Molecular techniques for clinical diagnostic mycology

Mikolojik klinik tanıda moleküler teknikler

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ABSTRACT

Diagnosing fungal infections remains a problem, particularly in the immunocompromised patient. The clinical manifestations of invasive fungal infections are usually not specific and can be produced by other organisms and colonization is difficult to distinguish from invasive disease. Existing diagnostic tools often lack sensitivity. Thus, the combination of various diagnostic tools is mandatory to allow earlier diagnosis of systemic fungal infections. Microscopy, culture based methods, antigen detection, and molecular techniques may help to facilitate and accelerate the diagnosis. Molecular methods are being developed for the detection and identification of fungi present in clinical samples at a very fast rate, whether it is done by nucleic acid amplification technology or by specific probes or nucleic acid sequencing. However, no comparative studies have been done to determine which are optimal and no standards for testing have been developped to date. Therefore, extensive validation and standardization is needed, before molecular assays can be used in a routine laboratory.

Key Words: Molecular diagnosis of fungal infections, fungus identification, molecular typing

ÖZET

Mantar hastalıklarının tanımı, özellikle bağışıklığı baskılanmış hastalarda bir sorun olmaya devam etmektedir. İnvaziv mantar enfeksiyonlarının klinik yönleri genellikle özgül değidir, başka mikroorganizmalar tarafından da oluşturulabilir ve kolonizasyonu invaziv hastalıktan ayırt etmek güçtür. Mevcut tanım yöntemleri ekseri duyarlılıktan yoksundur. Böylece, sistemik mantar enfeksiyonlarının erken tanımı için çeşitli tanım yöntemlerinin birlikte kullanılması gerekmektedir. Mikroskop incelemesi, kültüre dayalı yöntemler, antijen aranması ve moleküler teknikler tanımı kolaylaştırabilir ve hızlandırabilir. Klinik örnekte bulunan mantarın belirlenmesi ve tanımlanması için nükleik asit amplifikasyon teknolojisi, özgül problar ve nükleik asit sekanslama moleküler teknikleri çok hızla gelişmektedir. Ancak hangisinin en uygun olduğunu belirlemek için henüz karşılaştırmalı çalışmalar bulunmamaktadır ve bugüne kadar testlerin standartlaştılması geliştirilememiştir. Dolayısıyla, moleküler testlerin rutin laboratuvarlarda kullanılabilmesi için önce geçerli kılınmalarına ve standartlaştırılmalarına gereksinim bulunmaktadır.

Anahtar Kelimeler: Mantar enfeksiyonları moleküler tanı, mantarın tanımı, mantarların moleküler tiplendirilmesi

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INTRODUCTION

In the clinical mycology laboratory, identification of fungi to species level is important to determine the etiology of disease, to detect novel agents of diseae, to predict intrinsec resistance to antifungal agents, and to detect clusters of nosocomial infection among hospitalized patients. This information can be critically important in the management of fungal infection in the high risk patient. It is also important to recognize whether a fungal isolate recovered from a clinical sample do represent significant disease and the clinical relevance of that isolate.

Fungal infections which have emerged in recent years in the immunocompromised hosts, and different antifungal susceptibilities have emphasized the importance of early and accurate diagnosis (1). Clinical manifestations are seldom specific, a laboratory identification of the etiological fungus is, therefore, essential in establishing a defenitive diagnosis.

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The recovery of etiologic agents from clinical specimens is still considered to be the "gold standart". Methods used for identification of the fungi are a mixture of traditional and newer commercially available systems, e.g. yeast identification kits are commonly used fort his subject. Standard protocols to the laboratory diagnosis of invasive fungal infections depend upon (a) direct microscopic inspection of freshly obtained patient specimens for the presence of organisms, (b) recovery of fungi from cultures of blood, body fluids, tissues or other sites, and (c) histological identification of organisms morphologically consistent with certain species of fungi (2). Identification of emerging fungal pathogens by conventional methods is considerably difficult, time consuming and requires highly experienced laboratory staff for visual recognition of morphology. In some circumstances, whether the isolate displays atypical morphology, fails to sporolation, phenotypic results are nonspecific or especially confusing (3). The polymerase chain reaction (PCR) is a fairly simple but powerfull technique for molecular investigations of fungal phylogeny. Additionally, recent advances in molecular phylogenetic taxonomy have revealed cryptic species within morphologically indistinguishable isolates. Most serologic tests designed to detect specific serum antibodies are ineffective, because many patients who are at risk for fungal disease are not capable of mounting a specific antibody response to infection. Antigen detection for certain fungal agents can be useful for some patients suffering from invasive fungal infections but identification at species level is not achieved (4).

