[Frontiers in Bioscience 1, e72-77, 1 August 1996]

APPLICATION OF MOLECULAR BIOLOGY-BASED METHODS TO THE DIAGNOSIS OF INFECTIOUS DISEASES

Udo Reischl[1]

Institute of Medical Microbiology and Hygiene, University of Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany

TABLE OF CONTENTS

1. Abstract
2. Serodiagnosis of viral pathogens is improved by the availability of recombinant antigens.
3. PCR, the all-purpose tool in nucleic acid-based diagnostics.
4. The reliable and rapid diagnosis of tuberculosis is founded on a productive combination of traditional and modern methodologies.
5. Bacterial infection chains can be clarified with the help of DNA fingerprinting techniques.
6. The rapid and culture-independent assessment of antibiotic resistance is still a challenge which might be accessible on genomic level.
7. Future prospects
8. References

1. ABSTRACT

The basis for effective treatment and cure of a patient is the rapid diagnosis of the disease and its causative agent, which is founded on the analysis of the clinical symptoms coupled with laboratory tests. As we approach the 21st century, clinicians are becoming increasingly able to diagnose and treat diseases at the molecular level. The rapid development of new methods and techniques in the area of molecular biology has gained new insights into the genetic and structural features of a considerable number of human pathogens. These results obtained by intensive basic research are currently leading to improved diagnostic procedures.

Basically, there are four different possibilities for laboratory diagnosis of infections: 1. direct detection of the pathogens (*e.g.*, microscopy and/or culture), 2. detection of protein components of the pathogens with the help of specific antibodies (*e.g.*, antigen capture ELISA) 3. IgA-, IgM- and IgG-specific detection of antibodies directed against a given pathogen and changes in their corresponding titer, and as the most sensitive method, 4. specific detection of nucleic acids (*e.g.*, PCR) of the pathogens.

Here, the human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* are serving as examples to review the recent developments as well as the future perspectives in molecular biology-based laboratory diagnosis (Figure 1).

Received: 6/18/96; Accepted: 7/19/96

[1]To whom correspondence should be addressed at: Institute of Medical Microbiology and Hygiene, University of Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany, Tel #: ++49-941-944-6401; Fax: ++49-941-944-6402; E-mail: udo.reischl@klinik.uni-regensburg.de

2. SERODIAGNOSIS OF VIRAL PATHOGENS IS IMPROVED BY THE AVAILABILITY OF RECOMBINANT ANTIGENS.

Infection by the human immunodeficiency virus, for example, is routinely diagnosed by the identification of specific antibodies in the serum. ELISA (enzyme linked immunosorbent assay) is the most common test format for screening purposes (1).

First generation HIV tests are based on purified viral antigen preparations derived from virus culture (2, 3). Therefore, original test systems embody an appreciable rate of false-positive results, mainly due to the presence of residual cellular antigens which were incorporated into the virus particle during maturation. With the advent of "nucleic acid engineering" and recombinant technology, a number of strategies have been developed to produce recombinant proteins for diagnostic purposes. Several plasmid vectors and derivatives have been established for the high-level expression of foreign genes like recombinant antigens and antigenic peptides in prokaryotic (*e.g.*, *Escherichia coli*) and eukaryotic host microorganisms.

Once the genetic information for the antigenic viral protein of interest is introduced into the host cell with the help of a suitable expression vector, in addition to the proteins of the host microorganism, this foreign protein is also produced. Moreover, the production of recombinant antigens in undemanding bacteria like *E.coli* is usually a rapid,

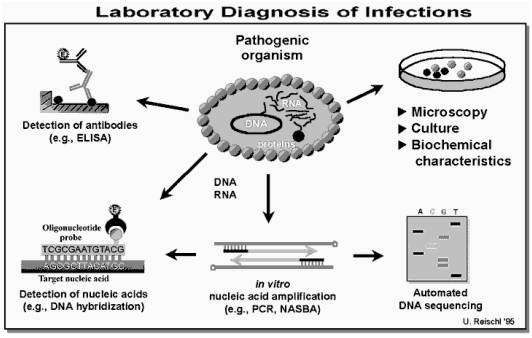

Figure 1: Modern laboratory diagnosis of infections is mainly based on refined traditional methods like microscopy and culture, specific detection by antibodies and, more recently, on the specific and high-sensitive detection methods for detection of nucleic acids.

cheap and simple alternative to the costly and hazardous virus culture associated with the production of native antigens.

