International Food Research Journal 24(6): 2689-2693 (December 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Analysis of *Salmonella enteritidis* in chicken meat and egg by real time-polymerase chain reaction

[1,2]Hayati, M., [1]Sudjadi and [1,3*]Rohman, A.

[1]Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, 55281 Indonesia
[2]Health Laboratory of South Kalimantan Province, Banjarmasin, Indonesia
[3]Research Center of Halal Products, Gadjah Mada University, Yogyakarta, 55281, Indonesia

Article history

Received: 12 July 2016
Received in revised form: 23 October 2016
Accepted: 26 October 2016

Keywords

Salmonella enteritidis Sdf1 gene
Real time PCR
Chicken meat
Egg

Abstract

The objective of this study was to develop analytical method for analysis of *Salmonella enteritidis* contamination in chicken meat and eggs using real time polymerase chain reaction (real-time PCR) method. A pair of primers have been designed using NCBI website and is proven to amplify *Sdf1* genes, a specific gene of *S. enteritidis*. The study involved pre-enrichment of bacterial culture of *S. enteritidis*, DNA isolation, validation real-time PCR method and application of validated method to analyze S. enteritidis in the commercial product. Isolation of DNA was performed using phenol-chloroform method. A range of temperature (51.0-62.2ºC) was examined to obtain optimum annealing temperature. The temperature of 60.2ºC was chosen based on melting peak profile. Specificity test performed toward four bacterial DNA (*S. enteridis, S. typhimurius, Escherichia coli* and *Listeria monocytogenes)* and chicken DNA showed that this primer specifically amplify *S. enteritidis* with melting peak point at 77.5-78.0ºC. Sensitivity test resulted the limit of detection at 12.5 pg/µL for *S. enteritidis* DNA. The results showed that no amplification peak in commercial products indicating that the tested commercial products do not contain *S. enteritidis.*

Introduction

Salmonella enteritidis is gram-negative bacterium, facultative anaerobic in nature, found mostly in the intestinal tract of animals like cattle, and transmitted by eggs and meat (Gast *et al.*, 2002). *S. enteritidis* has caused enteric pathogen which threat to public health, ranging from mild, watery diarrhea to life-threatening conditions, like dehydration, fever, vomiting, and in some cases, it can cause septicemia (Switt *et al*., 2009). *Salmonella* ranks the second or even the first of food poisoning around the worldwide (Lee *et al.*, 2015). National Standardization Body (Indonesian National Standard 7388: 2009) requires that *salmonella* should not contaminate 25 mg of food sample. European Commission (EC) Regulation 2073/2005, requires that foodstuffs should not be contaminated by any *Salmonella* or commonly known as "zero tolerance" (Feasey and Gordon, 2013). Therefore, it is necessity to develop a rapid, high sensitive and specific method of detection *S. enteritidis* for public health.

Several methods have been reported for identification of *S. enteritidis*. The most reported ones are culture-based methods according to ISO 6579:2002 (Maurischat *et al*., 2015) and immunological methods like enzyme-linked immunosorbent assay (ELISA) (Jaradat *et al.,* 2004). However it is not easy to detect low level of pathogens using culture methods and ELISA needs labelled antibody, therefore real-time polymerase chain reaction (real-time PCR) is massively developed for identification of pathogens including *S. enteritidis*.

Analysis of *Salmonella* with PCR on fish, chicken meat, eggs, milk and mayonnaise have been conducted (Chen *et al.*, 2010; Siregar *et al.*, 2013; (Almeida *et al*., 2013). Contamination of *S. enteritidis* in food (chicken, fish and dairy) with real-time PCR using TaqMan probe amplification as a marker showed 100% accuracy and more rapid than the conventional method, which only takes a test 24 hour, whereas with conventional methods, the test needs 4-5 days long (Malorny *et al*., 2004). Study of *S. enteritidis* detection was performed by Mallory *et al.* (2007) using specific oligonucleotide primers (Prot6e-5 and Prot6e-6) and *invA* gene as target of amplification. Several studies using ESR1, 2, 3 and saf A in *S. enteritidis* detection have been also carried out (Maurischat *et al*., 2015). In this study, *Sdf1* gene was used as primer target for *S. enteritidis* identification among four other DNAs (*S. typhimurium, E. coli, L. monocytogenes* and chicken). *Sdf1* (*Salmonella* difference fragment) is a gene which located in chromosome (Agron *et al.*,

*Corresponding author.
Email: *abdul_kimfar@ugm.ac.id*

2001).

