A Rapid and Sensitive Diagnosis of Typhoid Fever Based On Nested PCR-Voltammetric DNA Biosensor Using Flagellin Gene Fragment

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ABSTRACT

Typhoid fever caused by Salmonella typhi is an important issue for public health in the world. Laboratory methods for rapid and sensitive diagnosis are very important for disease management. The purpose of this study was to determine the performance of nested PCR–voltammetric DNA biosensor using flagellin gene (fla) of S. typhi as a marker. The differential pulse voltammetry using pencil graphite electrode was applied to measure the guanine oxidation signal of probes vs synthetic target stDNA and probes vs fla PCR product hybridizations. The probe DNA selectivity was examined by hybridized probes vs non-complementary sequence. The result showed that the first round nested PCR product can not be visualized by agarose electrophoresis, whereas using the voltammetric biosensor methods can be detected both for the first or second round nested PCR product. The average peak current of hybridized probe vs first and second round of PCR product was 2.32 and 1.47 µA respectively, at 0.9 V. Detection of the DNA sequences of the infectious diseases from PCR amplified real sample was also carried out using this voltammetric DNA biosensor methods.

Keywords: *Salmonella typhi; voltammetry; DNA biosensor; flagellin gene; PCR*

ABSTRAK

Demam tifoid yang disebabkan oleh Salmonella typhi merupakan salah satu masalah penting bagi kesehatan masyarakat di dunia. Metode laboratorium untuk diagnosis cepat dan sensitif menjadi sangat penting untuk pengelolaan penyakit ini. Tujuan dari penelitian ini adalah untuk menentukan kinerja PCR nested-biosensor DNA secara voltammetri berdasarkan gen flagellin (fla) S. typhi sebagai penanda. Voltammetri pulsa diferensial dengan menggunakan elektrode grafit pensil diaplikasikan untuk mengukur sinyal oksidasi guanin hibridisasi probe-target stDNA sintesis dan probe-fla produk PCR. Selektivitas DNA probe diuji dengan hibridisasi probe terhadap urutan non-komplementer. Hasil penelitian menunjukkan bahwa produk PCR nested pertama tidak dapat divisualisasikan dengan elektroforesis agarosa, sedangkan menggunakan metode biosensor voltammetri dapat dideteksi baik untuk produk PCR pertama maupun kedua. Rata-rata arus puncak dari hibridisasi probe-produk PCR pertama dan kedua masing-masing adalah 2,32 dan 1,47 µA, pada daerah potensial 0,9 V. Deteksi urutan DNA produk amplifikasi PCR penyakit infeksi ini juga dilakukan dengan menggunakan metode biosensor DNA voltammetri terhadap sampel nyata.

Kata Kunci: *Salmonella typhi; voltammetri; biosensor DNA; gen flagellin; PCR*

INTRODUCTION

Typhoid fever is an important health problem in many developing countries. Worldwide, an estimated 17 million persons had been suffering this disease annually. Most of this burden occurs among citizens of low-income countries, particular those in Southeast Asia, Africa, and Latin America. Typhoid fever is caused by *S. typhi* and is transmitted through the fecal-oral route by the consumption of contaminated water and food [1-2]. Typhoid fever is a contagious disease that can affect many people leading to outbreaks and can cause death. Therefore, detection method of *S. typhi* should be sensitive, precise and fast. Molecular techniques such as PCR (polymerase chain reaction) have proven to be specific and sensitive methods for detecting infectious pathogens [3-5].

