Development of a *Coccidioides immitis* Real Time PCR Assay using the BD MAX™ System

Marilyn Mitchell,a, # Dominic Dizon, b Robert Libke, b Michael Peterson, b David Slater, a Akashdeep Dhillon a

Microbiology Department, Community Medical Centers – Fresno, Fresno, California a; University of California – San Francisco, Fresno, California b

#Address correspondence to Marilyn Mitchell, mmitchell@communitymedical.org

Page 1 of 19
Development of a *Coccidioides immitis* Real Time PCR Assay using the BD MAX™ System

Marilyn Mitchell,¹# Dominic Dizon,² Robert Libke,² Michael Peterson,² David Slater,² Akashdeep Dhillon¹

Microbiology Department, Community Medical Centers – Fresno, Fresno, California ¹; University of California – San Francisco, Fresno, California ²

ABSTRACT

Rapid Real Time polymerase chain reaction (RT-PCR) can be performed in a community hospital setting for identification of *Coccidioides* species using the new Becton Dickinson molecular instrument, the BD MAX. Following sample preparation, both the DNA extraction and the PCR were performed on the BD MAX using the BD MAX ExK DNA-1 extraction test strip and a master mix prepared by BioGX (Birmingham, AL). Sample preparation took two hours and testing on the BD MAX took an additional two hours. 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 colony forming unit.
per microliter (cfu/ul). Testing with this method was validated using samples from various body sites including bronchial alveolar lavage (BAL), sputum, lung tissue, pleural fluid and spinal fluid.

Safety protocols were established and specimen preparation processes were developed for the various types of specimens. The range for cycle threshold (Ct) indicating adequate fluorescent signal to signify a positive result was established along with the acceptable Ct range for the internal standard. Positive controls run with each batch were prepared by spiking a pooled BAL specimen with a known dilution of *Coccidioides immitis* organism.

Our experience testing over 330 patient samples shows clinically relevant information can be available within 4 hours using a RT-PCR method on the BD MAX to identify *Coccidioides* spp. with sensitivity equivalent to culture.

**INTRODUCTION**

Valley Fever caused by the dimorphic fungi, *Coccidioides immitis* in the central valley of California and *Coccidioides posadasii* in other arid areas in the southwestern United States, continues to be an important illness in those areas (1, 2, 3). According to the Center of Disease Control, 4,431 cases of Valley Fever were reported in California for 2012 (4). For most healthy residents of the Central Valley who contract this infection, Valley Fever causes rather mild influenza-like illness. 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 leading 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 or
histopathology (6). Serology testing for Coccidioides is helpful but has its
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
could be prevented if the cause of the illness had been determined days or weeks
earlier.

Our goal was to develop a rapid RT-PCR assay that was sensitive and
required minimal hands-on time for laboratory personnel. We chose to develop
the assay on the BD MAX which automates both the extraction and the PCR
processes. Pretreatment steps of the sample are 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 of a biosafety hood. Sonicating,
autoclaving, then incubation with Proteinase K (PK) are the steps that safely
prepared the specimen for DNA extraction and RT-PCR testing.

The BD MAX platform includes a variety of extraction kits, 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 goal, and following consultation with 
the technical experts at Becton Dickinson, the options appropriate for our 
specimen types were evaluated. From this testing we determined BD MAX™ 
ExK™-DNA-1 worked consistently well for extraction of fungal Coccidioides 
DNA from all of the specimen types we tested. The sequences for the primers and 
probes used for the master mix in this study had been previously identified and 
published by Binnicker (9) namely using a 170-base pair sequence from within the 
ITS2 region of Coccidioides.

BioGX, a molecular assay design company, prepared the master mix by 
modifying the “Binnicker” sequences as mentioned above and adding a 5’-
nuclease dual-labeled probe fluorescent marker plus the sequences to identify the 
internal control into the snap-in micro tubes for use on the BD MAX™ System.

Following the initial specimen preparation as discussed above and using the 
BioGX prepared master mix, combining the extraction and RT-PCR processes on 
the BD MAX™ System provided a rapid method to reliably identify Coccidioides 
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 *Coccidioides 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’, the reverse primers 5’-CCT TCA AGC ACG GCT T-3’. The FRET Hybridization probes used by Binnicker were 5’-GAG CGA TGA AGT GAT TTC CC-3’ for the anchor probe with 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 640nm. BioGX modified these by adding a 5’-nuclease dual-labeled probe to change the hybridization probe to FAM, and also 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 ahead and frozen for safety and convenience and to provide a useful means 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 bronchioalveolar lavage (BAL) specimens from at least 5 *Coccidioides* culture- and PCR-negative patients for the matrix. Aliquots of 220uL of the pooled BAL are measured into sterile 2.0 mL microtubes with caps. Into each microtube 40uL of a 10^4 solution of live *Coccidioides* organism in sterile water is added. For safety purposes, the tubes are capped, sonicated for 15 minutes and autoclaved with steam at 100°C (1 atm) (10) for 30 minutes to kill the organism before being frozen for later use. One positive control is used for each test run. Sterile water or a known negative specimen is used as a negative control and is 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 both AFB and Fungal cultures. For many of the specimens which tested positive for the Direct Cocci PCR testing, the liquid NALC/NaOH preparation tube from the AFB culture was used for PCR once the Fungal culture was showing growth of *Coccidioides*. Additional respiratory specimens, spinal fluids and tissue specimens were randomly tested if they had fungal cultures ordered. Seven
Coccidioides posadasii samples from Arizona were tested to confirm that the test would work well for detection of Coccidioides spp. from other regions.

