Rapid Detection of *Shigella flexneri* on Egg Samples by the Real Time Polymerase Chain Reaction Method

Muktiningsih Nurjayadi, Noer Azizah, Ulfi Rahma Efrianti, Fera Kurniadewi, Dalia Sukmawati, Vira Saamia, I Made Wiranatha, Lidwina Nastassya

Abstract*— Shigella flexneri is one of the foodborne pathogen bacteria that caused food poisoning. Detection methods that are sensitive, specific and fast are very necessary in dealing with food poisoning, among these methods is Real Time PCR (RT-PCR). This study aims to apply the RT-PCR method to detect and quantify the Shigella flexneri bacteria in egg samples with the ipaH target gene. The amplicon of the target gene from the amplification process is 188 base pairs. Confirmation test of the ipaH primers pair with DNA template in concentration of Shigella flexneri culture of ±50 ng/μL gave the value of Cycle threshold (Ct) ±12. Primers sensitivity evaluation in detecting target bacteria gives the results that up to the smallest concentration of 8.05 pg/μL with a Ct value of 24.939. Specificity testing shows that the ipaH primers pair can differentiate Shigella flexneri bacteria significantly with some non-target bacteria as negative controls. Quantification of the number of bacteria found in egg samples using the flow of line equations by the RT-PCR method of 15.85 x 10^{-5} CFU/mL. These results provide more sufficiently information compared to the culture method. Based on the results, it can be concluded that the RT-PCR method was successfully applied in detecting Shigella flexneri bacteria with the target ipaH gene in egg samples quickly, sensitive and specific as well as can determine the number of bacteria accurately.

Keywords*— ipaH primers, Real Time PCR, Shigella flexneri, egg sample.

I. INTRODUCTION

Food is a basic need to support human survival, so food security is an important and increasing focus of attention. Lack of food security caused by processing, improper cooking and storage management are the main transmission lines for foodborne pathogenic microorganisms [1]. Food commodities that are often related to food poisoning cases are eggs, this is because eggs are a good medium for growth of bacteria [2]. One of the bacteria that caused food poisoning cases is *Shigella flexneri*. *Shigella flexneri* bacteria are included in the 500,000 cases of Shigellosis for each year in the United States [3]. The number of cases of food poisoning caused by bacteria is one reason that it is very necessary to develop a detection method that is fast, specific and accurate, so that handling food poisoning cases becomes more effective and efficient and can reduce the number of victims [4].

In this research, genomic level detection method which is Real Time PCR (RT-PCR) is used, a method that can amplify and quantify [5]. Testing using these methods provides faster and specific results for the types of pathogenic bacteria or causes of food poisoning in patients [6, 25, 26].

Previous research by Mokhtari *et al*., (2013) stated that the use of the primer pair of *ipa*H genes has successfully detected the presence of *Shigella* species with the Real Time PCR method in food samples and naturally contaminated stool samples [7]. In this study, the *ipa*H gene was used, but the primer's design in the position and sample of food was different from what the previous researchers did. The food samples used within this study were egg samples. So that it is expected to provide an alternative choice of detection devices for a variety of samples that vary in identification *Shigella flexneri* bacteria. Various primer's choices for this sample can be used as a benchmark for developing rapid, accurate, and more sensitive detection of *Shigella flexneri* bacteria. If the bacteria are quickly recognized, the treatment will be more focused. In the end, it can improve the quality of human life, and increase the repertoire of knowledge about the development of methods for rapid detection of food poisoning bacteria in Indonesia.

II. METHODOLOGY

Cultivate of Shigella flexneri Bacterial Culture

The culture of *Shigella flexneri* ATCC 9199 (Microbiologists) bacteria that have been rejuvenated and stored in the Luria-Bertani glycerol media (glycerol stock) was used as a test bacterium. Bacterial culture in glycerol stock was taken using 3 (three) ose sterile needles and planted using streak plate method McConkey Agar (Merck). Then the sample incubated for 24 hours at 37 ° C using the LM Series (Yihder Co., LTD) orbital shaking incubator at 200 rpm. Indications of the growth of *Shigella flexneri* bacteria are colorless colonies in the media McConkey Agar (Merck). One specific colony in each bacterium was taken and then replanted in liquid media Luria Broth (Deben Diagnostics, Ltd) incubated for 18 hours at 37 ° C.

Revised Manuscript Received on April 15, 2019.

