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PCR - A Promising Method for Detection of Fungi

Kavitha M K

Professor & HOD, Dept. of Medical Microbiology, CIHS, Thalassery

ABSTRACT

Early diagnosis of fungal infection is of importance as rapid initiation of antifungal therapy is crucial. In order to overcome the limitations of conventional diagnostic tests, molecular approaches are used for the detection and identification of pathogenic fungi. Methods employing polymerase chain reaction (PCR) are among the most promising alternatives and are increasingly applied in routine detection of pathogenic microorganisms. The ultimate sensitivity of PCR assay depends on the efficient lysis of fungal cells in the tissue sample and the purification of DNA that is free of PCR inhibitors. The target primer sequences must be unique in order to identify a specific organism. After implementation of the Real-time PCR test it is necessary to continue to monitor performance of the assay, equipment, reagents, and personnel. Daily quality control of reagents including positive and negative controls and/or extraction controls should be performed.
However, further studies are needed for standardization of such molecular procedures.

Key words: DNA extraction, PCR, RT-PCR.

1. INTRODUCTION

Early diagnosis of fungal infection is important as rapid initiation of antifungal therapy is crucial. Identification of yeasts cultured from various clinical specimens is critical for clinical laboratories. Nowadays, a large variety of *Candida* spp. identification methods are widely used, and they differ in principles. Generally yeast identification procedures are believed to start with a germ tube test, culture on cornmeal agar, carbohydrate fermentation and carbohydrate assimilation tests. It is well known that detection of growth patterns on cornmeal agar takes 24-72 h and sugar assimilation tests may take 72 h to two weeks. In order to facilitate rapid identification, several chromogenic substrates containing culture media also are increasingly becoming developed. These special media generate considerable interest as they give fungal colonies with varying colors and have the potential of improving identification of yeast, especially in mixed cultures. [1]

In order to overcome the limitations of conventional diagnostic tests, molecular approaches are useful for the detection and identification of pathogenic fungi. Methods employing polymerase chain reaction (PCR) are among the most promising alternatives and are increasingly applied in routine detection of pathogenic microorganisms. The greatest advantages of PCR are sensitivity, specificity and speed with results available within hours. Real-time PCR and microarray assays have been investigated for the detection and identification of pathogenic fungi. [2]

In general, PCR detection is completed in an hour or less, which is considered to be faster than conventional methods. Real-time PCR testing is considered to provide equivalent sensitivity and specificity as conventional PCR combined with Southern blot analysis. Since the nucleic acid amplification and detection steps are performed in the same closed vessel, the risk for release of amplified nucleic acids into the environment, and contamination of subsequent analyses, is

negligible compared with conventional PCR methods. Real-time PCR instrumentation requires considerably less hands-on time and testing is much simpler to perform than conventional PCR methods.

The combination of excellent sensitivity and specificity, low contamination risk, ease of performance and speed, has made Real-time PCR technology an attractive alternative to conventional culture-based testing methods used in the clinical microbiology for diagnosis of many infectious diseases. [3]

2. NUCLEIC ACID EXTRACTION

The ultimate sensitivity of PCR assay depends on the efficient lysis of fungal cells in the tissue sample and the purification of DNA that is free of PCR inhibitors. [4] As the DNA extraction process eliminates many interfering substances present in the biological material, it plays an important role in ensuring consistent test results. The availability of a rapid, low-cost, and reliable DNA extraction procedure for fungi would reduce the workload and the test turnaround time. [5] Hence, a critical pre analytical step for Real-time PCR assays is nucleic acid extraction. Extraction methods that work for one pathogen in a particular specimen type may not work for another pathogen in another specimen type. Extraction of clinical specimens can be accomplished either by manual or automated methods. [3]

Manual Extraction

Phenol-chloroform has been used successfully for the extraction of nucleic acids. However, phenol is a caustic and corrosive agent, and its use should be considered a safety hazard by clinical microbiology laboratories. Manual extraction is a laborious, time-consuming process which requires the undivided attention of the technologist performing this technique in order to ensure optimal results.

Automated Extraction

Automated extraction instruments are manufactured by a number of different companies, and vary in method, cost, and time requirements for extraction. Additionally, these instruments vary with specimen capacity per run and size. Automated extraction may be equivalent and in some instances superior to manual methods.

Automated extraction systems have certain inherent advantages over manual methods. Recovery of nucleic acids from automated instruments is consistent and reproducible. Automated extraction systems keep sample manipulation to a minimum, reducing the risk for cross contamination of samples. Many of the instruments are closed systems, further reducing the risk for contamination. Automated systems do not require constant attention, which permits personnel to perform other duties. Quality control monitoring is less intensive than that required for manual extraction.

While the benefits of automated extraction are considerable, there are potential drawbacks. It is most economical when instruments are fully loaded; therefore, a significant number of samples (50 to 100/day) should be processed in order to justify the capital investment required for these instruments. [3]

The choice of the most suitable technique is based on the criteria: target nucleic acid, source organism, starting material (tissue, leaf, seed, processed material, etc.), desired results (yield, purity, purification time required, etc.) and downstream application (PCR, cloning, labeling, blotting, RT-PCR etc.).

