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Isolation and Characterization of *fim*-C S. *typhi* Gene 0.8 Kilobase as a Preliminary Study to Discover a Candidate of Recombinant Vaccine for Typhoid Fever

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ABSTRACT: *Salmonella typhi* is bacteria that cause typhoid fever for human. In Indonesia, the morbidity rate of typhoid fevers suffer tends to increase. The common method to prevent typhoid fever is to weaken the vaccine of *S. typhi* bacteria. However this kind of vaccine has weaknesses that occur in several side effects, such as nausea, vomit, fever, and headache. Therefore this research is aimed to isolate and to characterize the gene structure of fim-C *S. typhi* local strain of 0.8 kilobase as an initial study to develop the recombinant vaccine. This research used explorative method that consists of (1) designing the specific primer of *fim*-C *S. typhi* gene; (2) synthesize the specific primer pair of *fim*-C *S. typhi* gene; (3) isolated the genome of S. typhi bacteria; (4) amplification the fim-C gene by PCR; (5) characterize the amplification result *fim*-C *S. typhi* gene. The specific primer from the designing process (Fw 1b-Rev 1a New) has succeeded to amplify the S. typhi genome showed by one band of 0.8 kilobase by using gel agarose electrophoresis. The characterization of 0.8 kilobase band from the amplification result by using sequencing technique succeeded to detect 766 base pairs. It is concluded that this research has succeeded to isolate and to characterize *fim*-C gene *S. typhi* local strain of 0.8 kilobase. This *fim*-C gene has possibility for developing a recombinant vaccine candidate in the further study.

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INTRODUCTION

Salmonella typhi is bacteria that cause typhoid fever for human. Typhoid fever that causes infection in human colon occurs because of *S. typhi* contamination in human body through food and drink. In Indonesia, the morbidity rate of typhoid fever's suffers tends to increase; neither to the good sanitary facility provided in both urban and rural areas.[1–3] Because of the increasing rate of morbidity, the alternative to overcome the typhoid fever or to prevent it from spreading is needed.[4,5]

Recently people used vaccine from the attenuated bacteria to overcome typhoid fever. This kind of vaccine had protection efficacy decreasing in a hyper-endemic area like Indonesia. Another weakness of this kind of vaccine is it only for adults; and children above 6 years old with repeat immunization in 5 years, which is less efficient. Furthermore, this kind of vaccine has side effects, such as nausea, vomit, stomach-ache, fever, and headache.[6]

According to some literatures, this research will discover the potency of *fim*-C *S. typhi* in the protein level as recombinant vaccine.[7,8] The recombinant vaccine is purer than the former discovered vaccine. It produces more specific immune response and also easy to store and produce.

Fim-C protein or *fimbriae* is a surface protein in both positive gram and negative gram bacteria, such as *S. typhi*. Fimbriae are used to attach it to the host and other objects.[9] Some literature suggests that those surface proteins may produce good immune response.

This research is aimed to amplify and characterize of *fim*-C *S. typhi* gene as a part of study about the potency of the bioactive molecule *fim*-C *S. typhi* for the vaccine candidates by using genetic engineering technology. It hopefully could help to prevent and to overcome the increasing number of typhoid fever suffers rate and its spreading. Hence, this research is necessary to be conducted.

The result will bring significances in: (1) helping to overcome the problems in developing recombinant vaccine; (2) as preliminary study of recombinant vaccine

discovery, which is expected better than the former methods in developing recombinant vaccine for other pathogen bacteria.

METHODS AND EXPERIMENTAL DETAILS

Designing and Synthesizing the Primer of Fimbrial-C *S. typhi*

The primer was directly designed from sequence of *fim*-C *S. typhi* CT18 gene from GenBank.[4] Primer design was conducted by standard procedure with computerized system and its parameters, among of them are (1) primer lengths of 20-30 nucleotides; (2) GC percentage above 50%; (3) 3' and 5' of the primer for both reverse and forward directions.

The primer design stage produced two primer pairs forward and reveres of *fim*-C *S. typhi* genes. There was each two primer design of Forward (FW-1b and FW-Int2) and Reverse (Rev-1a New and Rev-Int1 New). Fragment length of the amplification results FW-1b with Rev-1a New were 783 base pairs (bp) and Fw-Int2 with Rev-1a is 193 bp. Primer synthesis was conducted twice. The first was done by Company Euro fins MWG GmbH in Germany and facilitated by Immunology Laboratories of Justus Liebig Universitat Germany, whereas the second was conducted by First-Base Laboratory in Malaysia.