Electrochemical DNA biosensor has aroused great interest in recent years for its simplicity, higher sensitivity, selectivity, and cheaper equipments, and

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has enormous potential applications for the specific sequences detection in human disease, clinical test, biochemical analysis and so on [6-9]. Direct label-free electrochemical detection eliminated the external labels or indicators and greatly shortened the assay time, hence attracting increasing interest. In principal, such direct, in situ detection originates from the two following effects as a consequence of the DNA hybridization: 1) the introduced intrinsic redox activity of the nucleotide acid target; 2) changes in the electrochemical properties of the interface (e.g. capacitance, impedance, conductance as well as surface charge). The most redox active among the four nucleic acids bases is the guanine moiety [10-11]. However, few works have been done to detect the *S. typhi* bacteria via Vi gene using gold nanoparticles (AuNPs) modified SPE for DNA hybridization assay using methylene blue as redox hybridization indicator [12], graphene oxide (GO)-Chitosan (CHI) nano-composite [13], direct electro-deposition on gold nano-aggregates (GNAs) [8], and the amino modified salmonella ssDNA probe sequence was covalently linked with carboxylic group on the surface of nanoporous glassy carbon electrode to prepare the DNA biosensor [9]. The other detection methods of *S. typhi and S. typhimurium* generally focus on immunological methods and polymerase chain reactions [4-5,14-18].

Song et al., (1993) developed the detection method of *S. typhi* in the blood samples by using nested PCR method. The PCR product was characterized by agarose gel electrophoresis and Suthern Blot analysis which is time consuming and used dangerous radioactive compound [19]. In this paper we combined polymerase chain reaction (PCR) method with voltammetric DNA biosensor method, based on guanin signal target using graphite electrode (PGE) as a disposable transducer to the detected PCR product of *S. typhi's flagellin* gene fragment from *S. typhi* culture. This voltammetric method is more sensitive and faster than gel electrophoresis methods. The preparation step of the samples was the same as the agarose gel electrophoresis approach. The agarose gel electrophoresis measurement step typically takes about 30 min. However, in comparison our measurement step takes less than 1 min [20].

EXPERIMENTAL SECTION

Materials

S. *typhi* culture and blood samples of patient were obtained from Rajawali Hospital, Bandung. All of synthetic oligonucleotide sequence was synthesized by First Base Asia. Four synthetic oligonucleotide primers were used based on Song et al. (1993) method. The sequence of primers is, F1: 5'-ACTGCTAAAACCACTAC T-3', F2: 5'-AGATGGTACTGGCGTTGCTC-3', R1: 5'-TTAACGCAGTAAAGAGAG-3', and R2: 5'-TGGAGACT TCGGTGCGTAG-3'. The probe DNA sequence was design based on the sequence of *fla* gene, with the exchange of guanine base to inosine. Probe DNA is: 5'-IAICTITIAAATTTIITIIC-3'. Synthetic target DNA (stDNA) is complement of DNA probe. stDNA: 5'-CTCGACACTTTAAACCACCG-3'. Non complementary DNA (ncDNA) sequence is a sequence that is not complement to DNA probe. ncDNA: 5'–ACTTAAATTTT C-3'.

Other reagent was biomolecular grade such as *DreamTaq™ Green* PCR *Master Mix* (Fermentas), Wizard DNA genomic purification kit (Promega), TIANamp Blood DNA kit (Tiangen), 100 bp DNA ladder (Fermentas), 1x TAE (Tris-acetic acid 0,04M, EDTA 0.001M pH 8), TE (Tris-EDTA), agarose, ethidium bromide, acetate buffer (ABS) 0.50 M pH 5.0, phosphate buffer (PBS) 0.05 M pH 7.0,potassium chloride, sodium chloride, and nuclease free water.

Instrumentation

Instruments used in this research was rotring pencil, 2B ENO Pilot® pencil leads with 30 mm in length and 0.5 mm diameter, Metrohm® μAutolab type III potentiostat equipped NOVA software (Eco Chemie, The Netherlands). The three electrode system, consists of working electrode (pencil graphite leads), reference electrode (Ag/AgCl), and auxiliary electrode (Pt). Mastercycler Personal PCR (Eppendorf), Mini Sub Cell Horizontal Electrophoresis (Biorad), pH meters, Biophotometer (Eppendorf) and UV lamp.

