ELSEVIER

FEMS Immunology and Medical Microbiology 45 (2005) 355–360

FEMS Immunology and Medical Microbiology

www.fems-microbiology.org

MiniReview

Coccidioidomycosis and blastomycosis: Advances in molecular diagnosis

Ralf Bialek [a,*], Gloria M. González [b], Dominik Begerow [c], Ulrike E. Zelck [a]

[a] *Institut für Tropenmedizin, Universitätsklinikum Tübingen, Keplerstrasse 15, D-72074 Tübingen, Germany*
[b] *Departamento de Microbiología, Facultad de Medicina, Universidad Autónoma de Nuevo Léon, Monterrey, Nuevo Léon, México*
[c] *Institute of Biology I, University of Tübingen, Tübingen, Germany*

Received 20 April 2005; accepted 27 May 2005

First published online 14 July 2005

Abstract

Clinical isolates of *Coccidioides* spp. and *Blastomyces dermatitidis* can be identified by chemiluminescent DNA probes and PCR assays targeting multicopy genes. In fixed tissue samples, cells of the two fungi are specified by in situ hybridization and PCR assays targeting 18S rDNA but sequencing of the products is mandatory. Nested PCR assays targeting genes encoding species- or genus-specific proteins like proline rich antigen of *Coccidioides* spp. and *B. dermatitidis* adhesin facilitate amplification of specific DNA from fixed tissue samples. The value of DNA amplification from native specimens of suspected cases of coccidioidomycosis or blastomycosis still needs to be determined.
© 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Coccidioidomycosis; Blastomycosis; PCR assays; Antigen2/proline rich antigen; Blastomyces adhesin

1. Introduction

Coccidioidomycosis and blastomycosis belong to the group of deep-seated mycoses. They are caused by dimorphic fungi of the genus *Coccidioides* and *Blastomyces dermatitidis*, respectively. Coccidioidomycosis is restricted to arid and semi-arid areas of the Americas [1]. Blastomycosis is endemic in North America but has been described in circumscribed African regions and other parts of the world as well [2–4]. The mycelial or mold forms represent the saprophytic phases. Infection occurs by inhalation of mycelial fragments or micro-conidia of *B. dermatitidis* or arthrospores of *Coccidioides* spp. In the mammalian lung, i.e., at a temperature ⩾30 °C the parasitic stages develop, yeast cells and spherules, respectively. Coccidioidal spherules contain endospores that are released by rupture leading to new endospore-producing spherules, whereas yeast cells of *B. dermatitidis* grow by budding of new yeast cells. The parasitic stages are not infective and human-to-human transmission does not occur [1,2].

Primarily the lungs are affected but the infection might spread to other organs causing disseminated disease. However, most infections will cause either no symptoms or an unspecific flu-like illness with spontaneous resolution. Antimycotic treatment with azoles or amphotericin B is needed in persisting or severe symptomatic infections, in immunocompromised patients and in extrapulmonary or disseminated disease [5,6]. Clinical symptoms are unspecific and undistinguishable from other deep-seated mycotic, parasitic, bacterial or viral diseases. The infection is diagnosed by growing the fungus from clinical specimens. However, culture is restricted to biosafety level 3 laboratories. It may take

* Corresponding author. Tel.: +49 7071 2982367; fax: +49 7071 295267.
E-mail address: ralf.bialek@med.uni-tuebingen.de (R. Bialek).

0928-8244/$22.00 © 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.femsim.2005.05.011

weeks to grow the hazardous pathogens, sensitivity of the culture is low, and cultures require further identification.

Diagnosis is often achieved by detection of specific antibodies, which are usually absent at presentation with symptoms of an acute infection. Additionally, antibody production can be impaired in immunocompromised patients who are at special risk for invasive fungal infections [7]. Since therapy and prognosis depend on early and specific diagnosis, there has been considerable interest in molecular assays. However, excellent recent reviews on nonculture methods for diagnosis of invasive fungal infections with an emphasis on polymerase chain reaction-based assays did not mention molecular diagnosis of coccidioidomycosis and blastomycosis [7,8]. Here we review data on PCR-assays and other molecular diagnostic procedures for the two endemic mycoses either published or in review process by December 2004.

