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The Rate of Pulmonary Mycotic Infections with Coccidioidomycosis in An Najaf Province

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Abstract:

This study was aimed to evaluate the rate of infections with Coccidioidomycosis in An Najaf province and to demonstrate the importance of molecular diagnoses methods.

Ninety three respiratory specimens were obtained from patients with Coccidioidomycosis seen at Al-Sader Teaching Hospital and AL-Hakim general hospital in Al-Najaf, Iraq between January 2014 and June 2014. clinical isolates were examined for *Coccidioides immitis* and *C. posadasii* and inoculated onto sabouraud dextrose agar plates.

Nested PCR was used to confirm the diagnosis by screening for the presence of proline-rich antigen (PRA) which is a protein located in the spherule cell wall of *C. immitis* and *C. posadasii*. Twenty one clinical isolates determined to be *Coccidioides* spp. by microscopic examination from a total of 93 respiratory specimens, which were coexamined by general fungal culture. Nested PCR results showed a product of 526 bp was obtained from 23 of the 93 strains tested after the first PCR, whereas an expected 342 bp nested PCR product was detected by agarose electrophoresis from 25 strains tested.

Key words: Coccidioidomycosis, Nested PCR, proline-rich antigen

Introduction

Coccidioidomycosis (also known as valley fever) results from inhaling the spores (arthroconidia) of Coccidioides species (Coccidioides immitis or Coccidioides posadasii) [1]. *Coccidioides* spp. are native to arid and desert areas in North America (California, Arizona, Texas, Utah, Nevada, New Mexico, and northern parts of Mexico), Central America, and South America [2]. The manifestations of infection with either organism are

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assumed to be identical. *Coccidioides* spp. are found in lower elevation areas that have warm, sandy soil [3]. Organism growth is enhanced in areas of animal droppings, burial sites, and animal burrows [4]. Among persons living in coccidioidomycosis-endemic areas, 10%–50% have been exposed to *Coccidioides* spp. Each year in the United States, an estimated 150,000 new cases of coccidioidomycosis occur [5]. Symptomatic patients typically experience a self-limited influenza-like illness, but some develop severe or chronic pulmonary disease, and less than 1% of patients experience disseminated disease [6]. Coccidioidomycosis can be costly and debilitating, with nearly 75% of patients missing work or school because of their illness, and more than 40% requiring hospitalization [7].

Coccidioides spp. are dimorphic, soil-dwelling, fungi known to cause a broad spectrum of disease, ranging from a mild febrile illness to severe pulmonary manifestations or disseminated disease [8.9]. The genus *Coccidioides* is comprised of two genetically distinct species: *Coccidioides immitis* and *C. posadasii*. These two species cause similar clinical diseases, however they are present in different geographic regions of endemicity [10-12]. Symptoms of acute infection resemble bronchitis or pneumonia and are indistinguishable from other causes of community acquired pneumonia [13, 14]. In retrospective studies, patients with coccidioidomycosis were less likely to have cough and sputum production but more likely to have eosinophilia, pleurisy, myalgia, rash, and fatigue than patients without coccidioidomycosis [15].

This disease is mainly found in the Western Hemisphere, mainly in northwestern Mexico and southwestern United States. The incidence of the disease in the endemic area not known exactly but it relatively stable in the middle decades of the 20th century the reasons for that explained by population growth and migration and by increased the number of immunocompromised hosts, the absolute and relative frequency of primary disease and consequent dissemination have multiplied [16] .

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Clinical diagnosis of the disease can be difficult and cultivation of the microorganism is slow and risky, since direct manipulation of the fungal arthroconidia requires special care in the laboratory [17, 18]. Recent studies have focused on searching for faster and safer diagnostic methods, such as immunodiagnosis and molecular biology techniques [19].

This study was aimed to evaluate the rate of infections with Coccidioidomycosis in An Najaf province and to demonstrate the importance of molecular diagnoses methods.

Materials and Methods

Clinical isolates: One hundred and forty three respiratory specimens (bronchoalveolar lavage fluid, bronchial washings, and sputum) were obtained from patients with Coccidioidomycosis seen at Al-Sader Teaching Hospital and AL-Hakim general hospital in Al-Najaf, Iraq between January 2014 and June 2014. clinical isolates were examined of: *Coccidioides immitis* and *C. posadasii* from the fungal isolates were collected.

Culture of clinical specimens: Respiratory specimens were inoculated onto sabouraud dextrose agar plates (HIMEDIA, India). Plates were incubated at 30°C and held for up to 24 days. Colonies exhibiting morphologies consistent with those of *Coccidioides* spp. were examined microscopically for characteristic patterns of alternating arthroconidia.

DNA isolation: Genomic DNA was extracted with the Wizard® Genomic DNA Purification Kit (Promega, USA) for isolating Genomic DNA. According to the kit manual several colonies from each isolate were suspended in one ml of 50mM EDTA before proceeding with the kit protocol.

