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Blind Evaluation of the Microwave-Accelerated Metal-Enhanced Fluorescence Ultrarapid and Sensitive Chlamydia trachomatis Test by Use of Clinical Samples

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Accurate point-of-care (POC) diagnostic tests for Chlamydia trachomatis infection are urgently needed for the rapid treatment of patients. In a blind comparative study, we evaluated microwave-accelerated metal-enhanced fluorescence (MAMEF) assays for ultrafast and sensitive detection of C. trachomatis DNA from vaginal swabs. The results of two distinct MAMEF assays were compared to those of nucleic acid amplification tests (NAATs). The first assay targeted the C. trachomatis 16S rRNA gene, and the second assay targeted the C. trachomatis cryptic plasmid. Using pure C. trachomatis, the MAMEF assays detected as few as 10 inclusion-forming units/ml of C. trachomatis in less than 9 min, including DNA extraction and detection. A total of 257 dry vaginal swabs from 245 female adolescents aged 14 to 22 years were analyzed. Swabs were eluted with water, the solutions were lysed to release and to fragment genomic DNA, and MAMEF-based DNA detection was performed. The prevalence of C. trachomatis by NAATs was 17.5%. Of the 45 samples that were C. trachomatis positive and the 212 samples that were C. trachomatis negative by NAATs, 33/45 and 197/212 were correctly identified by the MAMEF assays if both assays were required to be positive (sensitivity, 73.3%; specificity, 92.9%). Using the plasmid-based assay alone, 37/45 C. trachomatis-positive and 197/212 C. trachomatis-negative samples were detected (sensitivity, 75.5%; specificity, 92.9%). The overall rates of agreement with NAAT results for the individual 16S rRNA and cryptic plasmid assays were 89.5% and 91.0%, respectively. Given the specificity, sensitivity, and rapid detection of the plasmid-based assay, the plasmid-based MAMEF assay appears to be suited for clinical POC testing.

Chlamydia trachomatis infection is the most common bacterial sexually transmitted infection (STI) in the world and the STI most frequently reported to the Centers for Disease Control and Prevention (CDC) (1). In 2011, a total of 1,412,791 chlamydial infections were reported to the CDC from the United States and the District of Columbia, representing an 8.0% increase from 2010 (1). Most chlamydial infections involve female patients 15 to 19 years (3,416.5 cases per 100,000 population) or 20 to 24 years (3,722.5 cases per 100,000 population) of age (1). Since chlamydial infections are most often asymptomatic, the CDC and other professional organizations recommend yearly screening for chlamydia among all sexually active women ≤25 years of age (2, 3). However, the Healthcare Effectiveness Data and Information Set (HEDIS) measure assessing screening coverage among female patients receiving medical care through private insurance or Medicaid indicated that only 43.1% of sexually active female patients 16 to 24 years of age in commercial plans and 57.5% of female patients covered by Medicaid received screening tests in 2010 (4). Barriers to screening include lack of awareness by clinicians and limited clinical resources.

There are several excellent commercial systems available for performing nucleic acid amplification tests (NAATs) for detection of chlamydia (5–8). However, new assays and new platforms that are able to be used at the time of the patient visit are urgently needed. Although NAATs are now recommended by the CDC as the tests of choice, these laboratory-based tests may require several days for final results, and many patients do not return for their results (9, 10). Additionally, delayed returns to clinics for treatment often contribute to pelvic inflammatory disease morbidity (11). The development of accurate rapid point-of-care (POC) tests is urgently needed in order to increase the ease of test performance and to provide treatment for patients before they leave the site of care. We report here the development of a new C. trachomatis test, a microwave-accelerated metal-enhanced fluorescence (MAMEF) test, and its performance with clinical specimens in a blind study. The MAMEF technology was developed by Geddes and colleagues (12–27). It combines the significant benefits of low-power microwave acceleration to accelerate biological reactions to completeness within seconds with those of metal-enhanced fluorescence (MEF), whereby the close proximity of silver nanoparticles (plasmon-supporting particles) amplifies the fluorescence or luminescence of labels in the near field, i.e., less than 1 wavelength of light away (28–35). The resulting technology, MAMEF, allows for combined ultrafast and ultrasensitive detection of DNAs (12–20), RNAs (36), and proteins (24–27).
MATERIALS AND METHODS