2. Molecular diagnosis – general considerations

In order to evaluate molecular assays the aim of the diagnostic procedure has to be defined. Molecular diagnosis can be used: (i) to identify fungal cultures, (ii) to specify fungal infection in tissue samples diagnosed by histopathology without possibility of further proving by culture because growing the infecting organisms is impossible after formaldehyde-fixation, and (iii) to detect specific DNA from appropriate specimens obtained from cases of clinically suspected endemic mycosis.

Multicopy genes, such as 18S rDNA, ITS-regions or 28S rDNA, are commonly used for diagnostic PCR assays [9–12]. Since several, up to hundreds of copies are present per genome, targeting these genes guarantees high sensitivity of the PCR assay. However, ribosomal genes are conserved and the primers amplify DNA from several fungal species, genera or even several fungal families. Thus, a limited specificity can be anticipated. As a consequence, PCR products have to be further specified by sequencing. Hybridization and restriction fragment length polymorphisms (RFLP) are considered to be alternatives. Diagnostic PCR assays can target gene sequences coding for immunodominant or otherwise unique, i.e., species- or genus-specific proteins. However, the number of copies of the encoding genes is limited to one or only a few leading to reduce sensitivity of highly specific PCR assays.

Another important issue is identification of PCR products. As stated before, the most appropriate approach is sequencing, and identifying the species by comparison with sequences published in databases such as GenBank. It has to be considered that only those fungal sequences, which have been sequenced and deposited in databases are found. Since ribosomal genes are conserved, many fungi and other organisms might have similar sequences. Thus, a 100% identity is mandatory in order to identify a fungal species by parts of a highly conserved ribosomal gene. For further illustration, experience with *Cryptococcus*-PCR assays is briefly summarized [13]. Specific primers were defined and a BLASTSearch in GenBank revealed identity only to 18S rDNA of *Cryptococcus neoformans*. A nested block cycler PCR and a LightCycler PCR were created showing excellent sensitivity in a mouse model of cryptococcosis. In addition, DNA from related species of the orders Tremellales and Filobasidiales within the fungus subclass Tremellomycetidae was tested. Phylogenetic relationships within this family of the phylum Basidiomycota had been estimated by sequences of the ribosomal large subunit (28S rDNA), whereas 18S rDNA sequences of most of these fungal species had not been entered into databases. DNA from 12 related species was amplified by the *Cryptococcus*-specific PCRs. Sequencing of the 278 bp nested products disclosed 6 sequences from related fungi to be 100% identical to the *C. neoformans* product. It was concluded that species-identification by a part of a ribosomal gene might not even be achieved by complete identity. Similar findings were reported for *Coccidioides immitis* by Millar et al. [14]. The authors found that 434 out of 435 bases of their PCR product within the 18S rDNA of *C. immitis* were identical to the sequence of *Chrysosporium keratinophilum*. Thus, less than complete identity does not allow species identification. Additionally, identification is limited to sequences available in databases and as long as ribosomal genes have not been sequenced from all fungi and deposited in databases some uncertainties remain. In view of these data, identification of PCR products targeting ribosomal genes by either hybridization or RFLP can only be even less specific. Due to increasing knowledge of gene sequences, PCR assays published some years ago might no longer be as specific as stated in the original paper. In 1997, a diagnostic PCR assay targeting the 18S rDNA was published using hybridization for identification of *Aspergillus*-specific DNA [10]. Fig. 1 shows a part of the amplified gene region and the complementary region of the hybridization probe which detects several species of different fungal genera – some are pathogenic to humans others might be contaminations leading to "false-positive" PCR results.

Targeting a gene encoding a species-specific protein avoids these problems because unspecific or cross-amplifications are highly unlikely to occur. However, sequencing remains mandatory to identify the PCR product. As stated before, this approach reduces overall sensitivity of the diagnostic PCR that can be increased by using a semi-nested or nested PCR assay.