Thus, HIV tests of the second or third generation are mainly based on recombinant antigens or synthetic peptides of selected immunodominant antigenic epitopes. Due to the absence of any contaminating cell-borne proteins, they are usually distinguished by an improved specificity and sensitivity (Figure 2).

Although such advanced antibody assays are reliable in most diagnostic situations, in certain selected cases, specific detection of nucleic acids turned out to be a valuable complement to the antibody detection method (4, 5). For example, in cases of primary HIV infection and, most importantly, in children born to HIV-positive mothers, an earlier diagnosis can be reached with the nucleic acid-based test assays (6).

3. PCR, THE ALL-PURPOSE TOOL IN NUCLEIC ACID-BASED DIAGNOSTICS.

Polymerase chain reaction (PCR), originally introduced by Saiki *et al.* (7) and subsequently automated by Mullis and Faloona (8), has emerged as a powerful tool in clinical medicine for the exponential *in vitro* amplification of specific sequences of interest from minute quantities of DNA or RNA and was rapidly applied to the diagnosis of pathogens in clinical material.

This method utilizes the essential portion of the cellular DNA replication machinery (DNA polymerase) in combination with two synthetic oligonucleotides (primers) to reproduce a specific

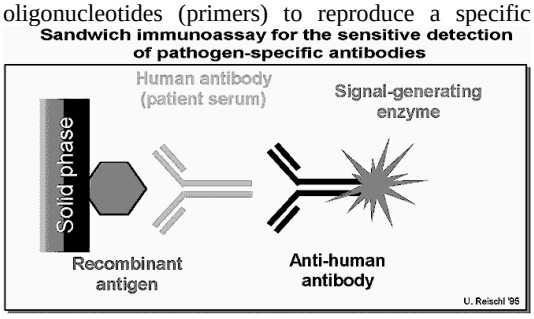

Figure 2: Recombinant antigens, usually accessible in high purity and large amounts, are improving the specificity of immunoassays like ELISA or Western-blotting.

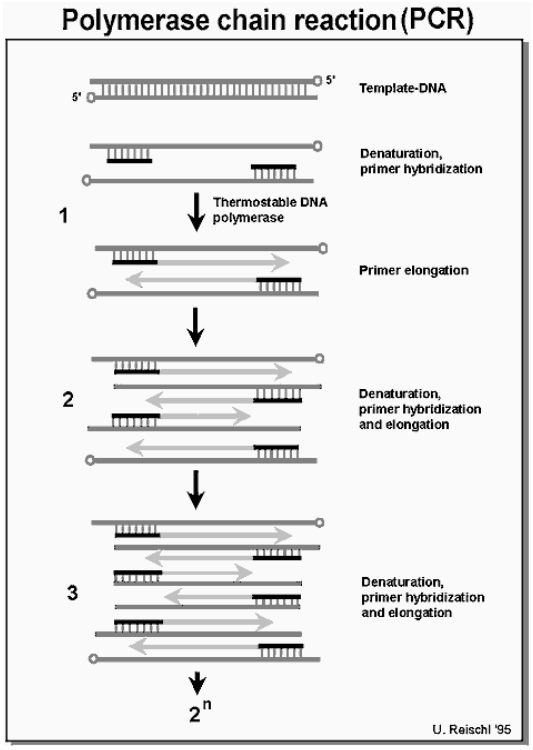

Figure 3: Schematic representation of the polymerase chain reaction (PCR).

target segment of the DNA *in vitro*. The reaction is cycled to produce an exponential increase in the target sequence yielding about 10^6 copies within a few hours (Figure 3). Due to its fundamental principle, PCR has been described as a technique that finds a genetic needle in a haystack and then builds its own haystack of needles. Because each species has DNA sequences that are unique, it is possible to select PCR-primers that specifically identify