Material and Methods

Primer design

The primer was designed in silico using website NCBI-Primer BLAST to obtain specific primer for *S. enteritidis* (taxid: 149539). Selected primers have G or C bases in the last 5 position of the 3´end, amounting to less than 3 G or C, which is expected to increase the specific binding at 3´ end, but not formed GC clamp folds. The primer sequence of target gene (*Sdf1* gene) is Sdf1 forward: 5' – CGAG CATG TTCTG GAAAGCC – 3', Sdf1 reverse: 5' – ATGG TGAG CAGA CAAC AGGC – 3' with amplicon length of 130 bp.

Bacterial Growth and DNA Extraction

S. enteritidis, S. typhimurium, E.coli and *L. monocytogenes* were inoculated in nutrient broth and were incubated at 37ºC for 16 ± 2 hours with gentle shaking. Eight milliliters of sample were centrifuged in 12.500 rpm for 6 minute. Supernatant was discarded and the pellet containing DNA was extracted using 750 µL lysis buffer and 10 µl proteinase K (20 mg/mL). The mixture was incubated at 55ºC for 30 minutes, followed by centrifugation. The supernatant was extracted using phenol-chloroform followed by DNA precipitation using two times volume of absolute ethanol. The DNA pellet was then washed using 70% ethanol and dissolved in TE buffer (10 mM Tris HCl pH 8 and EDTA 1 mM). Furthermore, the DNA samples were stored at -20ºC until being used for real-time PCR analysis.

Real-time PCR amplification of S. enteritidis *DNA*

Real time PCR was done using SsoFast®Evagreen®SUPERMIX with a pair of Sdf1 primers. The reaction was performed at 30 cycle with the condition of denaturation at 95ºC for 15 second, annealing at optimum temperature of 60.2ºC for 30 s, and extension at 72ºC for 30 s. optimization test was carried out using Sdf1 primers on S. enteritidis DNA at various annealing temperature (51.0-62.2ºC). This primer is subjected to validation procedure including specificity, sensitivity, efficiency, and repeatability.

Validation of real-time PCR for analysis of S. enteritidis *using Sdf1 primers*

Test of primer specificity was conducted on four species of bacteria (*S. enteridis, S. typhimurius, E.coli* and *L. monocytogenes*) and chicken meat DNA using optimum annealing temperature obtained. The limit of detection (LoD) was determined from standard curve of diluting series of *S. enteritidis* DNA (500, 250, 125, 50, 25 and 12.5 pg DNA). The value of LoD was expressed at the amount of DNA amplified by primer which resulting the last relative. These series of concentration were also used for making calibration regression to obtain efficiency value. The repeatability test was performed by computing coefficient variation (CV) of Ct during real-time amplification using certain concentration of wild boar DNA.

Detection of S. enteritidis *in reference sample of chicken egg and meat*

Reference sample was prepared by mixing the samples with dilution of bacteria, 100, 10^{-1}, 10^{-2} and 10^{-3}. A-900 mg chicken meat or 900 mL chicken eggs in 9 mL of buffered peptone water (BPW) was added to 100 mL of bacteria. The DNA is extracted and subjected to real-time PCR measurement.

Detection of contamination of S. enteritidis *in commercial products*

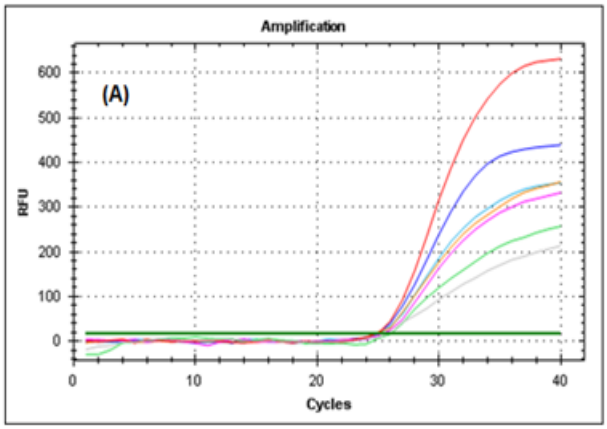
Identification of contamination in chicken meat, egg and nugget derived from traditional markets and supermarkets in Yogyakarta is performed using real-time PCR. The analysis was done using primers with condition as indicated on amplification procedure.

