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Research Article

PCR-Based Method for Rapid and Minimized Electrochemical Detection of *mec*A Gene of Methicillin-Resistant *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus epidermis*

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important pathogens that cause nosocomial infections. However, microbiological culture techniques take a few days to yield results; therefore, a simple, cost-effective, and rapid detection system is required for screening for MRSA and related bacteria: Methicillin-resistant *Staphylococcus epidermidis* (MRSE) carriers during the hospital admissions process. In this study, we described the simplified method using by one-time use and screen-printed carbon electrodes, relied upon current quantification of Hoechst dyes which bound with DNA amplified via polymerase chain reaction (PCR) targeted for MRSA *mecA* gene. Amount of DNA-bound Hoechst molecules were measured by the hand-held potentiostat within two minutes. We found that the peak of a Hoechst-mediated current depended upon the number of MRSA isolates, and successfully distinguished between carriers and a non-carrier based on nasal swabs from the patients. This method required only 10 μL for application, and the results could be obtained within total 60 min from sample collection when a minimum of 1×10^3 MRSA isolates was present. These results suggested that this minimized technique has the potential to become a useful system of active surveillance for MRSA/MRSE carriers.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a leading cause of infections in hospitals, and mortality from MRSA bacteremia is high [1-3]. Therefore, surveillance of patients during hospital admission for MRSA and related bacteria: Methicillin-resistant *Staphylococcus epidermidis* (MRSE), usually via nasal swab, is important [4,5]. Active surveillance involves the detection and tracking of patients who are asymptomatic, but carry MRSA/MRSE; moreover, compelling data support the practice of identifying carriers and using contact precautions for both carriers and infected patients to reduce spread of MRSA/MRSE within hospitals [6].

Usually, microbiological culture is used to identify and quantify MRSA/MRSE; however, this method is time-consuming, as bacterial growth requires overnight or a few days. Therefore, development of a highly sensitive detection system that is rapid, simple, cost-effective, and portable would be beneficial for the prevention of MRSA/MRSE transmission [4,5].

Polymerase chain reaction (PCR)-based DNA analysis has recently become routine in clinical diagnosis for the sensitive, rapid, and specific detection of viruses and bacteria [7]. Detection of PCR-amplified genes is commonly accomplished by gel electrophoresis and/or fluorescence staining based on TaqMan chemistry. In particular, fluorescence-based detection is highly sensitive and makes quantitative real-time PCR possible [8]. However, the apparatus for optical measurement of fluorescence intensity is not amenable to miniaturization; therefore, this technology is unsuitable for the construction of a portable system.

During studies of food preservation and food safety, we have investigated the development of electrochemical sensors for nucleic acids, including for DNA from *E. coli* [9]. Electrochemical-based DNA detection systems involve relatively simple technology that is highly amenable to miniaturization; such detection systems allow for rapid, label-free DNA measurement with low power consumption [10,11].

Here, we describe development of a simplified detection system for MRSA/MRSE targeted for *mec*A gene that involves a screen-printed carbon electrode and a hand-held potentiostat. This electrode could efficiently detect MRSA/MRSE by measuring DNA amplification using PCR with Hoechst; dyes used to stain DNA.

Materials and Methods

Patients and samples

This study was approved by the Research Ethics Committee of Osaka University and assigned accession number 11159-6. Two

patients provided written, informed consent for their participation in this study.

Upon hospital admittance, nasal swab samples were isolated from the patients, each of whom was admitted to Osaka University Hospital in Suita City, Osaka, Japan via the emergency room. Sample A was from a 37-year-old female patient who had been admitted because of severe hepatic failure that resulted from acute viral infection; sample B was from a 25-year-old female patient who had been admitted because of drug addiction. Samples A and C was isolated from the same patient, but sample C was collected five days after collection of Sample A.

