

The utility of molecular biology in the diagnosis of tuberculosis

FERNANDA CARVALHO DE QUEIROZ MELLO, JOSEANE FONSECA-COSTA

Since the 1950s, when the structure of deoxyribonucleic acid (DNA) was first described, a new branch of science - molecular biology - has appeared. Molecular biology techniques have proven to be useful tools for diagnosing infectious diseases through the identification of a specific gene sequence of a pathogenic agent that may be present in biological samples collected from patients. From early studies carried out in order to identify the *Mycobacterium tuberculosis* genome, the base sequences were discovered, thereby allowing us to determine the presence of *M. tuberculosis* through the identification of DNA or ribonucleic acid (RNA) sequences. In the presence of a known fragment of the *M. tuberculosis* genome, using a genetic probe (a sequence of a few complementary nucleotides of a specific sequence of the genome of the microorganism to be identified), the formation of duplexes of nucleic bases became possible, using base pairing in a process called hybridization. Probes were used in combination with enzymes or radioactive substances so that hybridization products could be identified. However, probes showed low sensitivity when directly applied to paucibacillary clinical samples, that is, with few copies of the *M. tuberculosis* DNA⁽¹⁾.

In 1983, Kary Mullis developed a technique that allowed the amplification of a sequence in very small quantities of genetic material from any organism. The new technique was dubbed polymerase chain reaction (PCR). In October of 1993, Kary Mullis was awarded the Nobel Prize in chemistry for having developed this process that allowed the artificial multiplication of DNA through repetitive cycles with the use of an enzyme, the DNA polymerase. The basic principle of the PCR technique is the amplification of a selected region of single-stranded or double-stranded DNA or RNA. The sample to be amplified, or target sequence, of a specific gene or gene portion, consists of a known

base sequence. This known sequence allows the synthesis of oligonucleotides that will become PCR primers. This technique copies in vitro DNA replication but in a small specific fragment. Initially, DNA is extracted from the microorganism present in the clinical specimen to be analyzed. Subsequently, a series of cyclic reactions are developed, each cycle consisting of three steps. The first, known as the denaturation step, consists of the dissociation of the double-stranded DNA into single-stranded DNA by heating. The second, known as the annealing step, consists of the annealing of the primer sequences to the complementary sequences of the target DNA. The third step is called the extension step and is developed through the polymerization of the new DNA strand with the help of the Taq polymerase enzyme. The entire process takes place in a thermocycler, which automatically makes the necessary changes in temperature for each reaction step. A DNA copy is synthesized from each primer, and the final product of the three steps is used as the substrate for new amplification reactions that occur sequentially. Therefore, exponential chain reactions occur so that new products of the specific DNA can be obtained⁽²⁾. Consequently, the target segment is amplified, generating multiple copies that can now be detected. Among the various detection techniques, agarose gel electrophoresis is one of the most common. Other techniques can also be used for the detection of the amplified material, and some, such as combining PCR with hybridization techniques for the detection of amplified products⁽³⁾, present higher detection sensitivity than does electrophoresis. Nucleic-acid amplification testing (NAT) methods other than the PCR were later created. These include transcription-mediated amplification, strand displacement amplification and ligase chain reaction⁽⁴⁾.

The need for rapid tests for the laboratory diagnosis of tuberculosis (TB) led to the development

of molecular methods for the detection and identification of *M. tuberculosis* directly from clinical samples or from isolated colonies from cultures.