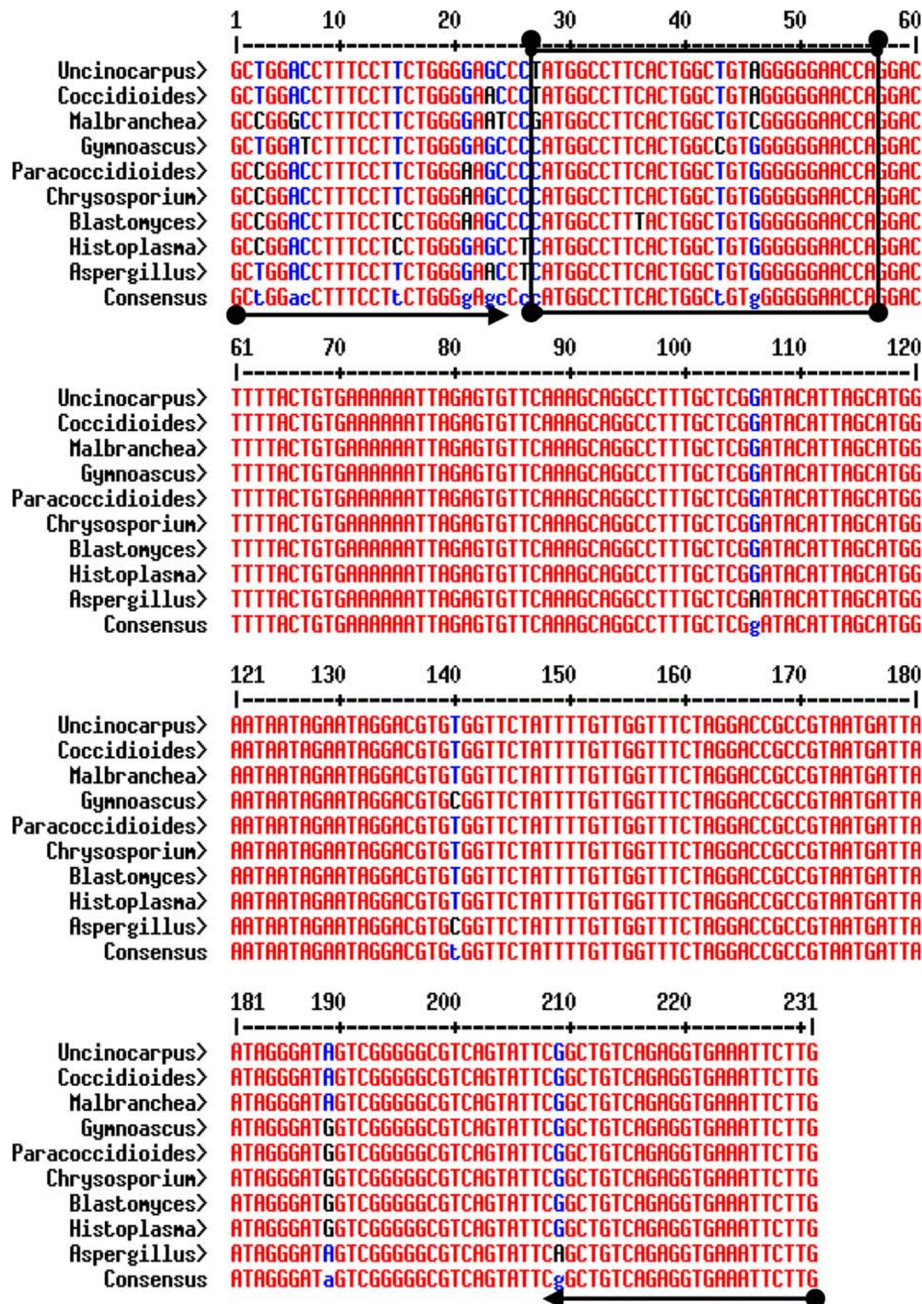

Fig. 1. Alignment of 18S rDNA sequences of Onygenales amplified by an inner primer set histo 1 (5′-GCC GGA CCT TTC CTC CTG GGG AGC-3′) and histo 2 (5′-CAA GAA TTT CAC CTC TGA CAG CCG A-3′) of a nested PCR assay to detect DNA of *Blastomyces dermatitidis* (and *Histoplasma capsulatum*) [30,31] based on a program according to Corpet [33] (http://prodes.toulouse.inra.fr/multialin/multialin.html). The complementary primer regions are indicated by black arrows. Blue and black nucleotides indicate low or missing consensus in contrast to red letters indicating identity of remaining or all sequences. The black box indicates the binding region of a hybridization probe formerly regarded to be *Aspergillus* specific [10]. Species and GenBank accession numbers used from top to bottom: *Uncinocarpus reesii* (U29394), *Coccidioides immitis* (X58571), *Malbranchea dendritica* (AY124496), *Gymnoascus petalosporus* (U29392), *Paracoccidioides brasiliensis* (AF227151), *Chrysosporium parvum* (U29390), *Blastomyces dermatitidis* (X59420), *Histoplasma capsulatum* (X58572), *Aspergillus fumigatus* (AB008401).

