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Development of a Real-Time PCR Assay for Identification of *Coccidioides immitis* by Use of the BD Max System

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Rapid real-time PCR (RT-PCR) can be performed in a community hospital setting to identify *Coccidioides* species using the new Becton Dickinson molecular instrument BD Max. Following sample preparation, DNA extraction and PCR were performed on the BD Max using the BD Max extraction kit ExK-DNA-1 test strip and a master mix prepared by BioGX (Birmingham, AL). Sample preparation took 2 h, and testing on the BD Max took an additional 2 h. Method sensitivity and specificity were evaluated along with the limits of detection to confirm that this convenient method would provide medically useful information. Using serial dilutions, the lower limit of detection was determined to be 1 CFU/μl. Testing with this method was validated using samples from various body sites, including bronchial alveolar lavage (BAL) fluid; sputum and lung tissue samples; and pleural and spinal fluids. Safety protocols were established, and specimen preparation processes were developed for the various types of specimens. The range for the cycle threshold (C_T) indicating adequate fluorescent signal to signify a positive result was established along with the acceptable range for the internal standard. Positive controls run with each batch were prepared by spiking a pooled BAL fluid specimen with a known dilution of *Coccidioides immitis* organism. Our experience with testing >330 patient samples shows that clinically relevant information can be available within 4 h using an RT-PCR method on the BD Max to identify *Coccidioides* spp., with sensitivity equivalent to culture.

Valley fever caused by the dimorphic fungi *Coccidioides immitis* in the Central Valley of California and *Coccidioides posadasii* in other arid areas of the southwestern United States continues to be an important illness in those areas (1–3). According to the Centers for Disease Control and Prevention, 4,431 cases of Valley fever were reported in California in 2012 (4). For most healthy residents of the Central Valley who contract this infection, Valley fever causes a rather mild influenza-like illness. However, the illness can be severe in some individuals, especially those with a compromised immune system. Without sensitive testing methods, it is usually not possible to distinguish the early symptoms of coccidioidomycosis from other causes of community-acquired pneumonia, which may lead to delayed or improper treatment (5).

Current laboratory testing methods rely on lengthy, labor-intensive protocols using experienced and highly skilled laboratory personnel. Methods include traditional serology testing for immunodiffusion tube precipitin (TP) or complement fixation (CF) antibody, enzyme immunoassays, culture, and histopathology (6). Serology testing for *Coccidioides* is helpful but has limitations for diagnosing current, active infections, since antibodies may be slow to increase to detectable levels, especially in immunocompromised patients (7, 8). Culture-based methods require several days or weeks to grow sufficient fungi for identification by molecular methods, posing additional safety threats to laboratory personnel working in the area. Especially for previously unidentified coccidioidomycosis patients, delays lead to additional testing and treatment that may have been prevented if the cause of the illness were determined days or weeks earlier.

Our goal was to develop a rapid real-time PCR (RT-PCR) assay that is sensitive and requires minimal hands-on time for laboratory personnel. We developed the assay on the BD Max, which automates the extraction and the PCR processes. Pretreatment of the sample is needed to ensure adequate cell lysis to remove the thick protective cell wall of the yeast spherule and to ensure safe handling of the specimen for PCR outside a biosafety hood. Sonicating, autoclaving, and then incubation with proteinase K (PK) are the steps taken to safely prepared the specimen for DNA extraction and RT-PCR testing.

The BD Max platform includes a variety of extraction kits (ExK), i.e., ExK-DNA-1, -DNA-2, -DNA-3, and -DNA-4, each validated for the automated extraction of nucleic acids from different specimen types. With sensitivity of the reaction and lack of interference in the PCR testing as the goals, and following consultation with the technical experts at Becton Dickinson, we evaluated the options appropriate for our specimen types. From this testing, we determined that the BD Max ExK-DNA-1 worked consistently well for extraction of fungal *Coccidioides* DNA from all of the specimen types we tested. The sequence for the primers and probes used for the master mix in this study, particularly, a 170-bp sequence from within the ITS2 region of *Coccidioides*, was previously identified and published by Binnicker et al. (9).

BioGX, a molecular assay design company, prepared the master mix by modifying the Binnicker et al. sequences and adding a 5′-nuclease dually labeled probe fluorescent marker plus the sequences, to identify the internal control, into the snap-in microtubes for use on the BD Max system. Following the initial speci-

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men preparation, discussed above and using the BioGX prepared master mix, the combination of extraction and RT-PCR processes on the BD Max system provided a rapid method by which to reliably identify *C. immitis* directly from various specimen types.