The first step of extraction of DNA from biological material requires the rupture of the cell and nucleus wall, inactivation of cellular nucleases and separation of the desired nucleic acid from cellular debris. The ideal lysis procedure must be rigorous enough to disrupt the complex starting material. Common lysis procedures include mechanical disruption (e.g. grinding),

Chemical treatment (e.g. detergent lysis) and enzymatic digestion (e.g. proteinase). [6]

3. TARGET NUCLEIC ACID SELECTION

The target primer sequences must be unique in order to identify a specific organism or an organism group, identify unique virulence genes or genes or mutations associated with antimicrobial resistance which can occur across strains or species. Moreover, the PCR primer must be able to identify with high efficiency and specificity the target primer sequences in the specimen of interest. A search for the intended primer sequence in a DNA database such as the National Center for Biotechnology Information (NCBI) database may reveal cross-reactivity. [3]

4. POSITIVE AND NEGATIVE CONTROLS

An acceptable positive control is pooled negative specimens spiked with whole organisms or if that is not available, a representative sample of the nucleic acid to be detected. The positive control should be at a concentration near the lower limit of detection of the assay.

A blank control such as water or buffer is often used as a negative control. [3]

5. IMPLEMENTATION OF REAL-TIME PCR TESTING IN THE CLINICAL MICROBIOLOGY LABORATORY

Quality Assurance

After implementation of the Real-time PCR test it is necessary to continue to monitor performance of the assay, equipment, reagents, and personnel. Daily quality control of reagents including positive and negative controls and/or extraction controls should be performed. Instrument performance should be assessed biannually, also as required by accrediting and regulatory agencies. Competency of personnel performing tests must also be evaluated. [3]

Facilities Requirements

A physical separation of processes, equipment, and reagents is recommended, to minimize the risk of specimen-to-specimen contamination. Four different work areas are suggested, including a reagent preparation area to prepare PCR master mix, a sample processing area where procedures, including nucleic acid extraction, occurs, a target loading area where the specimen is added to the PCR master mix in the reaction vessel, and an amplification area where thermo cycling and probe detection occurs.

The reagent preparation area should be kept free of all patient specimens and DNA extracts. Protocols for the sample preparation area should minimize the number of tubes that are simultaneously open. Each of the work areas should contain working materials, reagents, and pipetting devices. Reagents should be prepared and aliquoted into single use or small volumes. This ensures ease of use and less chance for contamination.

All working surfaces should be cleaned before and after use, with a reagent that destroys nucleic acid such as a 5% bleach solution. The manufacturer's recommendations should be followed for cleaning of instrument components, processing blocks, and other instrument surfaces and parts.

Gloves should be changed frequently, at least before beginning each of the separate tasks required in a work area and should always be changed if moving from one work area to another work area. The use of aerosol-resistant pipette tips and pipette tips long enough to prevent specimen contact with the pipetter aids in the prevention of specimen contamination. [3]

Personnel Requirements

Personnel should be trained in both the pre analytical and the analytical procedures. Well-written training materials, including training checklists and detailed standard operating procedures for each Real-time PCR test, should be available.

The training checklist serves to standardize the training of all personnel. Technologists are required to successfully complete a panel of unknown samples and perform the procedure under direct observation of the technical expert to ensure flawless manipulations throughout the procedure. They also are required to analyze a previous run of samples with a variety of unusual results. This allows them to perfect their skills manipulating the computer software associated with the Real-time PCR instrument and ensures consistent analysis and reporting of results.

The availability of resources for troubleshooting is a consideration when selecting a molecular platform for the clinical laboratory. Laboratory-developed tests require that the technical resources to resolve problems related to the assay are available within the laboratory. [3]

6. COSTS

The cost for Real-time PCR reagents is more than the cost for culture media used for traditional methods. However, costs for reagents and instrumentation may be obviated by savings in labor requirements in the laboratory and cost savings at the bedside due to higher sensitivity and more rapid turnaround time for results for Real-time PCR tests compared with traditional culture-based methods. Often the amount of labor required for performing Real-time PCR assays is considerably less than that required for culture based assays.

If a diagnosis can be provided sooner and more reliably, patients who require antimicrobial therapy will receive it sooner. Providing a negative result sooner can have important implications for the over prescription of antibiotics. More rapid results for bacterial identification and antimicrobial susceptibility can decrease the length of hospital stay for patients, decrease the mortality rate, and may result in cost savings. [3]

7. ISSUES TO CONCERN

One disadvantage of PCR technology is that it is sensitive. Even trace amounts of RNA or DNA contamination in the sample may produce misleading results. Another disadvantage is that the primers designed for PCR can only be used to identify the presence or absence of a known pathogen or gene. Another limitation is that sometimes the primers used for PCR can anneal to sequences that are similar, but not identical, to the target gene. [7]

8. CONCLUSION

A rapid and accurate identification of the pathogenic species of *Candida* is crucial for treatment. Conventional methods are laborious, time-consuming, and not reliable in identifying the broad spectrum of *Candida* species and usually require additional tests. Molecular strategies, such as PCR- have been used for providing more accurate results rapidly. Most of these methods could improve routine clinical laboratory identification of *Candida* species. However, further studies are needed to be done for standardization of such molecular procedures.

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