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), or proteins of an infection agent in clinical sample can be used to help identify the agent. Molecular techniques are being developped for the analysis of infectious fungal agents either present in clinical samples or grown on cultures. Molecular techniques such as restriction fragment lenght polymorphism (RFLP), electrophoretic karyotyping, multilocus enzyme electrophoresis (MLEE) are also useful biotyping tools to increase the knowledge of pathogenic fungi and develop prevention strategies (4-7).

1. DIRECT DETECTION OF THE NUCLEIC ACID OF FUNGI IN CLINICAL SPECIMENS

The use of molecular technologies in the detection and identification of fungi is still really in

its infancy compared with other areas of microbiology such as virology and bacteriology (8) but molecular diagnostics may offer mycologists many advantages such as sensitivity, specificity, simplicity and fastness. During traditional culture process, the disease may have progressed to be a point where therapy may be ineffective; molecular detection may allow for monitoring patients on a periodic basis for the presence of circulating nucleic acid of the infecting organism. Being able to start therapy at a much earlier time during the clinical course may significantly affect the survival rate of the patient with a lifthreatening fungal infection (5, 6).

1.1. In situ hibridization using specific nucleic acid probes

One of the simplest approaches used has been in situ hybridization using specific nucleic acid propbes for the identification of organisms in patient specimens. This method lacks amplification and is less sensitive than other assays but is useful for identifying fungi that can be seen in tissue and other clinical specimens (5). DNA probes can be used like antibodies as sensitive and specifc tools to detect, locate, and qantitate specific nucleic acid sequences in clinical specimens.

DNA probes are chemically synthesized or obtained by cloning specific genomic fragments. After chemical or heat treatments melt (separate) the DNA strands in the sample, the DNA prob is added and allow to hybridize (bind) with the identical or nearly identical sequence in the sample. The stringency (the requirement for an exact sequence match) of the interaction can be varied so that related sequences can be detected or different strains (mutants) can be distinguished. The DNA probes are labelled with radioactive or chemically modified nucleotids so that they can be detected and quantitated (6).

The DNA probes can detect specific genetic sequences, in fixed, permeabilized tissue biopsy specimens by in situ hybridization. When fluorescent detection is used it is called Fluorescent in situ hybridization (FISH) (8).

Assays for Aspergillus, Candida, Fusarium and many other fungi are helpful when a morphological identification can not be made (9-14). Identification of fungi in tissue sections can be difficult. In particular, species of Aspergillus, Fusarium, and Pseudallescheria all appear as septate, branched hyphae. While there was no ability to distinguish between the three groups of organisms by morphologic features, in situ hybridization may assist in rapidly distinguishing these organisms. Oligonucleotide DNA probes were directed against the 5S, 18S or 28S rRNA sequences of three groups of fungi with a high degree of specificity for each (8).

1.2 Amplification assays using the polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) amplifies single copies of fungal DNA millions of times over and allows for the detection of small amounts of target DNA in clinical specimens (15). In this technique, a sample is incubated with two short DNA oligomers, termed primers, that are complementary to the ends of a known genetic sequence within the total DNA, a heat-stable DNA polymerase (Taq or other polymerase obtained from thermophylic bacteria), nucleotids and buffers. The oligomers hybridize to the appropriate sequence of DNA, and act as primers for the polymerase, which copies that segment of the DNA (15). The sample is than heated to 95ºC to denature the DNA (separating the strands of the double helix) and cooled to anywhere from 42-75ºC. This allows the primers to bind (anneal) to their complementary sequence in the template DNA. The reaction is then heated to 72 ºC, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotids onto the primer in a sequential mannee, using the target DNA as a template. These steps are repeated many (20 to 40) times to amplify the original DNA sequence in

an exponential manner. A target sequence can be amplified 1,000,000-fold in a few hours using this method (6).