MATERIALS AND METHODS

Community Medical Center's Fresno flagship hospital has 641 licensed beds and is academically affiliated with the University of California, San Francisco (UCSF), Fresno Medical Education Program. The study was approved by the UCSF Fresno institutional review board.

Primer and probe preparation. BioGX (Birmingham, AL) modified the sequences we provided for *C. immitis* testing to make them appropriate for testing on the BD Max instrument. The original published forward primers were listed as 5′-CGA GGT CAA ACC GGA TA-3′ and the reverse primers as 5′-CCT TCA AGC ACG GCT T-3′. The fluorescent resonance energy transfer (FRET) hybridization probes used by Binnicker et al. were 5′-GAG CGA TGA AGT GAT TTC CC-3′ for the anchor probe, with a 3′-fluorescein label, and 5′-TAC ACT CAG ACA CCA GGA ACT CG-3′ for the donor probe, with 5′ LC Red labeling to read at 640 nm. BioGX modified these by adding a 5′-nuclease dually labeled probe to change the hybridization probe to 6-carboxyfluorescein (FAM) and added the primers and probes for the internal control contained in the BD ExK-DNA-1 reaction strip to be read using Cy5.5 fluorescence. This was then packaged into a convenient ready-to-use snap-in tube of lyophilized master mix to fit the BD Max platform. BioGX assisted in optimizing the reaction on the BD Max system to establish settings for time, temperature, and concentrations of primers and probes. Additionally, a small snap-in tube of reconstitution water was included for the reaction.

Quality control sample preparation. Quality control specimens were prepared in advance and frozen for safety and convenience and to provide a useful means by which to show reagent lot-to-lot consistency, since keeping live *Coccidioides* cultures in the laboratory incubators for use as control material is a safety hazard. Batches of inactivated control material were prepared by mixing together bronchoalveolar lavage (BAL) fluid specimens from at least 5 *Coccidioides* culture- and PCR-negative patients for the matrix. Aliquots of 220 μl of the pooled BAL fluid were measured into sterile 2.0-ml microtubes with caps. Into each microtube, 40 μl of a 10^4 solution of live *Coccidioides* organisms in sterile water was added. For safety purposes, the tubes were capped, sonicated for 15 min, and autoclaved with steam at 100°C (1 atm) for 30 min to kill the organism before being frozen for later use (10). One positive control was used for each test run. Sterile water or a known negative specimen was used as a negative control and processed from the beginning of the sample preparation procedure along with the patient samples.

Selection of samples for testing. The specimens tested by the direct PCR method were most commonly chosen from patient samples that were being tested with acid-fast bacillus (AFB) and fungal cultures. For many of the specimens that tested positive for the direct cocci PCR testing, the liquid *N*-acetyl-*l*-cysteine–sodium hydroxide (NALC-NaOH) preparation tube from the AFB culture was used for PCR once the fungal culture was showing *Coccidioides* growth. Additional respiratory specimens, spinal fluids, and tissue specimens were randomly tested if their fungal cultures were ordered. Seven *C. posadasii* samples from Arizona were tested to confirm that the test would work well for the detection of *Coccidioides* spp. from other regions.

Sample preparation before PCR. Bronchoalveolar lavage (BAL) fluid, bronchial washings, sputum, pleural fluid, cerebrospinal fluid (CSF), and lung tissue samples were tested by our method. We obtained very few results with interfering substances when we preprocessed the specimens to clean up the mucous, proteins, and lipids in the various matrices. Bronchial washings, BAL fluid, sputum, wound, and lung tissue samples were all preprocessed following the standard NALC-NaOH protocol for AFB culture setup using 3% NAC-PAC Red (AlphaTek, Vancouver, WA). Pleural fluids and bloody or cloudy CSF specimens were processed using MucoGest 50 (Hardy Diagnostics, Santa Maria, CA). Clear spinal fluid samples were tested directly, beginning with the sonication step, without NALC-NaOH or MucoGest preprocessing.