Assay description. When a liquid sample is deposited on metallic nanoparticles and exposed to microwaves, the small volume of fluid above the metal is quickly heated. However, the metallic nanoparticles remain cool because they do not absorb microwaves due to their subwavelength size (typically 100 to 200 nm in diameter), rapidly creating a temperature gradient between the cool nanoparticles and the warm fluid. This temperature gradient facilitates the mass transport of target DNA molecules toward the MEF assay surface, allowing for faster biorecognition kinetics observed near metallic nanoparticles (17, 18). The expedited transport of target DNA molecules to the assay surface not only accelerates the complementary binding of target sequences to fluorescently labeled tags but also increases assay sensitivity through the amplification of fluorescence signals (MEF) (17,18). As shown in Fig. 1, target DNA sequences bind to a fluorophore-labeled probe and to an anchor probe that is covalently bound to the assay surface. When the target DNA sequence is present, the three-piece assay construct (target DNA, fluorophore-labeled probe, and anchor probe) can form, thereby allowing the fluorophore label to come into close proximity to metallic nanoparticles and metal-enhanced fluorescence-based optical enhancement to occur (12–16). MEF assays are carried out in silver-coated microtiter plates (Fig. 2) containing the anchor probe and a fluorescent probe that includes a 6-carboxytetramethylrhodamine (TAMRA) dye. The TAMRA dye was selected to match specific wavelength requirements (32,37) as well as to enhance transmission through clinical samples (which may contain blood) for optical sensing, i.e., it emits in the therapeutic optical window (38, 39).

The sample preparation step associated with extraction of nucleic acids is a significant bottleneck in current PCR-based approaches, in addition to the time required for PCR-based detection (40–42). In contrast to the traditional lysis techniques, which can take several hours (43–45), our previously developed lysis approach can rapidly (typically in 5 to 10 s) lyse virtually any bacteria, enabling the genetic material (e.g., C. trachomatis DNA) to be rapidly collected for analysis with the MAMEF platform. This approach employs gold bowtie geometries (Fig. 2), which highly focus microwaves at 2.45 GHz onto eluted clinical samples. The sample lysis chambers have been theoretically designed and modeled using numerical simulations (finite different time domain) (12–14, 16). Thus, we can lyse samples using a $30 commercial low-power microwave oven with only a few slight modifications made inside for sample mounting. The rapid heating of the water (both around and within the C. trachomatis organ-
rapidly disrupts the membranes, allowing the sample lysate to be extracted and subsequently used for the MAMEF assay, where the extent of heating is controlled simply by the gap size between the triangles, the salt concentration, and the microwave exposure time (12–14, 16). Our lysis technology has been likened to how popcorn pops in a microwave oven; water, which absorbs at 2.45 GHz, heats rapidly, expands as a gas, and pops the corn. Our lysis approach also has the particular advantage of thermally fragmenting genomes into smaller sequences (<100 bp) (12–16), the extent of which is determined by the lysis conditions (including temperature, geometry, and salt concentration) and which is ideal for high-capacity DNA surface sensing (12–16). Further, this approach has the advantage that the high temperatures necessary for lysis (~95°C) also perturb enzymes that may destroy low-copy-number targets (26, 27).

**Microbial species.** The analytical sensitivity of the assay was tested through the use of 10-fold serial dilutions of *Chlamydia trachomatis* grown in McCoy cells, yielding final concentrations of 0 to 10³ inclusion-forming units (IFU)/ml. Each dilution was tested in triplicate. The limit of detection of the assays was calculated based on IFU/ml values. DNA from a masked panel of 18 microorganisms likely to be present in human genital samples or closely genetically related to *C. trachomatis* also was tested, to determine the analytical specificity of the assays.