Muktiningsih Nurjayadi, Associate Professor, Department of Chemistry, Universitas Negeri Jakarta, Indonesia. (muktiningsih@unj.ac.id)

Noer Azizah, Student Department of Chemistry, Universitas Negeri Jakarta, Indonesia. (noerazizah3@gmail.com)

Ulfi Rahma Efrianti, Student Department of Chemistry, Universitas Negeri Jakarta, Indonesia. (ulfirahma9@gmail.com)

Fera Kurniadewi, Senior Lecturer , Department of Chemistry, Universitas Negeri Jakarta, Indonesia. (fera@unj.ac.id)

Dalia Sukmawati, Senior Lecturer , Department of Biology, Universitas Negeri Jakarta, Indonesia. (Dalia-Sukmawati@unj.ac.id)

Vira Saamia, Senior Researcher, Pusat Laboratorium Forensik Badan Reserse Kriminal Kepolisian RI, Indonesia (virasaamia@yahoo.com)

I Made Wiranatha, Senior Researcher, Pusat Laboratorium Forensik Badan Reserse Kriminal Kepolisian RI, Indonesia (madewiranatha67@gmail.com)

Lidwina Nastassya, Senior Researcher, Balai Besar Uji Standar Karantina Pertanian, Jakarta, Indonesia (fankytassya@gmail.com)

The indication of the growth of these bacteria is the formation of turbidity in the media of Luria Broth (Deben Diagnostics, Ltd).

Preparation of Egg Samples

Chicken egg samples obtained from the market are boiled and crushed to a small size in sterile plastic. As much as 1 gram of egg sample was weighed in aseptic petri dishes, sterilized with 70% alcohol and irradiated by UV lamps in laminar air flow (ESCO) for 15 minutes.

Inoculation of Bacterial Culture in Egg Samples

1 (one) mL of *Shigella flexneri* bacterial suspension in LB media (Deben Diagnostics, Ltd), put into a test tube containing 9 (nine) mL of 0.85% NaCl and a dilution series was carried out until 10-7 dilution. The bacterial suspension at 10-4 to 10-7 dilutions was planted in the media to be specific to the spread plate method to calculate the number of bacterial colonies found in the suspension. The suspension that produces a number of colonies between 25-250 is chosen based on the FDA BAM (Food and Drug Administration Analytical Manual) count plate standard to calculate the number of pure culture bacteria contaminated with eggs. The bacterial suspension was contaminated to egg samples with a ratio of 1: 1 as a positive control. Whereas negative controls are only egg samples without being contaminated with bacteria. Then each sample was incubated for 18 hours at 37 ° C.

Isolation and characterization of DNA of Shigella flexneri bacteria

Each culture sample of *Shigella flexneri* (positive control), egg sample contaminated with *Shigella flexneri* bacterial culture (test sample), and egg sample without contaminated bacteria (negative control) of one mL were centrifuged at a speed of 5000 G for 5 minutes. The DNA pellet produced was isolated by the DNA protocol of QIAamp DNA Mini Kit (Qiagen) Gram-negative bacteria [8]. The concentration and purity of DNA from the isolation were measured at wave length the value of the A260 / A280 ratio with GE Nanovue Uv-vis Spetrophotometer.

Optimization of the Temperature of Annealing The ipaH Primer Pair of Shigella flexneri

Sequences of *Shigella flexneri*'s ipaH gene data were obtained from the NCBI GenBank. The area that shows the percentage of "Query Cover 0%" when compared to the bacteria *S. typhimurium*, *S. typhi*, and *E. coli* are the parameter chosen to be the DNA template which is then designed by the primer. The primer pair of ipaH gene's *Shigella flexneri* was designed with regard to good primer requirements such as primary length, amplicon length, melting temperature, % GC, Clamp GC, run and repeat in one base sequence [5]. The primers designed were subsequently synthesized at the commercial laboratory Macrogen, Inc. - Korea. Furthermore, the synthesized primer is optimized in the annealing temperature range (56 ° C, 57 ° C, 58 ° C, 59 ° C, 60 ° C, 61 ° C). Amplification was carried out using the appropriate method of the Dreamtaq Green Mastermix PCR reagent, namely at initial denaturation temperature of 95º C for 2 minutes, denaturation 95ºC for 30 seconds, annealing 5 ° C Tm for 30 seconds, extention at 72ºC for 1 minute and final extension at a temperature of 72ºC for 10 minutes, repeated in 40 PCR cycles [9].