Procedure

Amplification of S. typhi's flagellin gene fragment

S. typhi genomic DNA was isolated from the culture by using Wizard DNA genomic purification kit (Promega) following the protocol and used as a template for PCR. DNA from blood samples were isolated using TIANamp Blood DNA kit (Tiangen). The reaction mixture for the first round PCR contained 2 μL (~50 ng) of genomic DNA, 25 pmol of F1 dan R1 primer respectively, 20 μL of *DreamTaq™ Green* PCR *Master Mix* containing *DreamTaq™ green* buffer, *DreamTaq™* DNA polymerase, 10 mM dNTP, and $MgCl_2$. ddH_2O was added to the final volume 50 μL. The PCR cycle includes initial denaturizing for 1 min at 95 °C, 30 cycles; 1 min at 95 °C; 1 min at 63 °C; 3 min at 72 °C followed by a single period at 72 °C for 10 min. Five microlitter of first round PCR products were used as a template for second round PCR. The reaction mixture contain 25 pmol of F2 and R2 primers pairs, *DreamTaq™ Green* PCR *Master Mix* and ddH_2O in the final volume of 50 μL. The second round PCR was

performed for 35 cycles at 94 °C for 1 min, 61 °C for 1 min, and 72 °C for 7 min. The PCR product were characterized in 2% agarose electrophoresis and visualized by UV lamp.

Pretreatment of electrode

The pencil graphite electrodes was pretreated by applying + 1.4 V for 1 min in 0.05 M phosphate buffer solution (pH 7.0) containing 20 mM NaCl.

Immobilization of probe DNA

Pretreated pencil graphite electrode was dipped into 100 mL of probe DNA for 20 min by variation of time and probe concentration.

Hybridization of target stDNA and PCR product with probe DNA

Probe DNA was hybridized with target stDNA and PCR product respectively, with variation of time and concentration of stDNA and PCR product.

Characterization of PCR product

Product of first and second round PCR were characterized on 2% agarose gel electrophoresis in 1x TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA pH 8.0) following the protocol [21]. Electrophoresis was performed at 70 mV, ± 80 mA for 45 min. The result was visualized by UV lamp.

Measurement of guanine oxidation signals

Pretreated pencil graphite electrode was dipped into 100 mL of probe DNA, probe DNA-stDNA, and probe DNA-PCR product, then washed with 0.5 M acetate buffer pH 0.5. Each of DNA oxidation signals are measured using differential pulse voltammetry (DPV). Working conditions was setting for the reading potential between 0.3 V to 1.4 V with a cleaning time for 60 sec, and voltage reading level was 0.004 V and 0.400 V/s.

Measurement of hybridization signals of probe DNA-stDNA with the variation of stDNA concentration

Pretreated pencil graphite electrode was dipped into 100 µL of 10 µg/mL probe DNA for 20 min. Then the electrode was washed with 0.5 M acetate buffer (pH 5.0) containing 20 mM sodium chloride for 5 sec and dipped into 100 µL of 5-50 µg/mL of stDNA respectively, and the adsorption time was 20 min. Then the electrode was washed with 50 mM phosphate buffer (pH 7.0) containing 20 mM sodium chloride. The signal was observed with the differential pulse voltammetry in potential range between 0.3 to 1.4 V. In order to calculate the precision of measurement, each of oxidation signal (for every variation of the concentration of stDNA) are performed with 3 times repetition.