3. Molecular diagnosis of coccidioidomycosis

Until recently, coccidioidomycosis was entirely attributed to *C. immitis*. Work by Fisher et al. [15,16] has provided evidence of two species; *C. immitis*, formerly called Californian-strains, is found mainly in the central valley of California, and *Coccidioides posadasii* – in honour of Posadas, the author of the first description of coccidioidomycosis in Argentina 1892 [17] – is found mainly outside the central valley of California in the US states of California, Nevada, Utah, Texas, New Mexico and Arizona, in Northern Mexico and in

endemic areas of South America [15]. These two species are distinguished by length of defined microsatellite-containing loci and mutation in genes encoding fungal enzymes. Colony morphology, growth requirements and clinical disease are identical [16].

Coccidioides spp. grow within 2 to 5 days on several media used in mycology. Identification of the mycelial form requires conversion into the parasitic stage in an animal or by culture. These procedures are limited to specialised biosafety level 3 laboratories. Stockman et al. [18] showed a commercially available acridinium ester-labelled chemiluminescent DNA probe targeting the ribosomal RNA of *Coccidioides* spp. to be highly sensitive and 100% specific. A total of 164 strains from related and unrelated fungal species were tested to define specificity, and no cross-reaction was detected. The probe was considered to be used for spherule detection in tissue samples but further attempts have never been published. Probes targeting ribosomal RNA work perfectly when cultures are tested but they might fail to identify the fungus in tissue samples in the presence of human or animal RNA, and RNA from contaminating fungi and other microorganisms. Even if the probe does not react with non-fungal RNA, the presence of RNA molecules might inhibit hybridization, and liberation of RNA achieved by sonication and grinding with glass beads might be inappropriate for tissue samples. A growing culture is required, and in case of transportation to a reference laboratory inactivation by heat or formaldehyde is mandatory to minimize the risk of infection. However, fixation in formaldehyde reduces efficiency of this excellent and widely used identification system [19].

Sandhu et al. [11] published 21 nucleic acid probes complementary to the large subunit of ribosomal DNA considered to be specific after testing nearly 50 different human pathogens. Although not specified which of the described probes was regarded specific for *C. immitis*, it hybridized only with DNA extracted from 16 clinical isolates of *C. immitis*. A recent BLAST-Search in GenBank identified the specific probe but revealed as well complete identity to 28S rDNA of *C. keratinophilum*. Another major disadvantage for routine lab work mentioned already by Sandhu et al. was the use of radiolabelled probes. In another study species-specific primers complementary to the ITS region of *C. immitis* were designed by alignment of sequences from the pathogen and known related fungi of the genera *Uncinocarpus*, *Auxarthron* and *Gymnoascus* [20]. After testing against the above named members of the order Onygenales and several species of the genera *Penicillium* and *Aspergillus* the primers were found to be specific. They amplified DNA from all 30 *C. immitis* isolates examined in the study. The PCR was used to identify soil isolates and it was regarded positive if a band of the expected size was demonstrated by ethidium bromide stained agarose gel after electrophoresis. Nevertheless, identity was proven by sequencing the PCR products. Lindsley et al. [21] amplified the ITS1, 5.8 and ITS2 region of the fungal ribosomal DNA using the universal primers ITS1 and ITS4 complementary to the end of 18S and beginning of 28S rDNA described by White et al. [22] in 1990. Lindsley et al. concentrated on dimorphic and yeast-like fungal pathogens and developed a panel of 5′-dioxygenin-labeled probes used in an enzyme immunoassay to identify clinical isolates. However, there was some reactivity of the *B. dermatitidis* probe observed when tested against *C. immitis* DNA but the *C. immitis* probe did not cross-react with *B. dermatitidis*. Thus, preliminary identification by colony morphology reduces failures or several probes have to be tested to specify isolates of *C. immitis*. All methods described so far have been used solely to identify cultures. Therefore, a nested PCR and a real time PCR were developed targeting the genus-specific antigen2/proline rich antigen of *Coccidioides* spp. [23]. Both PCR assays correctly identified all 120 clinical isolates tested, which were identified as *C. posadasii* according to length of microsatellites. In addition, specific DNA was amplified by the conventional nested PCR from three microscopically spherule-positive paraffin-embedded tissue samples whereas 20 human samples positive for other dimorphic fungi remained negative. In an ongoing comparative study the PCR assay detected specific DNA from several clinical specimens, including bronchoalveolar lavage (BAL) and cerebrospinal fluid, obtained from suspected cases in accordance with microscopy and cultures (Gonzales et al., unpublished data). Although not tested in the study, the PCR assays should identify *C. immitis* as well because the targeted gene was demonstrated to be highly conserved among isolates from different geographical regions [24].

