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Sensitivity of enrichment-PCR method for *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis analysis in chicken carcasses

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Abstract

Salmonella spp. is Gram negative-pathogenic bacteria that usually found as a contaminant in chicken carcasses. This study was aimed to increase the sensitivity of PCR enrichment step and apply the enrichment-PCR combination to detect *Salmonella* in chicken carcasses. In this study were used *Salmonella enterica* serovar Hadar, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis with the target genes were *invA*, *STM4497*, and respectively. A total of 25 g of the chicken carcasses were artificially contaminated by approximately 0.96 and 3.33 MPN/mL for each serovar separately. Samples were incubated in pre-enrichment and enrichment media for 8 hrs prior to the DNA extraction. The pre-enrichment and enrichment media was Buffered Peptone Water and Rappaport-Vassiliadis-soya. The result showed that the target genes of *S. enterica* ser. Hadar, *S. enterica* ser. Typhimurium and *S. enterica* ser. Enteritidis were detected in chicken carcasses, indicated by the presence of DNA band with the size was 429 bp, 311 bp and 135 bp respectively. These result in line with analysis using ISO method and BLAST-comparison analysis of DNA amplicon sequences with GenBank references. Application of this method for *Salmonella* detection in chicken carcasses sold in the traditional market showed a higher prevalence than the previous result without enrichment. All samples (n = 100) from unsanitary practice sellers were positively contaminated by *Salmonella* spp. and also high prevalence for *S. enterica* ser. Typhimurium and *S. enterica* ser. Enteritidis. It can be concluded that enrichment is an important step to increase the sensitivity detection of PCR method.

1. Introduction

Salmonella spp. is facultative anaerobic bacteria, Gram-negative, and rod-shaped bacilli. This bacterium belongs to family E*nterobacteriaceae* (Kim *et al.,* 2006). More than 2600 serovars *Salmonella* spp. have been identified (Popoff *et al.,* 2003). *Salmonella* has been known as a foodborne pathogen and caused outbreaks in several countries. *Salmonella* infections occurred in America (Scallan *et al.,* 2011), China (Xiao *et al.,* 2015), Australia (Ford *et al.,* 2018), Canada (Morton *et al.,* 2019). *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis are the dominant contaminant serovars in chicken carcasses. Both serovars are reported being the main cause of salmonellosis in humans (Lee *et al.,* 2009).

Molecular detection is proposed to be an effective and reliable method to detect *Salmonella* spp. in this research. The basic principle of the PCR method is to amplify the DNA fragment of target bacteria (Joshi and Deshpande, 2011). The PCR method has widely used to detect *Salmonella* in food (Wang *et al.,* 2018). The *invA* gene is responsible for the virulence of *Salmonella* spp. and can be found in all serovar *Salmonella* spp. (Shanmugasamy *et al.,* 2011). The STM gene encodes fimbrial biosynthesis, which found in *S. enterica* ser. Typhimurium, particularly (Clavijo *et al.,* 2006). Fimbrial in *S. enterica* ser. Typhimurium has a function for adhesion, attachment of bacteria to the target cell, and start the infection (Malorny *et al.,* 2003). The Prot6E gene is 60 kb virulence gene and only found in in *S. enterica* ser. Enteritidis (Malorny *et al.,* 2007).

Previous developed molecular detection has low sensitivity, so it needs to be combined with the enrichment techniques (Yosua, 2018). This study aimed to increase the sensitivity of *Salmonella* spp. detection

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using a combination of PCR and enrichment steps then applying the method to detect *Salmonella* from chicken carcasses samples. The parameters of the optimized enrichment-PCR step are incubation time and media type. The method used in this study also confirmed by the ISO 6579: 2002 standard method and DNA sequencing.