Result and Discussion

Real-time PCR analysis

The identification of bacteria of *S. enteritidis* in food product is very essential in order to assure the safety of food products. Real-time PCR is an ideal technique for bacterial identification via DNA analysis. Some analyst designed primers which are specific for bacteria DNA. The designed primer of Sdf1 has the G-C base contents in the last 5 position of the 3' end less than 3. This composition can increase the specific binding at the 3' does not form GC clamp folds (Wang and Seed, 2006). Using this primer, the amplicon length is 130 bp. This primer is further used to amplify DNA extracted from chicken meat products and eggs which were contaminated by *S. enteritidis*. Primer Sdf1 is subjected to validation test (primer specificity, sensitivity, linearity, efficiency and repeatability) (Widyasari *et al*., 2015).

The selection of an appropriate primer and optimization of real-time PCR conditions are the important factor in real-time PCR (Zulkifli *et al.*, 2015).The optimization of annealing temperature of designed primer into DNA template (DNA extracted from samples) is an important part in PCR analysis. To determine the optimum annealing temperatures



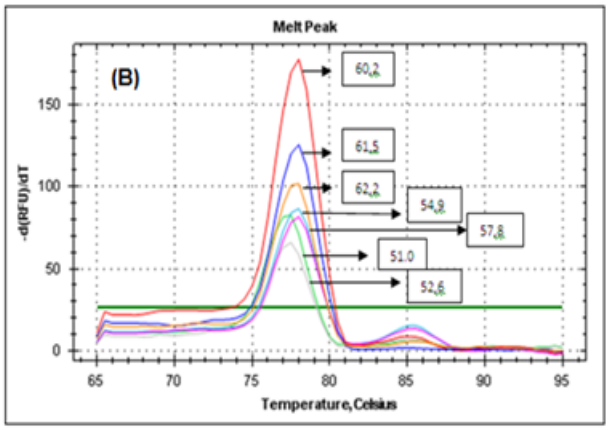
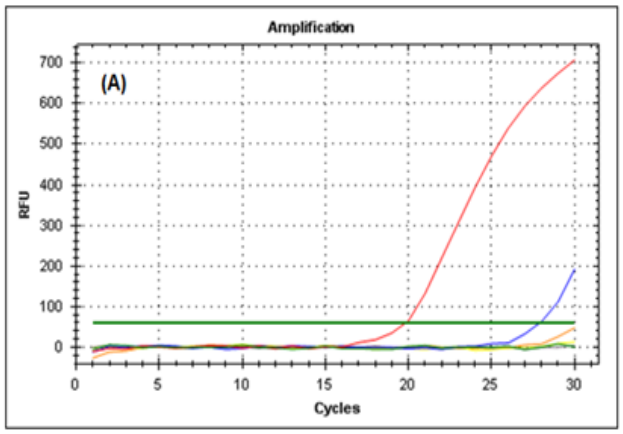


Figure 1. Amplification curve (A) and melting peak profile (B) of *Salmonella enteritidis* DNA using primer Primer Sdf1 at various annealing temperatures. The annealing temperature of 60.2°C is chosen because of its capability to exhibit the highest intensity.



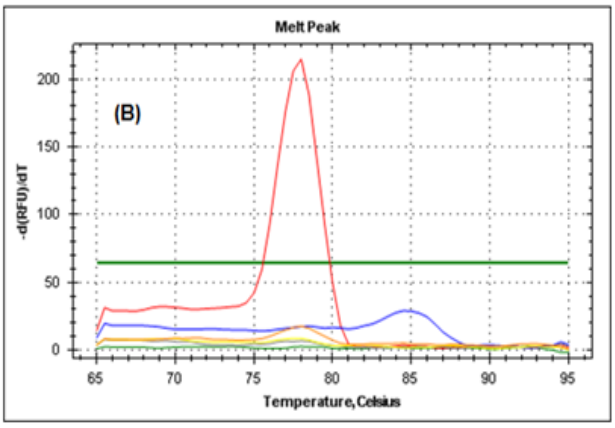


Figure 2. Amplification curve (A) and Melting peak (B) obtained during specificity test of Sdf1 primer on DNA from various bacteria and chicken meat. Red line: *S. enteritidis*; blue line: Chicken; orange line: *L. monocytogenes*; yellow line: *E.coli*; grey line: *S. typhimurium*; green line: non template control (NTC).

of primer, the primer was tested in various gradient temperatures, namely 51.0, 52.6, 54.9, 57.8, 60.2, 61.5 and 62.2ºC. The amplification curve revealed that melting peak, at annealing temperature of 62.2°C, showed the highest relative fluorescence unit (RFU) of 177.75 with the lowest number of cycles (24.74), as indicated in Figure 1. Therefore, annealing temperature of 62.2°C was selected during validation and analysis of samples using real-time PCR analysis.