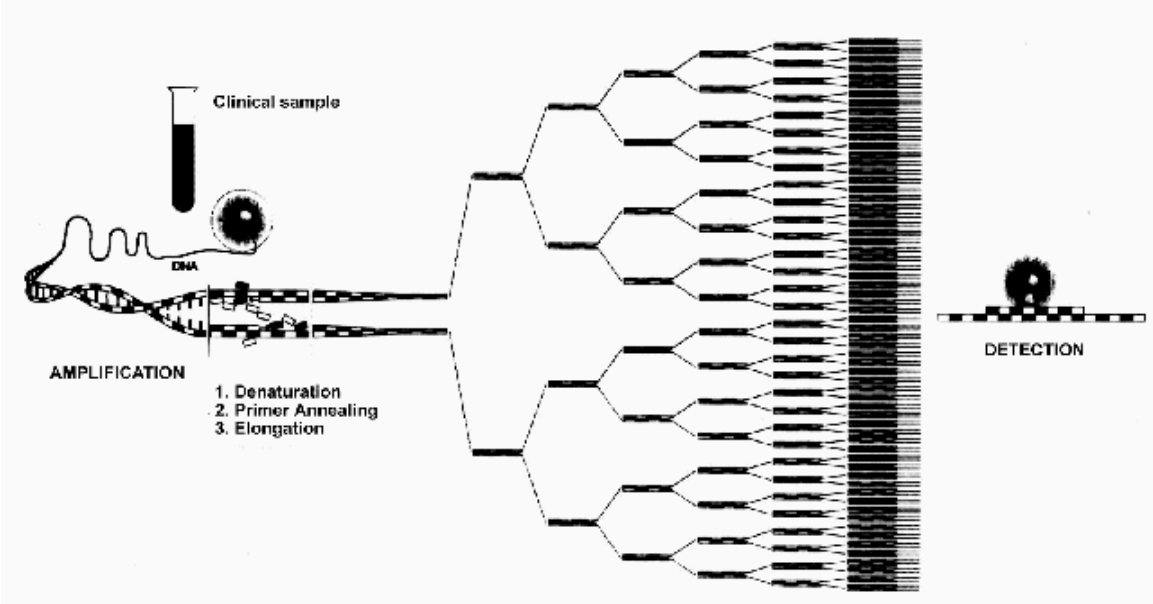

Figure 4: Illustration of the exponential nature of the amplification of target by PCR (reproduction with kind permission of Dr. Wehrle, Hoffmann-La Roche AG, Grenzach, Germany).

infectious agents like bacterial, viral, fungal, and parasitic microorganisms (see (9) for a comprehensive collection of laboratory protocols). Thus, presence of multiple microorganisms can be tested for in one clinical sample and results can be obtained usually within several hours (Figures 3 and 4).

4. THE RELIABLE AND RAPID DIAGNOSIS OF TUBERCULOSIS IS FOUNDED ON A PRODUCTIVE COMBINATION OF TRADITIONAL AND MODERN METHODOLOGIES.

Among the detection methods of bacterial pathogens, the laboratory diagnosis of mycobacterial infection is an outstanding example for highlighting some of the shortcomings of standard laboratory procedures. Despite a number of effective drugs like isoniazid, streptomycin, ethambutol, rifampin, or PZA, eradication of tuberculosis has not beeen achieved and, on the contrary, a resurgerence of tuberculosis has recently been observed (10, 11). According to the WHO, tuberculosis still kills 3 million individuals per year, making it the leading infectious cause of death (10). It is believed that one in every three individuals on the planet harbors the causative microorganism, *Mycobacterium tuberculosis*.

Classical culture and biochemical identification methods, when properly applied, detect *M. tuberculosis* in clinical samples, with reasonable sensitivity. However, primarily due to the slow growth of the bacteria, these methods usually require 4 to 8 weeks for completion (12). This results in numerous missed or delayed diagnoses, adversely affecting patient care and tuberculosis control and allows for the spread of infection. Although, more recently an automated radiometric system (BACTEC®) shortens the duration necessary for detection to 10 to 14 days, the only truly rapid and widely available diagnostic method is microscopy. Even with concentrated samples, a limited sensitivity (approximately 10^4 to 10^6 bacilli per ml) is observed, and *M. tuberculosis* can not be reliably differentiated from other mycobacteria in acid-fast stained smears.