Another successful approach to identify *C. immitis* in paraffin-embedded tissue sections is in situ hybridization. Hayden et al. [25] described oligonucleotide probes to identify yeast like organisms in tissue section. Their *C. immitis*-specific probe had a sensitivity of 94.3%, a 100% specificity and a positive predictive value of 100%. Identification of coccidioidal elements in tissue sections might be very difficult or impossible [26]. In those cases, in situ hybridization might be of essential help. However, identification by this method is limited to cases with microscopically visible fungal elements.

Recently, amplification of *Coccidioides* specific DNA from serum samples has been reported by Johnson et al. [27]. The authors used the primers described by Greene et al. [20] targeting the ITS region of *C. immitis*. They claimed to detect specific DNA in human and murine serum samples sent for antibody testing. Surprisingly identification of PCR products was done solely by fragment length estimation in an ethidium bromide or Vistra Green stained agarose gel instead of the appropriate denaturing

polyacrylamide gel electrophoresis. In contrast to the original description of the PCR, none of the products was identified by sequencing, hybridization or restriction enzyme digestion. In order to avoid frustration by scientists interested in newly published PCR assays it is recommended to run a BLASTSearch with the primers. This revealed no homologies to other fungal sequences but significant homologies to several human and murine genes were indicated. Therefore, the products found in serum samples could be due to cross-amplifications of human and murine gene sequences [28]. Consequently, until the products have not been appropriately identified this PCR assay can not be recommended for diagnostic purposes in humans nor in mice models of coccidioidomycosis.

4. Molecular diagnosis of blastomycosis

Isolates of *B. dermatitidis* can be identified by a chemiluminescent DNA probe [18]. A sensitivity of 87.4% and 100% specificity were reported after testing 74 target and 219 nontarget fungi. Specific probes complementary to the large ribosomal subunit or to the ITS-region have been successfully used for identification of isolates [11,21].

Yeast cells of *B. dermatitidis* are reported to resemble spherules of *Coccidioides* spp. in tissue section used in histopathology [29]. Further identification can be achieved by in situ hybridization. Hayden et al. [25] used a pair of two oligonucleotides complementary to the 18S and 28S rDNA. They reported a sensitivity of 95% and a specificity of 100% of these probes to identify *B. dermatitidis* yeast cells. We used a nested PCR targeting the 18S rDNA of *H. capsulatum* to amplify DNA of *B. dermatitidis* obtained from formalin-fixed canine tissue samples [30,31]. As shown in Fig. 1, there is only one mismatch in the forward primer binding region of the inner primer set but this does not affect the amplification of *B. dermatitidis* DNA. Differences to the other eight aligned species range from only two to a maximum of ten nucleotides per PCR product. Accordingly, a 100% identity of the sequence of the PCR product is required to identify *B. dermatitidis*. Since this appeared still insufficient a nested PCR assay was established targeting the gene encoding the species-specific *B. dermatitidis* adhesin (BAD), formerly called WI-1. As expected, this assay was specific and the detection limit of 0.1 fg cloned plasmid DNA was comparable to the 18S rDNA PCR. The latter is routinely used as a screening assay, and if positive, the specific nested PCR is added to confirm the diagnosis. This procedure has been successful especially in unusual manifestations of canine blastomycosis (Gabor et al., submitted; Caruso et al., submitted). However, concerning human blastomycosis it has to be considered that African strains of *B. dermatitidis* do not express this antigen [32]. It remains to be examined whether the protein is not expressed or the gene is completely absent. Up to now, we did not have the opportunity to screen native clinical specimens like BAL fluid or sputum by PCR assays. Additionally, we are not aware of published data on amplification of specific DNA directly from clinical specimens of human blastomycosis.