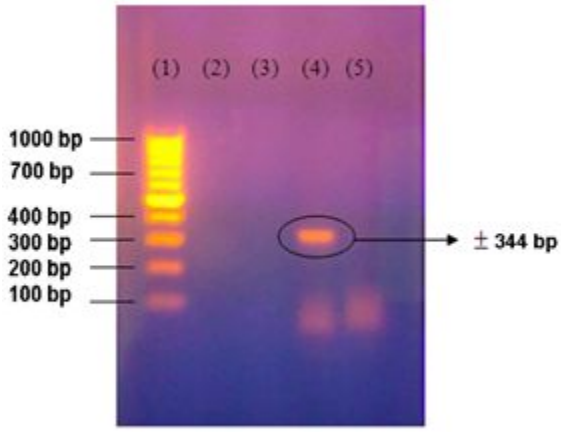

Fig 1. Characterization of the first and second round PCR product. Lane 1: 100 bp DNA ladder, lane 2: first round PCR product, lane 3: negative control of first round PCR, lane 4: 344 bp of second round PCR product, lane 5: negative control of second round PCR.

Application of voltammetric DNA biosensor for the PCR product

PCR products were diluted with sterile deionized water in the final concentration 20 µg/mL. Then denatured by heating at 95 °C for 5 min in a water bath, and then cooled in an ice bath for 2 min. Pretreated pencil graphite electrode was dipped into 100 µL of 10 µg/mL probe DNA for 20 min. Then the electrode was washed with 0.5 M acetate buffer (pH 5.0) containing 20 mM sodium chloride for 5 sec, and dipped into 100 µL of 20 µg/mL PCR product, the adsorption time was 20 min. Then the electrode was washed with 50 mM phosphate buffer solution (pH 7.0) containing 20 mM sodium chloride for 5 sec. The signal was observed with the differential pulse voltammetry with potential between 0.3 to 1.4 V.

RESULT AND DISCUSSION

Electroforegram of PCR Product

Fig. 1 shows the images of the first and second round PCR of *flagellin* gene fragment. It can be seen that the first round of PCR product cannot be visualized on a gel but the second round PCR was successfully visualized resulted in 344 pb fragment. The product of first round PCR should be 400-500 bp, but it was not visible on the gel, might be because the concentration was too low.

Guanine Oxidation Signals

Fig. 2 shows the difference in peak current of probe DNA, stDNA and probe DNA-stDNA after hybridization. It can be seen on Fig. 1 that the probe

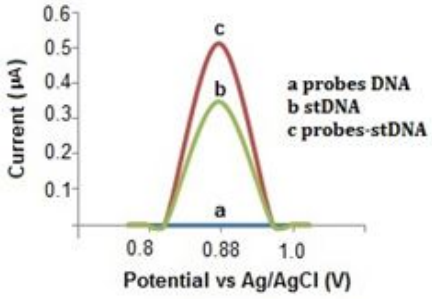

Fig 2. Differential pulse voltammograms for 20 µg/mL of probes DNA in acetate buffer saline, 20 µg/mL target stDNA in phosphate buffer saline, and the hybridization of probe-stDNA each 20 µg/mL in phosphate buffer saline, using pencil graphite electrodes. Measurement by using DPV, 50 mV amplitude and 4 mV step potential, between the potential range of 0.3 V-1.4 V.

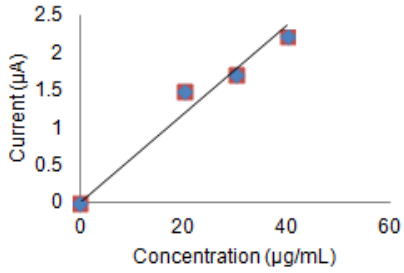

Fig 3. Linear relationship between peak current (Ip) with concentration of stDNA

DNA alone did not produce a peak current at potential about 0.9 V, because the probe DNA sequence did not contain any guanine base because the guanine bases in probes DNA had been replaced by inosine which had much smaller oxidation signal than guanine.

Measurement of Hybridization Signals with the Variation of stDNA Concentration

Linear relationship between peak currents with variations of stDNA concentration was shown in Fig. 3. Its shows that the hybridization of probe DNA-stDNA have a fairly good linearity till 40 µg/mL of stDNA with r = 0.9562 and a linear regression equation is y = 0.059x.