2. Materials and methods

2.1 Materials

The materials used in this study were in *S. enterica* ser. Hadar (BCC B2908), in *S. enterica* ser. Enteritidis (ATCC 13076) and in *S. enterica* ser. Typhimurium (ATCC 14028), Buffered Peptone Water (BPW) (Merck, Germany), Rappaport-Vassiliadis-soya (RVS) Broth (Merck, Germany), buffer TE 1X (Tris 10mM, 1 mM EDTA pH 7.5), TES bufer lisis (10 mM TRISbase pH 7.5; 1 mM EDTA pH 8.0; 0.5% SDS), proteinase K (Promega catalog number V3023 B), RNAse A (ThermoFisher Scientific catalog number EN0531), chelex100 (Sigma catalog number C7901), sodium acetate 3M, ethanol 95%, PCR GoTaq Green Master Mix (Geneaid), Nuclease Free Water, DNA ladder marker (Geneaid), 6x loading dye (Geneaid), *ethidium bromide* (BioBasic Canada Inc), agarose (ThermoFischer Scientific, Massachusetts, USA), (PCR primers (*invA* gene target, *STM4497, Prot6E*), Gel documentation (Bio -Rad, Bio-Rad Laboratories Pte. Ltd., Singapore), electrophoretic machines (Bio-Rad, Bio-Rad Laboratories Pte. Ltd., Singapore), Xylose Lysine Deoxycholate Agar (XLDA) (Merck, Germany), Triple Sugar Iron Agar (TSIA) (Merck, Germany), Muller-Kauffmann Tetrathionate-Navobiocin (MKTTn) (Merck, Germany), 100 chicken carcasses obtained from Bogor traditional markets. PCR instrument (Applied Biosystem Thermal Cycler 2720 Thermal Cycler).

2.2 DNA extraction

The DNA extraction of the deposition method by chelex100 resin was performed as described by Reyes *et al.* (2010). *Salmonella* bacterial culture was centrifuged for 20 mins at 3000 rpm to separate the bacterial cells. The formed sediment or bacterial cells were transferred into the microtube and recentrifuged for 5 mins at 8000 rpm at 4°C. The pellet that settles at the bottom of the tube was resuspended with a 1000 µL TE buffer solution (10mM TRIS-base pH 7.5; 1 mM EDTA pH 8.0). The mixture of the solution was centrifuged for 5 mins at 8000 rpm at 4°C. The pellet was resuspended with 100 µL TES buffer lysis (10 mM TRIS-base pH 7.5; 1 mM EDTA pH 8.0; 0.5% SDS), incubated at 65°C for 5 mins. Approximately, 7.5 µL proteinase and 2 µL RNAse were added to the solution, incubated at 65°C for 5 mins, and at room temperature for 2 mins. A total of 150 µL of chelex100 TE buffer was added into the tube, incubated at 65°C for 5 mins, centrifuged for 10 mins at 10000 rpm at 4°C. The aqueous phase of the solution was transferred into a clean microtube. 3M sodium acetate was added as much as 0.1 times the volume of the liquid layer, and 95% ethanol 2.5 times of the volume. The mixture was incubated in the freezer at -20°C for 20 hrs. After the centrifugation of the mixture for 5 mins at 8000 rpm, the supernatant was removed, and the tube containing the pellet at the bottom of the tube was dried at room temperature (26-27°C). Approximately, 75 µL of NFW was added into a tube containing the dry pellet and stored in a freezer for further analysis.

2.3 PCR amplification and gel electrophoresis

The mixture for the PCR reaction was 10 µL Master Mix (GeneAid), 1 µL of each primer forward and reverse, 7 µL Nuclease Free Water, and 1 µL DNA template. The PCR instrument was set for an initial denaturation at 95°C for 3 mins for 1 cycle, followed by denaturation temperature at 95°C for 1 mins for 35 cycles, annealing temperature at 52°C for 1 mins and extension temperature at 72°C for 1 mins, followed by a final extension temperature at 72°C for 5 mins. The running condition of the PCR instrument was set according to the Ta (Temperature annealing) and Tm (Temperature Melting) of a primer so the amplification process could occur properly. The primers and gene targets are detailed in Table 1. The PCR products were separated using electrophoresis gel for 45 mins at 90 V. The visualization was done using Gel Documentation after the gel was stained in EtBr for 30 mins.

