot specific for *S. typhi* detection

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ABSTRACT

Grass-jelly is one of the most popular plants consumed by people in various forms. Contamination can cause various diseases, one of those is typhoid fever by *Salmonella typhi*. The purpose of this study was to detect *S. typhi* in grass-jelly based on molecular detection by amplification of the *fliC* gene using PCR. Validation was done by culture methods on SSA media and biochemical testing. The *fliC* gene amplification results in grass-jelly samples (A1, A2, B1, B2, C1, and C3) showed the DNA fragments size of about 1500 bp. Colony and biochemical characters isolate Peterongan were lead to *S. typhi*, whereas another isolate was another *Salmonella* spp. Grass-jelly samples from the Peterongan market in Semarang were positively contaminated by *S. typhi* and isolate from Pedurungan and the minimarket was another *Salmonella* spp. Molecular-based food testing is fast enough and accurate for detecting types of bacterial contaminants but the amplification of only the *fliC* gene cannot specific for *S. typhi*.

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1. INTRODUCTION

Grass-jelly or commonly called cincau is a gel-like jelly that is processed from soaking certain plant leaves in water. The gel is formed because the carbohydrate content in the leaves is bind to water molecules.[1] In general, there are 2 types of grass-jelly on the market one of those is black grass-jelly, commonly called janggelan made from the leaves of *Mesona palustris* has fiber content that is useful for human digestion and green grass-jelly from the

leaves of *Premna oblongifolia* has several active compounds to relieve sore throat and keep blood pressure stable.[2] Both have a distinctive aroma and taste characters and both are popular drink mixes in the community.

The process of making grass-jelly recently is mostly done conventionally. It is food processing that was done traditionally with the usual procedures without good manufacturing practices such as the hygiene process. In order, the contamination of microorganisms can occur in grass-jelly due to several factors such as water for processing, pieces of equipment, and processing facilities, as well as people doing the processing.[3] This condition factors causing contamination by various pathogenic bacteria to black grass-jelly that sold in the market, one of which is *S. typhi* that causes typhoid fever.

A study on the identification of microbial contamination in grass-jelly at Wonodri Market, Semarang has been done.[4] The results of the study using culture methods and biochemical tests showed that there was contamination by *S. paratyphi A*, *Proteus mirabilis*, *Klebsiella oxytoca* and *Citrobacter diversus*.[5] stated that in Dr. Kariadi Hospital in Semarang city, population typhoid fever was associated with poor housing and inadequate food and personal hygiene. Based on the National Food and Drug Agency[6] Regulation No. 13 of 2019 concerning Maximum Limits of Microbial Contamination in Food should not contain *Salmonella sp*., in the Fruit-Based Dessert Includes Fruit-Based Water-Based Dessert Food that is black and green grass-jelly included in it. Therefore a fast and accurate detection of the *Salmonella spp* group is needed to be done.

Salmonella is a broad spectrum of disease-causing bacteria. They can cause significant morbidity, and in some cases, death in humans and animals.[7] *S. enterica serovar typhi* or commonly called *S. typhi* is a rod-shaped, solitary, flagellar bacteria (peritric type or around on the entire cell surface), Gram-negative, intracellular bacteria, including in the family *Enterobacteriaceae*, causes typhoid fever and gastroenteritis in humans, with mild to severe clinical symptoms.[8,9] This bacterium has somatic antigen O composed of lipopolysaccharides, flagellate antigens or H antigens composed of flagellin proteins, Vi capsule antigens composed of carbohydrates and fimbriae antigens composed of fillins protein.[8,10] The gene that codes for the flagellin protein in *S. Typhi* is the *fliC* gene.[11]

The *fliC* gene in *S. typhi* has a unique sequence and size. The *S.typhi* H1 antigen consists of 2 serovars namely H1-d which is widespread in the world and H1-j which is only found in Indonesia. Both are encoded by the *fliC* gene that is on the bacterial chromosome, but the *fliC* gene that encodes the j antigen has deletion 251 bp which results in changes in shape in the antigen epitope of the flagel.[12] The study by amplification *fliC* gene with primer LPW 1857 Forward and LPW 1857 Reverse showed DNA about 1260 bp using *S. typhi* isolates from Salatiga and different from 9 other strains about 1500 bp.[13] Similar studies have also conducted the results showed that the flagellin *fliC* gene had a DNA band size of 1260 bp.[12,14] Previous research conducted by results showed the flagellin *fliC* gene had a DNA size of 1260 bp.[12,14] The purpose of this study was to detect contamination of *S. typhi* in grass-jelly from a traditional market in Semarang based on molecular detection by amplification of the *fliC* gene using PCR.