Similar to the case of many other infectious diseases, nucleic acid amplification methods provide an alternative approach in the detection of microorganisms and thus offer new possibilities for a more rapid and accurate diagnosis of tuberculosis. As mentioned before, rather than to increase the number of microorganisms by culture, amplification methods, like PCR, directly increase the amount of nucleic acid target *in vitro* (13, 14).

Primers used in the amplification process are either species-specific or genus-specific and, once amplified, the characteristic nucleic acid sequences of mycobacteria can be rapidly identified. Using PCR, by direct determination of the nucleotide sequence within the amplification products, specific primers annealing to a well-conserved sequence but flanking a

hypervariable region of the 16S rRNA gene, even allow precise differentiation of mycobacteria at the species level (15).

Although the detection limit of purified DNA by PCR has been reported to be as low as 1 fg (equivalent to one-fifth of an organism), in clinical specimens such as sputum, the detection limit has varied from 10 to 1000 microorganisms (16). Due to the mycobacteria's waxy coat, which is quite resistant to simple disruption procedures, specimen processing is the most difficult aspect of assay development and probably is the limiting factor in the test sensitivity. It also should be emphasized that every microorganism has its own special peculiarites, which are presenting a strong challenge to applied research.

5. BACTERIAL INFECTION CHAINS CAN BE CLARIFIED WITH THE HELP OF DNA FINGERPRINTING TECHNIQUES.

Apart from the high-sensitive detection of bacterial pathogens, amplified DNA segments can be used for a variety of purposes. The number and position of repetitive DNA elements present in the genome of *M. tuberculosis*, for example, are representing strain-specific markers and the information has been used in epidemiologic studies of tuberculosis (17, 18, 19).

Epidemiologic studies are an important component of disease surveillance in defining the origin and spread of tuberculosis in the community, which can facilitate effective disease prevention and control measures. Bacterial strains can be either identified or fingerprinted by growing the microorganisms, extracting the genomic DNA, digesting the DNA with restriction enzymes, blotting and then probing the blot for the particular repetitive element (17). The resulting fingerprint patterns are also highly valuable markers for providing important insights into the global transmission, identification of strains with particular properties, such as high infectivity, high virulence, multi-drug resistantance, and in answering questions of a reactivation versus a reinfection of the disease (18, 19).

6. THE RAPID AND CULTURE-INDEPENDENT ASSESSMENT OF ANTIBIOTIC RESISTANCE IS STILL A CHALLENGE WHICH MIGHT BE ACCESSIBLE ON GENOMIC LEVEL.

The alarming trend of coinfection of TB with HIV, has been associated with the worsening of the urban and social conditions, and changes in immigration patterns. Widespread emergence of multidrug-resistant tuberculosis (MDR-TB), especially in institutional settings, has made it difficult to control the spread of TB (20). The emergence of multidrug-resistant strains has reduced the efficacy of treatment almost to the level of the pre-antibiotic era (21). Recent MDR-TB outbreaks have been characterized by mortality rates of 50 - 80% and a duration of only 4-16 weeks from the time of diagnosis to the time of death. Resistance to rifampin, for example, involves alterations of the RNA polymerase.