**Clinical samples.** Vaginal swabs collected at the Cincinnati Children’s Hospital Medical Center Teen Health Center (Cincinnati, OH) in December 2010 through March 2012 were included in the study. Duplicate swabs were tested locally with the ProbeTec assay (Becton Dickinson, Sparks, MD). The specimens for MAMEF assays were collected and stored frozen at −80°C as dry swabs, as part of a study looking at the accuracy of new *POC Chlamydia* test devices among adolescents and young women. The frozen vaginal swabs initially were shipped to the Johns Hopkins School of Medicine (Baltimore, MD) and then were given to the Institute of Fluorescence as masked samples for testing with the *Chlamydia MAMEF* assays.

**DNA extraction and fragmentation.** Vaginal swabs were transferred frozen to 15-ml conical tubes, and 2 ml of autoclaved deionized water was added to each swab. Following a 20-min incubation, the swabs were vortex-mixed for 10 s and excess liquid was removed by pressing the swab against the side of the tube. A 200-μl aliquot of each sample was transferred to Gen-Probe medium and stored frozen for future testing in the event of discordant results between the ProbeTec assay and the MAMEF assay. DNA from the rest of the sample (approximately 1.5 ml) was extracted using the previously described gold-bowtie/focused-microwave lysis approach, with minor modifications (12, 16). Briefly, two equilaterial gold triangles (12.5 mm long and 100 nm thick) were deposited on glass slides using a vapor disposition system, and a self-adhesive silicon isolator (31 mm by 9 mm) was placed over the bowtie region, creating a lysis chamber (Fig. 2). The swab eluate was then placed in the lysis chamber and exposed to 35 s of microwave irradiation, at a power corresponding to 270 W, over the entire microwave cavity. The lysed sample was then collected and centrifuged at 4,580 × g for 3 min before undergoing MAMEF testing. Gels confirmed that all sample DNA had been fractured into <100-bp fragments, which is ideal for high-capacity MAMEF-based sensing (12–16).

**Design of DNA probes.** Two different MAMEF assays were used during this study. The first assay targets the *C. trachomatis* 16S rRNA gene, and the details of this assay have been reported previously (16). A second assay targeting the *C. trachomatis* cryptic plasmid was developed to increase the sensitivity of the MAMEF platform and for use as a confirmatory assay. Similar to the 16S rRNA assay, the *C. trachomatis* cryptic plasmid assay involves two probes; the anchor probe is composed of 26 nucleotides with a terminal thiol group that readily binds to the silver surface, and the fluorescent probe is composed of 27 nucleotides and is labeled with a TAMRA N-hydroxysuccinimide (NHS) ester dye at the first nucleotide, which corresponds to the position closest to the metal surface. When *C. trachomatis* DNA is present, the 3-piece DNA assay construct is complete (Fig. 1). In both assays, the DNA probes are complementary to the *C. trachomatis* target sequence. The anchor and fluorophore-labeled probes were designed to bind to the negative strand.

**MAMEF-based *C. trachomatis* DNA assay.** MAMEF-based DNA detection is mediated by the complementary binding of two probes to the target DNA sequence. The anchor probe is chemically linked to the silver nanoparticles on the wells of the microtiter plate via a thiol group. The fluorophore-labeled probe is added to the anchor probe-containing wells with the sample prior to microwave irradiation. In the presence of the target DNA sequence, the 3-piece assay construct is complete, resulting in an enhanced fluorescence signal due to the close proximity of the fluorescent label to the silver nanoparticles (Fig. 1). MAMEF-based DNA detection involves four steps: (i) elution of the sample from the swab, (ii) microwave-based cell lysis and DNA fragmentation, (iii) separation of DNA and cellular debris by centrifugation, and (iv) MAMEF-based DNA detection. Following the previously described sample elution and centrifugation steps, sample testing was carried out in silver-coated microtiter plates (37) (Fig. 2). DNA detection was carried out by combining 50 μl of 50 nM fluorescent probe with 200 μl of sample in the anchor probe-containing wells and heating the sample in a microwave cavity for 3 min. All samples were tested in duplicate, using both the 16S rRNA and cryptic plasmid *C. trachomatis* MAMEF assays. A negative-control sample, consisting of pooled *C. trachomatis*-negative specimens, and a *C. trachomatis*-positive sample were tested in parallel with the unknown samples. Prior to fluorescence detection, the silver-coated wells were subjected to a primary washing step with deionized water to remove excess unbound fluorescent probe and sample. A secondary washing step was performed for all samples with elevated fluorescence signals, as outlined below.