Various NAT methods have been devised for the direct detection of the *M. tuberculosis* complex in clinical samples. Some consist of systems developed in research laboratories, manually operated and known as in-house systems, whereas others are already commercially available in the form of semi- or completely automated standardized kits: the AMTD test and its successor, the enhanced AMTD test, from Gen-Probe Inc (San Diego, CA, USA); the Amplicor and Cobas Amplicor tests (the latter being the completely automated version of the Amplicor kit) from Roche Molecular Systems (Branchburg, NJ, USA); the LC test (Abbot Laboratories, North Chicago, IL, USA); and the SDA test (Biosciences, Sparks, MD, USA). However, the FDA has approved only the AMTD, Amplicor and enhanced AMTD tests for use in respiratory samples with positive smear test results. We should also point out that the enhanced AMTD test was the only test approved for use in respiratory samples with negative smear test results. In addition, the enhanced AMTD test was the only kit whose sensitivity and specificity were systematically evaluated among HIV-positive patients, presenting a lower yield in such patients than that found in HIV-negative patients. In summation, these testing kits were approved only for routine use when there is clinical suspicion of pulmonary TB in adult patients not infected with HIV and who had not been previously treated within 12 months prior to the current event. Although, on average, these tests have presented high sensitivity (95%) and specificity (98%) in samples with positive smear tests, their yields are low⁽⁵⁾. In a meta-analysis on the role of the PCR technique in the diagnosis of pulmonary TB with negative sputum smear results, Sarmiento et al. concluded that the PCR technique does not consistently present sufficient accuracy to be routinely recommended for the diagnosis of paucibacillary pulmonary TB⁽⁶⁾.

Guidelines for the interpretation of the results of these tests have been established by the Centers for Disease Control and Prevention. If the results of smear testing and the NAT are both positive in the first sputum sample, the diagnosis of TB is confirmed. If the smear results are positive and the NAT is negative, a technical evaluation must

be carried out in order to determine if there are amplification inhibitors in the sample. If no inhibitors are detected, the diagnosis of nontuberculous mycobacteriosis is confirmed. If the sputum sample presents negative smear results and positive NAT, another sample must be analyzed. If both tests are then positive, the diagnosis of active pulmonary TB is confirmed. If both the smear testing and the NAT are negative, a new sputum sample must be submitted to amplification. If this sample tests negative, the diagnosis of "non-transmissible" pulmonary TB is made, although the final confirmation of the diagnosis must be based on a contextualized clinical decision⁽⁷⁾. We should emphasize that these tests are not applicable to routine treatment monitoring and are not intended to be used as a replacement for culture testing⁽⁵⁾. We should also point out that in-house testing has been described in our milieu, with promising results in samples with negative smear results. However, such tests are still under interlaboratory validation⁽⁸⁾.

The application of molecular biology through the use of the described genetic probes and combined with hybridization techniques, such as the AccuProbe[®] enzymatic system (Gen-Probe), which uses acridinium ester labeling to identify the hybridization product and thereby identify the mycobacteria grown in culture (using solid or liquid media, such as BACTEC[®] 460 or MGIT[®] 960), are alternatives to the biochemical tests for the identification of the species. The molecular biology techniques are rapid, are easy to use and do not require very sophisticated equipment. The Accuprobe system is a validated system that allows the identification of *M. tuberculosis* complex, *M. avium* complex, *M. avium*, *M. intracellulare*, *M. kansasii* and *M. gordonae* within a few hours. The importance of this application is highlighted in the Second Brazilian Guidelines for Tuberculosis, in which mycobacterium identification is recommended if there is suspicion of TB caused by atypical mycobacteria, especially in immunocompromised patients. However, because of their high cost and current phase of development, it was suggested that these molecular tests should be implemented only in referral laboratories⁽⁹⁾.

The study conducted by Spada et al.⁽¹⁰⁾ and published in the present issue is of significant relevance because it presents an example of the possible benefits of rapid identification of

mycobacteria and the feasibility of implementing this technique in a referral laboratory. Their findings take on added relevance in Brazil, where there are great numbers of immunocompromised patients under suspicion of having pulmonary TB, furthering the idea that such tests are recommended under these operational conditions. In addition, this study indicates the need to take the clinical and epidemiological context into consideration when interpreting the results of these tests. It also shows the importance of using the clinical and radiological evolution as a benchmark in the evaluation of molecular tests because of the well-known limitations of the traditional methodologies available. In conclusion, this study demonstrates the need for cost-effectiveness studies so that a strategic plan for TB control can be established, especially in terms of incorporating new diagnostic methodologies in referral laboratories.

FERNANDA CARVALHO DE QUEIROZ MELLO
Adjunct Professor at the Universidade
Federal do Rio de Janeiro
School of Medicine

JOSEANE FONSECA-COSTA
Doctoral Student in the Postgraduate course in Clinical Medicine
Department of Respiratory Sciences
Universidade Federal do Rio de Janeiro

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