Application of Voltammetric DNA Biosensor for Nested PCR Product

The DVP measurement shows the guanine oxidation signals at about 0.9 V for probe-first round PCR product hybridization signals of *fla* gene fragment of *S. typhi*, gave a mean average signal of 2.32 µA with a relative standard deviation value of 0.33. DVP measurement of probe-second round PCR product hybridization signals of *fla* gene fragment of *S. typhi*

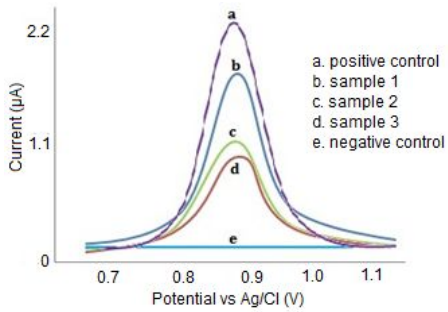

Fig 4. Differential pulse voltammograms for hybridization between probes DNA and first round PCR product of real samples, positive control, and negative control; other conditions are same as in Fig. 2.

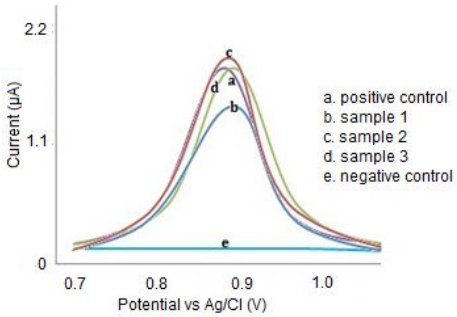

Fig 5. Differential pulse voltammograms for hybridization between probes DNA and second round PCR product of real samples, positive control, and negative control; other conditions are same as in Fig. 2.

gave a mean average signal of 1.47 µA with a relative standard deviation value of 0.07. It can be seen that voltammetric DNA biosensor can be used to detect *S. typhi*'s *flagellin* gene fragment from bacterial culture, both for the first and the second round PCR product. If we compared to the characterization using electrophoreses gel agarose, voltammetric DNA biosensor is more sensitive. The limit detection was obtained at 0.5 µg/mL (data were not shown).

In this study, detection of the DNA sequences of the infectious diseases from PCR amplified real sample was also carried out by using voltammetric DNA biosensor. The three subsequent experiments for the detection of hybridization between the probe DNA and the target stDNA sequence from the positive real samples gave reproducible results. Fig. 4 shows the guanine signal obtained from the hybridization between the probe DNA and the first round nested PCR of sample 1, sample 2, sample 3, positive control, and blank solution. The positive control is PCR product of *S. typhi* culture. The mean average signal of sample 1, sample 2, sample 3 are 1.82, 1.06, and 1.12 µA, respectively.

Fig. 5 shows the peak current of hybridization between the probe DNA and the second round nested

PCR of sample 1, sample 2, sample 3, positive control, and blank solution. The mean average signal of sample 1, sample 2, sample 3 and positive control are 1.29, 1.74, 1.50, and 1.47 µA, respectively.

The selectivity of biosensor was optimized using non complementary sequences as a target, and the hybridization between the probe DNA and the non complementary sequences as a target gave no signal (data was not shown).

CONCLUSION

Voltammetric DNA biosensor can be used to detect the *flagellin* gene fragment of *S. typhi* from nested PCR product using pencil graphite electrodes. The guanine oxidation signal of hybridization between probe-first round PCR product and probe-second round PCR product showed the mean average peak current of 2.32 and 1.47 µA, respectively. The limit detection obtained was 0.5 µg/mL. The first round nested PCR product can not be visualized by agarose electrophoresis, whereas using the voltammetric biosensor methods can be detected either for the first round or second round nested PCR product. The experiments for the detection of hybridization between the probe DNA and the target stDNA sequence from the positive real samples gave reproducible results.

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