Table 1. Future prospects of PCR-based methodology

- Amplification of ribosomal RNA-copding genes in combination with DNA sequencing for a rapid and precise species- and strain-typing of bacteria
- Specific amplification of particular messenger RNA's to characterize the clinical state of viral infections (*e.g.*, differentiation between active and latent infections)
- Specific amplification of nucleic acids within the cellular compartments to localize pathogenic events (*in situ* PCR)
- Development of quantitative procedures to enhance the derived diagnostic expressiveness significantly

With the help of specific PCR primers and sequencing of the amplified DNA, a small region within the RNA polymerase subunit ss (rpoB) gene was identified which contributes to the high-level resistance to rifampin (22). These very promising findings provide the basis for a rapid and culture-independent detection of rifampin resistance, which, at the moment, has to serve as a MDR-TB surrogate marker. The primary mechanism for emergence of the multidrug-resistance in tuberculosis is the development of different mutations in the genes that the drug targets Therefore, a continued investigation of the sites of drug interactions and the molecular basis of drug resistance at the genomic level is of utmost importance. Application of molecular biology-based technology, however, will significantly contribute to the development of rapid and reliable diagnostic protocols and, as a consequence, the production of more effective antimycobacterial drugs.

The development of resistance is unlikely to be a problem peculiar to tuberculosis. Therefore, the constant mutations in infectious pathogens which confer resistance to the host of previously effective drugs and to the immunological surveillance is a major dilemma that we continuously face (table 1).

7. FUTURE PROSPECTS

Improvements in antibiotic and antiviral therapy for specific microorganims will require development of increasingly more accurate method of diagnosis. Nucleic acid-based technology has the

potential to become one of the most powerful tools in clinical microbiology since it essentially circumvents the necessity for the microorganism to be isolated in pure culture prior to its definitive identification.

PCR or other methods for the direct detection of pathogens can be used in conjunction with more traditional techniques and is likely that it will not completely replace microscopy, culture or antibody assays in the near future. Although amplification methods were established for an increasing number of infectious agents, conventional culture techniques for most bacterial pathogens are often just as sensitive, rapid, and inexpensive; they also allow detection of multiple microorganisms in a single procedure.

For infectious diseases, modern serological techniques, more and more based on recombinant antigens, are being proven to be specific, rapid and less labor intensive. The decision to use a nucleic acid-based method is likely to be dictated by the sensitivity and specificity of the respective *in vitro* nucleic acid amplification procedure as compared to the low-cost, time-proven conventional method, and the clinical need for definitive results.

Above all, PCR may prove very useful in clinical situations where conventional methods turned out to be either too insensitive (*e.g.*, during the asymptomatic stage of HIV infection), too slow (*e.g.*, mycobacterial culture) or too cumbersome to be used on a large scale (*e.g.*, virus isolation). Another important application of PCR is to monitor the emergence of mutations in the genome, for instance, the selection of resistant variants during antiviral/antibiotic therapy.

This can be achieved by identifying specific mutations directly by PCR or by determining the nucleotide sequence of a DNA fragment generated by PCR. The recently developed RT-PCR will allow sophisticated evaluation techniques for the evaluation of the effectiveness of antibiotic therapy and the molecular regulation of disease by the selective amplification of messenger RNA.

A number of unsettled questions, including the true clinical significance of PCR results and the associated costs, have delayed the wide adoption of these methods. However, in the face of the current problems in laboratory diagnosis of infectious diseases, it is apparent that the continued use of older and slower methods of detection is unacceptable. Especially in the case of severe infections, the diagnostic capabilities of molecular biology-based techniques will have major positive impact on health care costs, as well as on the associated morbidity and mortality. If, for example, generation of a report that a given patient is infected with an antibiotic-resistant pathogen, can help the physician to better tailor therapy to that person's needs. Undoubtedly, the next few years will show which of the competing methods, all embodying their individual strengths and weaknesses in terms of sensitivity, ease of use, costs and reliability, will prove successful in the clinical laboratory practice.

8. REFERENCES

1. W.F. Vann, A. Sutton & R. Schneerson: Enzyme-linked immunosorbent assay. *Meth Enzymol* 184, 537-41 (1990)

2. D.A. Cooper: Antibody response to human immunodeficiency virus after primary infection. *J Infect Dis* 155, 1113-8 (1987)

3. G.E. Tegtmeier: Current tests for serologic detection of HIV-infection. *J Clin Immunoassay* 11, 112-24 (1988)

4. A.J. Whetsell, J.B. Drew, G. Milman, R. Hoff, E.A. Dragon, K. Adler, J. Hui, P. Otto, P. Gupta & H. Farzadegan: Comparison of three nonradioisotopic polymerase chain reaction-based methods for detection of human immunodeficiency virus type 1. *J Clin Microbiol* 30, 845-53 (1992)