**Sample preparation before PCR.** Bronchioalveolar lavage (BAL), bronchial washings, sputum, pleural fluid, CSF fluid, and lung tissue samples were tested by our method. We obtained very few “Interfering Substance” results if we preprocessed the specimens to clean up the mucous, proteins or lipids in the various matrices. Bronchial washings, BAL, sputum, wound and lung tissues were all preprocessed following the standard NALC/NaOH protocol for Acid Fast Bacillus (AFB) culture set up 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, the steps were taken to lyse the yeast cell wall and make the sample safe for handling outside of the biosafety hood. The steps used for lysis and safety were to pipette 250uL of the preprocessed specimen into a 2.0ml microtube with cap, sonicate for 15 minutes, autoclave with steam at 100°C (1 atm) for 30 minutes, add 250ul Proteinase K (1mg/mL) (Qiagen, Germantown, MD) to each tube, incubate in a 56°C waterbath for 1 hour, inactivate the PK with a 5 minute incubation at 95°C, then cool to room temperature for use.
Extraction set-up 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, BioGX Master Mix microtube into position 2, and the BioGX reconstitution water into position 3.

Sample loading onto BD MAX™ System. Following the previously described specimen clean-up and preprocessing steps, 350uL of the cooled samples are added to the BD MAX Sample Buffer Tubes. The Sample Buffer Tubes are vortexed and placed into the testing rack for the BD MAX™ System. The loaded testing rack is placed onto the BD MAX instrument and the run is started.

Testing on the BD MAX™ System. BD MAX runs were set up according to BD instructions. Initial denaturation occurred at 99°C for 120 seconds to activate the *Taq* polymerase. This was followed by 45 cycles for amplification which alternated 99°C for 12 seconds followed by 62°C for 36.7 seconds. The change in fluorescence was measured on the 475/520 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 channel (Cy5.5). Extraction and PCR are complete within approximately 2 hours 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* dymorphic fungi, we chose to make a close approximation of the sensitivity using the conidial and hyphal growth from a Sabouraud dextrose agar plate. Preparation for a 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 also the CLSI document for yeasts. We accepted this well-documented concentration value as the starting suspension density for our testing with *Coccidioides immitis* diluted in sterile water (11, 12, 13). Tenfold dilutions were prepared for initial screening.

Once the approximate range was established, further testing showed 95% positive results at the 1 cfu/μL level when testing 20 test dilutions. 56% of results were detected positive in concentrations as low as 0.01 cfu/μL.

Specificity of the testing method was confirmed by testing a panel of organisms (Table 1). 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 had tested an extensive list of organisms without cross-reactivity, we accept their study 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, Santa Maria, CA) followed by incubation at 30°C for up to four weeks. Confirmation testing of the culture growth was performed on colonies suspicious for *Coccidioides* using the GenProbe AccuProbe *Coccidioides immitis* Culture Identification Test (Gen-Probe Incorporated, San Diego, CA). Standard histopathology microscopic review was performed on many of the tissues or 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 *Coccidioides immitis* and to help rule out *Coccidioides* species as the probable cause of community acquired pneumonia. Our investigation used over 330 patient samples to evaluate the potential use of this method for testing specimens from bronchioalveolar lavage, lung tissue, sputum, wounds, pleural fluid, and spinal fluid (Table 2). These results were then compared to culture or histopathology results which are the current reference methods for *Coccidioides* diagnosis. Monitoring of the Ct value of the internal control as well as the Ct values of the prepared positive and negative controls were used to provide
evidence that the instrument and reagents were working properly. The Ct range for positive Coccidioides detection of the test samples was set at \( \leq 37 \) for Positive, 38-45 low positive or indeterminate, and greater than Ct of 45 indicated no evidence of infection. The acceptable Ct range for the internal control was 29.5 +3 based on the average value when performing over 430 tests including patient samples, limit of detection, reproducibility and interfering substance testing.

In testing over 330 patient samples from various types of specimens, there were no false positive or false negative results when compared to culture or histopathology as is shown in Table 3.