MRSA culture

MRSA strain GTC 01186 (obtained from Gifu University, Japan) and isolates isolated from the patients described above were used in this study. These MRSA was cultured in brain heart infusion (BHI) broth overnight at 30°C with reciprocal shaking at 110 strokes/ min; isolates were harvested by centrifugation at 8000 xg for 5 min at 30°C. The bacterial pellet was kept at -20°C until use. The number of bacterial isolates was calculated by plating MRSA on BD MRSA-selective agar (Beckton Dickinson, Franklin Lakes, NJ, USA). The automated identification system (MicroScan WalkAway; Siemens, Munich, Germany) was ultimately used to definitively identify the MRSA/MRSE present in the patient samples [12].

PCR amplification

A primer pair, MecA1 (5'-GTAGAAATGACTGAACGTCCGATAA -3') and MecA2 (5'-CCAATTCCACATTGTTTCGGTCTAA -3'), was used for selective amplification of the MRSA/MRSE *mecA* gene(13). Each 20-μL PCR mixture contained 10×Fast Buffer, 1.5 U of SpeedSTAR HS DNA Polymerase (Takara Bio. Inc. Shiga, Japan), 200 μM of each dNTP, 1.0 μM of primers, and 1 μL of diluted culture suspension; DNA was not purified from the culture suspension; the suspension was used at each of the six indicated concentrations and added directly to each reaction mixture as template. PCR was performed in a Gene Atlas 322 thermal cycler (Astec Co., Ltd., Japan). The cycling conditions included a single initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 5 s (denaturation) and 60°C for 18 s (annealing and extension); the total time was ~60 min. For the negative control reactions, nuclease-free water was added instead of culture suspension to the PCR mixture. After PCR, electrophoresis through a 4%-agarose gel or electrochemical measurement were used to confirm amplification of target sequences.

Electrochemical detection of PCR amplification

Hoechst 33258 [2'-(4-hydroxyphenyl)-5-(4- methyl-1-piperazinyl)-2, 5'-bi-1H-benzimidazole), H33258] (Sigma Aldrich Co., MO, USA) was used as a reporting element for electrochemical detection as previously described [9-14]. H33258 is an electro-active molecule with high affinity for nucleic acids; H33258 can intercalate into the DNA double helix. Binding of H33258 molecules to amplified DNA causes the peak current to decrease because H33258–PCR-amplified DNA complex diffuse more slowly than free H33258 to the electrode surface.

A screen-printed carbon electrode chip was used for the electrochemical measurements (Figure 1A). The carbon-based chip contained a three-electrode system that comprised a working electrode, counter electrodes, and the Ag/AgCl reference electrode [9-15]. The working electrode area was 3.04 mm², and the total size, including the connection part and the carbon barrier that prevented solution from flowing into the chip connector, was 12.5 mm×4 mm×0.3 mm [15]. A small volume of solution (10 μL) was measured by direct application onto the electrode surface, and each chip was discarded after a single use. The screen-printed chips were affordable, and insertion of a new chip into the connector was fast and easy [9,10]. Furthermore, each chip was discarded after one measurement; therefore, cross-contamination over multiple measurements was avoided.

A USB-powered hand-held Potentiostat (BDTminiSTAT100; Bio device Technology Co., Ltd., Ishikawa, Japan) was used to collect electrochemical signals via the linear sweep voltammetry (LSV) technique [10] (Figure 1B). Since this portable potentiostat can be controlled and powered by connecting it to the USB port of a laptop computer, it was suitable for the development of bedside-use detection system [9,10]. After each amplification reaction, 3 μL of the respective PCR mixture was mixed with 10 μL of 40 μM H33258 solution (H33258 in HEPES buffer, pH 7.0). For each measurement in this study, 10 μL of the mixed solution was applied to the screen-printed electrode. The conditions used for LSV measurements of peak current intensity of H33258 oxidation were as follows: initial potential, 0 V; end potential, 700 mV; and scan rate, 50 mV/s.