2.4 Enhancement of sensitivity and Salmonella spp. detection using enrichment technique

A total of 25 g of the chicken carcasses were put in a

Table 1. Gene targets and primer sequences

Serovar	Gene target	Primer	Primer sequence (5' – 3')	Size (bp)	Reference
Salmonella spp.	*invA*	ST11-*f*	GCC AAC CAT TGC TAA ATT GGC GCA	429	Soumet *et al.* (1999)
		ST15-*r*	GGT AGA AAT TCC CAG CGG GTA CTG G		
S. enterica ser. Typhimurium	*STM4497*	STM4497M2-*f*	AAC AAC GGC TCC GGT AAT GAG ATT G	311	Park *et al.* (2009)
		STM4497M2-*r*	ATG ACA AAC TCT TGA TTC TGA AGA TCG		
S. enterica ser. Enteritidis	*Prot6E*	438-*f*	GGC ACC GCA GCA ATG GTT GG	135	Hadjinicolaou *et al.* (2009)
		572-*r*	GGT CGA GCT ACA GAG AGT CAC AC		

sterile plastic bag and it was dipped in boiling water for 30 s to reduce the number of initial bacteria. Approximately, 1 mL pure cultured of each *S. enterica* serovars was added into plastic bags separately with 1 to 10 CFU/mL. Then, 225 mL of BPW media was added to the plastic bag containing the sample and bacterial culture and incubated for 2, 4, 6, and 8 hrs at 37°C. The fastest incubation time was chosen with a 100% positive percentage, then it was combined with another incubation using RVS media. An aliquot (1 mL) of the obtained culture was transferred to 10 mL RVS then incubated at 42°C. The DNA was extracted from RVS media. Gel electrophoresis was used to visualize the PCR product. The analysis was carried out five times for each serovar to check the stability of this method. It also needs to be confirmed using a standard ISO 6579:2002 method. Confirmation of the PCR products was done by DNA sequencing.

2.5 Confirmation by ISO 6579: 2002 and DNA sequencing

The confirmation by ISO 6579:2002 method was performed using ten chicken carcasses samples. A total of 25 g of the sample was put into a sterile plastic bag containing 225 mL BPW and was incubated for 18 hrs at 37°C. As much as 0.1 mL of suspension was transferred into a tube containing 10 mL RVS media, and was incubated for 18 hrs at 42°C. A loopful of RVS suspension was streaked aseptically onto XLDA and TSIA slant and the slants were incubated for 18 hrs (ISO, 2002).

PCR products that showed positive result of each gene of *invA, STM4497*, and *Prot6E* were sent to First BASE Laboratories in Malaysia for DNA sequencing. Sequencing results were analyzed using the Bioedit 7.2.5 program (http://bioedit.software.informer.com/7.2/). Then, the results were matched with data in the GenBank data center via the NCBI website (https:// www.ncbi.nlm.nih.gov/) using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.Cgi).

2.6 Application of confirmed methods in chicken carcasses

The enrichment-PCR method that had been confirmed in the previous stages was used in detecting *Salmonella* spp. in chicken carcasses. The sample was 100 chicken carcasses from nineteen traditional markets in Bogor, Indonesia. The selected sellers have not applied good sanitary practices for handling their products based on previous observations.

3. Results and discussion

3.1 Enhancement of sensitivity and Salmonella spp. detection using enrichment technique

All three serovars *Salmonella* spp. could be detected at very low bacterial counts (0.96-3.33 MPN/mL) with incubation times from 4 to 8 hrs on BPW media (Table 2). The incubation time of 2 hrs was not enough to detect these bacteria at a low number. Therefore, the incubation time for 4 hrs was chosen. The number of *Salmonella* spp. was increased from 10^2 to 10^8 CFU/mL, it was detected using the fibre-optic method with a combination of enrichment in BPW and RV for 4 hrs incubation in each media (Abdelhaseib *et al.,* 2016). The log number of in *S. enterica* ser. Enteritidis and in *S. enterica* ser. Typhimurium were increased from 10 to 10^5 CFU/mL with an incubation time of 8 hrs on BPW (Josefsen *et al.,* 2007).