Amplification DNA target by Real Time PCR Method

Each isolation DNA sample and annealing temperature data in stage E are then used as templates and conditions in the Real Time PCR (Applied Biosystem) process. Amplification of DNA by Real Time PCR was carried out in the following steps: (1) The primer confirmation test of the ipaH gene in egg samples; (2) the primer sensitivity test with dilution of target DNA isolates, (3) the specificity test by the primer cross linking of the IpaH gene against non-target bacteria. The preparation of the reaction mixture (positive control, test sample, and negative control) for the RT PCR process was carried out through mixing several components. That are consisting of a pair of IpaH primer, SYBR Green I master mix, DNA template, and Nuclease Free Water (NFW) with a total volume of 20 microliter (the composition of the mixture components and the primer sequence of the ipaH from this study is in the process of being patented). Each mixture was distributed into each well on the 96 well reaction plate, then the plate was closed with PCR ™ sealer and inserted into a real time PCR device using the PCR protocol as follows: initial denaturation temperature 95ºC for 3 minutes, denaturation 95ºC for 15 seconds, annealing 60ºC for 30 seconds, extention at 72ºC for 30 seconds, repeated in 40 PCR cycles. [10, 27, 28].

Characterization of DNA samples with Agarose Gel Electrophoresis

The isolated DNA was characterized by 0.7% agarose gel concentration while the amplified DNA was characterized by a 2% agarose gel concentration in TAE 1X buffer (Tris-acetat-EDTA) (Thermo Scientific) with etidium bromide (Promega). Electrophoresis was carried out at 70V for 60 minutes, then DNA bands were observed with UV transiluminator. DNA size is compared to a ladder (Thermo Scientific) to determine the size of the sample DNA [11].

III. RESULTS AND DISCUSSION

Cultivation of Shigella flexneri bacterial culture

The culture of *Shigella flexneri* culture produces colorless colonies in the McConkey Agar media as shown in Fig. 1. This indicates that the bacteria grown are actually the target bacteria of *Shigella flexneri*. In the McConkey Agar has a combination of lactose and neutral red indicator, which can indicate bacterial growth. *Shigella flexneri* bacteria cannot ferment lactose, therefore, utilizing the content of peptone as a source of nitrogen in the media to ferment, thus producing ammonia and increasing the pH of the media which causes the formation of white or colorless colonies. In addition, an increase in pH causes the neutral red indicator to change the color of the media so that from red to yellow. Another content such as crystal violet and bile salt in MCA media to prevent the growth of gram-positive bacteria and accelerate the growth of gram-negative bacteria. Furthermore, there is NaCl which functions to maintain osmotic balance in the medium [12].

Published By:
Blue Eyes Intelligence Engineering
& Sciences Publication

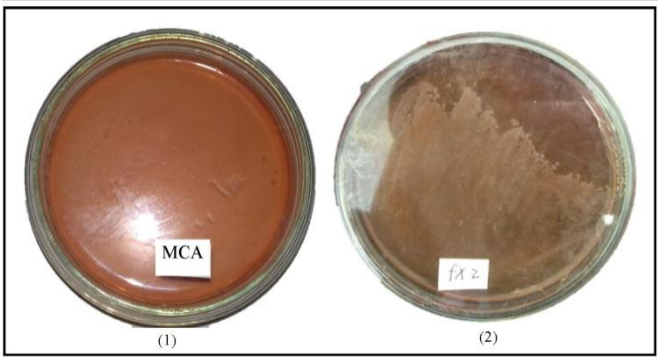

Fig 1. Characterization of *Shigella flexneri* bacteria on MCA media. (1) Before *Shigella flexneri* bacteria are grown, (2) After growing *Shigella flexneri* bacteria.

According to research conducted by Surjawidjaja (2007) [13] selective media are recommended for culturing and isolating *Shigella* species bacteria namely Xylose Lactose Dectrose (XLD) media> McConkey Agar (MCA) media> *Salmonella Shigella* Agar (SSA) media.

In this study, a single colony produced from the McConkey media was subsequently grown in Luria's broth media to obtain more bacteria so that the optimum DNA readily in the isolation process, and bacterial culture can be contaminated in egg samples.

The results of bacterial culture growth are indicated by turbidity in LB media that was previously transparent yellow. Turbidity formed in the media indicates that the *Shigella flexneri* bacteria can grow well with the media of Luria Bertani, which is rich in nutrients and the existence of a shaking culture process that can help optimize the supply of oxygen to bacterial growth compared to the absence of the process [14].