Results

Electrochemical detection of PCR amplification of a MRSA/MRSE mecA gene

For quantitative analysis, culture medium containing MRSA GTC 01186 isolates was diluted to attain concentrations from 3×10^6 to 3×10^1 of MRSA isolates per PCR mixture; these dilute solutions were used as the template for PCR.

Each of six dilutions of culture media and water were used as the template or no-template control (NTC), respectively, for PCR amplification of the MRSA/MRSE *mecA* gene; after 40 PCR cycles, products were subject to agarose gel electrophoresis (Figure 2A). A specific amplification product of 310bp was clearly evident with four solutions, those with 3×10^6 to 3×10^3 MRSA isolates; notably, the same
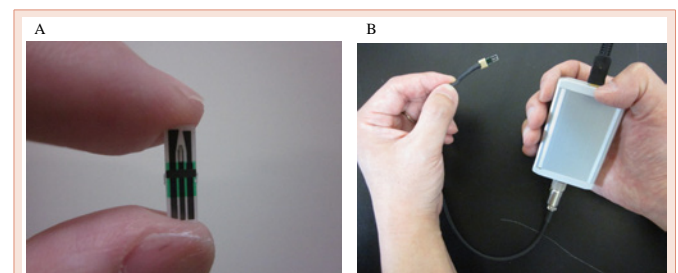

Figure 1: Photograph of the devices used for semi-real-time electrochemical monitoring. (A) Screen-printed electrodes. (B) USB powered portable Potentiostat.

product was barely evident in the sample containing 3×10^2 MRSA isolates.

LSV was used to measure currents resulting from free H33258 in PCR mixtures; current decreased as bacterial concentration increased because, as more DNA duplexes were amplified via PCR, the amount of DNA-bound H33258 molecules increased; conversely, the amount of free electro-active H33258 decreased, as did the associated current (Figure 2B). The average H33258 peak currents at a peak potential of approximately 0.4 V decreased depending on the number of MRSA isolates used as PCR templates; the peak current values were as follows: 1060, 915, 800, 650, 430, and 1045 for 3×10^2, 3×10^3, 3×10^4, 3×10^5, 3×10^6 cells, and negative control, respectively.

The standard curve generated from the relationship between peak current signals of H33258-PCR and the number of MRSA isolates is shown in Figure 2C. The LSV measurements for allowed determination of the dependence of the decrease in H33258 peak current on the number of MRSA isolates. The reliable and linear range of the standard curve representing the relationship between number of MRSA isolates and electrochemical current was from 3×10^3 to 3×10^6 MRSA isolates, and the square of the regression coefficient (R^2) for the linear regression was 0.98535 (Figure 2C). The results suggested that PCR amplification and subsequent H33258-mediated electrochemical measurement could be used for a highly sensitive and accurately quantitative analysis of MRSA/MRSE number without DNA extraction process. The total time of amplification was <55 min and electrochemical measurement took <2 min.

Electrochemical detection of MRSA carriers based on nasal swab taken from patients being admitted to a hospital

Next, we used nasal swab samples from patients to investigate the potential of this method for clinical applications. PCR and subsequent H33258-mediated electrochemical measurement were used to measure the amount of colonizing MRSA in nasal swabs taken from two patients that were being admitted into the hospital (Figure 3). Nasal swab samples A, B, and C and an NTC were subject to PCR and subsequent gel electrophoresis (Figure 3A). The specific 310-bp amplification product was clearly evident with Sample A and C, but evidently absent with NTC and Sample B.

LSV current measurements from the same PCR mixtures to which H33258 had been added were as follows: control, 1020; Sample A, 595; Sample B, 1030; and Sample C, 680 nA. The average H33258 peak presumably depended upon the number of template MRSA present in the sample; based on the standard curve (Figure 2C), samples A, B, and C contained 3.8 x10^5, none, and 1.0 x10^5 CFU, respectively. These results were qualitatively consistent with semi-quantitative results generated via culture in the hospital laboratory (Table 1).