Validation of analytical method

Validation of analytical method of real-time PCR is intended to its purpose for identification of *S. enteritidis* in chicken meat and eggs through determination of several parameters namely, primer specificity, sensitivity, efficiency and repeatability as recommended by Codex Allimentarius Commission (2010). The specificity test of Sdf1 primer is conducted by amplifying the DNA of *S. enteritidis, S. typhimurium, E.coli, L. monocytogenes* and chicken DNA. Figure 2 revealed the amplification

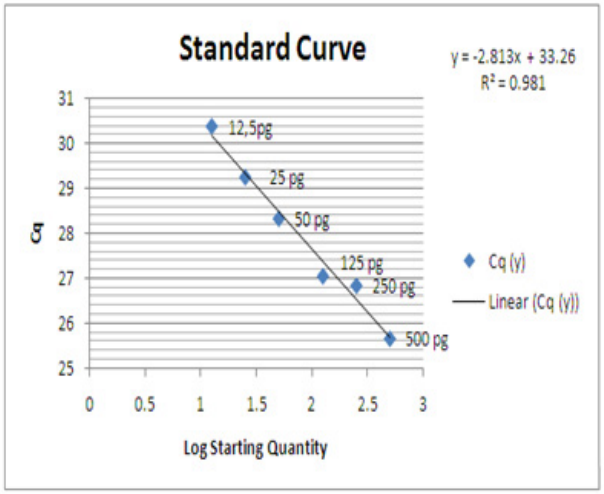
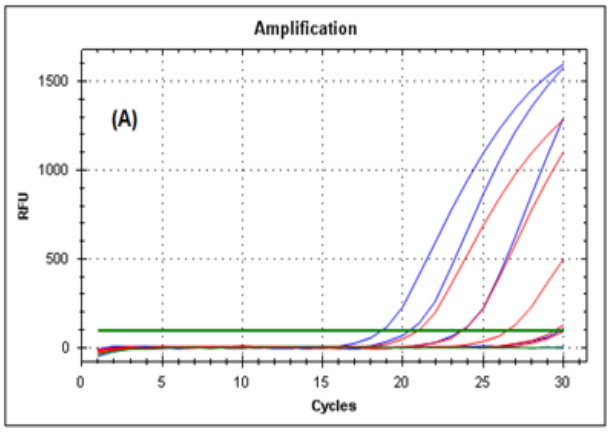


Figure 3. Standard curve relating the log concentration of *S. enteritidis* DNA and Cq value.

curve of Sdf1 primer. DNA from *S. enteritidis* is amplified at Cq value of 19.80, while DNA from chicken is amplified at Cq value of 27.88, but with different melting peak point. Therefore, DNA from S. enteritidis and chicken meat can be distinguished



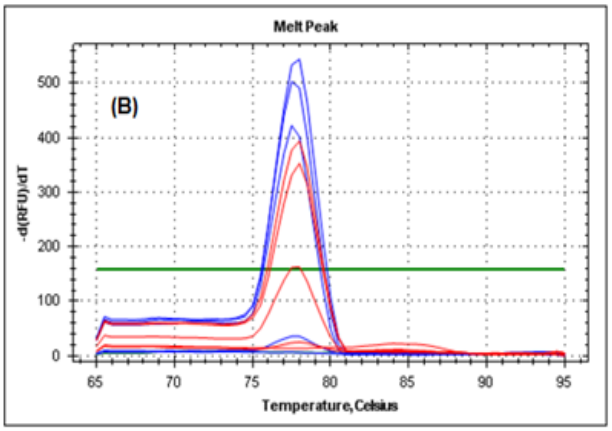
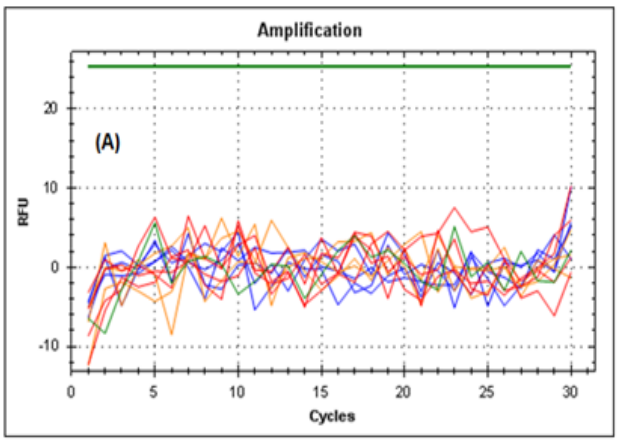


Figure 4. Amplification curve (A) and Melting peak (B) of sample reference containing *S. enteritidis* in chicken egg and meat; blue line: chicken egg; red line: chicken meat.



