Rapid Solid-Phase Immunoassay for Detection of Methicillin-Resistant Staphylococcus aureus Using Cycling Probe Technology

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A Cycling Probe Technology (CPT) assay with a lateral-flow device (strip) was developed for the detection of the mecA gene from methicillin-resistant Staphylococcus aureus (MRSA) cultures. The assay uses a mecA probe (DNA-RNA-DNA) labeled with fluorescein at the 5′ terminus and biotin at the 3′ terminus. The CPT reaction occurs at a constant temperature, which allows the probe to anneal to the target DNA. RNase H cuts the RNA portion of the probe, allowing the cleaved fragments to dissociate from the target DNA, making the target available for further cycling. The strip detection step uses a nitrocellulose membrane with streptavidin and immunoglobulin G antibody impregnated on the surface. In the absence of the mecA gene, the uncut probe is bound to an antifluorescein-gold conjugate and is then captured by the streptavidin to form a test line. In the presence of the mecA gene, the probe is cut and no test line is formed on the strip. A screen of 324 S. aureus clinical isolates by the CPT-strip assay showed a 99.4% sensitivity and a 100% specificity compared to the results of PCR for the detection of the mecA gene. Specificity testing showed that the CPT-strip assay did not exhibit any cross-reactivity with a panel of mecA-negative non-S. aureus isolates. The CPT-strip assay is simple and does not require sophisticated equipment. Furthermore, the assay takes 1.5 h starting from a primary culture to the time to detection of the mecA gene in S. aureus isolates.

The increased use of broad-spectrum antibiotics in recent years has led to the proliferation of antibiotic-resistant strains of common bacteria. Methicillin-resistant Staphylococcus aureus (MRSA) is an antibiotic-resistant variant of S. aureus commonly found in hospitals around the world. Since it was first reported, the prevalence of MRSA has increased dramatically, with infections caused by MRSA becoming one of the most commonly acquired types of nosocomial infections (9). Thus, there is a need for rapid and efficacious methods for the detection of MRSA.

Traditional methods of screening for MRSA use susceptibility tests that are dependent on the phenotypic expression of resistance (6). However, these tests are time-consuming, requiring an initial culture period of 18 to 24 h followed by an additional 18 to 24 h for antibiotic susceptibility testing (16, 17). DNA-based assays for the detection of antibiotic resistance provide a rapid method for the detection of MRSA (4). These assays test for the presence of genes that confer antibiotic resistance and thus have an inherent time advantage over culture-based tests that require phenotypic expression of the genes. Although PCR can detect the mecA gene (1, 5, 15), the potential for cross contamination, long turnaround times, and the expense and availability of specialized equipment and trained staff may deter some laboratories from using PCR for clinical diagnosis.

Cyclic Probe Technology (CPT) can be used to detect specific DNA sequences (8). CPT is an isothermal reaction that is not prone to cross contamination because the target is not amplified. The principle of CPT is outlined in Fig. 1a. An excess of a chimeric DNA-RNA-DNA probe specifically hybridizes to the target DNA in solution. RNase H cleaves the RNA portion of the probe-target duplex, thus allowing the cut probe fragments to dissociate from the target due to their lower melting temperatures. The target is now free to hybridize with another intact probe molecule while the cut probe fragments accumulate. With an isotopic detection format, CPT has been used for the detection of a tandem repeat DNA sequence in Mycobacterium tuberculosis (2) and has recently been successfully used for the detection of the mecA gene in MRSA (7) and the vanA and vanB genes in vancomycin-resistant enterococci (VRE) (12). The mecA assay was modified to a nonisotopic enzyme immunoassay (EIA) format that would be practical for use in clinical laboratories where such a diagnostic kit would be most valuable for the identification of MRSA (3). In the present study, we describe a new format of the CPT assay in which a lateral flow device (strip) is used to further simplify the procedure. The CPT-strip assay format significantly reduces the amount of hands-on manipulation by replacing the multistep, multireagent EIA detection step with a simple single-step procedure. The CPT-strip assay requires 90 min to process 24 samples after the initial culture, whereas the CPT-EIA requires 2 h. The hands-on time of the CPT-strip assay is approximately 25 to 30 min when processing 24 samples.

