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The utility of real-time polymerase chain reaction in detecting *Coccidioides immitis* among clinical specimens in the Central California San Joaquin Valley

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Abstract

Coccidioidomycosis, the fungal infection caused by dimorphic *Coccidioides* species, is typically diagnosed by histopathologic identification of spherules, by culture, or by serology. These tests are reliable but time-intensive, delaying diagnosis and treatment. Rapid real-time polymerase chain reaction (RT-PCR) can be performed and was validated to identify *Coccidioides immitis* using an in-house developed assay for the Becton Dickinson molecular instrument (BD MAX™). These studies were performed using patient samples that had been shown to be positive on previously set up fungal cultures. To evaluate this new RT-PCR test in the clinical setting, we conducted a retrospective chart review of patients (N = 1160) who underwent *Coccidioides* PCR (Cocci PCR) on clinical samples between March 1, 2014, and Dec 31, 2016. We abstracted clinical, microbiologic, serologic, radiographic, treatment, and follow-up data. Specimens of cerebrospinal fluid (CSF), bronchioalveolar lavage fluid (BAL), lung tissue biopsy (LTB), sputum, and pleural fluid were evaluated to determine sensitivity and specificity. Of the 113 specimens that tested positive for Cocci PCR, all had clinical disease defined by traditional clinical criteria, yielding 100% specificity. Overall sensitivity was 74% versus 46% for fungal culture and was available in 4 hours rather than 1–2 weeks. Sensitivities varied by source material and clinical setting. CSF had a sensitivity of 59%, BAL for acute pneumonia 91%, sputum for acute pneumonia 94%, pleural fluid 86%, but LTB for lung nodules only 44%. Overall positive predictive value (PPV) was 100%, while negative predictive value (NPV) was 96%, but again this varied by specimen and clinical setting. Our experience with clinical testing of >1160 specimens over 2–3 years shows we can utilize this technology to improve our ability to diagnose disease but that the sensitivity varies by specimen source and clinical setting.

Key words: Valley fever, Coccidiodomycosis, San Joaquin Valley, Cocci PCR, BD MAX.

Background

Coccidioidomycosis is an endemic illness caused by the dimorphic fungi *Coccidioides immitis* in the Central Valley of California and *Coccidioides posadasii* in arid areas of the Southwestern United States as well as Central and South America.[1] The laboratory methods most frequently used to diagnose coccidioidomycosis include serology, direct smear, histopathology, and culture with confirmation by DNA probe analysis. Various serologic assays have demonstrated reliable sensitivity and specificity, especially when used in combination (e.g., complement fixation and immunodiffusion).[2] However, serology may take 1 to 2 weeks following the onset of symptoms to become positive and may remain negative despite infection in immunosuppressed patients.[3] Direct smear and histopathology have also been used in the diagnosis of *Coccidioides* disease, but these methods lack sensitivity and specificity.[4,5]

At present, the gold standard for the diagnosis of coccidioidomycosis is culture of the organism from clinical specimens.[6] However, growth in culture may take several days to several weeks. This often delays the diagnosis and initiation of appropriate treatment in infected individuals.

Recently, a collaborative study between the University of California San Francisco (UCSF) Fresno and the Microbiology department of Community Regional Medical Center (CRMC) in Fresno, developed and published a real-time PCR assay for *C. immitis* using the BD MAX instrument.[7] The experience demonstrated that clinically relevant information can be available within 4 hours using an RT-PCR method on the BD MAX to identify *C. immitis* in laboratory samples with 100% sensitivity and 100% specificity, when compared with fungal culture. The results mirror earlier studies in Mayo Clinic Arizona by Binnicker and colleagues[8] wherein they applied real-time PCR using the same ITS2 base-pair sequence but via a LightCycler instrument and demonstrated 100% sensitivity and 98% specificity for *C. posadasii* when compared with fungal culture. In a follow-up Mayo Clinic study assessing the performance characteristics of the LightCycler PCR assay after implementation as a standard test, the clinical sensitivity was 56% versus 44% for fungal culture, similar to the sensitivity of fungal culture (*C. posadasii*) in past studies.[9]

In California, the incidence of coccidioidomycosis is rapidly increasing beginning in 2015.[10] The 8182 cases reported in 2017 is a 255% increase from 2306 cases reported in 2014.[11] A majority of the cases were from the Central Valley, with Kern, Fresno, and Tulare counties on top.[11]

Our study analyzes the benefit of using our in-house developed BD MAX Cocci PCR assay when used as a test to detect *C. immitis* in the Central California San Joaquin Valley. This is the first study to our knowledge to use the BD MAX instrument as a test for Cocci PCR and the first to test it clinically on the species *C. immitis*. This study also looked specifically at different specimens in the clinical setting and assessed accuracy and utility of the test. We evaluated variability in the utility of these tests depending on the specimen type and clinical setting.