Inoculation of Shigella flexneri culture on Egg Samples

Bacterial cultures that have been grown on LB media were serially diluted from 10^{-1} to 10^{-7} using NaCl salt solvents with a concentration of 0.85%. The use of these concentrations is because at 0.85% NaCl concentration is the optimum condition for the growth of *Enterobactericeae* [15]. The results of the spread of the bacterial suspension plate at 10^{-4} dilutions to 10^{-7} and their coloni counts using the total plate count method, showed that in bacterial suspension 10^{-6} produced colonies according to the FDA's Food and Drug Administration Analytical Manual (BAM) which produced 77 bacterial colonies (Fig. 2).

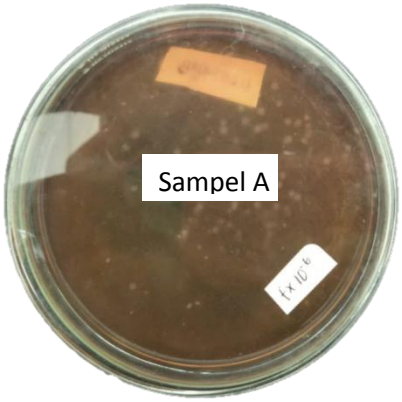

Fig 2. The spread results of the bacterial suspension plate at 10^{-6} dilutions.

The culture concentration of *Shigella flexneri* bacteria contaminated in egg samples (test samples) was calculated as by the total plate account method so that the number of bacterial cultures in one milliliter was obtained: 77 x 10^5 CFU /mL.

Isolation, Quantitative and Qualitative Evaluation of DNA extracted.

The process of bacterial DNA isolation in pure culture samples (A), eggs contaminated with bacteria (B) and uncontaminated egg samples (C) was used commercially by the kit, QiAamp DNA Mini Kit. Each sample obtained 200 microliter DNA isolates stored at -20 ° C in the ependorf tube before quantitative and qualitative testing. Measuring the concentration and purity of isolated DNA using GE Nanovue UV-Vis Spetrophotometer measurement data is presented in Table 1.

Table 1. Measurement of concentration and purity of *Shigella flexneri* bacterial DNA

	Sample	Purity $A_{260/280}$	concentration (ng/µL)
A	Pure Culture	1,852	25,16
B	Non dilute + Egg sample	1,976	39,83
C	Eggs without bacterial culture	2,012	18,5

The purity of DNA can be seen by measuring the ratio A 260nm/A 280nm and the measurement results are in the range 1.8-2.0 for good purity values [16]. In table 1 shows that all samples that have been isolated produce good purity. A good DNA template concentration for genomic testing, it is 0.1 ng-1000ng [17], so that the DNA isolated from both pure culture and test samples produced a good template concentration value. This is also justified in a study conducted by Abdelhai (2016) which compared the methods of pathogenic bacterial DNA isolation using the kit, boiling, phenol/ethanol, and physical method, which showed better DNA purity using the kit method than other standard methods [18]. The presentation of qualitative data on the isolation of the bacterial genome is shown in Fig 3.

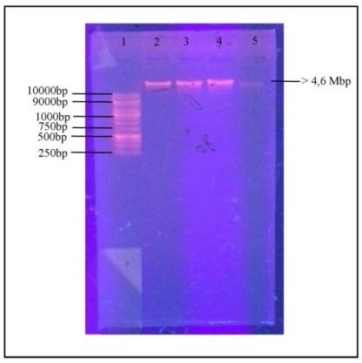

Fig 3. Characterization of the results of genomic isolation of *Shigella flexineri* bacteria by 0.7% agarose gel electrophoresis. (1) Ladder DNA 1 Kb; (2) isolates of sample A DNA; (3) isolates of sample B DNA; (4) Sample DNA isolates C

Based on the data in Fig.3 provides information that the genome of *Shigella flexneri* bacteria from pure and inoculated cultures in egg samples was successfully isolated using a QIAamp DNA Mini Kit commercial kit with the emergence of bands. That matched the size of the whole genome of the *Shigella flexneri* bacterium 4,6 Mb [19] This is evidenced by the appearance of DNA bands, which are located higher than the first line of DNA ladder with a size of 1.5 Kb and DNA bands that appear as single bands indicates that bacterial genome DNA is not degraded [20].