Growth of *S. typhi* Culture

S. typhi, *S. typhimurium* and *E. coli* bacteria which used in this research were obtained from Microbiology Laboratories University of Indonesia. It was then bred and rejuvenated in Biochemistry–Biotechnology Laboratory of Mathematics and Natural Sciences in Universitas Negeri Jakarta as culture bacteria.

Bacteria culture in liquid Nutrient Broth medium. The culture bacteria obtained in UI's Microbiology Laboratories were on slanted gel medium. In this research, the rejuvenations of bacteria were implemented in the following way: culture bacteria in sloped gel were taken by using an aseptic wire loop (one colony). Then the sterile wire loop was dipped in 5 mL of liquid nutrient broth media sterile in the test tube.

Bacteria culture in nutrient gel. Sterile wire loop was dipped in liquid culture bacteria from nutrient broth. Then the wire loop was scratched with zigzag in the prepared sterile nutrient gel media. Nutrient gel media, which contained with bacteria, was afterwards incubated for 16-18 hours at 37°C. The result of bacteria growth was checked after the incubatory process for 16-18 hours.[10]

Isolation of Genome of *S. typhi* Bacteria and Its Characterization

Isolation of genome of *S. typhi* bacteria. 1 mL of the grown culture bacteria for about 16-18 hours (overnight culture) was put in the 1.5 mL microcentrifuge Eppendorf tube. The Eppendorf tube then centrifuged in 13,000-16,000 g for 2 minutes until the cell pellets produced. The supernatant was discarded by pouring the rest of the breaker glass media to a disinfected container. Then the resulted pellet was added with 600 µL of *Nuclei Lysis Solution*. The pellet was suspended by pipetting it up and down from the mixture until it turned homogeny. The mixture was then incubated at 80°C for 5 minutes to lysis the bacteria cell. Then the bacteria were cooled at room temperature. After reaching room temperature, the bacteria were added with 3µl *RNase Solution* in the lysate liquid of bacteria. The tube was shaken 2-5 times to homogenize the mixture. Then the tube was incubated at 37°C for 15-60 minutes to optimize the work of *RNAse* enzyme. Next, the sample is cooled at room temperature again. The mixture of bacteria cell which had been treated by RNAse and been cooled in room temperature was once again added with 200µl *Protein Precipitation Solution*. The mixture was vortexed for 20 seconds in high speed until homogenized the protein precipitation solution with lysate cell of the bacteria. Then the mixture was incubated in ice for 5 minutes.

After being incubated, the mixture was centrifuged at 13,000–16,000 g for 3 minutes. The supernatant was then carefully inserted into sterilized 1.5 mL Eppendorf tube that has been filled with 600µl isopropanol at room temperature. The tube, which contained in the mixture was shaken until the soft thread appeared. Then the tube was centrifuged again at 13,000-16,000 g for 2 minutes. The supernatant was carefully taken out. Then the tube was dried by rotating the tube on the absorbent paper, such as clean tissue paper. The tube which contained DNA pellet was added with 600µl of 70% ethanol at room temperature, and then the tube was shaken 2-5 times to wash the DNA pellet. Next, the tube with DNA lysate was centrifuged at 13,000-16,000 g in 2 minutes. The ethanol was then carefully taken out from the tube using pipette. The centrifuge can be done more than once to make sure that there is no ethanol left in the tube. The Eppendorf tube contained DNA pellet was dried by rotating it on the clean tissue paper. The pellet allowed drying for 10-15 minutes. Then the tube contained DNA pellet was added with 100µl *DNA Rehydration Solution* to dissolve the DNA and to be incubated in 65°C for an hour.