5. C.-Y. Ou, S. Kwok, S.W. Mitchell, D.H. Mack, J.J. Sninsky, J.W. Krebs, P. Feorino, D. Warfield & G. Schochetman: DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. *Science* 239, 295-7 (1988)

6. G. Scarlatti, V. Lombardi, A. Plebani, N. Principi, C. Vegni, G. Ferraris, A. Bucceri, E.M. Fenyo, H. Wigzell & P. Rossi: Polymerase chain reaction, virus isolation and antigen assay in HIV-1 antibody positive mothers and their children. *AIDS* 5, 1173-8 (1991)

7. R.K. Saiki, S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich & N. Arnheim: Enzymatic amplification of ss-globin sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350-4 (1985)

8. K.B. Mullis & F.A. Faloona: Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol* 155, 335-50 (1987)

9. D.H. Pershing, T.F. Smith, F.C. Tenover & T.J. White: *Diagnostic Molecular Microbiology - Principles and Applications.* American Society for Microbiology, Washington, D.C. (1993)

10. WHO Report: *TB - a global emergency.* World Health Organisation, Geneva, Switzerland (1993)

11. B.R. Bloom & C.J.L. Murray: Tuberculosis: commentary on a re-emergent killer. *Science* 257, 1055-63 (1992)

12. G.D. Roberts, E.W. Konemann & Y.K. Kim: Mycobacterium. In*:* A. Balows, W.J. Hausler Jr., K.L. Herrmann, H.D. Isenberg & H.J. Shadomy (eds.). *Manual of Clinical Microbiology. 5th ed.,* American Society for Microbiology, Washington, D.C., 304-39 (1991)

13. D.V. Cousins, S.D. Wilton, B.R. Francis & B.L. Gow: Use of polymerase chain reaction for rapid diagnosis of tuberculosis. *J Clin Microbiol* 30, 255-8 (1992)

14. J.T. Crawford: New technologies in the diagnosis of tuberculosis. *Sem Resp Infect* 9, 62-70 (1994)

15. B. Böddinghaus, T. Rogall, T. Flohr, H. Blöcker & E.C. Böttger: Detection and identification of mycobacteria by amplification of rRNA. *J Clin Microbiol* 28, 1751-9 (1990)

16. U. Reischl, M. Pulz, W. Ehret & H. Wolf: PCR-based detection of mycobacteria in sputum samples using a simple and reliable DNA extraction protocol. *BioTechniques* 17, 844-6 (1994)

17. D. van Soolingen, P.W.M. Hermans, P.E.W. de Haas, D.R. Soll & J.D.A. van Embden: Occurence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DMA polymorphism as a tool in the epidemiology of tuberculosis. *J Clin Microbiol* 29, 2578-86 (1991)

18. W.H. Haas, W.R. Butler, C.L. Woodley & J.T. Crawford: Mixed-linker polymerase chain reaction: a new method for rapid fingerprinting of isolates of the *Mycobacterium tuberculosis* complex. *J Clin Microbiol* 31, 1293-8 (1993)

19. M.D. Cave, K.D. Eisenach, G. Templeton, M. Salfinger, G. Mazurek, J.H. Bates & J.T. Crawford: Stability of DNA fingerprint pattern with IS6110 in strain of *Mycobacterium tuberculosis. J Clin Microbiol* 32, 262-6 (1994)

20. T.R. Frieden, T. Sterling, A. Pablos-Mendez, J.O. Kilburn, G.M. Cauthen & S.W. Dooley: The emergence of drug-resistant tuberculosis in New York City. *N Engl J Med* 328, 521-6 (1993)

21. B.R. Bloom: Tuberculosis - back to a frightening future. *Nature* 358, 538-9 (1992)

22. A. Telenti, P. Imboden, F. Marechesi, D. Lowrie, S. Cole, M.J. Colston, L. Matter, K. Schopfer & T. Bodmer: Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis. Lancet* 341, 647-50 (1993)