Characterization of Results of Primer Annealing Temperature Optimization

This study succeeded in designing a forward *ipa*H-F and reverse *ipa*H-R primer *Shigella flexneri* with an amplicon 188 base pairs (bp) length. Determination of the temperature of primers annealing is done by PCR Gradient before being used for the Real Time PCR process. Optimization of *ipa*H-F and *ipa*H-R primers in the annealing temperature range of 57° C-61 ° C the selection of annealing temperature based on ±5° C from the temperature of the Tm the primary pair of ipaH-F and ipaH-R can be calculated with the formula Tm = 4 (G+C)+ 2 (A+T) [21].

Based on the data in Fig. 4, there is a specific DNA band of *Shigella flexneri* with an amplicon size of 188 bp in all conditions of the temperature of 57°C-61°C, so that the product has been successfully amplified. This is because the amplicon size of the amplification results is in accordance with the size of the primers design results. In Fig. 4 also provides information that in all these temperatures, conditions produced a band that is relatively the same and indicate that in all conditions, annealing temperature produces optimum amplification. So that it can be used for the Real Time PCR process. However, in this study annealing temperature of 60 ° C was used, the choice of temperature because at 60°C produced the most optimum amplification based on the literature [7, 22].

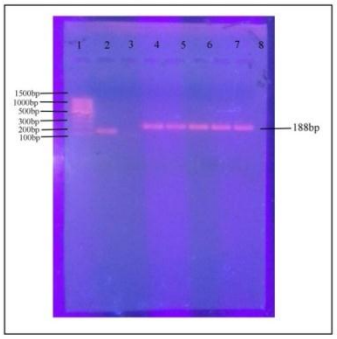

Fig 4. Characterization of the results of temperature optimization of the *ipa*H gene annealing. (1)DNA Ladder 100 bp ; (2) Positif controle of *pef Stpm*; (3) NTC; (4) fragment of *ipaH* gene at temperature 57°C; (5) fragment of *ipaH* gene at temperature 58°C; (6) fragment of *ipaH* gene at temperature 59°C; (7) fragment of *ipaH* gene at temperature 60°C; (8) fragment of *ipaH* gene at temperature 61°C

Primer Specificity and Sensitivity Test of Genes of Shigella flexneri

Specificity testing using DNA templates *Shigella flexneri* as target DNA and DNA template *Salmonella typhi* as non-target DNA shows that the *ipa*H gene primer *Shigella flexneri* can recognize the target DNA of *Shigella flexneri* (red line) in the PCR 11,762 cycle and can distinguish non target DNA (purple line) amplified in the PCR cycle 19,368. It can be stated that the primers used are specific for detecting *Shigella* bacteria, this is because there are differences about the value of Ct ±8 (eight) cycles between target and non-target bacteria [23].

In addition to producing a specific Ct value, the real time PCR process also generates typical melting curve data. The melting curve data of *Shigella flexneri* bacteria are presented in Fig. 6, where the data can confirm whether a product or sample has been successfully amplified or the presence of a primary dimer interference in a reaction [5].

The melting curve analysis shown in Fig. 6 produces a high-intensity *Shigella flexneri* peak (red line) obtaining a Tm value of 80.403 °C, the formation of one peak indicates that the primer only recognizes the target with the Tm temperature and does not occur dimer primers. In contrast to the non-target peaks of *Salmonella typhi* (purple lines) produce peaks with lower intensity and Tm values, which are also different from the bacterial Tm values of Shigella flexneri's target of 76.288 °C.

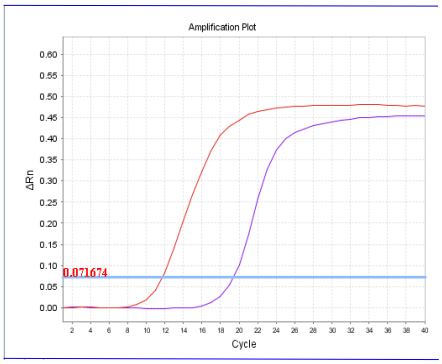

Fig 5. The amplification curve of the specificity test results. Primer of *ipaH Shigella flexneri Shigella flexneri* against bacteria targeted at *Shigella flexneri* and nontarget *Salmonella typhi.*

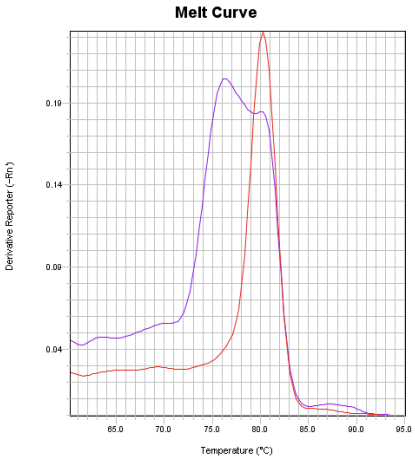

Fig 6. Melting curve specificity test. The *ipa*H primer of Shigella flexneri against bacteria targeted *Shigella flexneri* and non targeted *Salmonella typhi.*