Periodically in the incubation process, homogenization is done by tapping the tube or touching Eppendorf using the index finger until the solution becomes homogeneous. Another alternative is to incubate a DNA solution at 4oC overnight. The results of bacterial genomic DNA isolation were stored at 2–8°C.[11]

Characterization of DNA genome *S. typhi* with gel agarose electrophoresis. The characterization of bacterial genome was done by electrophoresis gel 1.0 % agarose with ethidium bromide dye. Each of 5 µL DNA genome was mixed with 7 µL loading dyes buffer. The electrophoresis was done in the condition of 80 volt in an hour or 70 volt in 1.5 hour. The visualization of electrophoresis was done by the light of 260 nm UV lamp.[10]

Amplification of Genome *S. typhi* by PCR and Its Charactarization

Amplification of genome *S. typhi*. In this research, the amplification of DNA genome was done using the thermal cycle machine (Bio-Rad), primers pair FW-1b (5' CGC TGG TTT ACC CAT GAA TTT TCT TGC CGA 3') and Rev-1a-New (5' GGT TGT CTT CTT CAT CGT GTT CTC GTG TGG 3'), nuclease-free water and also Master Mix PCR (Bio-Rad). The total of reaction was 25 µL with these following compositions: Master Mix PCR (Bio-Rad) 12.5 µL, primer FW-1b 1.25 µL (12.5 pmol), primer Rev-1a-New 1.25 µL (12.5 pmol), nuclease-free water 9 µL, and DNA genome of S. typhi 1 µL was put into PCR tube and homogenized. Then the tube contained the mixture were placed into the thermal cycle machine with the initial condition of heating at 95°C for 5 minutes, denaturation at 57.5°C for a minute, with the chain extension stage at 72°C for a minute and product finishing stages at 72°C for 7 minutes, in 35 cycles and at room temperature (25°C).

The characterization of PCR result with electrophoresis agarose gel. The characterization of amplication result was done with electrophoresis agarose gel of 2% with ethydium bromide dye. Electrophoresis was done in the condition of 80 volts for an hour. The visualisation of electrophoresis result was done under the light of 260 nm UV lamp.[10]

Characterisation PCR Product by Sequencing Technique

Purification of *fim*-C *S. typhi* gene from PCR product. The stages to the purification process are as follows: (1) the empty Eppendorf micro tube was weighted and noted the weight (m0), (2) gel from electrophoresis results, which contains DNA was located on the 260 nm UV trans-iluminator, (3) the DNA band was cut from the gel, and make it smaller, after that put the gel into Eppendorf tube which its initial weight was measured, (4) the Eppendorf tube that contained the gel was weighted the second time (m1), the weight of purified gel is the differences between Eppendorf tube containing gel and empty tube (m1-m0), (5) membrane binding solution was added into the tube by the ratio of 10 µL : 10mg weight of the gel, (6) the mixtures were vortexed and incubated in 50-65°C for 10 minutes until it is completely dissolved, (7) centrifuge the tube at room temperature to make sure the position of mixture at the bottom of the tube, (8) the mixture of gel solution was moved to SV mini column, which is put above Collection Tube and incubated at room temperature for 1 minute, (9) SV Mini column was centrifuged at 16,000 g (14,000 rpm) for 1 minute, and then SV Mini column was moved from the collection tube. The rest of liquid inside the collection tube was disposed. Next SV Mini column was set at collection tube, (10) column was washed with 700µL membrane wash solution (it has been diluted with ethanol 95%) then SV Mini column was centrifuged at 16,000 g for 1 minute, (11) SV Mini column was washed with 500µL membrane wash solution, and centrifuged at 16,000 g for 1 minute, (12) Repeat the centrifugation step for 1 minute and move SV Mini column to above sterile Eppendorf tube, (13) 50µL nuclease free water was added in the centre of SV Mini column and incubated at room temperature for 1 minute, (14) the SV Mini column was out and the Eppendorf tube that contains DNA was taken at 4°C until -20°C.[12]

Characterization of purification results by electrophoresis agarose gel. The characterization of purification result was done by electrophoresis 2% gel agarose with dye Ethidium Bromide. Each of 5 µL DNA solution was mixed with 7 mL loading dye buffer. The electrophoresis was done in the condition of 80 volts in an hour or 70 volt in 1.5 hour. The visualization of electrophoresis was done under the light of UV lamp with 260 nm wavelength.[10]

Sequencing of *fim*-C gene from amplification results. The sequencing of *S. typhi fim*-C gene from amplification results in this research was done at Molecular Biology of Eijkman Institute, Salemba-Jakarta.