Table 2. Optimization of incubation time in BPW media

Serovar	Incubation time (hrs)	Positive test percentage (%)
S. enterica ser. Hadar	2	50
	4	100
	6	100
	8	100
S. enterica ser. Typhimurium	2	0
	4	100
	6	100
	8	100
S. enterica ser. Enteritidis	2	0
	4	100
	6	100
	8	100

BPW used as non-selective pre-enrichment media and RVS used as selective enrichment media. In non-selective media, the injured cells of both pathogen bacteria target and other bacteria are usually repaired (Chen *et al.,* 2013). RVS media should promote the growth of the recovered bacteria target (Margot *et al.,* 2015). RVS has more nutrients resulting in better growth of individual *Salmonella* strains (Gorski, 2012). RVS components such as $MgCl_2$ will increase osmotic pressure along with malachite green and low pH of this media inhibit the growth of non-*Salmonella* (Rappaport *et al.,* 1956; Vassiliadis *et al.,* 1978). The enrichment-PCR analysis with a combination of BPW and RVS for a total of 8 hrs gave a 100% positive percentage in detecting *Salmonella* spp. with low detection limit (Table 3).

3.2 Confirmation with ISO 6579: 2002 and DNA sequencing.

Confirmation by the ISO 6579:2002 standard procedure was done simultaneously with the enrichment-PCR method and used the same chicken carcass samples.

Table 3. Analysis of enrichment-PCR method in chicken carcasses

Salmonella spp. serovars	Total count (MPN/mL)	Positive result test	Positive test percentage (%)
S. enterica ser. Hadar	3.33	5/5	100
	0.96	5/5	100
S. enterica ser. Typhimurium	2.08	5/5	100
	1.00	5/5	100
S. enterica ser. Enteritidis	2.38	5/5	100
	1.00	5/5	100

XLDA was used as a detection media for *Salmonella* because it is more likely to yield black colonies from a wide variety of *Salmonella* isolates (Gorski *et al.,* 2011). TSIA contained lactose, sucrose, and dextrose used to detect bacteria that can ferment those sugars and produce H2S (Majeed, 2016). The result shows all samples (n = 10) were positively contaminated with *Salmonella* spp. not only with the enrichment-PCR but also with the ISO 6579:2002 method. Positive results were marked by the growth of black colonies on the XLDA plate. *Salmonella* spp. is a bacteria that can ferment sugars, so the positive result was marked by a change in color and the formation of gas in TSIA slant.

Another confirmation was performed by sequence of PCR product (amplicon) of 2 positives samples for *Salmonella* spp., in *S. enterica* ser. Typhimurium, and in *S. enterica* ser. Enteritidis with *invA*, *STM4497*, and *Prot6E* primer respectively. The results were analyzed using BioEdit to find consensus region of the base sequence before further analysis with BLAST NCBI (Table 4). *S. enterica* ser. Typhimurium and *S. enterica* ser. Enteritidis appeared in BLAST analysis of sequenced samples (Table 5). The BLAST analysis of the sequenced sample with *invA* primer showed that there were *S. enterica* ser. Enteritidis and *S. enterica* ser. Typhimurium as serovar matched with the genebank database. The *invA* gene located in a chromosome known as *Salmonella* Pathogenicity Island (SPI) I, a protein encoder, in the inner membrane of bacteria (Boddicker *et al.,* 2013). The majority of *Salmonella* virulence genes distributed in a chromosome called SPI (Pal *et al.,* 2017). The *invA* gene is responsible for the virulence of *Salmonella* spp. and found in all serovar *Salmonella* spp. (Shanmugasamy *et al.,* 2011). Several bacteria genus were detected simultaneously by the multiplex PCR method. The *invA* gene was used as a gene target for the detection of *Salmonella* spp. (Kim *et al.,* 2007). The identification of *Salmonella* spp. in poultry meat was carried out using *invA* primers (Sharma and Kashmiri, 2016). Compared to other virulence genes, *invA* gene was detected in all *Salmonella* isolates (Thung *et al.,* 2018).

S. enterica ser. Typhimurium and *Klebsiella pneumoniae* appeared on the BLAST result. Further analysis was performed to confirm these findings. Based on the percent identity to sequenced samples, *S. enterica ser.* Typhimurium had 96.76%, while *Klebsiella pneumoniae* had lower percent identity, which was 96.46%. Alignment analysis conducted between the two bacteria and the sequenced sample also showed that the matched number of bases was higher in *S. enterica ser.* Typhimurium. The *STM* gene encodes fimbrial biosynthesis and only found in *S. enterica ser.* Typhimurium (Clavijo *et al.,* 2006). Fimbrial in *S. enterica ser.* Typhimurium has a function for adhesion, attachment of bacteria to the target cell, and start the infection (Malorny *et al.,* 2003).