The validation of *ipa*H-F and *ipa*H-R *Shigella flexneri* primers were also carried out by sensitivity testing, which results in a standard curve so that the lowest detection limit of the primer can be accurately. This standard curve was made by conducting a dilution series on the *Shigella flexneri* pure culture DNA template which then each dilution was tested by the real time PCR method.

The amplification curve of sensitivity testing shows that the primers pair of *ipa*H can detect up to the smallest DNA concentration of 8.05 pg/µL with a Ct value of 24.939 (Table 2). The amplification curve also shows that bacterial DNA concentration is inversely proportional to the Ct value (Fig 7). The lower concentration of bacterial DNA requires a longer time for the product to produce a signal of fluorescence so that the resulting cycle is greater [24] so that the amplification curve produced in the sensitivity test is appropriate.

If the amplification curve is extended by the value of the x, axis is the number of cycles (Ct), and the y-axis value is the sample concentration, then the standard curve can be made Ct relationship and concentration with the curve equation y = -2.4741x + 27.14 with the value R^2 = 0, 9956 or value regression which shows excellent linearity [5]. Good linearity is influenced by pure DNA isolates as well as a fairly good dilution and pippeting process so that test points are generated for some samples that have a relatively close distance to the line, according to standard curve requirements.

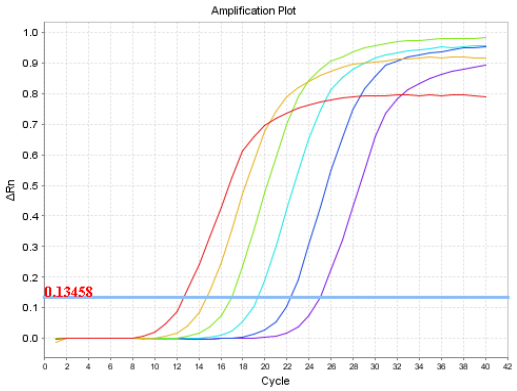

Fig 7. Amplification curve of sensitivity evaluation. The *ipa*H gene primer *Shigella flexneri* detected the target DNA sample to the smallest concentration of 8.05pg /□L at Ct 24.939.

Tabel 2. The results of the primer sensitivity evaluation of the ipaH gene *Shigella flexneri*

Primer	Concentration DNA (pg/ µL)	Sigmoid Line	Treshold Cycle(Ct)
ipaH Shigella flexneri	25160		12,660
	5032		14,725
	1006,4		16, 908
	201,28		19,341
	40,256		22,313
	8,05		24,939
	NTC		25,5
Slope : -2,4741; R^2 : 0,9956; y-Int : 27,14			

Confirmation of the ipaH Primer Pair and Quantification of Shigella flexneri on Egg Samples

Confirmation of the primer pairs of *ipa*H-F and *ipa*H-R evaluation in the two target bacterial samples, including pure culture of *Shigella flexneri* (A) bacteria, eggs contaminated with aseptic culture of *Shigella flexneri* bacteria (sample B), non target samples of eggs without contaminated bacterial samples (sample C) and No Template Control or NTC (sample D) produced the amplification curve shown in Fig. 8.

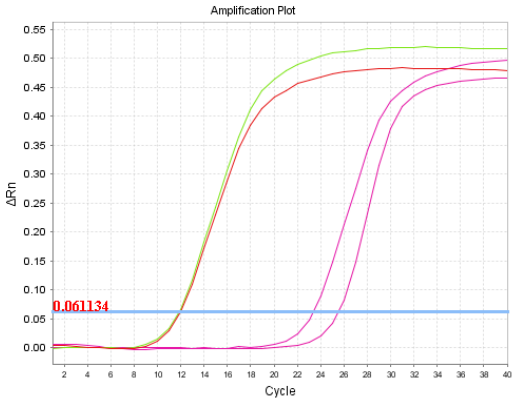

Fig 8. The amplification curve from the test confirms the egg sample. The primer pair of ipaH gene Shigella flexneri in the pure culture (red line), egg sample contaminated with bacteria (green line), egg without bacterial and NTC (purple line).