Discussion

We have described a method for electrochemical detection of MRSA/MRSE following PCR amplification of unpurified MRSA/MRSE mecA DNA; this method involved single-use, screen-printed carbon electrodes [9,15]. The techniques that we developed were versatile, and integratable in nature, utilizing gene analysis for high-
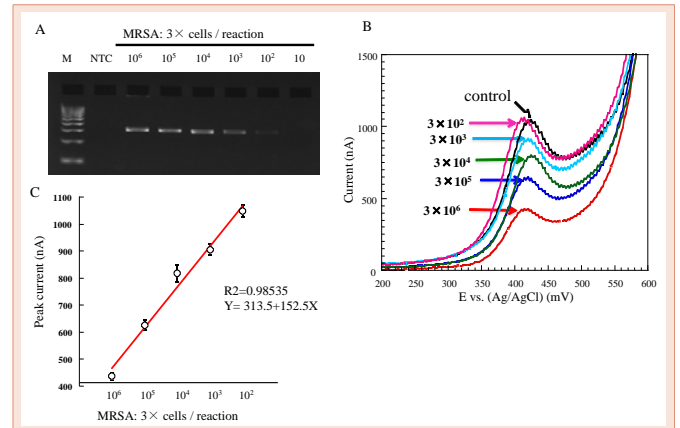

Figure 2: Quantitative electrochemical detection of MRSA (A) Agarose gel electrophoresis. The amplicon from the MRSA/MRSE mecA gene is 310 bp. The numbers of initial input MRSA cells numbers were from 3 ×106 to 3×100. *100-bpDNA ladder/marker. *NTC=Non-template control. (B) LSV-based electrochemical measurements for determination of MRSA numbers. Each measurement was taken with a new, previously unused electrode and expressed as linear sweep voltammogram. Scan rate: 50 mV/s. (C) Plots of peak currents for the initial number of MRSA cells. Error bars indicate the standard deviation of three individual PCR measurements. R2=0.98535, Y= 313.5+152.5X.
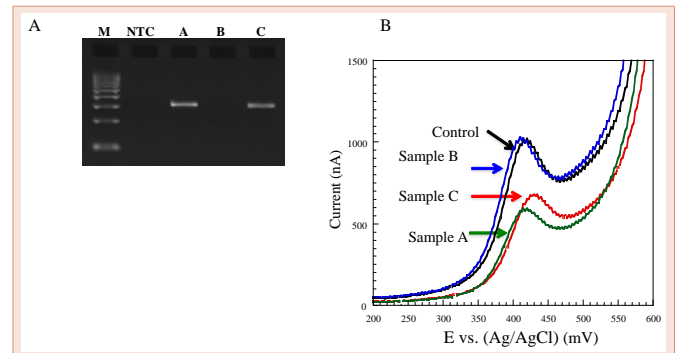

Figure 3: Quantitative electrochemical detection of MRSA in samples from. (A) Agarose gel electrophoresis. The amplicon from the MRSA/MRSE mecA gene is 310 bp and is visible in lanes representing Sample A and C, but not in lanes representing Sample B or the negative control. *100-bpDNA ladder/marker. *NTC=Non-template control. (B) LSV-based electrochemical determination of MRSA numbers in samples colonized patients. Each measurement was taken with a new, previously unused electrode and expressed as linear sweep voltammogram. Scan rate: 50 mV/s.

Table 1: Comparison between LSV and Cultures regarding estimate of MRSA number in clinical samples.

Sample	Estimated MRSA number(cells/mL)	Culture results
A	3.8×10^5	++
B	Negative	-
C	1.0×10^5	+
+: < 100 colonies /plate, ++: 100-500 colonies/plate, +++: 500> colonies /plate		

sensitivity detection for simplified [9]. Furthermore, use of disposable electrodes should minimize cross-contamination, and handheld USB-powered potentiostats could be used to develop as bedside-use detection systems for MRSA surveillance [10].