Salmonella enterica serovar 4,[5],12:i and 1,4,

Table 4. Result of the sequenced sample

Primer	Size (bp)	Sequences
invA	429	TTTGCCAACCAATGCTAAATTGGCGCACAACCTTCGACACAGACAGMAAATCGCTATTTTC GTCCGGCATGACGATGGTAACACTAAAAAAAGGGAGATTGCATCACTCTGTAGCAAATCAA GACCCTTGACAGGGTTTCTCCATTATCTTTCTACGCGCCGTATAGCGCTTCTCATCGACAAC CTAACTTCTGCACCAGACTCCCCTTTGTCGTTTTCACCTCGCTGGCTACCGCTTCAGGCAAG GCAGACCACAGCGTCAGCACTGGCTTCGTTTTATGTTCGATAGCCTCCACGGTAACCTGATA GTCGCAAAAATTCGCCGAACGGGGCGTATTCTGATCCATAATAAACGCAGCGTAATTTCCT TCACTTTTTGACGGCGCCGTTCCGGTGAATAWWACCTTCCAGTACCCGCTGGGAATTTCTA CCANA
STM4497	311	TGGGAACAACGGCTCCGGTAATGAGATTGGGTTCTGGATTTTTGATTATCCTGCTCAGAATG AGCTGCAGGTACGTGAACACCTGAAGTATCTGGTTCCGTAATCTGGAAAAGGACCACAAGT TCGCGCACCTCAACATCTTTCAGATCATYATCGACATGCTCACTGAACGTGGGTTATTTGAG CGCGTCTGTCAGCAGGAAGTGAAAGTCGGTACCGAAGCGCTGAAAAAACAACTCGTTGGC TTGTTAGAATCAGAAAAAGATCGCRGATTACATAGCAAAAWWAGTCGATCTTCAGAATCA AGAGTTTGTCNAANT
Prot6E	135	TGGCACCGCAGCAATGGTTGGGTTCGGGGGRGACTATWCCTACAGGGGCACAATAACCGT AACCGGAGAGGCGCTCATCGGTCCTGCTGTAGATGCAAGGGTGCCTAAGGTTAGTGTGACT CTCTGTAGCTCGACCATTGCTGCGGTGCCNA

Table 5. Confirmation the amplicon sequences with GenBank organism references using BLAST

Sample	Primer	Identified Ac. Numbers	Species	Subspecies	Serovar	Identified Serovars
A	*invA*	100	*Salmonella enterica*	subsp. enterica	Enteritidis	91
			Salmonella enterica	subsp. enterica	Typhimurium	1
			Salmonella enterica			8
	STM4497	100	*Salmonella enterica*	subsp. enterica	Typhimurium	47
			Salmonella enterica	subsp. enterica	1,4,[5],12,i	30
			Salmonella enterica	subsp. enterica	4,[5],12,i	8
			Salmonella enterica	subsp. enterica		12
			Salmonella enterica	subsp. enterica	Rough-O	2
			Klebsiella pneumoniae			1
	Prot6E	60	*Salmonella enterica*	subsp. enterica	Enteritidis	52
			Salmonella enterica			8
B	*invA*	100	*Salmonella enterica*	subsp. enterica	Enteritidis	91
			Salmonella enterica	subsp. enterica	Typhimurium	1
			Salmonella enterica			8
	STM4497	100	*Salmonella enterica*	subsp. enterica	Typhimurium	48
			Salmonella enterica	subsp. enterica	1,4,[5],12,i	27
			Salmonella enterica	subsp. enterica	4,[5],12,i	11
			Salmonella enterica	subsp. enterica		12
			Salmonella enterica	subsp. enterica	Rough-O	2
	Prot6E	60	*Salmonella enterica*	subsp. enterica	Enteritidis	53
			Salmonella enterica			7

[5],12:i are monophasic serovars of *S. enterica* ser. Typhimurium. The serovar was similar to *S. enterica* ser. Typhimurium, but has a genetic mutation (Ido *et al.,* 2014). *Salmonella* 4,[5],12:i was the result of a genetic mutation of *S. enterica* ser. Typhimurium that lacks a second phase flagella antigen (Switt et al. 2009). The virulence gene in *S. enterica* ser. Typhimurium was found in serovar 4,[5],12:i to prove that the serovar belongs to the *S. enterica* ser. Typhimurium variant (Palma *et al.*, 2018). *Salmonella* serovar Rough-O is a Typhimurium serovar that has an antigen-O deficiency in its genetic structure (Park *et al.,* 2018).