2. MATERIAL AND METHOD

The samples in this study were black grass-jelly from two traditional markets in Semarang, namely Pedurungan Market, Peterongan Market, and one of the branded packaging products from a minimarket. The research was conducted at the Laboratory of Molecular Biology and Microbiology of Program of Study in Medical Laboratory Technology, Universitas Muhammadiyah Semarang.

DNA extraction

The sample came from 1 g of grass-jelly homogenized with 9 ml physiological NaCl then inoculated to the Brain Heart Infusion (BHI) slant agar medium and incubated 24 hours in 37ºC. The growing colonies were then inoculated into 15 ml BHI broth media with 24-hour incubation in 37ºC. After that, centrifuged 3000 RPM for 15 minutes to get pellets.

The DNA extraction protocol uses Phenol Chloroform Isoamyl Alcohol (PCIA) with composition 25:24:1. Pellets biomass added 750 µl lysis buffer (100 mM NaCl, 100 mM Tris-HCl pH 8, 50 mM EDTA, 2% SDS) and added 20 proteinase-K 10 mg/ml, then incubated at 55 ^{o}C in a water bath for 1 hour with shaking every 10 minutes. After incubation, the solution was centrifuged at 3000 RPM for 15 minutes. The supernatant was taken and added 700 µl PCIA (1:1) then centrifuged at 3000 RPM for 15 minutes. Furthermore, the aquous phase in the supernatant is transferred to new conicles and cold absolute ethanol (1:1) was added then thread-like DNA will form. The DNA was transferred to a new microtube tube. Then washed with 500 µl 70% ethanol 3 times. After that, let dry and added with 50 µl Tris-EDTA (TE).

Analyze quality of genomic DNA by 1% agarose electrophoresis

Agarose gel was made with a concentration of 1%. The DNA dye used SBYR safe. Buffer electrodes used Tris Acetic-EDTA (TAE). The sample was mixed with loading dye and then put in a gel pit. The electrophoresis was run with a voltage of 100 volts for 30 minutes.

DNA amplification

The Polymerase Chain Reaction (PCR) mix was made in a total volume of 25 µl containing 12.5 µl master mix (Promega) kits, Forward primers (5'-TTAACGCAGTAAAGAGAGGACGTT-3') and Reverse (5`-ATGGCACAAGTCATTAATACAA-3`)[14] each 2 µl, DNA template 1 µl and nuclease-free water 7.5 µl. The thermal cycler settings are predenaturation 95 °C for 4 minutes, denaturation 95 °C for 30 seconds, annealing 48 °C for 30 seconds, extension 72 °C for 2 minutes, with the PCR cycle repeated 35 times. Post extension 72 °C for 10 minutes, then temperature 4 °C for 10 minutes to maintain PCR product stability.

Analysis of PCR results with 2% agarose electrophoresis

Agarose gel was made with a concentration of 2%. The DNA dye used is SBYR safe. Buffer electrodes used TAE. The PCR sample was inserted into the wells and used a 100 bp (Vivantis) DNA marker. The electrophoresis was run with a voltage of 100 volts for 60 minutes

Inoculation and culture in *Salmonella Shigella* Agar (SSA) media

Grass-jelly samples are homogenized first. The homogenate sample was put into a test tube and added physiological NaCl in the ratio of 1:9 (sample 1 g, physiological NaCl 9 ml). After being homogeneous it is put into BHI broth media. Incubated for 24 hours at 37 °C. Then cultured on SSA agar, incubated for 24 hours at 37 ° C. Morphological characters of the growing colonies were observed, such as color, shape, size, edge, consistency, and elevation.

Biochemical testing

S. typhi suspected colonies with a black center in SSA were cultured on biochemical test media, those were Indol, Methyl Red (MR), Voges Proskauer (VP), Citrate, Motility, Urea and Triple Sugar Iron Agar (TSIA) incubated at 37 ° C for 24 hours.

Insilico PCR

In silico PCR analysis used website http://insilico.ehu.eus/ [15] then the sequences from the primer pair were added. These primers analyze in All strains of Genus *Salmonella*.

