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Coupling electrochemical response of a DNA biosensor with PCR for *Neisseria gonorrhoeae* detection ☆

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ABSTRACT

Early diagnosis of gonococcal infections is important with regard to a patient's health and stage of infection. In this context, we report the development of an *opa*-gene–based electrochemical DNA biosensor for detection of *Neisseria gonorrhoeae* by monitoring redox peak of methylene blue indicator. The fabricated biosensor has been shown to be highly sensitive and specific when evaluated with complementary, non-complementary, and 1-base mismatch DNA sequences and polymerase chain reaction (PCR) amplified products (amplicons) of standard strain of *N. gonorrhoeae* (ATCC49226). The biosensor has been further evaluated using amplicons of known positive and negative clinical samples, and cut-off for positives has been determined using receiver operating characteristic curve. The sensitivity (SN), specificity (SP), positive predictive value, and negative predictive value of the biosensor have been found to be 96.2%, 88.2%, 92.6%, and 93.8%, respectively. We conclude that the combination of PCR amplification with electrochemical detection shows distinct advantage of high SN and increased SP for gonococcal detection.

1. Introduction

In developing countries, sexually transmitted infections (STIs) and their complications are amongst the top 5 disease categories for which adults seek health care (UNAIDS, 1998). The currently available diagnostic testing algorithms require 2–14 days of turnaround time, thus contributing to low rates of patient return for test results, reinfection of the presenting patient, and ongoing transmission of infection in the patient's partner(s). Further, the stigma, privacy, and confidentiality issues associated with STIs emphasize the need for development of point-of-care (POC) or near POC diagnostics for these infections (Bissonnette and Bergeron, 2012; Hsieh et al., 2010). An ongoing revolution in this area is the development of biosensors that have the potential to be used as an integral component in POC test (POCT) devices.

DNA-biosensing methodologies are usually based on detection of DNA hybridization, which is both specific and robust (Drummond et al., 2003). However, very often, the amount of DNA to be detected (in femtomolar or attomolar ranges) is lower than the detection limit of conventional analytical techniques (Pedrero et al., 2011). One approach to overcome this problem would be amplification of signal using innovative routes of electrochemistry. However, it is pertinent to mention that amplification of the signal alone for detection of DNA, which may be present in a very low concentration in clinical samples, continues to be a challenge. Amplification of the target by means of polymerase chain reaction (PCR) before biosensor analysis is commonly required to overcome the problems imposed by the low abundance and extreme complexity of the non-amplified DNA in the clinical samples (Loaiza et al., 2009; Pedrero et al., 2011; Zhu et al., 2008).

Many attempts have been made worldwide for the development of electrochemical biosensors for pathogen detection (Gabig-Ciminska et al., 2004; Liao et al., 2006; Meric et al., 2002; Pedrero et al., 2011). However, most of these make use of synthetic DNA (complementary, non-complementary, and 1-base mismatch [OBM] sequences) for evaluation of the biosensor. Not much effort has yet been made on application of these methods to clinical samples. In a recent work

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(Singh et al., 2010), we reported fabrication of an electrochemical DNA biosensor based on self-assembled monolayer of thiolated DNA probe on gold electrode targeting *opa* gene of *N. gonorrhoeae*. Mercaptohexanol was used as a blocking agent, and methylene blue (MB), as the electro-active DNA hybridization indicator. The biosensor was used for successful detection of synthetic complementary DNA, PCR amplified DNA, and sonicated genomic DNA from gonococcal culture. In the present study, we have extended the approach to disposable screen printed gold electrodes for detection. Moreover, in this study, the performance of the biosensor has been validated using PCR amplified DNA (amplicons) from multiple known positive and negative clinical samples. During the study, it was observed that when the biosensor was evaluated on clinical samples, a quantitative variation in signal intensity was observed. This may perhaps be due to the variability in the bacterial load in clinical samples due to the host-pathogen interaction. In addition, a background current was detected, which was perhaps due to non-specific adsorption on the matrix. Keeping the above in mind, the present manuscript focuses on the study of the response characteristics of the fabricated bioelectrode with regard to the amplicons to arrive at a cut-off point using the receiver operating characteristic (ROC) curve to distinguish the infected from non-infected individuals (Akobeng, 2007). The diagnostic accuracy and the clinical utility index of the test have also been determined for validation of the assay.

2. Materials and methods

2.1. Primers and probe

Primers targeting *opa* gene of *N. gonorrhoeae* were used for PCR amplification, as described earlier (Verma et al., 2012) (GenBank accession no. PUID 9716120 SNUM 2706 Ng_opa). 5′-thiolated 19-mer oligonucleotide modified with a 6-carbon (C6) spacer arm was used as the probe (thssDNA). The sequences of oligonucleotide primers, probe, complementary, non-complementary, and OBM sequences used in this study are as follows:

Forward primer: 5′ CGGTGCTTCATCACCTTAG 3′
Reverse primer: 5′ GGATTCATTTTCGGCTCCTT 3′
Probe: 5′ S-S-C6-CGGTGCTTCATCACCTTAG 3′
Complementary sequence: 5′ CTAAGGTGATGAAGCACCG 3′
OBM: 5′ CTAAG**T**TGATGAAGCACCG 3′
Non-complementary sequence: 5′ ACGGTTCACGCGGTACTTA 3′

The primers were procured from Sigma-Aldrich (Co., St. Lious, MO, USA). The probe, complementary, non-complementary and OBM sequences were procured from Midland Certified Reagent Company (Midland, TX, USA).