The result of sequencing analysis with Prot6E primer showed that only *S. enterica* ser. Enteritidis in BLAST analysis from a total of 60 Ac. identified numbers. The enrichment-PCR method using Prot6E primers for the detection of *S. enterica* ser. Enteritidis obtained specific detection. The *Prot6E* is a 60kb virulent gene that only found in *S. enterica* ser. Enteritidis. Detection of *Salmonella* PCR tests other than *S. enterica* ser. Enteritidis showed negative results using the *Prot6E* gene target (Malorny *et al.,* 2007).

3.3 Application of confirmed methods in chicken carcasses

Chicken carcasses sample were obtained from several traders in traditional markets in Bogor region, Indonesia. The selection of traditional markets was based on their poor sanitation of facilities and sanitary behaviour of the traders. Most of the traders stored chicken carcasses outdoors and did not store in refrigerator temperatures. Samples were whole chicken carcasses, not frozen, and estimated it had been on display for 2-5 hrs. The result of the analysis using the enrichment-PCR method shows a high prevalence of contamination. In contrast, previous studies using the non-enrichment method showed 60% contamination for *Salmonella* spp., 16% for *S. enterica* ser. Typhimurium and 60% for *S. enterica* ser. Enteritidis (Melati, 2019). All chicken samples from traditional markets (n = 100) were positively contaminated by *Salmonella* spp., 96 samples were contaminated with *S. enterica* ser. Typhimurium and 98 samples were contaminated with *S. enterica* ser. Enteritidis. Positive results were marked by

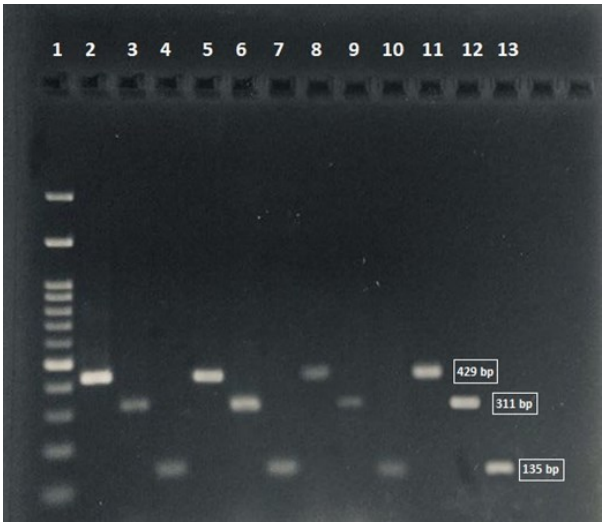

Figure 1. Visualization of sample amplicons using electrophoresis gel on agarose; (1) DNA ladder (2-10) Amplicon of samples (11-13) Positive control of primers

the appearance of DNA bands on agarose through gel electrophoresis gel (Figure 1). The amplicons size were matched with positive primer control, 429 bp for *invA*, 311 bp for *STM4497* and 135 bp for *Prot6E*.

4. Conclusion

Enrichment for 4 hrs on non-specific pre-enrichment media and 4 hrs on specific enrichment media can increase the sensitivity of PCR detection. Confirmation of sequenced amplicon to Genebank indicated the specific primer sequences used, *invA* gene for the *Salmonella* genus (429 bp), *STM4497* for *S. enterica* ser. Typhimurium (311 bp) and *Prot6E* (135 bp) for *S. enterica* ser. Enteritidis, are specific primers referring to those serovars. This method can be applied to detect contamination in chicken carcasses by showing a high prevalence of findings.

Conflict of interest

The authors declare no conflict of interest.

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