The strip is composed of four main components: (i) the sample pad, (ii) the conjugate pad that contains mouse antifluorescein antibodies conjugated to gold particles (Anti-F-GP), (iii) the nitrocellulose membrane imprinted with a streptavidin line and an immunoglobulin G (IgG) line, and (iv) an absorbent pad (Fig. 2). The CPT-strip assay uses the same mecA chimeric probe labeled with fluorescein at the 5′ terminus and biotin at the 3′ terminus as the EIA (3). When the CPT reaction is finished, the strip is placed in the reaction tube and the CPT reaction is absorbed into the sample pad and onto the strip. The sample then flows through the conjugate pad where the fluorescein of the chimeric probe binds to Anti-F-GP, an antifluorescein-gold conjugate, and is then captured by the streptavidin to form a test line. In the absence of the mecA gene, the uncut probe is bound to an antifluorescein-gold conjugate and is then captured by streptavidin to form a test line. In the presence of the mecA gene, the probe is cut and no test line is formed on the strip. A screen of 324 S. aureus clinical isolates by the CPT-strip assay showed a 99.4% sensitivity and a 100% specificity compared to the results of PCR for the detection of the mecA gene. Specificity testing showed that the CPT-strip assay did not exhibit any cross-reactivity with a panel of mecA-negative non-S. aureus isolates. The CPT-strip assay is simple and does not require sophisticated equipment. Furthermore, the assay takes 1.5 h starting from a primary culture to the time to detection of the mecA gene in S. aureus isolates.

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GPs. The complex of uncut probe and Anti-F-GP, if present, will be captured by the streptavidin via the probe’s biotin and result in the development of a “test” line. This test line indicates the absence of the \textit{mecA} target in the sample and identifies the isolate as a methicillin-sensitive \textit{S. aureus} (MSSA) isolate. Conversely, the absence of a test line indicates an MRSA strain. A second line is used as a control for the proper sample flow in the strip and develops as the result of the binding of excess Anti-F-GP to the rabbit anti-mouse IgG line.

An outline of the CPT-strip format is shown in Fig. 1.

To validate the CPT-strip assay, 324 clinical isolates of \textit{S. aureus} were tested by the CPT-strip test. The assay was also tested for cross-reactivity against 19 \textit{mecA}-negative non-\textit{S. aureus} isolates. In addition, we tested the effect of growing a subset of the clinical isolates on three commonly used culture media prior to testing by the CPT-strip assay.

**MATERIALS AND METHODS**

**Bacterial strains.** Reference strains of MSSA (ATCC 29213) and MRSA (ATCC 33592) were used as negative and positive controls, respectively, for the assay. Clinical isolates of staphylococci used in the blind screen were obtained from Cleveland Clinic Foundation (Cleveland, Ohio), Wishard Memorial Hospital (Indianapolis, Ind.), Hospital of the University of Pennsylvania (Philadelphia, Pa.), Primary Children’s Medical Center (Salt Lake City, Utah), Sunnybrook Health Science Centre (North York, Ontario, Canada), Veterans Affairs Medical Center (Nashville, Tenn.), University of Alabama (Birmingham, Ala.), and Vancouver General Hospital (Vancouver, British Columbia, Canada). A total of 324 \textit{S. aureus} isolates, including 6 borderline-resistant \textit{S. aureus} strains, were tested. As well, 12 non-\textit{S. aureus} staphylococcal organisms from the American Type Culture Collection (ATCC; \textit{S. capitis} ATCC 35661, \textit{S. cohnii} ATCC 35662, \textit{S. epidermidis} ATCC 14990, \textit{S. haemolyticus} ATCC 29970, \textit{S. hominis} ATCC 27844, \textit{S. lugdunensis} ATCC 43809, \textit{S. saprophyticus} ATCC 15305, \textit{S. sciuri} ATCC 29980, \textit{S. schleiferi} ATCC 43808, \textit{S. simulans} ATCC 27851, \textit{S. warneri} ATCC 27836, and \textit{S. xylosus} ATCC 29971, and 7 nonstaphylococcal organisms from ATCC (\textit{Candida albicans} ATCC 10231, \textit{Enterococcus faecalis} ATCC 29212, \textit{Enterococcus faecium} ATCC 51299, \textit{Escherichia coli} ATCC 25922, \textit{Lactobacillus acidophilus} ATCC 43808, \textit{S. hominis} ATCC 27844, \textit{S. lugdunensis} ATCC 43809, \textit{S. saprophyticus} ATCC 15305, \textit{S. sciuri} ATCC 29980, \textit{S. schleiferi} ATCC 43808, \textit{S. simulans} ATCC 27851, \textit{S. warneri} ATCC 27836, and \textit{S. xylosus} ATCC 29971) were tested to determine the cross-reactivity of the CPT-strip assay.