3. RESULTS AND DISCUSSION

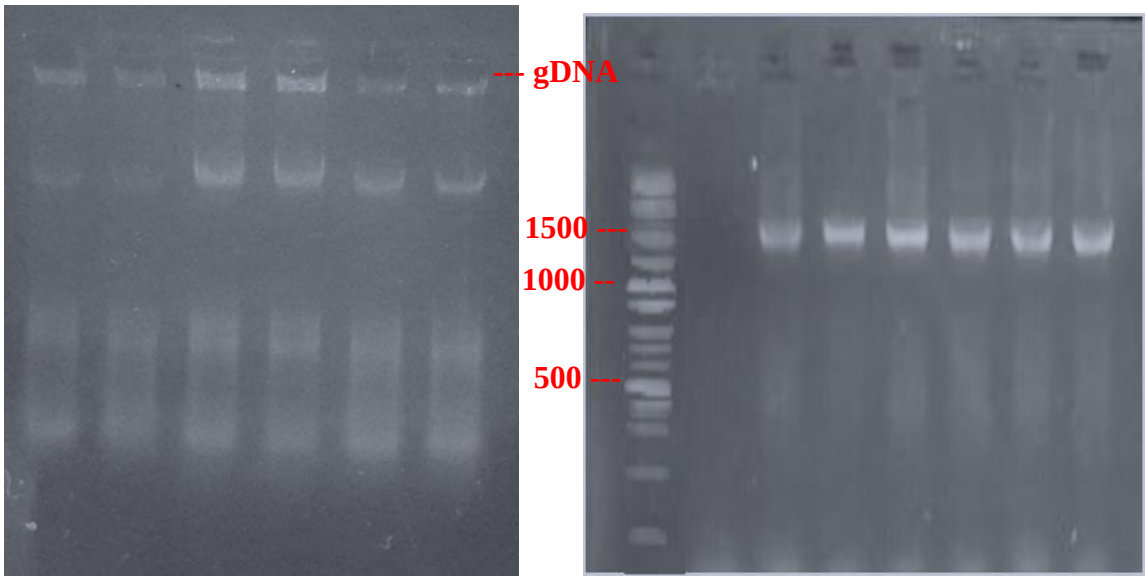
Identification of *S. typhi* contamination in grass-jelly samples is needed to provide protection to consumers and prevent typhoid fever. In the two traditional markets of Semarang City (Pasar Pedurungan and Peterongan) that we observed, the process of selling black grass-jelly was placed in an open condition, exposed to dirty market air, dust, and flies attached to grass-jelly. This allows the contamination of microorganisms.

Grass-jelly samples were taken from Pedurungan Market (A1 and A2), Peterongan Market (B1 and B2), and one of the branded packaging products from minimarket (C1 and

C2) in Semarang City as a comparison of grass-jelly in factory packaging. This market location was chosen because it is the place most visited by the people to buy grass-jelly.

There were 2 steps carried out in this study, the molecular identification stage and the validation stage. The molecular identification stage was by testing the DNA of *S. Typhi* in grass-jelly samples using PCR based on the *fliC* gene. The validation stage was a bacterial culture method from grass-jelly samples using selective media and biochemical tests.

The results of genomic DNA extraction got genomic DNA (gDNA) isolates obtained from suspected *Salmonella* culture from grass-jelly samples visualized using agarose electrophoresis 1% in 100 volts for 30 minutes. The genomic DNA is closest to the good gel because of its large DNA size (Figure 1. A). DNA fragments under the genome bands were might be extrachromosomal DNA such as plasmids. DNA fragments that migrate to the bottom of the gel were suspected debris from cell proteins or protease enzymes while the smears formed are likely short pieces of DNA or RNA.



(A) (B)

Figure 1. Visualization of genomic DNA (A) and PCR products (B) from isolates culture

Primers that are used have specificity on the sequenced of *fliC* gene. The *fliC* and *fliB* genes play a role in the synthesis of flagellin proteins that compose bacterial cell flagella.[16] The results of amplification DNA isolates from grass-jelly based on *fliC* gene primer visualized using agarose electrophoresis 2% showed a single DNA fragment size around 1500 bp (Figure 1. B). This is in correspondence with another research.[12,13,14]

In silico PCR analysis from http://insilico.ehu.eus/ [15] using sequences from the primer pair, showed the results of amplified the *fliC* gene with a size of 1521 bp in *S. typhi* strain (Figure 2). The sequence database is derived from the Multiple Drug Resistant (MDS) *Salmonella enterica serovar typhi* CT18.[17] We used *in silico* PCR to matched the size of the PCR product that resulted in this research with the database reference.