Methods

We evaluated specimens from many sources including bronchioalveolar lavage (BAL) fluid, thoracentesis fluid, cerebrospinal fluid (CSF), lung tissue biopsies (LTB), and sputum. Samples were from patients for whom a diagnosis of coccidioidomycosis was considered based on clinical presentation. Patients who underwent BD MAX Cocci PCR testing of their samples based on clinical grounds at the Microbiology Laboratory of CRMC from March 1, 2014, to December 31, 2016, were identified using a computerized search of the institutional laboratory records (Epic Systems). A retrospective review was conducted of the medical records of all patients who had such testing, and data were abstracted concerning their clinical, microbiologic, serologic, radiographic, treatment, and follow-up information. This study was approved by the UCSF Fresno Institutional Review Board.

Serologic testing

The serologic tests included enzyme immunoassay (EIA) (IMMY, Norman, OK, USA) done at the CRMC lab and using the package insert. Any EIA tests positive for immunoglobulin M (IgM) or immunoglobulin G (IgG) are then sent to UC Davis Microbiology lab for confirmation using immunodiffusion-based tests for traditional tube precipitin (IDTP) or complement-fixing (IDCF) anticoccidioidal antibodies.

Polymerase chain reaction assay

Polymerase chain reaction testing was performed as previously described.[7] In brief, following sample preparation, DNA extraction and PCR were performed on the BD MAX using extraction kit BD MAX^{TM} ExK^{TM} –DNA test strip and a master mix prepared by BioGX (Birmingham, AL, USA). The BD MAX provided a platform for amplification of the internal transcribed spacer 2 (ITS2) region of *Coccidioides* species. Sample preparation took 2 hours, and testing on the BD MAX took an additional 2 hours.

Fungal cultures

Cultures were performed by plating specimens onto Sabouraud Dextrose Agar (Hardy Diagnostics, Santa Maria, CA, USA) and Inhibitory Mould Agar supplemented with chloramphenicol (Hardy Diagnostics) followed by incubation at 30°C for up to 4 weeks. Confirmation testing of the culture growth was performed on colonies suspected to be *Coccidioides* using the Gen-Probe AccuProbe *C. immitis* culture identification test (Gen-Probe Incorporated, San Diego, CA, USA). Standard histopathology microscopic review was performed on many of the tissues and fluids to identify the presence or absence of spherules consistent with *Coccidioides*.[7]

Definition of positives

Our reference positive results in Table 1 (either Proven, Probable or Possible) were based on the Infectious Diseases Society of America (IDSA) clinical practice guidelines,[12] American Thoracic Society (ATS) systematic review[13] and expert opinion laboratory algorithms on the diagnosis of Coccidioidomycosis.[14,15]

Given that it is often difficult to definitively diagnose coccidioidomycosis, this table was not intended as a firm rubric in defining true positives but as reference for clinical comparison. Each case is cross referenced with the clinical discharge diagnosis indicated by the attending providers in the patients' charts,

Table 1. Clinical and laboratory criteria used to define reference positive coccidioidomycosis disease.

	Pathology	Body fluids	BAL	Sputum
Cocci proven	Presence of spherules on histopathology **OR** positive culture **AND** Clinical discharge diagnosis of coccidioidomycosis	Presence of spherules on histopathology **OR** positive culture **AND** Clinical discharge diagnosis of coccidioidomycosis	Presence of spherules on histopathology **OR** positive culture **AND** Clinical discharge diagnosis of coccidioidomycosis	Presence of spherules on histopathology **OR** positive culture **AND** Clinical discharge diagnosis of coccidioidomycosis
Cocci probable	Negative culture **AND** granulomatous inflammation suggestive of coccidioidomycosis on histopatology **AND** Clinical discharge diagnosis of coccidioidomycosis	No spherules **AND** negative culture **AND** Positive serology (positive IgM or IgG on EIA **AND** positive IDTP or IDCF) **AND** Clinical discharge diagnosis of coccidioidomycosis	No spherules **AND** negative culture **AND** Positive serology (positive IgM or IgG on EIA **AND** positive IDTP or IDCF) **AND** Clinical discharge diagnosis of coccidioidomycosis	No spherules **AND** negative culture **AND** Positive serology (positive IgM or IgG on EIA **AND** positive IDTP or IDCF) **AND** Clinical discharge diagnosis of coccidioidomycosis
Cocci possible	Negative culture **AND**Fibrosis/nonspecific inflammation on histopathology **AND** Clinical discharge diagnosis of coccidioidomycosis	No spherules **AND** negative culture **AND** Negative serology (No IgM or IgG on EIA **OR** Negative IDTP plus Negative IDCF) **AND** Clinical discharge diagnosis of coccidioidomycosis	No spherules **AND** negative culture **AND** Negative serology (no IgM or IgG on EIA **OR** negative IDTP plus negative IDCF) **AND** Clinical discharge diagnosis of coccidioidomycosis	No spherules **AND** negative culture **AND** Negative serology (no IgM or IgG on EIA **OR** negative IDTP plus negative IDCF) **AND** Clinical discharge diagnosis of coccidioidomycosis