Once the preprocessing cleaned up the samples, steps were taken to lyse the yeast cell wall and make the sample safe for handling outside the biosafety hood. The steps used for lysis and safety were to pipette 250 μl of the preprocessed specimen into a 2.0-ml microtube with a cap, sonicate for 15 min, autoclave with steam at 100°C (1 atm) for 30 min, add 250 μl proteinase K (1 mg/ml) (Qiagen, Germantown, MD) to each tube, incubate in a 56°C water bath for 1 h, inactivate the PK with 5 min incubation at 95°C, and then cool to room temperature for use.

Extraction setup on the BD Max system. Following the addition of the BD Max ExK-DNA-1 reaction strip into the testing rack, the extraction reagent microtube from the kit, which also contained the nucleotide sequence from *Drosophila melanogaster* as an internal control, was snapped into position 1, the BioGX master mix microtube was snapped into position 2, and BioGX reconstitution water was put into position 3.

Sample loading onto BD Max system. Following the previously described specimen clean-up and preprocessing steps, 350 μl of the cooled samples were added to the BD Max sample buffer tubes. The sample buffer tubes were vortexed and placed into the testing rack for the BD Max system. The loaded testing rack was placed onto the BD Max instrument, and the run was started.

Testing on the BD Max system. BD Max runs were set up according to BD instructions. The initial denaturation occurred at 99°C for 120 s to activate the *Taq* polymerase. This was followed by 45 cycles for amplification that alternated between 99°C for 12 s and 62°C for 36.7 s. The change in fluorescence was measured on the 475/520-nm channel (FAM) at the end of each annealing cycle and plotted against the cycle number. The internal control followed the same process but was measured on the 680/715-nm channel (Cy5.5). Extraction and PCR were complete within approximately 2 h for up to 24 samples.

Analytical sensitivity and specificity. Analytical sensitivity of 95% was determined to be approximately 10^3 CFU/ml (1 CFU/μl) for this method. Since it is difficult to prepare spherules from a culture of the *Coccidioides* dimorphic fungi, we made a close approximation of the sensitivity using the conidial and hyphal growth from a Sabouraud dextrose agar plate. Preparation for 0.5 McFarland solution from a mixture of conidial and hyphal fragments growing on the Sabouraud dextrose agar is accepted to be 10^6 CFU/ml per the Clinical and Laboratory Standards Institute (CLSI) for filamentous fungi and the CLSI document for yeasts. We accepted this well-documented concentration value as the starting suspension density for our testing with *C. immitis* diluted in sterile water (11–13). Tenfold dilutions were prepared for the initial screening. Once the approximate range was established, further testing showed 95% positive results at the 1-CFU/μl level for 20 test dilutions. Of the results, 56% were positive in concentrations as low as 0.01 CFU/μl.

Specificity of the testing method was confirmed by testing a panel of organisms, including *Cryptococcus neoformans*, *Cryptococcus gattii*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Acremonium* spp., *Gliocladium* spp., *Penicillium* spp., *Mycobacterium tuberculosis*, *Mycobacterium avium* complex, *Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida utilis*, *Candida ciferrii*, *Pichia ohmeri*, and *Rhizopus* spp. No false-positive or cross-reaction results were noted in this testing. Many of the specimens contained bacterial organisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and various respiratory and skin flora with no interference. Since the Binnicker group used the same DNA ITS2 sequence and tested an extensive list of organisms without cross-reactivity, we accept their study's success as additional evidence of specificity using the ITS2 sequence.

Standard mycology culture or histopathology methods. Fungal cultures were performed by plating specimens onto Sabouraud dextrose agar (Hardy Diagnostics, Santa Maria, CA) and inhibitory mold agar supplemented with chloramphenicol (Hardy Diagnostics) followed by incubation at 30°C for up to 4 weeks. Confirmation testing of the culture growth

TABLE 1 Comparison of the *Coccidioides* RT-PCR assay on the BD Max with reference method for detection of *C. immitis* in various body samples

BD Max RT-PCR result	No. of specimens with indicated culture/ histopathology reference method result		Total no. of specimens
	Positive	Negative	
Positive	77	0	77
Negative	0	258	258
Total	77	258	334

was performed on colonies suspicious for *Coccidioides* using the Gen-Probe AccuProbe *C. immitis* culture identification test (Gen-Probe Incorporated, San Diego, CA). Standard histopathology microscopic review was performed on many of the tissues and fluids to identify the presence or absence of spherules consistent with *Coccidioides*.