Amplification assays using the PCR or similar methods allow for the detection of small amounts of target DNA in clinical specimens. Specific primers with or without specific probes have been used with or without specific probes have been used with some success. A few reviews (2, 3, 16-19) and several papers (20-23) summerize specific targets used and detection methods. Assays have been developped to detect DNA of Candida (24-26), Aspergillus (27, 28), Fusarium (29), Cryptococcus (30), Histoplasma (31), Blastomyces (32), Coccidioides (33), Mucormycetes (34), Paracoccidioides (35), *Penicillium marneffei* (36) and dermatophytes (37).

Quantitative real-time PCR can be used to quantitate the amount of DNA or RNA after it is converted to DNA by reverse transcriptase (6). Simply put, the more DNA in the sample, the faster new DNA is made in a PCR reaction, and the reaction kinetics are proportional to the amount of DNA (6). The proportion of double stranded DNA is measured by the increase in fluorescense of a molecule bound to the amplified double-strand DNA molecule or by other means (6). Results are available within one hour of testing and sensitivity is exquisite. This technology was used to develop assays for Aspergillus, Candida, and the zygomycetes (26). Real-time PCR was also used fort he identification of culture isolates of *Histoplasma capsulatum* (38). The Light Cycler may also be used to identify organisms to the species level using specific primers and probes (5).

3. MOLECULAR METHODS AVAILABLE FOR IDENTIFICATION OF ISOLATED FUNGI

Recent improvements in technology and the availability of whole genome sequences for many fungi have made DNA sequence-based methods useful for both research and clinical microbiology application (3). Today it is possible to obtain a sequence-based identification of an unknown fungus grown in culture. The choice of locus depends on the type of fungi studied and the level of identification required. In general, the conserved 18S or 28S regions are appropriate for analyses at the genus level and above, while the ITS regions and the variable D1/D2 domains are used for analyses at the clade or species level and below (38). Although the ITS region and the D1/D2 domains remain the most commonly sequenced fungal loci, they suffer from several disadvantages, including failur to distinguish closely related species due to few variable nucleotide sites. Alternate markers that have been evaluated as possible “universal loci” for use with a wide range of fungi include translation elongation factor 1 α (EF-1), ß-tubulin, and RPB2, the gene that codes second largest RNA polymerase subunit (3).

After the target locus is decided and a sequencing product is obtained, the next step is to compare DNA sequence of the unknown with DNA sequences in a database such as BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The species identification of the unknown isolate can be determined by comparison to the similarity scores of the database sequences. However, there can be problems in interpreting the results when the percentage similarity is lower (3). GenBank is the US National Institutes of Health (NIH) genetic sequence database, an annotated collection of all publicly available DNA sequences. The GenBank database is designed to provide and encourage access within the scientific community to the most up to date and comprehensive DNA sequence information.

4. MOLECULAR TYPING METHODS

In the past, phenotypes of fungi were determined by the morphology of the colonies when grown on specific media, by biochemical tests, by serology, by killer toxin susceptibility and by resistotyping. However, these systems did not show enough reproducibility, were often evcesively cumbersome, and above all, had limited discriminatory power.

Furthermore, some fungi, such as *Candida albicans, C. tropicalis, C. glabrata* and *Cryptococcus neoformans* can switch phenotype, thus rendering the phenotyping techniques unable to answer these questions (7). The use of molecular methods is important to investigate the epidemiology and environmental sources of fungi that infect immunocompromised patients and, in some instances, immunocompetent patients (5, 40).

The development of DNA-fingerprinting techniques has enabled us to compared the genomes of the strains and selected areas of the genome, named genetic markers (7). Several criteria have been proposed for assesing the discriminatory power of a fingerprinting method in determining genetic relatedness (7). Epidemiologic typing can determine whether or not organisms share the same DNA profile and this can be related to environmental isolates to determine the point source. Most of the studies have been related to isolates of Candida, Cryptococcus, Aspergillus and Fusarium. The use of molecular tools has allowed for the reduction of hospital-acquired infections and their spread (5).

Techniques based on restriction fragment lenght polymorphism (RFLP) with or without hybridization probes (Southern), PCR-based techniques, electrophoretic karyotyping (EK) and multilocus enzyme electrophoresis (MLEE) and the newers that are being developped for the fingerprinting of fungal strains (7).