Based on the results from the amplification curve in Fig. 8 it can be stated that the Ct value of the egg food tested sample (sample B) produces a value of Ct, which is almost the same as pure culture of *Shigella flexneri* (sample A) which is consistent with Ct ±12 so that both samples it is true that the target bacteria is *Shigella flexneri*. The test data is presented in table 3.

Table 3. The Treshould cycle value of confirmation test of the primer pair of ipaH gene's Shigella flexneri in food samples

	Sample	*Treshould cycle (Ct)*
A	Pure bacterial culture	*11,930*
B	Non dilute + egg sample	*11,858*
C	Eggs without bacterial contamination	*23,314*
D	NTC	*25,499*

In sample C produces adjacent Ct which is only 2 cycles different with sample D so that it can be stated that the two samples are non-target. The difference of more than 10 cycles between food test samples and negative controls indicates that the design results of the ipaH-F and ipaH-R primers can be applied to detect Shigella species bacteria found in food samples, namely in this study egg.

In the sensitivity test obtained standard curve line equations used for quantification of Shigella flexneri bacteria, which were deliberately contaminated into egg samples namely y = -2.4741x + 27.14; R2 = 0, 9956. The y value is Ct (threshold cycle) and x is the log 10 bacterial concentration in the egg sample. R^2 value shows good linearity. The value of Ct obtained in the egg sample (sample B), which is 11,858, is included in the standard curve equation so that the log 10 values as the concentration of Shigella flexneri bacteria found in the contaminated egg are 6.176. The number of bacteria obtained by the Real Time PCR $10^{6.2}$ method which is 15.85 x 10^5 CFU/mL is equivalent to the DNA concentration of the test sample obtained which is 39.83 ng /µL.

IV. CONCLUSION AND FURTHER RESEARCH

Based on the results obtained it can be concluded that the Real Time PCR Method uses the ipaH gene primer *Shigella flexneri* can be used to detect *Shigella flexneri* from egg samples quickly, sensitively and specifically. Minimum detection level The Real Time PCR method in detecting *Shigella flexneri* bacteria is 8.05 picograms, which is equivalent to 15.85 x 10^{-5} CFU/mL. The next study was the development of the RT PCT method to detect other food poisoning bacteria.

V. ACKNOWLEDGMENT

Our gratitude deliver to the DRPM Ministry of Research and Technology of Higher Education for providing funding through the 2018 PDUPT research scheme with contract numbers: 7 / SP2H / DRPM / LP-UNJ / PUPT / II / 2018.