**Sample preparation.** The strains were grown overnight (18-to 24-h culture) at 37°C on tryptic soy agar (TSA) with 5% sheep blood (PML Microbiologicals, Wilsonville, Ore.). Other media tested were mannitol-salt agar and Columbia agar with 5% horse blood (all from PML Microbiologicals), and these media were used in the same overnight culture procedure with 96 of the clinical isolates chosen at random. Samples were prepared by using a 1-μl BAC-LOOP inoculum.

![FIG. 1. Schematic diagram of CPT-strip assay used for the detection of \textit{mecA} in \textit{S. aureus} cultures. (a) CPT. A single-stranded target (I) serves as a template for CPT. In the presence of probe (F-DNA-RNA-DNA-B) (II) and RNase H (III), the RNA portion of the probe-target complex (IV) is cleaved by RNase H. The shorter cleaved probe fragments dissociate from the target, thereby regenerating the target DNA for further cycling (V). (b) Strip detection. Upon placement of a strip into the CPT reaction, the probe binds to Anti-F-GP and the complex of Anti-F-GP with uncut probe binds to the streptavidin test line (VI). Excess Anti-F-GP binds to rabbit anti-mouse IgG to form the control line (VII). A test line indicates the presence of uncut probe and identifies the isolate in the sample as MSSA, whereas the absence of a test line indicates that the probe was cleaved and that the sample contains MRSA. The presence of the control line confirms a normal flow of the liquid through the strip.](http://jcm.asm.org/)
Probes, Eugene, Oreg.) and was scanned with a FluoroImager (Molecular Dynamics). The amplified DNA was resolved on a 1.5% agarose gel with 5 mM Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; pH 8.2, 0.05% Triton X-100, 100 µM EGTA, 200 U of a chromopeptidase (Wako Bioproducts, Richmond, Va.) per ml, 100 mM trehalose, and the preservative ProClin 300 (Supelco Park, Bellefonte, Pa.) at 20 ppm. Achromopeptidase is a bacteriolytic hydrolase enzyme that lyases the bacterial cell wall. Cell suspensions were lysed by incubation for 20 min in a 55°C heating block (Barnstead Thermolyne, Dubuque, Iowa).

**CPT-strip assay.** (i) CPT. The contents of microcentrifuge tubes that contained 50 µl of crude lysate were allowed to denature in a heating block (Barnstead Thermolyne) for 5 min (to achieve an in-tube temperature of 96 ± 1°C) and were then transferred back to the 55°C heating block. A 50-µl aliquot of cycling reagent was added to each tube. The 100-µl reaction mixture contained 20 mM TES (pH 7.2), 0.05% Triton X-100, 2 mM MgCl₂, 0.625 mM spermine tetrahydrochloride, 100 mM trehalose, 0.1% polynvinlypyrrolidone, 2 µg of bovine serum albumin per ml, 10 fmol of F-mecA945-29-B probe, and 1.0 µg of RNase H. RNase H and the mecA probe were as described elsewhere (3). Cycling reactions were incubated for 25 min in the 55°C heating block.

(ii) Strip detection step. After the 25-min incubation, one 4-mm-wide strip (Mizuho USA, San Diego, Calif.) was placed into each reaction tube at 55°C, and the tube was immediately removed from the heating block and placed at room temperature. The strips were kept in the reaction tube for 15 min at room temperature to allow sample flow, and then the strips were observed for the presence of a control line and a test line. The presence of a control line indicated a normal flow of the liquid through the strip; if the control line was not present, the test was considered invalid and the assay was repeated. The interpretation of results is summarized in Table 1.

**PCR.** The presence or absence of the mecA gene was determined by PCR as described previously (7). Briefly, 30 PCR cycles were used to amplify a 227-bp fragment with the following primers: mecA938-25 (5′-TGTTAAAAAGGGAAGGAC TGAAAAACCTT-3′) and mecA1039-22 (5′-GTTGATAGCAGTACCCGGC-3′). The amplified DNA was resolved on a 1.5% agarose gel with 5 µl of the 100-µl PCR mixture. The gel was stained with Sybr Green I dye (Molecular Probes, Eugene, Oreg.) and was scanned with a Fluorolmager (Molecular Dynamics, Sunnyvale, Calif.).

## RESULTS

**Screens of clinical isolates.** The 324 staphylococcal isolates were analyzed by PCR. A single 227-bp band was observed with 174 isolates; these isolates were designated mecA positive. No band was observed with 150 isolates; these isolates were designated mecA negative (data not shown). A screen of the 324 staphylococcal isolates was performed by the CPT-strip assay. The ATCC reference strains were tested in parallel as positive and negative controls. The results of the screen are as follows: 173 isolates were CPT-strip assay positive and PCR positive, 150 isolates were CPT-strip assay negative and PCR negative, and 1 isolate was CPT-strip assay negative and PCR positive. No isolate was CPT-strip assay positive and PCR negative. All samples showed a control line on the strips as expected. An example of a strip used with a sample that contained MSSA (presence of a test line) and a strip used with a sample that contained MRSA (no test line) is shown in Fig. 3.