4.1. Restriction fragment lenghth polymorphism (RFLP) or restriction enzyme analysis (REA)

Purified DNA is restricted by selected endonucleases, and the yielded fragments are separated in an agarose gel (7). This generates a banding pattern based on different fragment lenghts determined by the restriction sites identified by the particular endonuclease used. Variations among strains can ocur as a result of mutations in restriction site sequences. This technique had the advantage of being rapid, easy and inexpensive (7).

4.2. RFLP with hybridization

The fragments generated by RFLP, can be transferred to a membrane and hybridized by Southern blot with a probe that can recognize one or more fragments of the restricted DNA (7). In this manner, only certain fragments of the RFLP are selected for visualisation, thus ignoring the rDNA and the mitocondrial DNA, and increasing the resolution. The advantage of this method is that, if a probe is carefully selected, it can have high discriminatory power (7).

4.3. PCR-based techniques

These methods are similar to RFLP, because they evaluate DNA sequence variation in short regions, but instead of analyzing restriction endonuclease recognition sequences, they focus on PCR priming regions. This can prevent primer annealing and PCR amplification and can detect insertions and deletions in genome. The main advantage of this technique is that it is rapid, easy and relatively inexpensive (7).

PCR-based techniques have been broadly used to compare isolates of several fungal species, such as *C. albicans, C. tropicalis, C. glabrata, C. lusitaniae, C. dubliniensis, C. krusei* (41), *Aspergillus fumigatus* (42), *Cryptococcus neoformans* (43) and *Fusarium solani* (44).

Random amplified polymorphic DNA (RAPD) uses a random single primer of approximately ten bases. It is easily designed, technically simple and often detects variation among isolates that are invariant with RFLP analysis, with or without Southern blot hybridization (7).

Sequence-specific DNA polymorphism (SSDP) uses specific primers and PCR at higher stringency, thus avoiding the the drawback of the lack of reproducibility of the RAPD method (45).

Microsatellites are tandemly repeated stretches of two tos ix nucleotides that ocur between codifying regions of eukaryotic genomes, including fungi (42).

Microsatellite lenght polymorphism (MLP) uses specific primers for these sequences in order to amplify the microsatellite locus by PCR (46).

4.4. Electrophoretic karyotyping (EK)

In this technique, intact DNA molecules migrate through an agarose gel matrix under the influence of pulsed fields, which permits easy seperation of DNA molecules of several megabases. Chromosome-lenght polymorphism is evaluated by EK analysis, which uses electric fields of alternating orientation to move intact chromosomes through an agarose gel matrix. The analysis of chromosomal binding patterns, known as electrophoretic karyotypes, and the detection of karyotipic variations within the species. EK has been extensively used to fingerprint *C. albicans* and other Candida species. It has a moderate discriminatory power, however, shows good reproducibility (7).

4.5. Multilocus enzyme electrophoresis (MLEE)

MLEE evaluates the polymorphism of isoenzymes or alloenzymes of the isolates. Proteins from cell extracts are separated by electrophoresis under native conditions, and the enzymes are visualized by specific enzyme-staining procedures (7). The main advantage of this method is its high discriminatory power when a sufficient number of enzymes is evaluated, and the very low probability of homoplasy in clonal organisms (7).

4.6. Identification of single-nucleotid polymorphisms (SNPs)

SNPs are single-base variations at a unique physical location. A variety of techniques is available for SNP identification, such as confirmation-based polymorphism scanning single-strand confirmation polymorphism analysis (SSCP) (7).

4.7. DNA microarray genotyping

The DNA microarray is a hybridization-based genotyping technique that offers simultaneous analysis of many polymorphisms. High-density microarray (or DNA chips) are prepared by attaching hundreds of thousands of oligonucleotids to a solid silicon surface in an ordered array. Microarrays produce a very large amount of sensitive and accurate data, and can analyze a large number of polymorphisms at a time. However, at present, there are difficulties in the management and analysis of the high amount of data generated (7, 47).

4.8. DNA sequencing

DNA sequencing is the most accurate way to compare two strains by compairing the sequences of their genomes. This method displayed a high discriminatory power, and the ability to discriminate heterozygotes (46).