Lung tissue biopsy (LTB) was considered under pathology, while CSF and pleural fluid were under body fluids. EIA, enzyme immunoassay; IDCF, immunodiffusion-based tests complement-fixing; IDTP, immunodiffusion-based tests for traditional tube precipitin; IgG, immunoglobulin G; IgM, immunoglobulin M.

considering all radiologic data and clinical symptoms. Hence, the authors decided that a Cocci Probable or Cocci Possible case will still be considered reference positive and addressed as a false negative if the BD MAX Cocci PCR test was negative. For the purposes of this study, all reference positive cases (proven, probable, and possible) shared the criterion that there has to be a clinical discharge diagnosis of coccidioidomycosis in the chart. For completely outpatient cases, the clinical discharge diagnosis is the visit diagnosis documented on Epic Systems for that particular clinical encounter.

Data analysis

All analyses were conducted using OpenEpi, version 3.01 (www.OpenEpi.com). Simple descriptive statistics (numbers, percentages, and means) and inferential statistics (confidence intervals) were used to analyze the data obtained in the study. To determine specificity and sensitivity of the BD MAX Cocci PCR test, we compared these results with those deemed proven or probable positive using fungal culture, histopathology, and serology. The 95% confidence interval was computed on each of the specimen type sensitivity values using the Wilson score

Table 2. Patients with Cocci PCR testing done on clinical samples.

		False negatives			True positives (reference)						
Source	True neg	Proven	Probable	Possible	Proven	Probable	Possible	Sensitivity	95% CI	NPV	PPV
CSF	282	0	13	0	16	3	0	59%	42%–74%	98%	100%
BAL	259	1	3	0	42	0	0	91%	80%–97%	96%	100%
Lung tissue Bx	258	16	4	0	16	0	0	44%	30%–60%	93%	100%
Sputum	113	0	1	1	30	0	0	94%	80%–98%	98%	100%
Pleural fluid	95	0	1	0	5	1	0	86%	49%–97%	99%	100%
Total/avg	1007		40			113		74%	66%–80%	96%	100%

BAL, bronchioalveolar lavage fluid; CI, confidence interval; CSF cerebrospinal fluid; NPV, negative predictive value; PPV, positive predictive value.

method.[16] The χ^2 tests were used to examine statistically significant differences in sensitivity values between specimen sources as paired comparisons.

Results

We identified a total of 1160 BD MAX Cocci PCR tests conducted on patients' clinical specimens from March 1, 2014, through December 31, 2016, which involved CSF, BAL, LTB, sputum, or pleural fluid. The rest of the Cocci PCR testing involved abscess fluid (16), lymph node biopsy (11), and synovial fluid (3). Data from these last three types of specimen sources were excluded due to small sample sizes.

Out of these 1160 tests, 1047 were negative and 113 positive. All 113 specimens that tested positive for BD MAX Cocci PCR had either Cocci Proven (96%) or Cocci Probable (4%), yielding 100% specificity and 100% positive predictive value for all specimens. There were no Cocci Possible cases among these 113 specimens and they all shared the criterion mentioned in Table 1 of clinical diagnosis of coccidioidomycosis in the chart. There were no false positives observed.

Of the 1047 negative, 1007 were true negatives and 40 had reference positive results, meaning they had proven, probable or possible coccidioidomycosis based on Table 1. Hence, all of these 40 had discharge diagnosis of coccidioidomycosis. From our Table 2, you can see that 17 of these were proven (histology or fungal culture), 22 were probable (EIA and IDCF or IDTP), and 1 had discharge diagnosis of based purely on clinical grounds and without histology, culture, or serologic markers. Therefore, for calculation of sensitivity, our denominator was indeed all cases with discharge diagnosis of coccidiodomycosis, regardless of whether they tested positive or negative for BD-MAX Cocci PCR, had spherules on histology, had positive fungal culture, or tested positive for serology (EIA, IDCF, IDTP). This number was 40 + 113 = 153. This yielded a sensitivity of 74% for all specimens.

Of the total 153 that were identified as either Cocci Proven, Cocci Probable or Cocci Possible, fungal culture was positive for 70, yielding a sensitivity of only 46%. Besides sharing the clinical diagnosis of coccidiodomycosis in the chart, all of these 153 specimens were in clinical cases whereby treatment for the disease was initiated either inpatient or in the ambulatory setting.