Genomic-based microbiological methods, including PCR, are widely used currently because these methods are rapid and usually highly sensitive. Previously, we used real-time PCR (SeptiFast: BD) to investigate pathogen detection system for blood samples from patients with sepsis; we found that the rapid multiplex pathogen detection system complemented traditional culture-based methods and offered some additional diagnostic value for the timely identification of causative pathogens [7]. This system showed high sensitivity for MRSA/MRSE, similar to traditional blood culture; moreover, the PCR-based method took only an hours, instead of days, to accurately confirm the presence or absence of the bacteria.

Active surveillance involving PCR-based sensitive and rapid methods for detection of MRSA/MRSE, could reduce MRSA/MRSE transmission in wards by more detailed and rapid intervention of infection control team (ICT) in hospitals [4,5]. Taguchi H et al. report that they investigated active screening of patient for colonization with MRSA upon admission to the hospital and during weekly follow-up surveillance after admission to a tertiary care center [4]. Of the 42 MRSA positive cases found in their study, 52% (22/42) were detected as MRSA positive by both PCR and culture; however, 19 (45%) of the 42 were found as PCR positive but culture negative cases. MRSA detection cases were decreased week by week. These findings suggested that active surveillance based on PCR methods might be highly sensitive and potentially useful for the early detection of MRSA/MRSE colonization during the hospital admission. In addition, Ghazal SS et al. performed preemptive screening in emergency departments; specifically, they used PCR testing of nasal swab specimens collected from patients 1) who had been transferred from other hospitals, 2) who had a history of prior hospitalization in the last 6 months, or 3) who had a history of infection or colonization with MRSA to identify active carries [6]. Maximum-contact precautions were employed for any patients who had positive PCR test results. They report that hospital acquired (HA)-MRSA infection rate decreased significantly, from 0.17 cases per 1,000 patient-days in 2007 to 0.03 cases per 1,000 patient-days in 2009, and the rate of HA-MRSA bloodstream infections decreased from 0.1 cases per 1,000 patient-days in 2007 to 0 cases per 1,000 patient-days in 2009 [6]. These results also suggested the PCR-based surveillance might be possible for early interventions by ICT, and they could efficiently reduce the nosocomial transmission and infection of MRSA/MRSE.

With the system describe here, detection of MRSA/MRSE carries could be rapid and sensitive, and also simple and easy, because our PCR systems did not require extraction of template DNA or agarose gel electrophoresis of PCR products. We needed only to mix the PCR products with H33258 dye, apply the mixtures to screen-printed electrodes, and measure peak currents. As with our previously reported detection systems [10], only two minutes were needed after PCR for the final measurements. The assay time from sample collection to interpretation of final results is estimated to be less than 1 hour. Moreover, the simplified and rapid process might be easy, and sample handling may not require advanced technical skills. Risk of contamination will be also reduced because of the simple handling processes. Clinical staff will be able to get results at point of care without need of expert assistance from special laboratories and equipment; clinicians could immediately implement more strict precautions for patients who are colonized with MRSA/MRSE.

In conclusion, we have described methods for electrochemical detection of PCR-amplified MRSA/MRSE-specific sequences; these methods involve a screen-printed carbon electrode and do not necessitate DNA purification or culturing of patient samples. The techniques were highly sensitive and constituted a simple detection system. The results showed that the peak of a H33258-mediated current decreased depending on the number of MRSA isolates in a PCR sample, and that the system could successfully detected MRSA/MRSE carriers based on nasal swabs taken from patients. Use of disposable electrodes will presumably minimize cross-contamination, and the handheld USB-powered potentiostat can be utilized to develop an easy-handling detection system. We should perform more detailed investigations using by more MRSA/MRSE positive samples, and next to analyze another target genes, including femA to distinguish MRSA and MRSE [16]. However, this minimized PCR-based active surveillance mechanism in combination with the implementation of contact precautions for patients with MRSA/MRSE-positive results may contribute to further reductions of MRSA/MRSE transmission in hospitals in near future.

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