Another fingerprinting technique that uses sequencing of certain areas of the genome is multilocus sequence typing (MLST) which is similar in concept to MLEE, but it uses nucleotide sequence determination to identify the alleles of housekeepng genes. It is much faster and easier to perform since it is based on a PCR technique and shows very good reproducibility (48, 49).

5. CONCLUSION

Considering the large number of fungi in the environment that are capable of causing human disease, it may be difficult to believe that molecular methods will replace conventional methods anytime soon. However, a limited number of fungi may be identified to genus and species using PCR and specific probes. Yeasts in blood cultures have been identified and most were species of Candida, Aspergillus, zygomycetes, dermatophytes, and several filamentous fungi may be identified using amplification and probes. Nucleic acid sequencing has been use with great success for the identification of fungi in culture (5).

Overall, molecular methods have and will continue to have a majör impact on the diagnosis

and appropriate treatment of fungal infection. Analytical parameters of these methods need to be standardized to optimize sensitivity and specificity and comparative studies need to be performed to determine which are best to use in the laboratory. Ideally tests should be as simple as possible to perform so that most clinical laboratories can use them. The most important element, clinical corelation, must be established for methods. If these criteria are met, most of the newly developped molecular-based tests will be available to all of the patients with fungal infection (5). They will be especially useful for non-culturable, slow growing, pleomorphic opportunistic fungi. The time required to achive the molecular skills is minimal compared to the time required to be trained as a classical mycologist. However, regarding the vast number of fungi exists in the environment which may infect particularly immunocompromized patients, a strong partnership between classical mycology and molecular biology is needed.

REFERENCES

1. Khot PD, Ko DL, Fredricks DN. Sequencing and analysis of fungal rRNA operons for development of broad-range fungal PCR analysis. Appl Envir Microbiol, 2009; 75(6): 1559-65.
2. Yeo SF, Wong B. Current status of nonculture methods for diagnosis of invasive fungal infections. Clin Microbiol Rev, 2002; 15(3): 465-84.
3. Balajee SA, Sigler L, Brandt ME. DNA and the classical way: identification of medically important molds in the 21st century. Med Mycol, 2007; 45(6): 475-90.
4. Stevens DA. Diagnosis of fungal infections: current status. J Antimicrob Chemother, 2002; 49 (Suppl S1): 11-9.
5. Roberts GD, Goodman NL. Laboratory diagnosis. In: Merz WG, Hay RJ. (Eds) Medical Mycology, ASM Press, London, 10th ed, 2005, pp. 82-96.
6. Murray PR, Rosenthal KS, Pfaller MA. Molecular diagnosis. In: Medical Microbiology, Elsevier, Philadelphia, 7th ed, 2013, pp. 25-8.
7. Gil-Lamaignere C, Roillides E, Hacker J, Müller FMC. Molecular typing for fungi- a critical review of the possibilities and limitations of currently and future methods. Clin Microbiol Infect, 2003; 9(3): 172-85.
8. Stone NR, Gorton RL, Barker K, Ramnarain P, Kibbler CC. Evaluation of PNA-FISH yeast traffic light for rapid identification of yeast directly from positive blood cultures and assessment of clinical impact. J Clin Microbiol, 2013, 51(4): 1301-2.
9. Hayden RT, Qian X, Roberts GD, Lloyd RV. In situ hybridization for the identification of yeastlike organisms in tissue section. Diagn Mol Pathol, 2001; 10(1): 15-23.

10. Hayden RT, Qian X, Procop GV, Roberts GD, Lloyd RV. In situ hybridization for the identification of filamentous fungi in tissue section. Diagn Mol Pathol, 2002; 11(2): 119-26.

11. Hayden RT, Isolato PA, Parret T, Walk DM, Qian X, Procop GV, et al. In situ hybridization for the detection of Aspergillus, Fusarium and Pseudallescheria species in tissue section. Diagn Mol Pathol, 2003; 12(1): 21-6.

12. Shinozaki M, Okubo Y, Sasai D, et al. Identification of Fusarium species in formalin-fixed and paraffin-embeded sections by in situ hybridization using peptide nucleic acid probes. J Clin Microbiol, 2011; 49(3): 808-13.