RESULTS AND DISCUSSION

Designing and Synthesizing the Specific Primer of *fimbrial*-C *S.typhi*

The result of primer designed that are the pair *fim*-C *S. typhi* primers showed in Table 1. This research used the primer pair FW-1B and Rev-1A-new, which in scipto it can amplification the DNA fragment into 783 pairs of base (bp) or 0.8 kilobase (kb) *S. typhi fim*-C primers are designed immediate-

Table 1. Result of primer design of gene *fim*-C *S.typhi* C.T 18 Protein (MS 3602)

No.	Primer	Length of base pair	Sequence	Base Pair	Scale	%G C	TM °C	MW
1.	FW-1B	783	5' CGC TGG TTT ACC CAT GAA TTT TCT TGC CGA 3'	30	0,01 µmol	46.7	66.8	9138.9
2.	REV-1A NEW	783	5' GGT TGT CTT CTT CAT CGT GTT CTC GTG TGG 3'	30	0,01 µmol	50	68.1	9207.9
3.	Rev-Int1 New	165	5' GCG TCT TCC GGC GTC TGC GGC AAA T 3'	25	0,01 µmol	64	69.5	7649.9
4.	FW-Int2	223	5' GGT CAA TAT CAG CGT GGG TGC GAA A 3'	25	0,01 µmol	52	64.6	7771.1

ly from the start codon to the stop codon so that the sequence can be translated into functional proteins. In addition, at the S.typhi C primer added ten amino acids encoding histidine and the nucleotide sequence identifying the HindIII restriction enzyme at tip 3 'while at the end of 5' the BamHI restriction enzyme sequence was added according to the restriction enzyme found in the pGEM-T cloning vector and pET 31 expression vector.[13,14] The addition of ten Histidine amino acids aims to facilitate the purification process to be carried out at a later stage.[15,16]

Growth of Bacteria Culture

This research has succeeded in growing the *S. typhi* bacteria under the good and sufficient conditions in both liquid and solid media. The bacteria that grow in the liquid mediums are marked by more turbidities in its medium. Meanwhile, the result of bacteria growing in solid mediums showed by the growth of bacteria colonies on the surface of a gel medium (picture not be displayed).

Isolation of Genome *S. typhi* Bacteria and Its Characterization

The DNA bacteria genome isolation by using Wizard kit has been successfully done for *Salmonella typhi* bacteria in local strain to be used as the research sample. The results of bacteria genome of *S. typhi*, *S. typhimurium* and *E. coli* isolation are displayed in Figure 1.

According to Figure 1, the DNA of tested bacteria has two bands, with both bands are higher than the first band on DNA marker 2 kb Ladder. It shows that the size of DNA of tested bacteria is bigger than 2 kb. This is consistence with the result of information searching towards the three bacteria genome from several genomes in the database and publications on bacteria genome determination.

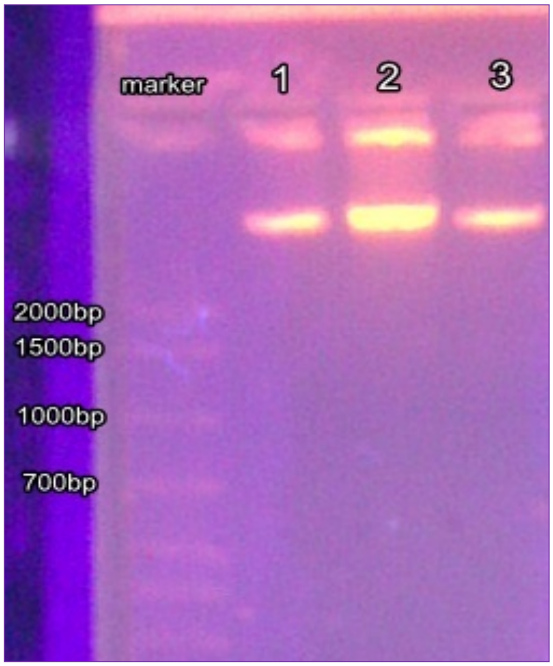

Figure 1. The results of Genome Electrophoresis of Tested Bacteria. Marker DNA Ladder; (1). DNA genome *S. typhi*, (2). DNA genome *S. typhimurium*, (3). DNA genome *E. coli*.
Note: Electrophoresis is conducted in 80 volt for 1 hour, with ethydium bromide color, and the visualization by 256 nm UV lights

The result of data analysis some of the literature reviews showed that the size of each DNA genome of the three bacteria respectively as follow: *S. typhi* 4.81 mega base (mb), *S. typhi*murium 4.85 mb, and *E. coli* 4.69 mb. The two bands showed that DNA genomes of the three tested bacteria are circular. The form of agarose electrophoresis has two bands bands so that the result obtained is in accordance with the standard of the bacterial genome. Based on the above data, this study has been successfully performing the isolation of genomic DNA in the following bacteria, such as *S. typhi*, *S. typhi*murium, and *E. coli*, in its proper sizes.[17–21] Descriptions of each circular bacterial genome and its size are presented in Figure 2.