**Post-MAMEF analysis.** All samples were tested masked. For determination of positivity, fluorescence data from the unknown sample were compared to fluorescence data from the *Chlamydia*-negative control sample, as shown in Fig. 3. Samples with fluorescence signals equal to or below the value for the standardized negative-control sample were reported as *C. trachomatis* negative and were not subjected to the secondary washing step. All samples with fluorescence signals above the value for the standardized negative-control sample were subjected to a secondary washing step to remove residual unbound fluorescent probe. Initial characterization of the assay revealed that the secondary washing step can help to remove excess unbound probe, the presence of which can result in false-positive results. Furthermore, the secondary washing step does not disrupt the 3-piece DNA construct when target DNA is present (Fig. 3). Samples were considered to be *C. trachomatis* positive if the level of fluorescence was above the threshold for negativity following the secondary washing step.

**MEF detection.** Our MEF reader consists of a 532-nm continuous-wave laser (LaserMate), for which the excitation power is adjusted using an absorbing neutral-density filter wheel (Edmund Optics, Barrington, NJ), with focusing optics (Thorlabs) in a 600-μm bitruncated fiber (Ocean Optics, Dunedin, FL). A 532-nm notch filter blocks the excitation light through the emission channel of the bitruncated fiber, which falls incident onto an Ocean Optics HD2000 spectrometer (Fig. 4).

**Patient demographics.** A total of 260 vaginal swabs were collected from 248 subjects, and MAMEF assay results were available for 257 samples. Twelve subjects had swabs from two different visits. The age distribution of the 245 subjects was as follows: 17.1%, 14 to 16 years of age; 58.4%, 17 to 19 years of age; 24.5%, 20 to 22 years of age. The majority of subjects were African-American (88.6%), followed by Caucasian (8.9%), Hispanic (1.6%), and other (0.8%). The majority of subjects with NAAT-confirmed chlamydial infections were African-American (95.6%).

**RESULTS**

The limit of detection (3 × the standard mean value) of the cryptic plasmid MAMEF assay was determined by testing serial dilutions of *C. trachomatis*, in the form of cultured *C. trachomatis* tested in tissue culture, by visualization of inclusion-forming units (IFU).
The assay showed high analytical sensitivity, on the order of 10 IFU/ml (Fig. 5). The 16S rRNA-based assay correctly identified all of the C. trachomatis strains tested, but it also showed cross-reactivity with two different strains of Chlamydia pneumoniae. The cryptic plasmid-based assay also correctly identified its target, but it did not show cross-reactivity against any other microbial species (Table 1).