DISCUSSION

Because of the high prevalence of Coccidioides infections in the San Joaquin valley of Central California, we are part of the ongoing quest to find better diagnostic methods for patients with this illness. Several past studies have shown PCR to provide great promise for accurate and rapid methods for identification of Coccidioides species. Studies by Aguiar Cordeiro, et al, (14) showed success with direct PCR using amplification of the antigenic gene Ag2/PRA to successfully detect Coccidioides posadasii from highly contaminated sputum samples. The Binniker study from Mayo had published the gene sequence used for the primers and probes to successfully detect the ITS2 region of the Coccidioides gene (9). An article by Burt (10) suggested the use of the autoclave at 100° at 1atm to make use of steam for added safety when handling the specimen.
A significant challenge for our laboratory when reviewing the available literature discussing *Coccidioides* PCR methods was the personnel time required to perform the separate extraction step (6, 9, 14) and how to make this testing fit into our daily workflow. An additional issue was that the extraction and the PCR testing from other researchers was 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 any 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* sp. detected *C. immitis*, a species indigenous to California, as well as *Coccidioides posadasii* from other regions of the southwest United States. The analytical sensitivity of $10^{3} \text{ cfu/mL}$ (1 cfu/µL) and results showing 100% concordance with culture and/or histopathology results for detection of *C. immitis*, confirm that this method could 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 pre-treatments, there were fewer results exhibiting the effects of interfering or inhibitory substances if these pre-treatment steps were performed. No discrepancies or incorrect results were noted when samples were tested by various testing 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 micro tubes, the whole testing process from a direct specimen could be accomplished within 4 hours. The speed and convenience of this RT-PCR method is a great improvement compared with the gold standard of fungal culture, which takes 1-2 weeks.

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

We acknowledge the technical support for use of the BD MAX provided by Becton Dickinson. In addition, we thank the Microbiology department staff of Community Regional Medical Center for their expertise and participation in this important study.

We thank Michael Saubolle from the Division of Infectious Diseases, Laboratory Sciences of Arizona/Sonora Quest Laboratories, Banner Health and the Department of Medicine at the University of Arizona College of Medicine in Arizona, USA, for supplying *Coccidioides posadasii* isolates.

This project was made possible by a grant obtained through the UCSF Central California Faculty Medical Group Intramural Grant program. We are grateful for that support.

REFERENCES


Table 1. Organism panel tested to rule out cross-reactivity on BD MAX RT-PCR assay for *Coccidioides*.

<table>
<thead>
<tr>
<th>Organism tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptococcus neoformans</td>
</tr>
<tr>
<td>Cryptococcus gattii</td>
</tr>
<tr>
<td>Aspergillus nidulans</td>
</tr>
<tr>
<td>Aspergillus niger</td>
</tr>
<tr>
<td>Aspergillus fumagatus</td>
</tr>
<tr>
<td>Histoplasma capsulatum</td>
</tr>
<tr>
<td>Acremonium species</td>
</tr>
<tr>
<td>Gliocladium sp.</td>
</tr>
<tr>
<td>Penicillium sp.</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>Mycobacterium avium complex</td>
</tr>
<tr>
<td>Candida albicans</td>
</tr>
<tr>
<td>Candida dubliensis</td>
</tr>
<tr>
<td>Candida glabrata</td>
</tr>
<tr>
<td>Candida krusei</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
</tr>
<tr>
<td>Candida utilis</td>
</tr>
<tr>
<td>Candida ciferri</td>
</tr>
<tr>
<td>Pichia ohmeri</td>
</tr>
<tr>
<td>Rhizopus spp.</td>
</tr>
</tbody>
</table>

Table 2. Source of specimens used for *Coccidioides* RT-PCR on BD MAX

<table>
<thead>
<tr>
<th>Type of specimen</th>
<th>Number tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory: BAL, Bronchial wash, sputum, Endotrachial Aspirate (ETA)</td>
<td>173</td>
</tr>
<tr>
<td>Tissues: Lung, Wound, Bone</td>
<td>79</td>
</tr>
<tr>
<td>Fluids: Pleural Fluids, Spinal fluid</td>
<td>82</td>
</tr>
<tr>
<td>Total</td>
<td>334</td>
</tr>
</tbody>
</table>
Table 3. Comparison of the *Coccidiodes* RT-PCR assay on the BD MAX with Reference Method for Detection of *C. immitis* in Various Body Samples

<table>
<thead>
<tr>
<th>BD MAX RT-PCR</th>
<th>Culture/Histopathology</th>
<th>Reference Method</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td></td>
<td></td>
<td>77</td>
<td>0</td>
<td>77</td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
<td>0</td>
<td>258</td>
<td>258</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>77</td>
<td>258</td>
<td>334</td>
</tr>
</tbody>
</table>