13. Montone KT. Differentiation Fusarium from Aspergillus species by colorimetric in situ hybridization in formaline-fixed, paraffin-embeded tissue sections using dual fluorogenic-labeled LNA probes. Am J Clin Pathol, 2009; 132(6): 866-70.

14. Perez-Jaffe LA, Lanza DC, Loevner LA, Kennedy DV, Montone KT. In situ hybridization for Aspergillus and Penicillium in allergic fungal sinusitis: a rapid means of speciating fungal pathogens in tissues. Laryngoscope, 1997; 107(2): 233-40.

15. Chen SCA, Catriona LH, Meyer W. A review of nucleic acid-based diagnostic tests for systemic mycoses with an emphasis on polymerase chain reaction-based assays. Med Mycol, 2002; 40(4): 333-57.

16. Walther G, Pawlowska J, Alastruey-Izquierdo A, et al. DNA barcoding in Mucorales: an inventory in biodiversity. Persoonia, 2013; 30: 11-47.

17. Atkins SD, Clark IM. Fungal molecular diagnostics: a mini review. J Appl Gen, 2007; 7(1): 3-5.

18. Hay RJ, Jones RM. New molecular tools in the diagnosis of superficial fungal infections. Clin Dermatol, 2010; 28(2): 190-6.

19. Kline BC, Sandhu GS, Roberts GD. Mycology with molecular probes. Rev Iberoam Mycol, 1997; 14(1): 4-5.

20. Campa D, Tavanti A, Gemignani F, et al. DNA microarray based on arrayed-primer extension technique for identification of pathogenic fungi responsible for invasive and superficial mycoses. J Clin Microbiol, 2008; 46(3): 909-15.

21. Miller MB, Tang Yi-Wei. Basic concepts of microarrays and potential applications in clinical microbiology. Clin Microbiol Rev, 2009; 22(4): 611-33.

22. Rickerts V, Khot PD, Myerson D, Ko DL, Lambrecht E, Fredricks DN. Comparison of quantitative real time PCR with sequencing and ribosomal RNA-FISH fort he identification of fungi in formalin fixed, paraffin embedded tissue specimens. BMC Infect Dis, 2011; 11: 202-13.

23. Huang A, Li J-W, Shen Z-Q, Wang X-W, Jin M. High-throughput identification of clinical pathogenic fungi by hybridization to an oligonucleotide microarray. J Clin Microbiol, 2006; 44(9): 3299-305.

24. Hays C, Duhamel C, Cattoir V, Bonhomme J. Rapid and accurate identification of species belonging to the Candida parapsilosis complex by real-time PCR and melting curve analysis. J Med Microbiol, 2011; 60(4): 477-80.

25. Mirhendi H, Bruun B, Schonheyder HC, et al. Molecular screening for Candida ortophysilosis and Candida metapsilosis among Danish Candida parapsilosis group blood culture isolates: proposal of a new RFLP profile for differentiation. J Med Microbiol, 2010; 59(4): 414-20.

26. Loeffler J, Henke N, Hebart H, et al. Quantification of fungal DNA by using fluorescense resonance energy transfer and the light cycler system. J Clin Microbiol, 2000; 38(2): 86-90.

27. Lass-Flörl C, Rath P, Niederwieser D, et al. Aspergillus terreus infections in haematological malignancies: molecular epidemiology suggests association with in-hospital plants. J Hosp Infect, 2000; 46(1): 31-5.

28. Ferns RB, Fletcher H, Bradley S, Mackinnon S, Hunt C, Tedder RS. The prospective evaluation of a nested polymerase chain reaction assay fort he early detection of Aspergillus infection in patients with leukemia or undergoing allograft treatment. Br J Haematol, 2002; 119(3): 720-5.

29. Hue FX, Huerre M, Rouffault MA, de Bievre C. Specific detection of Fusarium species in blood and tissues by a PCR technique. J Clin Microbiol, 1999; 37(8): 2434-8.

30. Hendolin PH, Paulin L, Koilula-Kahköla P, et al. Panfungal PCR and multiplex liquid hybridizatiom for detection of fungi in tissue specimens. J Clin Microbiol, 2000; 38(11): 4186-92.

