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Review

Towards a nucleic acid-based diagnosis in clinical parasitology and mycology

Stéphane Bretagne [a,*], Jean-Marc Costa [a,b]

[a] *Laboratoire de Parasitologie-Mycologie and UMR BIPAR 956, Hôpital Henri Mondor, Créteil, and CNRMA Institut Pasteur Paris, France*
[b] *Laboratoire de Biologie Moléculaire, hôpital américain de Paris, Neuilly sur Seine, France*

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Abstract

Background: Multiple in-house polymerase chain reaction (PCR) assays for the diagnosis of parasitic and fungal diseases have been reported. Encouraging results have been published to anticipate or improve the diagnosis. However, the absence of standardized methods has led to discrepant results. As a consequence, these tests are not recognized as consensual diagnostic criteria.
Methods: The major breakthrough for improving the results of these methods is the emergence of real-time technologies. This markedly improves the reliability of the PCR results by dramatically decreasing the risk of false positive results due to PCR products carryover. Moreover, the quantitative results provided by these techniques allow to compare rapidly the efficiency of primers, probes, and DNA extraction. Therefore, one can expect a more consensual method to implement comparisons between laboratories. Automated DNA extraction should also be useful to achieve this goal. Whatever sophisticated technology is used, the meaning of detecting nucleic acids in a given clinical sample still needs to be defined. This requires well-designed studies with clinical consensual criteria and PCR techniques that are as similar as possible.
Conclusions: The development of real-time technology should improve our knowledge in order to give the clinicians informative clues for decision-making.

Keywords: Molecular diagnosis; Parasitic diseases; Fungal diseases

Contents

* Corresponding author. Laboratoire de Parasitologie-Mycologie, Hôpital Henri Mondor, 41 avenue du Maréchal De Lattre de Tassigny, 94000, Créteil, France.
E-mail address: stephane.bretagne@hmn.ap-hop-paris.fr (S. Bretagne).

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1. Introduction

Polymerase chain reaction (PCR) has entered into the microbiology laboratories to address a wide range of issues such as identification, genotyping, drug susceptibility, mutation detection, route of infection, taxonomy, and epidemiology of a given species. However, the main interest in routine laboratories lies in the detection of slow or nongrowing microorganisms or microorganisms requiring a huge work load or a considerable expertise. The reliability of PCR has increased to the point where it is now accepted as the standard method for detecting nucleic acids from some viruses and bacteria. We have not reached the same point for parasites and fungi.

This lack of confidence in parasitology and mycology can have several reasons. Among them are technical issues. The first step in increasing acceptance in routine laboratories is to have a more homogenous technique to compare its performance between different teams on the same patient populations. The advent of real-time PCR assays should reach this goal. The molecular diagnosis could then be objectively compared with conventional diagnostic tools such as microscopy, culture and/or antigen detection.

Other expected difficulties in implementing a routine molecular diagnosis arise from the diseases themselves. As several parasites and fungi are opportunistic, the detection of DNA is not systematically synonymous with overt disease. Therefore, there is a need for a close dialogue between clinicians and biologists to validate the technique for routine diagnosis.

2. Limits of the conventional PCR assays

The main reason of the poor acceptance of PCR in parasitology and mycology lies in the absence of commercial kits with quality controls, as most of the microorganisms implicated are not considered of commercial interest. Therefore, all the current publications deal with in-house PCR assays. The sensitivity and the specificity of a PCR assay is highly dependent on every step of the amplification, including the type and the preparation of the sample, the DNA extraction method, the choice of the DNA target and the primers, the use of a hot-start method and an enzymatic prevention of contamination, and the means used to check the specificity of the amplified products [1,2]. Moreover, the reproducibility must be assumed with every clinical specimen from very different patients. The nested PCR assays, when the tubes are open between the two runs of amplifications, are particularly prone to contamination and should not be used for diagnosis. The very divergent sensitivity and specificity of reported PCR assays reported have lead to recommendations not to include PCR results in the diagnostic criteria, as exemplified by the consensual definitions of invasive fungal diseases [3].

3. Real-time technology

The lack of standardization has technical reasons which should be solved, at least in part, in future years by the advent of real-time detection of PCR products [4]. The detection of amplicon as the amplification progresses provides insight into the kinetics of the PCR. The role of each parameter in the reaction, from the nucleic acid extraction method to the last cycle through the hybridization of the primers and probes, can be evaluated by analyzing the amplification curves.

The absence of post-PCR processing after amplification has many advantages. With real-time PCR, the only source of contamination after sample preparation is pipetting the prepared specimen into the reaction mixture and loading the instrument. Therefore, the main cause of false positive reactions, i.e. the previously amplified products potentially aerosolized in the environment, is eliminated. Nevertheless, there is still a risk of breaking the tubes or capillaries during handling after amplification, hence contamination of the environment. This can be avoided by enzymatic prevention based on the substitution of dTTP for dUTP in the reagent mix and the use of the enzyme uracyl-DNA-glycosylase (UDG) in real-time PCR [5]. The simultaneous use of real-time PCR and enzymatic prevention increases result reliability. Moreover, all currently available real-time PCRs can give the results in <2 h, a requirement for timely clinical decision-making. Consequently, results of studies with classical PCR warrants confirmation by new studies using real-time PCR assays.

The real-time monitoring of accumulating amplicons in has been made possible by the labeling of primers, oligonucleotide probes or the amplicon itself with molecules capable of fluorescing. The simplest method employs SYBR Green dye that increases in fluorescencœ when bound to double strand DNA allowing quantification. The analysis of the melting curve is indicative of the nature of the amplified fragment. However, for diagnostic purposes, it is of utmost importance to check the specificity of the amplified products. This can be achieved by using specific hybridization probes.

False negative results, due to PCR inhibitors in the clinical samples, should also be taken into account in the procedure. This possibility can be recognized by co-