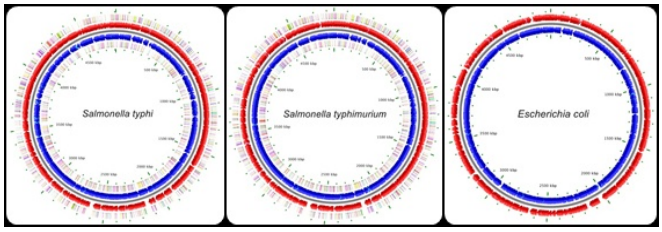

Figure 2. The circular representation three bacterial genomes. *S. typhi* bacteria has genome's size of 4.81 megabase (mb), *S. typhimurium* 4.85 mb, and *E. coli* 4.69 mb.

Amplification and Characterization of fim-C *S. typhi* Bacteria Gene

The process of amplification by polymerase chain reaction technique which was under the described conditions in materials and method's chapter and used template of *S. typhi* chromosome of the primer pair of Fw-1b-Rev-1a-new has been succeeded to amplify the DNA fragment size of 783 base pairs (bp) or 0.8 kilo base (kb). The availability of DNA bands which detected by agarose gel electrophoresis from the amplification showed that the primer pair of *Fw-1b-Rev-1a-new* design and synthesis results at previous step can be used for amplification process. These primer pairs were designed to limit the fimbriae-C of *S. typhi* with size 783 base pairs.

The compatibility between in scripto design and amplification result showed success in designing and synthesizing primer, and also in amplification process. All at once it is as the first step to conduct the advance research, both at genomic or proteomics level. The documentation of PCR result is presented in Figure 3.

Characterization of *fim*-C *S. typhi* Gene by Sequencing Technique

The aim of sequencing step is to make sure whether the amplification results from *S. typhi fim*-C gene with size 0.8 kb from previous step has performed the sequence in accordance with the database from GenBank and in order to definite every prior step in this research has been done correctly. Before conducting the sequencing, the amplification results of the *S. typhi fim*-C gene with size 0.8 kb was purified by using DNA purification from gel kit.[12] The function of the purification process is to dispose the rest of mixture from PCR process. So that the results will be just the DNA band with size 0.8 kb. To check the purification process DNA solution from purification was done by electrophoresis agarose gel 2% contained with ethidium bromide dyes and visualized by 256 nm UV light. According to data, the result of purification has 1 DNA band with size of 0.8 kb. It means that purification process was done successfully.

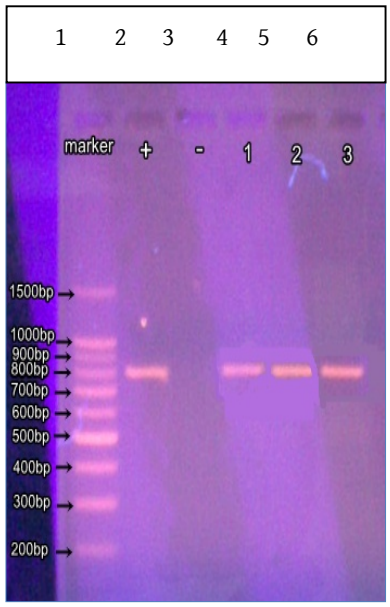

Figure 3. The PCR Product of fim-C Gene with primer pair FW-1b-Rev-1a-new.
Note: (1) Marker DNA Ladder 5 µL, (2) PCR Result DNA S. typhi Genome 0.8 kb from previous research as a positive control, (3) ddH2O as negative control (4) PCR Result DNA S. typhi genome as a tested bacteria 0.8 kb, (4) PCR Result DNA S. typhimurium genome 0.8 kb, (5) PCR Result DNA E. coli genome 0.8 kb. The result carried out with 2 % agarose gel electrophoresis containing 0.1% ethidium bromide. The visualization of electrophoresis result performed under UV light at a wavelength of 256 nm.