5. Conclusion

Molecular identification of fungal isolates of *Coccidioides* spp. and *B. dermatitidis* can be achieved by chemiluminescent DNA probes or PCR assays targeting multicopy ribosomal genes. Fungal cells and specific DNA can be detected and identified in fixed tissue samples by in situ hybridization and PCR assays targeting genes encoding species- or genus-specific proteins. However, comparative studies using native clinical specimens from patients in defined stages of disease are needed to determine sensitivity and specificity of diagnostic PCR assays in the molecular diagnosis of coccidioidomycosis and blastomycosis.

References

[1] Chiller, T.M., Galgiani, J.N. and Stevens, D.A. (2003) Coccidioidomycosis. Infect. Dis. Clin. N. Am. 17, 41–57.

[2] Salvo di, A.F. (1999) *Blastomyces dermatitidis* (Chapter 18). In: Topley & Wilson's microbiology and microbial infections (Collier, A., Balows, A. and Sussman, M., Eds.), 9th Edn. In: Medical Mycology (Ajello, L. and Hay, R.J., Eds.), Vol. 4, pp. 337–355. Arnold, London.

[3] Vandepitte, J. and Gatti, F. (1972) La blastomycose nord-americaine en Afrique son existence en Republique du Zaire. Ann. Soc. Belge Méd. Trop. 52, 467–480.

[4] Davidson, L. and Gelfand, M. (1972) North American blastomycosis in Rhodesia. Cent. Afr. J. Med. 18, 129–137.

[5] Chapman, S.W., Bradsher Jr., R.W., Campbell Jr., S.D., Pappas, P.G. and Kauffman, C.A. (2000) Practice guidelines for the management of patients with blastomycosis. Clin. Infect. Dis. 30, 679–683.

[6] Galgiani, J.N., Ampel, N.M., Catanzaro, A., Johnson, R.H., Stevens, D.A. and Williams, P.L. (2000) Practice guidelines for the treatment of coccidioidomycosis. Clin. Infect. Dis. 30, 658–661.

[7] Yeo, S.F. and Wong, B. (2002) Current status of nonculture methods for diagnosis of invasive fungal infections. Clin. Microbiol. Rev. 15, 465–484.

[8] Chen, S.C.A., Halliday, C.L. and Meyer, W. (2002) A review of nucleic acid-based diagnostic tests for systemic mycoses with an emphasis on polymerase chain reaction-based assays. Med. Mycol. 40, 333–357.

[9] van Burik, J.-A., Myerson, D., Schreckhise, R.W. and Bowden, R.A. (1998) Panfungal PCR assay for detection of fungal infection in human blood specimens. J. Clin. Microbiol. 36, 1169–1175.

[10] Einsele, H., Hebart, H., Roller, G., Löffler, J., Rothenhöfer, I., Müller, C.A., Bowden, R.A., van Burik, J.-A., Engelhard, D., Kanz, L. and Schumacher, U. (1997) Detection and identification

of fungal pathogens in blood by using molecular probes. J. Clin. Microbiol. 35, 1353–1360.

[11] Sandhu, G.S., Kline, B.C., Stockman, L. and Roberts, G.D. (1995) Molecular probes for diagnosis of fungal infections. J. Clin. Microbiol. 33, 2913–2919.

[12] Haynes, K.A., Westerneng, T.J., Fell, J.W. and Moens, W. (1995) Rapid detection and identification of pathogenic fungi by polymerase chain reaction amplification of large subunit ribosomal DNA. J. Med. Vet. Mycol. 33, 319–325.

[13] Bialek, R., Weiss, M., Bekure-Nemariam, K., Najvar, L.K., Alberdi, M.B., Graybill, J.R. and Reischl, U. (2002) Detection of *Cryptococcus neoformans* DNA in tissue samples by nested and real-time PCR assays. Clin. Diagn. Lab. Immunol. 9, 461–469.

[14] Millar, B.C., Jiru, X., Walker, M.J., Evans, J.P. and Moore, J.E. (2003) False identification of *Coccidioides immitis*: do molecular methods always get it right? J. Clin. Microbiol. 41, 5778–5780.

[15] Fisher, M.C., Koenig, G.L., White, T.J. and Taylor, J.W. (2002) Molecular and phenotypic description of *Coccidioides posadasii* sp. Nov., previously recognized as the non-California population of *Coccidioides immitis*. Mycologia 94, 73–84.

[16] Fisher, M.C., Koenig, G.L., White, T.J., Sans-Blas, G., Negroni, R., Gutiérrez Alvarez, I., Wanke, B. and Taylor, J.W. (2001) Biogeographic range expansion into South America by *Coccidioides immitis* mirrors New World pattern of human migration. Proc. Natl. Acad. Sci. 98, 4558–4562.