Sensitivity did vary by specimen sources and clinical setting. Of the 1160 samples, 314 (28%) were CSF, 305 (26%) were BAL, 294 (25%) were LTB, 145 (12%) were sputum, and 102 (9%) were pleural fluid. Sputum, BAL, and pleural fluid had the highest sensitivities at 94%, 91%, and 86% respectively, while CSF and LTB had the lowest sensitivities at 59% and 44%, respectively. The sensitivities, confidence intervals, negative predictive values and positive predictive values are as shown in Table 2.

There are statistically significant differences when comparing sensitivities for CSF (59%) versus BAL (91%) with $P < .001$, CSF (59%) versus sputum (94%), with $P = .00117$, for lung tissue (44%) versus BAL (91%) with $P < .001$, lung tissue (44%) versus sputum (94%) with $P < .001$, and lung tissue (44%) versus pleural fluid (86%) with $P = .045$.

Discussion

Coccidioidomycosis is a significant illness in the Central California San Joaquin Valley, and in the Southwestern regions of the United States.[1] It appears it is increasing in frequency and geographic reach. Delays in treatment often occur due to delays inherent in current laboratory testing[17]. Besides delays in results, serologic testing can be insensitive in early infection or negative in immunocompromised patients such as those undergoing chemotherapy or afflicted with human immunodeficiency virus.[18,19] Although *Coccidioides* spp. are relatively easy to grow and identify, the sensitivity of fungal culture is low (<50%),[13,14,20] and the isolation of these organisms is time-consuming and can pose a risk of infection to laboratory personnel.[18,19,21–23]

In the present study, we reviewed the results of 1160 Cocci PCR tests performed on clinical specimens of patients who were undergoing testing for diagnostic purposes. For all patients, Cocci PCR was performed because either symptoms, physical examination, laboratory data or radiographic abnormalities were

such that coccidioidomycosis was included in the differential diagnosis. The specimen sources were derived from either bronchoalveoalar lavage, sputum, pleural fluid, cerebrospinal fluid, or lung tissue biopsy. There were 113 total specimens that were positive for the Cocci PCR and all of these had proven or probable coccidioidomycosis. Regardless of specimen source, the Cocci PCR assay performed on the BD MAX has now become a viable tool in our facility when used to detect *C. immitis* in the San Joaquin Valley.

The overall sensitivity of 74% for BD MAX Cocci PCR compared with 46% for fungal culture shows a statistically significant improvement. However, we still need to put this study into the appropriate context of current clinical practice. Due to the fact that 26% of cases of coccidioidomycosis were still missed with a negative BD MAX Cocci PCR, it should not be used as a stand-alone test in any given clinical scenario. Clinical judgment should still be used when evaluating a negative BD MAX Cocci PCR result and other diagnostic tools should be examined.

The sensitivities varied significantly based on sample source and clinical setting. The lung tissue biopsies were primarily bronchoscopic or transthoracic needle biopsy specimens derived in the outpatient setting. This type of clinical setting and specimen source produced the lowest sensitivity at 44%. Inadequate tissue sampling could play a role in this low sensitivity. CSF also produced low sensitivity at 59% and a majority of these cases were done in the clinical setting of altered mental status and possible meningitis. Validation studies previously done in the laboratory[7] showed minimum concentrations of 1 cfu/ul or 1000 cfu/ml needed for positive Cocci PCR results. Pathologists need only see one good spherule, whereas the BD MAX Cocci PCR may require dozens or hundreds of spherules to have 1000 copies of the DNA target in 1 ml of specimen to turn positive. This may not occur routinely in CSF and probably not in a thin needle used in lung tissue biopsies.

This study has the limitation of relying more on laboratory criteria for the diagnosis of proven or probable coccidioidomycosis. While this study only saw one Cocci possible case out of 1160 specimens tested, it could have certainly missed other cases of coccidioidomycosis based purely on clinical grounds.

In conclusion, we were able to demonstrate that the Cocci PCR test using the BD MAX system in an academically affiliated community hospital laboratory is a viable and useful test for coccidioidomycosis in the clinical setting here in Central California. This could offer patients an earlier diagnosis, as the result comes back within 4 hours rather than days to weeks and the patient can be started on appropriate treatment regimens which could ultimately affect outcomes. The cost of the test is not prohibitive and comparable to serology and fungal culture. For the past 3 years, it has been readily ordered and used by clinicians in their workup for patients considered to have the disease.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper. Due to the spirit of collaboration encouraged in the awarding of the intramural grant, there were no commercial patents or intellectual proprietorships pursued by the authors during the entirety of this project. The objective was to share any knowledge and clinical benefits derived from this study with other local, regional, and international institutions who are also dealing with the problem of coccidioidomycosis.

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