The PCR product 0.8 kb, which is purified, was then characterized by sequencing. The sequencing process did by deoxy Sanger principle at Molecular Biology Eijkman Institute, Salemba-Jakarta. From the process, the sequence of the nucleotide *fim*-C *S. typhi* gene was obtained. A part of electroforegram of sequencing result is shown in Figure 4 and the results of sequence data shown in Figure 5.

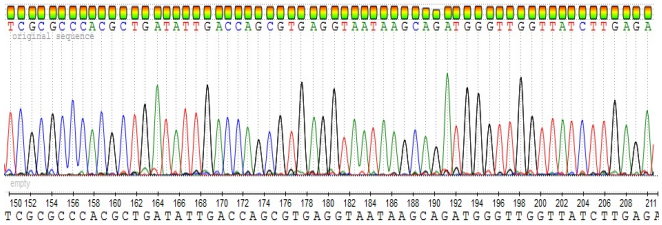

Figure 4. Electrophoregrame the sequencing results from a part of *fim*-C *S. typhi* gene. The complete sequence is showed at Figure 5.

1	TTCGGGTGGT	TTACCCATGA	AATTTCTTGC	CGACGTGACT	CAATGCAAAA
51	GGTATTTTTT	TACCATGCTG	AATAGTATAA	AATTAGGCTT	TATTGTTCTT
101	CTCACGTTAT	TTACTTCGCT	GAACGTACAG	GCGGCGGGGG	GGATTGCATT
151	AGGCGCCACG	CGAGTTATTT	ATCCCTCGGC	GGCGAAGCAG	ACTTCTCTGG
201	CAATCAGTAA	TAGCGATACT	CAAGAACGTT	ACCTCGTCAA	TTCATGGATC
251	GAAAATAGCG	CCGGGCAGAA	AGAAAAAACG	TTTATCGTTA	CGCCGCCTTT
301	ATTCGTCAGC	GAGCCCAAAA	GCGAAAACAC	GCTGCGTATT	ATCTACGCCG
351	GGCAACCGCT	ACCCGGGGAT	CGGGAGTCGT	TATTCTGGAT	GAACGTGAAA
401	GCCATCCCGT	CGGTCGATAA	AAGTCATATT	GAAGGAAAAA	ACGTTCTGCA
451	ACTGGCGATT	CTGTCGCGCA	TCAAACTGTT	CGTGCGTCCG	GCGAATTTGC
501	CGCAGACGCC	GGAAGACGCG	CCGACCTTGC	TGAAATTTTC	CCGTGTCGGC
551	AACCATCTCA	AGATAACCAA	CCCATCTGCT	TATTACCTCA	CGCTGGTCAA
601	TATCAGCGTG	GGCGCGAAAA	AGATTGATAA	CGTGATGATC	GCGCCAAAAA
651	GCGACATGCA	AATTCCCTTA	CCGACTGGCG	CGCAGGGCAG	CGTGACATTT
701	CAGACCGTCA	ATGATTACGG	CGCATTGACG	TCGGCGACAA	CGGCCATTTC
751	GGTAAGCCGA	GAGGGG			

Figure 5. The sequence results from sequencing process of fim-C S. typhi gene. According the electrophoregram data was detected amount of 766 base pairs of *fim*-C *S. typhi* gene.

The result of sequencing gene *fim*-C which obtained from this research lately known that it has similarity with the sequence of *fim*-C gene *S. typhi* in GenBank from strain CT18 and Ty2a.[4,19] The homology analysis by using BLAST program (www.ncbi.nlm.nih.gov) showed that the *S. typhi* bacteria sample of local strain has 98% homology with the DNA sequence of CT18 and Ty2a.

The result of this research can be used as reference for other research in discovering recombinant vaccine to prevent typhoid fever. The discovered molecule of *fim*-C *S. typhi* gene will be used as an insert into the vector of the genetic engineering process. It will be used as a basic stage in discovering the recombinant vaccine.

CONCLUSION

This research succeeded in isolation and amplification process towards gene fimbrial-C from *S. typhi* bacteria genome local strain using the primer pair FW-1B-REV-1A NEW in accordance to the size of DNA band which is 0.8 kb. Characterization of DNA amplification to local strain *S. typhi* size 0.8 kb with the sequence technique gave good reference to the result. It can be shown by 98% (766 base pairs) detected from expected 783 bases. Analysis of fragment homology from the result of amplification of DNA *S. typhi* local strain 0.8 kb has 99% homology with sequence of gene *fim*-C *S. typhi* strain Ty2 and CT18 in Genbank.

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