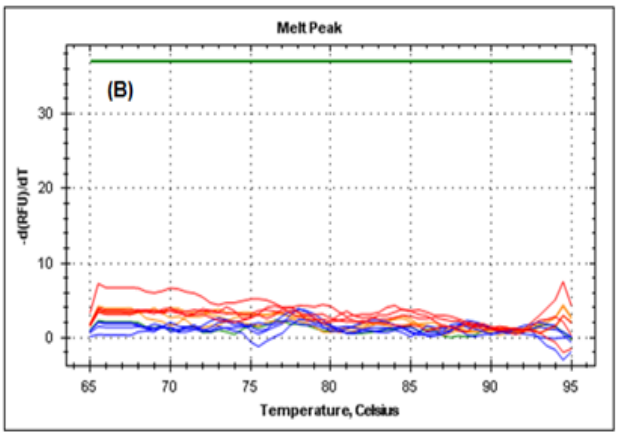


Figure 5. Amplification curve (A) and Melting peak (B) of commercial samples of chicken egg and meat; blue line: chicken egg; red line: chicken meat.

based on its melting peak. Whereas other bacterial DNAs are not amplified, as indicated by the absence of a Cq value. Thus, the primer of Sdf1 is specific among tested bacteria and chicken meat.

The sensitivity of real-time PCR is evaluated by diluting concentration series of DNA, and the standard curve of S. enteritidis relating between the amount of DNA and Cq value was built, as in Figure 3. Standard curve has coefficient of determination (R^2) of 0.981, slope -2.813 and y-intercept of 33.26. The value of amplification efficiency (E) was 126.67%. The value E is above 110%, indicating that there is interference during PCR reaction, such as a low quality DNA and carry over during DNA purification (Biorad, 2006; Muhammad et al., 2015). Primer Sdf1 able to amplify DNA as low as 12.5 pg at Ct value of 30.39. This value (12.5 pg) is detection limit of real-time PCR to be used for identification of *S. enteritidis* DNA.

The repeatability test showed positive amplification from *S. enteritidis* DNA with concentration 125 pg. Replication of this concentration shows similar Cq value. The coefficient variation (CV) of Cq values are 12.63%, which is lower than required by Codex Allimentarius Commission (2010), i.e. CV must ≤ 25%. Therefore it can be concluded that the real-time PCR is precise (Rahmawati *et al.*, 2016).

Detection of S. enteritidis *in reference samples and commercial samples*

DNA derived from reference samples contained *S. enteritidis* with dilution 100, 10^{-1} and 10^{-2} was amplified, while sample with bacteria dilution 10^{-3} was not amplified both in meat and chicken eggs (Figure 4). Amplification of DNA extracted from sample products of chicken (4 meats, 4 eggs and 3 nuggets) is shown in Figure 5. There are no amplification peaks on each samples. This result indicated that evaluated commercial samples do not contain *S. enteritidis.*

Conclusion

Real-time PCR using primer targeting on Sdf1 *gene* has been successfully used for detection of *S. enteritidis* with optimum primer annealing temperature is 60.2ºC with limit of detection (LoD) of 12.5 pg. Standard curve showed coefficient of determination (R^2) 0.981 and amplification efficiency

of 26.67%. Amplification of sample products showed no *S. enteritidis* detected, which mean that there were no *S. enteritidis* contamination on the evaluated products.

Acknowledgement

The authors thank to the Ministry of Research and Higher Education, Republic Indonesia for financial assistance during conducting this research via scheme Penelitian Unggulan Perguruan Tinggi (PUPT) with contract number: 624/UN1-P.III/LT/DIT-LIT/2016.

References

Almeida, C., Cerqueira, L., Azevedo, N.F. and Vieira, M.J. 2013. Detection of *Salmonella enterica* serovar Enteritidis using real time PCR, immunocapture assay, PNA FISH and standard culture methods in different types of food samples. International Journal of Food Microbiology 161: 16–22.