31. Bialek R, Feucht A, Aepinus C, et al. Evaluation of two nested PCR assays for detection of Histoplasma capsulatum DNA in human tissue. J Clin Microbiol, 2002; 40(5): 1644-7.

32. Bialek R, Cirera AC, Herrmann T, Aepinus C, Schearn-Bocsler VI, Legendre AM. Nested PCR assays for detection of Blastomyces dermatitidis DNA in paraffin embedded canine tissue. J Clin Microbiol, 2003; 41(1): 205-8.

33. Sandhu GS, Kline BC, Stockman L, Roberts, G.D. Molecular probes for diagnosis of fungal infections. J Clin Microbiol, 1995; 33(11): 2913-9.

34. Bernal-Martinez L, Buitrago MJ, Castelli MV, Rodriguez-Tudela JL, Cuenca-Estrella M. Development of a single tube multiplex realtime PCR to detect the most clinically relevant Mucormycetes species. Clin Microbiol Infect, 2013; 19(1): 1-7.

35. Gomes GM, Cisalpino CS, Taborda CP, de Camargo ZP. PCR for diagnosis of paracoccidioidomycosis. J Clin Microbiol, 2000; 38(9): 3478-80.

36. Vanittanakom N, Vanittanakom P, et al. Rapid identification of Penicillium marneffei by PCR based detection of specific sequences on the rRNA gene. J Clin Microbiol, 2002; 40(5): 1739-42.

37. Gutzmer R, Mommert S, Küttler U, Werfel T, Kapp A. Rapid identification and differentiation of fungal DNA in dermatological specimens by LightCycler PCR. J Med Microbiol, 2004; 53(12): 1207-14.

38. Martagon-Villamil J, Sherestha N, Sholtis M, et al. Identification of Histoplasma capsulatum from culture extacts by real-time PCR. J Clin Microbiol, 2003; 41(3): 1295-8.

39. Kurtzman CP. Recognition of yeast species from gene sequence comparisons. Open Appl Inform J, 2011; 5(Suppl1-M4): 20-9.

40. Kiraz N, Akgün Y. Typing methods for Candida albicans strains. Turkish J Infect, 2000; 2: 297-306.

41. McCullough MJ, Clemons KV, Stevens DA. Molecular and phenotypic characterization of genotypic Candida albicans subgroups and comparison with Candida dubliniensis and Candida stellatoidea. J Clin Microbiol, 1999; 37(2): 417-21.

42. Anderson MJ, Gull K, Denning DW. Molecular typing by random amplification of polymorphic DNA and M13 southern hybridization of related paired isolates of Aspergillus fumigatus. J Clin Microbiol, 1996; 34(1): 87-93.

43. Cogliati M, Allaria M, Tortorano AM, Viviani, MA. Genotyping Cryptococcus neoformans var. neoformans with specific primers designed from PCR-fingerprinting bands sequenced using a modified PCR-based strategy. Med Mycol, 2000; 38(2): 97-103.

44. Anaissie EJ, Kuchar RT, Rex JH, et al. Fusariosis associated with pathogenic fusarium species colonization of a hospital water system: a new paradigm fort he epidemiology of opportunistic mold infections. Clin Infect Dis, 2001; 33(11): 1871-8.

45. Mondon P, Brenier MP, Symoens F, et al. Molecular typing of Aspergillus fumigatus strains by sequence- specific DNA primer (SSDP) analysis. FEMS Immunol Med Microbiol, 1997; 17(2): 95-107.

46. Botterel F, Desterke C, Costa C, Bretagne S. Analysis of microsatellite markers of Candida albicans used for rapid typing. J Clin Microbiol, 2001; 39(11): 4076-81.

47. Kurella M, Hsiao LL, Yoshida T, et al. DNA microarray analysis of complex biologic processes. J Am Soc Nephrol, 2001; 12(5): 1072-8.

48. Maiden MC, Bygraves JA, Feil E, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci USA, 1998; 95(6): 3140-5.

49. Feavers IM, Gray SJ, Urwin R, et al. Multilocus sequnce typing and antigene gene sequencing in the investigations of a meningococcal disease outbreak. J Clin Microbiol, 1999; 37(12): 3883-7.