Among the 260 swabs tested with the ProbeTec NAAT (Becton, Dickinson, Sparks MD), 42 were C. trachomatis positive and 218 were C. trachomatis negative. Additional testing of MAMEF assay-positive samples with a second NAAT (Aptima Combo 2; Hologic Gen-Probe, San Diego, CA) identified four additional C. trachomatis-positive swabs that were negative by the first NAAT. Overall, 46 swabs were considered C. trachomatis positive and 214 were C. trachomatis negative by either of the two NAATs (ProbeTec or Gen-Probe). The overall prevalence of STIs in this sample set was 17.7% for C. trachomatis, 14% for Trichomonas vaginalis, and 5.1% for Neisseria gonorrhoeae; 8.5% of samples were positive for two STIs. MAMEF assay results were available for 257 (98.8%) of the 260 swabs. Three samples were excluded from the analysis due to loss of sample during the lysing procedure. One of the excluded samples was C. trachomatis positive and two were C. trachomatis negative, resulting in 45 C. trachomatis positive and 212 negative swabs. As shown in Table 2, of the 45 samples identified as C. trachomatis positive and 212 samples identified as C. trachomatis negative in NAATs, 33/45 and 197/212 samples were correctly identified by both MAMEF assays, i.e., 16S rRNA and C. trachomatis cryptic plasmid assays. The calculated...
Clinical sensitivities and specificities of the two MAMEF assays required to be positive in comparison to NAATs were 73.3% (33/45 samples) (95% confidence interval [CI], 60.4 to 86.2%) and 92.9% (197/212 samples) (95% CI, 89.8 to 96.0%), respectively. Eighteen percent of samples (35/197 samples) determined to be C. trachomatis negative in NAATs and MAMEF assays were positive for at least one STI. The 16S rRNA-based MAMEF assay (Table 2) had a sensitivity of 75.5% (34/45 samples) (95% CI, 62.9 to 88.1%) and a specificity of 92.9% (197/212 samples) (95% CI, 89.8 to 96.0%). The cryptic plasmid-based MAMEF assay had a sensitivity of 82.2% (37/45 samples) (95% CI, 71.0 to 93.4%) and a specificity of 92.9% (197/212 samples) (95% CI, 89.8 to 96.0%) (Table 2). The overall agreement of MAMEF assay results with NAAT results was 89.5% (95% CI, 85.4 to 93.4%) for the 16S rRNA-based assay and 91.0% (95% CI, 87.3 to 94.5%) for the cryptic plasmid-based assay. The total time to detection was <9 min, which included DNA extraction by microwave lysis (35 s), centrifugation (3 min), probe hybridization, and MAMEF detection by a human operator (5 min) (Fig. 2).

**TABLE 2 MAMEF assay results versus NAAT results**

<table>
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<tr>
<th>MAMEF assay result</th>
<th>No. of samples with a NAAT (ProbeTec or Gen-Probe) result of:</th>
<th>Total no. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Combination of 16S rRNA- and cryptic plasmid-based MAMEF assays</td>
<td>Positive</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>45</td>
</tr>
<tr>
<td>16S rRNA-based MAMEF assay</td>
<td>Positive</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Total</td>
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<td></td>
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</tr>
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<td></td>
<td>Total</td>
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**DISCUSSION**

Although the use and acceptability of NAATs for detection of C. trachomatis have increased significantly over the past decade, the utility of NAATs as point-of-care tests in clinical settings is limited and they are cost-prohibitive in low-resource settings. The World Health Organization Sexually Transmitted Diseases Diagnostics Initiative has developed the ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to those in need) criteria as benchmarks to determine whether a diagnostic test addresses certain disease control needs (46). According to a qualitative study of focus group discussions with medical care providers, C. trachomatis was identified as the priority organism for the development of a new POC test and the ideal POC test for C. trachomatis detection should be accurate and rapid (<20 min) (47). A recent review of available POC tests for detection of STIs reported disappointing sensitivity for tests for Chlamydia (3). A recent cost-effectiveness study has demonstrated that implementation of POC tests meeting the ASSURED criteria can be cost-effective, compared with traditional NAATs (48). Additionally, a POC test with moderate sensitivity (65%) can help to treat more Chlamydia-positive cases than a NAAT alone when the rate of patients returning for results and treatment is lower than 65% (49).