Agron, P.G., Walker, R.L., Kinde, H., Sawyer, S.J., Hayes, D.C., Wollard, J. and Andersen, G.L. 2001. Identification by Subtractive Hybridization of Sequences Specific for *Salmonella enterica* Serovar Enteritidis. Applied Environmental Microbiology 67: 4984–4991

Bio-Rad. 2006. Real-time PCR application guide. Retrieved on December 20, 2015 from Biorad website: *http://www.biorad.com/webroot/web/pdf/lsr/literature/Bulletin_5279.pdf*

Chen, J., Zhang, L., Paoli, G.C., Shi, C., Tu, S.-I. and Shi, X. 2010. A real-time PCR method for the detection of *Salmonella enterica* from food using a target sequence identified by comparative genomic analysis. International Journal of Food Microbiology 137: 168–174.

Codex Alimentarius Commission. 2010. Codex Guidelines on Performance Criteria and Validatin of Methods for Detection, Identification, and Quantification of Specific DNA Sequences and Specific Proteins in Foods (CAC/GL 74). Retrieved from *http://www.fao.org/fao-who-codexalimentarius/standards/list-of-standards/en/*

Feasey, N.A. and Gordon, M.A. 2013. Salmonella infections. Manson's Tropical Disease 2: 342-346.

Gast, R.K., Guard-Petter, J. and Holt, P.S. 2002. Characteristics of *Salmonella enteritidis* contamination in eggs after oral, aerosol, and intravenous inoculation of laying hens. Avian Disease 46: 629–635.

Jaradat, Z.W., Bzikot, J.H., Zawistowski, J. and Bhunia, A.K. 2004. Optimization of a rapid dot-blot immunoassay for detection of *Salmonella enterica* serovar Enteritidis in poultry products and environmental samples. Food Microbiology 21: 761–769.

Lee, K.M., Runyon, M., Herrman, T.J., Phillips, R. and Hsieh, J. 2015. Review of *Salmonella* detection and identification methods: aspects of rapid emergency response and food safety. Food Control 47: 264–276.

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A. and Helmuth, R. 2004. Diagnostic Real-Time PCR for detection of *Salmonella* in Food. Applied Environmental Microbiology 70: 7046–7052.

Maurischat, S., Baumann, B., Martin, A. and Malorny, B. 2015. Rapid detection and specific differentiation of *Salmonella enterica* subsp. *enterica* Enteritidis, Typhimurium and its monophasic variant 4,[5],12:i: − by real-time multiplex PCR. International Journal of Food Microbiology 193: 8–14.

Muhammed, M.A., Bindu, B.S.C., Jini, R., Prashanth, K.V.H. and Bhaskar, N. 2015. Evaluation of different DNA extraction methods for the detection of adulteration in raw and processed meat through Polymerase Chain Reaction-restriction Fragment Length Polymorphism (PCR-RFLP). Journal of Food Science and Technology 52: 514-520.

Rahmawati, Sismindari, Raharjo, T.J., Sudjadi and Rohman, A. 2016. Analysis of pork contamination in Abon using Mitochondrial DLoop22 Primers using Real Time Polymerase Chain Reaction Method. International Food Research Journal 23(1): 370-374.

Siregar, T.H., Elliman, J. and Owens, L. 2013. Development of real time polymerase chain reaction for detection of *Salmonella typhimurium* and *Salmonella enteritidis* in fish. SQUALEN Bull. Mar. Fish Postharvest Biotechnology 7: 51–58.

Standar Nasional Indonesia (Indonesian National Standard). 2009. Batas Maksimum Cemaran Mikroba dalam Pangan (The maximum limit of microbes in Food, SNI 7388). Retrieved from *http://sisni.bsn.go.id/index.php/sni_main/sni/detail_sni/9806*

Switt, A.I.M., Soyer, Y., Warnick, L.D. and Wiedmann, M. 2009. Emergence, distribution, and molecular and phenotypic characteristics of *Salmonella enterica* serotype 4, 5, 12:i. Foodborne Pathogens and Disease 6: 407–415.

Wang, X. and Seed, B. 2006. High-Throughput Primer and Probe Design. In Dorak, M.T. (Ed). Real-Time PCR, p. 93-104. New York: Taylor and Francis.

Widyasari, Y.I., Sudjadi and Rohman, A. 2015. Detection of Rat Meat Adulteration in Meat Ball Formulations Employing Real Time PCR. Asian Journal of Animal Sciences 9 (6): 460-465.

Zulkifli, Y., Alitheen, N.B., Son, R., Raha, A.R., Samuel, L., Yeap, S.K. and Nishibuchi, M. 2009. Random amplified polymorphic DNA-PCR and ERIC PCR analysis on *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia. International Food Research Journal 16: 141-150.