2.2. Reagents

Screen-printed gold electrodes have been procured from DropSens (Oviedo, Spain). Tris buffer, ethylene diamine tetra acetic acid, potassium monohydrogen phosphate, potassium dihydrogen phosphate, and MB have been purchased from Sigma-Aldrich and 6-mercapto-1-hexanol (MCH) from Fluka (Gillingham, UK). All solutions and glassware have been autoclaved prior to being used. Solutions have been prepared in autoclaved deionized water (MilliQ 10TS, Millipore, Bedford, MA, USA).

2.3. Bacterial isolates and clinical samples

The bacterial strains used to test the specificity (SP) of the biosensor included *N. gonorrhoeae* (ATCC 49226), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Neisseria meningitidis* (ATCC 13077 serogroup A), and *Neisseria sicca* (ATCC 29193). The diagnostic stability of the probe sequence was further evaluated using 16 *N. gonorrhoeae* strains comprising of WHO B, WHO C, WHO F, WHO G, WHO K, WHO L, WHO M, WHO N, WHO O, WHO P, and 6 quality control strains derived from WHO Collaborating Centre for STD, The Prince of Wales Hospital, Sydney, Australia, and received from Regional Reference Laboratory, Safdarjung Hospital, New Delhi.

Patients in the age group of 15–45 years attending the STD Clinic, Dermatology OPD of AIIMS, New Delhi, India, with symptoms suggestive of gonorrhoea were included in the study. The inclusion criteria for patients were urethral, cervical/vaginal discharge, or lower abdominal pain. In addition, history of symptomatic partner or positive risk factors or partner with risk factors was taken into consideration. The swabs were collected in triplicate using standard techniques by a trained physician (WHO, 1999); one was used to prepare a smear for microscopic examination, second for culture, and third for DNA extraction for PCR followed by biosensor evaluation. This preliminary study with biosensor was performed on DNA extracted from 26 gonorrhoea-positive patient samples (i.e., positive by culture and PCR), 16 negative patient samples (i.e., negative by culture and PCR), and 1 negative control (without DNA). The study has been approved by the Ethics Committee, All India Institute of Medical Sciences, New Delhi, India.

2.4. Sample processing

Microscopic examination of smear was done following Gram's staining. Swabs were plated on standard culture media (Saponin lysed blood agar and chocolate agar), and identification was done as per the standard microbiological protocol (WHO 1999). DNA was extracted from isolated strains of *N. gonorrhoeae* and the patients' swab samples using QIAamp DNA mini kit (Qiagen Sciences Inc., Germantown, MD, USA) according to the manufacturer's instructions.

2.5. Fabrication of the bioelectrode

Screen-printed gold electrodes (Au) procured from DropSens, Spain, were used for the study. Before self-assembled monolayer formation, the electrodes were treated with 50 μL of 0.5 mol/L H_2SO_4 solution containing 10 mmol/L KCl by cycling 10 times the potential between 0.0 and +1.7 V with a scan rate of 100 mVs^{-1} (Loaiza et al., 2008). The characteristic oxidation/reduction peaks were obtained at +1.35 V and +0.65 V, respectively. The thiolated probe DNA was activated using 0.1 mol/L dithiothreitol as per the manufacturer's instructions. The pre-treated electrodes were incubated overnight with 10 μL of 1 μmol/L activated probe in an inert atmosphere in humid chamber with a humidity of 80% to form self-assembled monolayer. The bioelectrodes were layered with 40 μL of 1 mmol/L MCH for 2 h to prevent any non-specific binding of DNA on the surface. The electrodes were thoroughly rinsed with deionized water and dried at each step. These electrodes were stored at 4 °C till further use. Fig. 1 depicts the graphic representation of the above-mentioned protocol. Electrochemical characterization of the bioelectrodes (Pristine gold electrode, electrode immobilized with probe DNA, and electrode hybridized with complementary DNA) was performed using cyclic voltammetry (CV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (EIS) in phosphate buffer saline electrolyte (PBS) (50 mmol/L, pH 7.0, 0.9% NaCl) containing 5 mmol/L $[Fe(CN)_6]^{3-/4-}$ (Potentiostat/Galvanostat; Autolab Eco Chemie, Utrecht, The Netherlands). Surface characterization was done using scanning electron microscopy (SEM, LEO-440).

2.6. Hybridization studies with synthetic DNA

The hybridization detection studies were performed on thssDNA-Au electrode by incubating with 10 μL complementary, non-