PCR assay: Nested Polymerase chain reaction (PCR) was used to screen all isolates by using the following primers: The outer primers Cocci I 5´-GTA CTA TTA GGG AGG ATA ATC GTT-3´ and Cocci II 5´-GGT GTC AAC TGG TGG GAT GTC AAT-3´ (amplicon size 526 bp) coding for the proline-rich antigen (PRA) which is a protein also known as antigen 2 [20-

22]. PRA is a heavily glycosylated protein which is located in the spherule cell wall [23] that is unique to *C. immitis* and *C. posadasii* [24] (accession number AF013256, Gen-Bank).

The inner primer set Cocci III 5´-ATC CCA CCT TGC GCT GTA TGT TCG A-3´ and Cocci IV 5´-GGA GAC GGC TGG ATT TTT TAA CAT G-3´ (amplicon size 342 bp). The primers synthesized by AccuOligo® Bioneer Corporation USA were published previously [24]. According to the Experimental Protocol of AccuPower® TLA PCR PreMix tube, the PCR reaction mixture was performed using 5 µl of the template DNA, 4 µl of each primer (10pmole/µl, 2 µl forward and 2 µl reverse), were added to each AccuPower® TLA PCR PreMix tube. Distilled water was added to the tubes to a final volume of 20 µl.

Reaction mixtures with the outer primer set were thermally cycled once at 94°C for 5 min, 35 times at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, followed by a single terminal extension at 72°C for 5 min. For the nested PCR products, reaction mixtures were thermally cycled once at 94°C for 5 min, 30 times at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final elongation at 72°C for 5 min.

Electrophoresis: The PCR products were electrophoresed through 1% agarose (Sigma Chemical Co., St. Louis, Mo.) dissolved in Tris-borate-EDTA buffer TBE (0.1 M Tris, 0.09 M boric acid, 0.001 M EDTA pH 8.4), stained with ethidium bromide. Electrophoresis was conducted at 80 V for 60 min; the bands were visualized with a UV transilluminator.

Results

Clinical sensitivity and specificity: Twenty one clinical isolates determined to be *Coccidioides* spp. by microscopic examination from a total of 93 respiratory specimens, which were coexamined by general fungal culture.

The distribution of *Coccidioides* spp. isolates among all of the clinical specimens were 9 (21.4%) from 42 bronchoalveolar lavage fluid specimens,

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8 (16.6%) from 48 bronchial washings specimens, and 4 (7.5%) from sputum specimens, with significant difference (P> 0.05) between them (Table 1).

Table 1: The distribution of *Coccidioides* spp. isolates among the clinical specimens

Clinical Specimens	*Coccidioides* spp. Isolates	Non *Coccidioides* spp. Isolates	Total
Bronchoalveolar lavage fluid	9	33	42
Bronchial washings	8	40	48
Sputum	4	49	53
Total	21	122	143

***P <0.05 significant**

Nested PCR: A product of 526 bp was obtained from 23 of the 143 strains tested after the first PCR (Figure1), whereas an expected 342 bp nested PCR product was detected by agarose electrophoresis from 25 strains tested. There was no significant difference (P> 0.05) between the cultural identification results and the Nested Pcr identification results (Figure2). These results were agreed with Al-Attraqchi et al [25] by the detection of coccidioidomycosis in Iraq.

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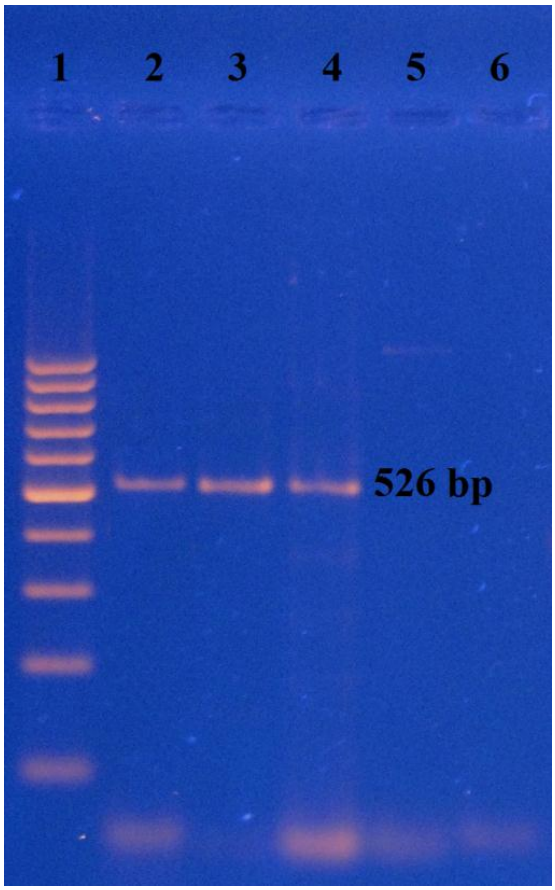

Figure 1: Agarose gel electrophoresis of PCR products after amplification of proline-rich antigen gene. Lane 1: molecular weight marker (GeneRuler™ 100 DNA Ladder, SM0243-Fermentas); Lanes 2-4: Cocci I-Cocci II primer pair (526bp); Lane 5,6: Negative control.