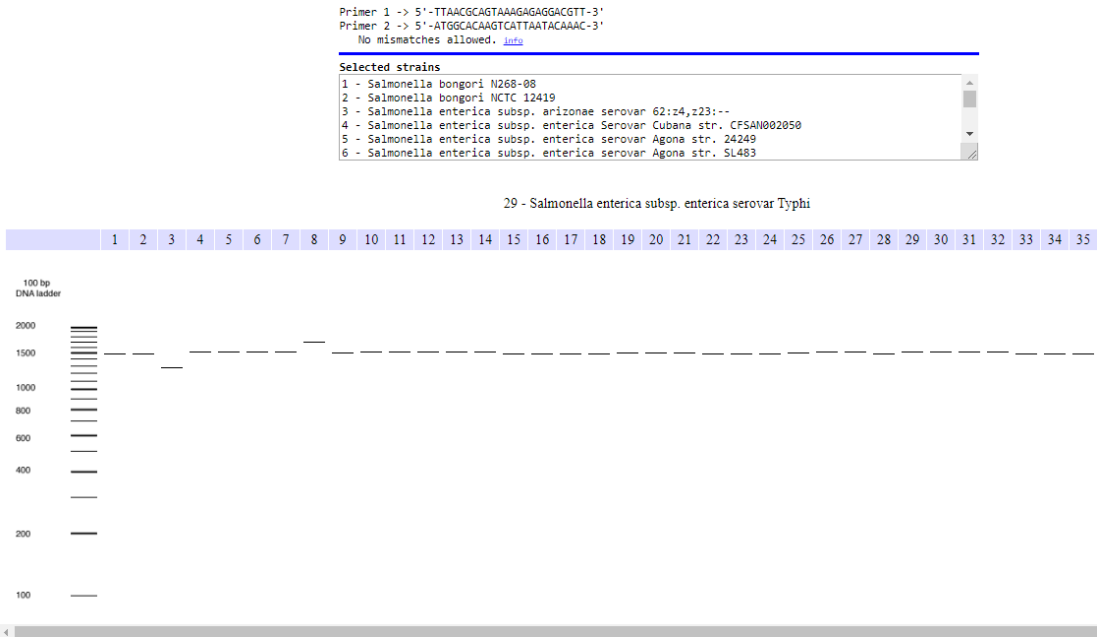


Figure 2. *In silico* amplification of *FliC* gene using primer pairs LPW 1857 from all *Salmonella* genome in a database (http://insilico.ehu.eus/)

Molecular identification of *Salmonella* contamination in samples of juice drinks at the Wonodri traditional market, Semarang, was previously conducted it resulted in 3 of 16 fruit juice samples in Gunung Pati District of Semarang were Positive of *Salmonella*.[18] The test uses primers for *invA*1 and *invA*2 genes that produce 244 bp DNA amplification. Therefore, the study of development primers target for detecting specific genes in certain pathogenic bacteria needs to be carried out. Developing specific primers is important to detect specific bacteria such as the pathogenic strains, so the molecular detection results are precise on target.

Figure 3. Morphology of culture colonies resulted on SSA media

SSA is a bacterial medium that uses lactose as a source of carbohydrates, contains a color indicator of pH changes is Neutral Red, and contains ferric citrate as an indicator of H_2S gas formation. Interpretation of the results of bacterial culture samples on SSA media that were colorless or transparent colony because does not ferment lactose and was able to produce black sediment due to producing H_2S (Figure 3). A colony that had the character of *Salmonella typhi* which is the black center was used for biochemical testing.[9,19]

Interpretation of the biochemical results of three samples with double repetitions showed differences (Table 1). The indole test was negative because a pink ring did not form when the Kovach reagent was added. Kovac or Erlich reagents containing hydrochloric acid and p-dimethylaminobenzaldehyde in amyl alcohol will react with indole derived from tryptophan hydrolysis by showing pink color.[9] MR test result in isolates from Pedurungan and

minimarket were negative because there was no red color when Methyl Red reagent was added but in Peterongan isolates were positive. Methyl red is an indicator of organic acids at pH 4.5 and below to indicate the fermentation of glucose. All *S. typhi* isolate was negative in the indole test result and *S. typhi* isolates from Peterongan Market had positive Methyl Red result while others were negative.