RESULTS

Real-time PCR using the BD Max system was tested for ease of use and accuracy to improve the availability of results from specimens suspected to contain *C. immitis* and to help rule out *Coccidioides* species as the probable cause of community-acquired pneumonia. Our investigation used 334 patient samples to evaluate the potential use of this method for testing respiratory specimens from bronchoalveolar lavage fluid, bronchial wash, sputum, and endotracheal aspirate ($n = 173$); samples from lung, wound, and bone tissue ($n = 79$); and samples from pleural and spinal fluids ($n = 82$). These results were then compared to culture or histopathology results, which are the current reference methods for *Coccidioides* diagnosis. Evaluation of the C_T values of the internal controls from each reaction read from the 680/715 channel, along with the C_T values of the positive and negative quality control samples read on the 415/520 channel, were used to provide evidence that the instrument and reagents were working properly. The C_T range for *Coccidioides* detection in the test samples was set at $\leq$37 for positive results, 38 to 45 for low positive or indeterminate results, and $\geq$45 for no evidence of infection. The acceptable C_T range for the internal control was 29.5 $\pm$ 3 based on the average value of >430 tests, including patient sample, limit-of-detection, reproducibility, and interfering-substance testing.

In testing >330 patient samples from various types of specimens, we found no false-positive or false-negative results compared to culture or histopathology, as shown in Table 1.

DISCUSSION

To address the high prevalence of *Coccidioides* infections in the San Joaquin Valley of central California, our study is part of the ongoing quest to find better diagnostic methods for patients with this illness. Several past studies showed that PCR assays provide great promise in accurate and rapid methods for the identification of *Coccidioides* species. Studies by de Aguiar Cordeiro et al. (14) had success with direct PCR using amplification of the antigenic gene Ag2/PRA (antigen 2/proline-rich antigen) to successfully detect *C. posadasii* from highly contaminated sputum samples. Binnicker et al. (9), from the Mayo Clinic, published the gene sequence used for the primers and probes to successfully detect the ITS2 region of the *Coccidioides* gene. An article by Burt et al. (10) suggested use of the autoclave at 100°C (1 atm) to make use of steam for added safety when handling the specimen.

A significant challenge for our laboratory, found when reviewing the available literature on *Coccidioides* PCR methods (6, 9, 14), was the personnel time required to perform the separate extraction step and how to fit this testing into our daily workflow. An additional issue was that the extraction and PCR testing from other researchers were performed on instruments not available in our laboratory. Responding to these concerns, and using the groundwork presented by previous investigators, we used the BD Max to automate the extraction step and move directly into PCR on the same instrument without further handling.

The results from our study support the use of this practical method for automated molecular testing on the BD Max for *Coccidioides* species. It shows that previously tested probe and primer sequences for *Coccidioides* spp. detected *C. immitis*, which is indigenous to California, and *C. posadasii*, from other regions of the southwestern United States. The analytical sensitivity of 10^3 CFU/ml (1 CFU/μl) and results showing 100% concordance with culture and/or histopathology results for detection of *C. immitis* confirm that this method can potentially provide medically useful information for patient management in this population. However, since every specimen submitted to the laboratory for fungal culture was not tested by PCR for *Coccidioides*, we do not have data to show if our method is more sensitive than culture, only that it seems to be equivalent to culture. Current and future studies will determine if this PCR method is more sensitive than culture.

Although many samples worked well without NALC-NaOH or MucoGest pretreatments, fewer results exhibited the effects of interfering or inhibitory substances when the pretreatment steps were performed. No discrepancies or incorrect results were noted when samples were tested by various personnel since the steps of the method are easy to follow and convenient to perform (data not shown).

By modifying the previously published probe sequences for compatibility with the BD Max system, adding an internal control, formulating the PCR master mix, and packaging into snap-in microtubes, the whole testing process from a direct specimen could be accomplished within 4 h. The speed and convenience of this RT-PCR method is a great improvement compared with the gold standard of fungal culture, which takes 1 to 2 weeks.

Although the implications of a faster, practical diagnostic tool such as this are enormous, we do not propose to use this as a stand-alone test but rather as an adjunct to the current armamentarium of tests, including serology, histopathology, and fungal cultures, to diagnose coccidioidomycosis. A practical advantage is this tests speed and accuracy compared with culture. Further studies to delineate clinical applications are needed and are under way at our facility, where coccidioidomycosis is still a major health threat. The hope is that these clinical studies will pave the way toward development of an algorithm for early diagnosis and treatment of the disease using currently available diagnostic methods.

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