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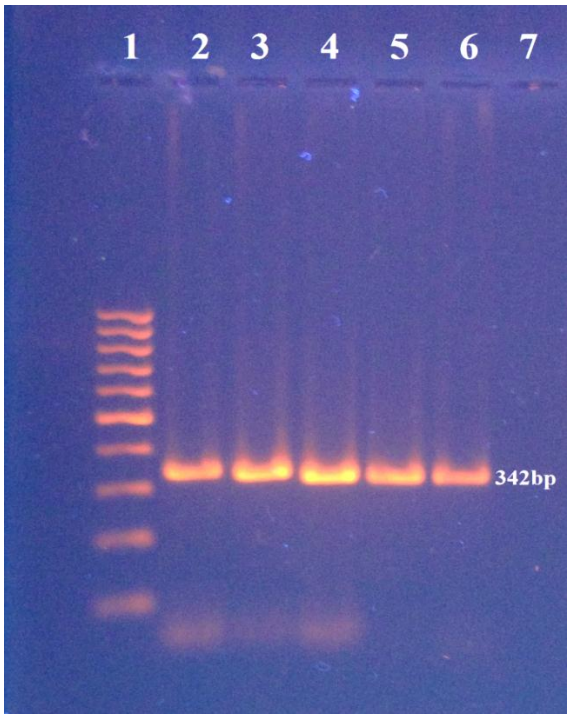

Figure 2: Agarose gel electrophoresis of PCR products after amplification of proline-rich antigen gene. Lane 1: molecular weight marker (GeneRuler™ 100 DNA Ladder, SM0243-Fermentas); Lanes 2-6: Cocci III-Cocci IV primer pair (342bp); Lane 7: Negative control.

Discussion

Several previous reports have described the identification of *Coccidioides* spp. by PCR, either from environmental sources [26], clinical isolates [27]. However, most of the *Coccidioides* spp. PCR assays described in the literature have utilized conventional PCR technology [28], a method which possesses limited utility as a diagnostic tool due to it being an open system and therefore having an increased potential for contamination events. In addition, to date, no report has included a thorough clinical verification in which large numbers of clinical specimens have been tested. This study was described the development of a Nested PCR assay for the accurate detection of *Coccidioides* spp. directly from clinical specimens.

Based on our findings, the PCR assay does not differentiate between the two known species of *Coccidioides*, *C. immitis* and *C. posadasii*. The two species differ in single-nucleotide polymorphisms, microsatellite size, and geographical distribution, with *C. immitis* and *C. posadasii* being historically known as the Californian and non-Californian strains, respectively [29].

It is often reported that Nested PCR displays greater sensitivity over conventional detection methods, such as culture [30, 31]. Therefore, it was of interest to us that four respiratory specimens were positive by Nested PCR but were negative by culture. These discordant results may indicate evidence of the enhanced sensitivity of Nested PCR over culture for certain pathogens.

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معدل الاصابات الفطرية الرئوية بمرض الفطار الكرواني في محافظة النجف

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الخلاصة:
هدفت هذه الدراسة لتقييم معدل الاصابات الرئوية بمرض الفطار الكرواني في محافظة النجف و لأظهار اهمية الطرق الجزيئية في تشخيصه.

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تم الحصول على مائة و ثلاث و اربعين عينة من المرضى المصابين بالفطار الكرواني في مستشفى الصدر التعليمي و مستشفى الحكيم العام في مدينة النجف للفترة ما بين كانون الثاني و حزيران 2014 . فحصت العينات السريرية للبحث عن الفطرين *Coccidioides immitis* و *C. posadasii* و لقحت على وسط السابرود دكستروز اكار. اجري اختبار Nested PCR لتاكيد التشخيص من خلال البحث عن بروتين proline-rich antigen (PRA) و هو البرو تين الموجود في جدران الخلايا الكروية لكلا الفطرين اعلاه. حددت احدى و عشرين عزلة من اصل 143 على انها *Coccidioides* spp. بالفحص المجهري و الزرعي. أظهرت نتائج Nested PCR وجود ناتج البلمرة ذو الطول 526 زوج قاعدي في 23 من مجموع العزلات البالغ 143 بعد مرحلة التضاعف الاولى في حين تم الحصول على ناتج البلمرة ذو الحجم 342 زوج قاعدي في 25 عزلة من مرحلة التضاعف الثانية لاختبار Nested PCR.