The one mecA-positive strain that was identified by PCR but that the CPT-strip assay did not detect was difficult to suspend; i.e., the sample formed a visible clump that could not be homogenized. This strain with a false-negative result showed a faint test line instead of the absence of a test line by the CPT-strip assay. Retesting of this sample again gave a false-negative result. The other 173 MRSA strains did not show a test line, indicating that the probe was cleaved in the presence of the specific target. All 150 MSSA strains showed a test line. These results show that the CPT-strip assay correctly identified all 150 MSSA isolates and 173 of 174 MRSA isolates. Thus, the sensitivity and specificity of CPT-strip compared to the results of PCR were 99.4 and 100%, respectively.

**Cross-reactivity testing of CPT-strip assay.** Twelve non-S. aureus isolates and seven nonstaphylococcal clinical isolates tested by the CPT-strip test showed no cross-reactivity with the mecA probe. As expected, all of these samples showed test lines, indicating the absence of the mecA gene. However, testing of methicillin-resistant S. epidermidis (MRSE) did not consistently identify the presence of the mecA gene (data not shown).

**Testing of alternate media for cell culture.** A subset of the S. aureus clinical isolates from the previous screen were plated onto two media commonly used in clinical microbiology laboratories as alternatives to TSA with 5% sheep blood. The two new media tested gave results comparable to those obtained with TSA with sheep blood. The results are summarized in Table 2.

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<th>Control line</th>
<th>Test line</th>
<th>Interpretation</th>
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<tr>
<td>Present</td>
<td>Present</td>
<td>Negative for mecA gene, MSSA</td>
</tr>
<tr>
<td>Present</td>
<td>Absent</td>
<td>Positive for mecA gene, MRSA</td>
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<tr>
<td>Absent</td>
<td>Present or absent</td>
<td>Improper flow of liquid through the strip; repeat test</td>
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FIG. 3. A typical result of the CPT-strip assay for mecA detection. Control MSSA (lane S) and MRSA (lane R) strains from ATCC are tested in parallel with clinical isolates of MSSA (lane 1) and MRSA (lane 2). The test line (T) is present for the samples with MSSA and is absent for the samples with MRSA due to cleavage of the probe. The control line (C) shows a normal flow of the liquid through the strip.
We recommend TSA with 5% sheep blood as the culture medium for use with the CPT-strip assay. However, some clinical laboratories may routinely use other types of media for primary culture. Therefore, we tested two other commonly used media in combination with the CPT-strip assay. Mannitol-salt agar and Columbia agar with 5% horse blood were shown to perform comparably to TSA with 5% sheep blood. Thus, it would likely not be necessary for most clinical microbiology laboratories to alter their normal primary culture method to successfully run the CPT-strip assay.

There are, however, some limitations to the technique described here. Studies on detection of the mecA gene in MRSE by the CPT-strip assay did not consistently identify the presence of the mecA gene. Since the mecA sequence is homologous in MRSA and MRSE (15, 18), we would expect that the CPT-strip assay should detect the target sequence. The most likely reason for the failure to identify MRSE is insufficient lysis of the S. epidermidis cells. Thus, the assay could be modified to improve the lysis of S. epidermidis.

We have shown that the CPT-strip assay is a rapid, simple, and accurate new method for detection of MRSA. Previous studies have used gene-based systems for detection of the mecA gene. For example, a DNA hybridization assay with chemiluminescence detection has been developed (10). This assay requires a luminometer and takes 3.5 h to process 25 samples after isolation by culture. Alternatively, the enzymatic detection of PCR products from colonies of clinical isolates can be completed within 3 h (18). More recently, a branched-DNA assay based on probe signal amplification was shown to accurately detect the mecA gene in MRSA and coagulase-negative staphylococci. The branched-DNA assay uses a luminometer during the detection step and takes 6 h to complete (11). By comparison, after an initial 24-h culture, the CPT-strip assay described here would require 1.5 h to discriminate between mecA-positive and mecA-negative strains for 24 S. aureus isolates. Because the CPT-strip assay is rapid and the results are determined visually, we expect that the CPT-strip assay will be a valuable tool for the rapid detection of methicillin resistance in clinical staphylococci as well as for other gene-based detection systems.

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REFERENCES