[17] Wernicke, R. (1892) Ueber einen Protozoenbefund bei Mycosis fungoides (?). Centralbl. Bacteriol. Parasitenkd. 12, 859–861.

[18] Stockman, L., Clark, K.A., Hunt, J.M. and Roberts, G.D. (1993) Evaluation of commercially available acridinium ester-labeled chemiluminescent DNA probes for culture identification of *Blastomyces dermatitidis*, *Coccidioides immitis*, *Cryptococcus neoformans*, and *Histoplasma capsulatum*. J. Clin. Microbiol. 31, 845–850.

[19] Gromadzki, S.G. and Chaturvedi, V. (2000) Limitation of the AccuProbe *Coccidioides immitis* culture identification test: false-negative results with formaldehyde-killed cultures. J. Clin. Microbiol. 38, 2427–2428.

[20] Greene, D.R., Koenig, G., Fisher, M.C. and Taylor, J.W. (2000) Soil isolation and molecular identification of *Coccidioides immitis*. Mycologia 92, 406–410.

[21] Lindsley, M.D., Hurst, S.F., Iqbal, N.J. and Morrison, C.J. (2001) Rapid identification of dimorphic and yeast-like fungal pathogens using specific DNA probes. J. Clin. Microbiol. 39, 3505–3511.

[22] White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: A Guide to Methods and Applications (Innis, M.A., Gelfand, D.H., Sninsky, J. and White, T.J., Eds.), pp. 315–322. Academic Press, New York.

[23] Bialek, R., Kern, J., Herrmann, T., Tijerina, R., Cecenas, L., Reischl, U. and Gonzalez, G.M. (2004) PCR assays for identification of *Coccidioides posadasii* based on the nucleotide sequence of the antigen2/proline-rich antigen. J. Clin. Microbiol. 42, 778–783.

[24] Peng, T., Orsborn, K.I., Orbach, M.J. and Galgiani, J.N. (1999) Proline-rich vaccine candidate antigen of *Coccidioides immitis*: conservation among isolates and differential expression with spherule maturation. J. Infect. Dis. 179, 518–521.

[25] Hayden, R.T., Qian, X., Roberts, G.D. and Lloyd, R.V. (2001) In situ hybridization for the identification of yeastlike organisms in tissue section. Diagn. Mol. Pathol. 10, 15–23.

[26] Kaufman, L., Valero, G. and Padhye, A.A. (1998) Misleading manifestations of *Coccidioides immitis* in vivo. J. Clin. Microbiol. 36, 3721–3723.

[27] Johnson, S.M., Simmons, K.A. and Pappagianis, D. (2004) Amplification of coccidioidal DNA in clinical specimens by PCR. J. Clin. Microbiol. 42, 1982–1985.

[28] Bialek, R. (2005) Amplification of coccidioidal DNA in clinical specimens by PCR. J. Clin. Microbiol. 43, 1492–1493.

[29] Watts, J.C., Chandler, F.W., Mihalov, M.L., Kammeyer, P.L. and Armin, A. (1990) Giant forms of *Blastomyces dermatitidis* in the pulmonary lesion of blastomycosis. Potential confusion with *Coccidioides immitis*. Am. J. Clin. Pathol. 93, 575–578.

[30] Bialek, R., Feucht, A., Aepinus, C., Just-Nübling, G., Robertson, V.J., Knobloch, J. and Hohle, R. (2002) Evaluation of two nested PCR assays for the detection of *Histoplasma capsulatum* DNA in human tissue. J. Clin. Microbiol. 40, 1644–1647.

[31] Bialek, R., Cascante Cireira, A., Herrmann, T., Aepinus, C., Shearn-Bochsler, V.I. and Legendre, A.M. (2003) Nested PCR assays for detection of *Blastomyces dermatitidis* DNA in paraffin-embedded canine tissue. J. Clin. Microbiol. 41, 205–208.

[32] Klein, B.S., Aizenstein, B.D. and Hogan, L.H. (1997) African strains of *Blastomyces dermatitidis* that do not express adhesin WI-1. Infect. Immun. 65, 1505–1509.

[33] Corpet, F. (1988) Multiple sequence alignment in hierarchical clustering. Nucleic Acids Res. 16, 10881–10890.