We previously reported on the development of a 16S rRNA-based MAMEF assay for rapid detection of C. trachomatis (16). In the present study, we have developed an additional MAMEF assay for detection of the C. trachomatis cryptic plasmid and have shown that the MAMEF assays can detect as little as 10 IFU/ml of Chlamydia trachomatis. The combined performance of the two assays (16S rRNA and cryptic plasmid) was initially evaluated to determine the diagnostic utility of a dual-assay approach. Compared to
NAATs, the sensitivity was moderate (73.3%) when both MAMEF assays were required to be positive. This sensitivity was similar to that of the 16S rRNA-based assay alone (75.5%). Compared to NAATs, the sensitivity of the cryptic plasmid-based assay was higher (82.2%) than that of the 16S rRNA assay alone or of both assays if required to determine positivity. The increased sensitivity of the cryptic plasmid assay in comparison to the 16S rRNA gene. It has been estimated that *C. trachomatis* has an average of only 2.1 copies of the 16S rRNA gene, which is less than the 10 copies of cryptic plasmid commonly present in *C. trachomatis* cells.

There were several cases of discordant MAMEF assay and NAAT results. Of the 257 samples, 27 had discordant MAMEF assay and NAAT results (Table 2). Twelve samples identified as *Chlamydia* positive by the ProbeTec assay were MAMEF assay negative by both the 16S rRNA assay and the cryptic plasmid assay. All 12 samples were confirmed as *Chlamydia* positive by the Gen-Probe assay. This equates to 12 missed positive results (false-negative results). There were a total of 11 false-negative results by the 16S rRNA MAMEF assay and 8 samples that were missed by the cryptic plasmid assay (Table 3). Fifteen samples identified by the ProbeTec assay as *Chlamydia* negative were positive by both the 16S rRNA and cryptic plasmid MAMEF assays. All 15 samples also tested negative with the Gen-Probe assay. This equates to 15 false-positive results (Table 2). The exact reason for these false-positive results is unknown. Cross-reactivity of the probes with other STIs is unlikely, as only 20% of the NAAT-negative/MAMEF assay-positive samples (3/15 samples) were positive for another STI. Additionally, no cross-reactivity with other STIs was noted in *C. trachomatis*-negative samples, as 18% (35/197 samples) of the NAAT/MAMEF assay-negative samples were positive for another STI.

Several rapid tests for *C. trachomatis* detection have been developed and evaluated. The Clearview *Chlamydia* immunoassay test (Inverness, Princeton, NJ) has been primarily evaluated against culture, and lack of sensitivity has been reported repeatedly (48). The *Chlamydia* Rapid Test has shown promising results for the sensitive detection of *C. trachomatis* in 25 min (50). However, a recent evaluation study in Suriname found that the assay lacks sensitivity (51). Our MAMEF assays, especially the cryptic plasmid-based assay, have shown moderate to good sensitivity and acceptable specificity in a high-prevalence population.

One of the limitations of our study is the large number of false-positive samples. Unfortunately, we were unable to do further testing on these samples due to sample availability.

In this study, we successfully demonstrated that two *C. trach-
matis MAMEF assay has substantial agreement ($\kappa = 0.64.6\%$ and 70.8\% for the 16S rRNA and plasmid assays, respectively) with NAATs for 257 vaginal swab samples. The new cystic plasmid assay is more sensitive than the original C. trachomatis 16S rRNA assay (16). The total time for our assays was <9 min, with assays able to be run in parallel. Additionally, the cystic plasmid-based MAMEF assay has the ability to detect the Swedish C. trachomatis variant, as the target sequence for the MAMEF probes is located outside the 377-bp deletion region (52). We have estimated that the cost of each assay is $1.00, with an additional $1.00 per lysing procedure. Consequently, our C. trachomatis MAMEF assay is a low-cost, rapid-turnaround, specific, sensitive test for C. trachomatis detection. While our current detection device (Fig. 6) is about the size of a shoebox, work is under way to reduce the size and cost of the reader, to enable the approach to provide additional benefits in low-resource settings. Additionally, we are currently working on developing a chip-based assay for the simultaneous lysis and detection of C. trachomatis DNA with a single platform, as well as the multiplex detection of multiple STIs. We are hopeful that further improvements to our assay platform will meet most of the ASSURED criteria when the platform comes to market.

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