REFERENCES

1. WHO. *Who estimates of the global burden of foodborne diseases*. Switzerland: World Health Organization Press. 2015.
2. CDC. Surveillance for foodborne disease outbreaks - United States, 1998-2008. *Surveillance Summaries*, *62*(2), 1–34. 2013.
3. Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V, Widdowson, M., Roy, S. L., … Griffi, P. M. Foodborne Illness Acquired in the United States — Major Pathogens. *Emerging Infectious Diseases*, *17*(1), 7–15. 2011.
4. Laude, A., Valot, S., Desoubeaux, G., Argy, N., Nourrisson, C., Pomares, C., … Morio, F. Is real-time PCR-based diagnosis similar in performance to routine parasitological examination for the identification of Giardia intestinalis, Cryptosporidium parvum/Cryptosporidium hominis and Entamoeba histolytica from stool samples? Evaluation of a new. *Clinical Microbiology and Infection*, *22*(2), 190.e1-190.e8. 2016.
5. Dorak, M. T. *Relative quantification*. (E. Owen, Ed.), *Real-time PCR*. New York: Taylor & Francis Group. 2006.
6. Saeed, K. Real-Time Polymerase Chain Reaction : Applications in Diagnostic Real-Time. *International Journal of Medical Students*, *1*(1), 28–36. 2013.
7. Mokhtari, W., Nsaibia, S., Gharbi, A., & Aouni, M. Real-time PCR using SYBR Green for the detection of Shigella spp . in food and stool samples. *Molecular and Cellular Probes*, *27*(1), 53–59. 2013.
8. Qiagen. Blood Mini Handbook QIAGEN Sample and Assay Technologies (fifth edit). Hilden: qiagen. 2016.
9. ThermoScientific. *DreamTaq Green PCR Master Mix (2x)* (Vol. 5). California: Thermo Fisher Scientific Inc. 2016
10. SMOBIO. Product Information ExcelTaq Series 2X Q-PCR Master Mix (SYBR, ROX). Retrieved from www.smobio.com. 2017.
11. Magdeldin, S. *Gel Electrophoresis - Principles and Basics*. (T. Smiljanic, Ed.). Croatia: InTech. 2012.
12. Tecnova Science matters. MacConkey Agar (MAC). Retrieved November 7, 2018, from
13. https://www.teknova.com/category-s/504.htm. 2018
14. Surjawidjaja, J. E., Salim, O. C., & Bukitwetan, P. Perbandingan agar MacConkey, Salmonella-Shigella, dan xylose lysine deoxycholate untuk isolasi Shigella dari usap dubur penderita diare. *Universa Medicina*, *26*(2), 57–63. 2007.
15. Lu, Z., Guo, W., & Liu, C. Isolation , identification and characterization of novel Bacillus subtilis. *Veterinary Medical Science*, *80*, 427–433. 2018.
16. Delamare, A. P. L., Costa, S. O. P., Silveira, M. M. Da, & Echeverrigaray, S. Growth of Aeromonas species on increasing concentrations of sodium chloride. *Applied Microbiology*, 57–60. 2000.
17. Sambrook, J. and D. W. Russell. Moleccular Cloning: A Laboratory Manual 2nd edition. New York: Cold Spring Harbor Laboratory Press. pp. 165. 2001.
18. Bonab, M. M., Alimoghaddam, K., Talebian, F., Ghaffari, S. H., Ghavamzadeh, A., Nikbin, B., … O'Shea, K. S. Real-time PCR Handbook. *Life Technologies*, *4*(2), 189–200. 2015.
19. Abdelhai, M. H., Hassanin, H. A. M., & Sun, X. Comparative Study of Rapid DNA Extraction Methods of Pathogenic Bacteria. *American Journal of Bioscience and Bioengineering*, *4*(1), 1–8. 2016.
20. Wei, J., Goldberg, M. B., Burland, V., Venkatesan, M. M., Deng, W., Fournier, G., … Mmun, I. N. I. Complete Genome Sequence and Comparative Genomics of *Shigella flexneri* Serotype 2a Strain 2457T †. *Infection and Immunity*, *71*(5), 2775–2786. 2003.
21. Omar, B. A., Atif, H. A., & Mogahid, M. E. Comparison of three DNA extraction methods for polymerase chain reaction (PCR) analysis of bacterial genomic DNA. *African Journal of Microbiology Research*, *8*(6), 598–602. 2014.
22. Innis, M. A. Optimization of PCRs. *Science*. New York: Academic Press, Inc. 1997.
23. Mustapha, A. Simultaneous Detection of Escherichia coli O157 : H7 , Salmonella , and Shigella in Apple Cider and Produce by a Multiplex PCR † AND. *Journal of Food Protection*, *67*(1), 27–33. 2004.
24. Bustin, S. A., & Nolan, T. Pitfalls of Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction. *Journal of Biomolecular Techniques*, *15*(3), 155–166. 2004.
25. BioRad. Real Time PCR Application Guide. BioRad Laboratories, Inc, USA. 2006.
26. StephenA. Bustin, Vladimir Benes, Jeremy,A. Garson, Ja n Hellemans, Jim Huggett, Mikael Kubista, Reinhold Mu eller, Tania Nolan, MichaelW. Pfaffl, Gregory,L. Shipley , Jo Vandesompele, Carl T. Wittwer. 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Journal Clinical Chemistry. DOI: 10.1373/clinchem.2008.112797.

Published By:
Blue Eyes Intelligence Engineering
& Sciences Publication

27. Law *et al*. 2015. Review. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. Frontiers in Microbiology. Vol. 5, 770
28. M Nurjayadi, N Islami, YP Pertiwi, V Saamia, IM Wirana, 2018. Evaluation of primer detection capabilities of fimC Salmonella typhi using real time PCR for rapid detection of bacteria causes of food poisoning. IOP Conf. Series: Materials Science and Engineering 434 (2018) 012097 doi:10.1088/1757-899X/434/1/012097.
29. M Nurjayadi, YP Pertiwi, N Islami, V Saamia, IM Wiranatha, 2018. Evaluation of Primers Detection Capabilities of the pef Salmonella typhimurium Gene and the fimC Eschericia coli Gene Using Real-Time PCR to Develop Rapid Detection of Food Poisoning Bacteria. IOP Conf. Series: Journal of Physics: Conf. Series 1097 (2018) 012049 doi:10.1088/1742-6596/1097/1/012049.