The results of the VP test were negative in isolates from Peterongan and minimarket because no red color was formed when the α-Napthol reagent and 40% KOH were added but in Pedurungan isolates were positive. VP test to see the presence of acetyl-methyl carbinol (acetoin) production from glucose fermentation. Acetoin will be reacted with 40 % KOH to form diacetyl then diacetyl and the guanidine group in the media will be reacted with α -naphthol which will produce condensation showing pink color. Citrate test results were negative because all isolate could not turn media to blue due it uses a color indicator for changing the pH of Bromothymol Blue (BTB). The citrate test is to determine the ability of microorganisms to use citrate for carbon compounds as an energy source. When a bacterium uses citrate, the ammonium salt will be converted to alkaline ammonia so that the pH in the media increases, and the BTB indicator will show blue.[9] *S. typhi* isolates from Pedurungan Market had a positive result in VP test while others were negative. All *S. typhi* isolates had a negative result in the citrate test.

Motility test using semisolid media with 0.4% agar and Triphenyltetrazolium Chloride (TTC), the results were positive because the media turned turbid and red color formed on the puncture marks from the TTC reaction that showed cell movement. Urea test results were negative because all parts of the media did not turn pink. TSIA results were Alkalis/Acid and H_2S (+), which means red on the slope because it does not ferment sucrose and lactose, while a yellowish black color at the bottom of the media due to glucose fermentation and H_2S formation. Whereas gas formation in Pedurungan and minimarket isolates were positive but in Peterongan isolates were negative.

Table 1. Result of biochemical testing from colony cultures such Indol, MR, VP Citrate, motility, urea, and TSIA test

Biochemical test	Pedurungan Market		Peterongan Market		Minimarket		*S. typhi* (Hall and Woods, 2017)
	A1	A2	B1	B2	C1	C2	
Indol	-	-	-	-	-	-	-
MR	-	-	+	+	-	-	+
VP	+	+	-	-	-	-	-
Citrate	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Urea	-	-	-	-	-	-	-
TSIA	Alkaline/ Acid and H_2S (+), gas (+)	Alkaline/ Acid and H_2S (+), gas (+)	Alkaline / Acid and H_2S (+), gas (-)	Alkaline/ Acid and H_2S (+), gas (-)	Alkaline / Acid and H_2S (+), gas (+)	Alkaline/ Acid and H_2S (+), gas (+)	Alkaline/ Acid and H_2S (+), gas (-)

Biochemical test results can represent the physiological characteristics of certain bacteria. Based on the results of biochemical tests in this study, isolates from the Peterongan market matched with the characteristics of *S. typhi* [9,19] supported by the *fliC* gene amplification result. Biochemical identification test results and colony culture on SSA media were used as a comparison of the results of the amplification *fliC* gene in DNA isolated from grass-jelly samples. Identification of *S. typhi* using the *fliC* gene only without biochemical test is less specific because all other *Salmonella* isolates can also amplify the fliC gene by PCR.

Molecular-based food testing is fast enough and accurate to detect types of pathogenic bacteria contaminants qualitatively in food compared to culture methods and biochemical tests but the accuration has to increase. As a result of Table 1, we can see that isolates from Pedurungan and the minimarket have different biochemical characteristics with reference. For

increases the specificity we can use multiplex PCR with more that one primer such for detecting S. Typhi we can use genes such *rfbE*, *fliB*, *fliC* and *invA1*, and *invA2*.[20,21] However, the use of conventional PCR methods has limitations that can only be used to detect types of bacteria but are unable to count the number of bacterial cells. The method for detecting and calculating the predicted number of bacteria can be done by Quantitative PCR (qPCR).[22,23,24,25]

Based on the results of this study grass-jelly samples from two traditional markets and one branded packaging product, both were positive for *S. typhi*. In traditional markets it was observed that the process of selling black grass-jelly was placed in an open condition, exposed to air, dust, and the presence of flies attached to grass-jelly so that it was suspected to be a source of contamination beside from its conventional processing. Whereas the branded grass-jelly sample from a minimarket should not contain *S. typhi* because it is more controlled by the regulation of the Good Manufacturing Practice (GMP) system of industrial companies.[26] Suspected contamination in branded food samples was predicted comes from suppliers, storage processes, mixing, filling, or packaging processes in the company.[27] From the findings of *S. typhi* in the sample, manufacturing companies must improve and pay attention to the hygiene in the manufacturing process to maintain product safety.

4. CONCLUSION

In this study it can be concluded that the three grass-jelly samples from the Peterongan Market were contained *S. typhi* contamination and isolate from Pedurungan and minimarket were *Salmonella* spp. The method of identification pathogenic bacteria of S. typhi on food used amplification of *fliC* gene cannot be specific in *S. typhi* because isolates that have different biochemical characters from the *S. thypi* reference remain amplified so reducing the accuracy of the results.

DISCLOSURE STATEMENT

The authors declare that they have no conflict of interest.

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SHORT BIOGRAPHY

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