

## Molecular Approaches for Epidemiological Analysis of Infectious Diseases

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### Abstract

The continuing presence and burden of infectious agents still cause substantial illness and death in many parts of the world, particularly where periodic outbreaks of infectious diseases occur frequently. The potential spread of infectious diseases underscores the need for improved epidemiological surveillance, identification of genetic basis of infectious disease and documentation of the extent of genetic variations among infectious agents which exacerbate the burden of disease. These innovations have led to the development of DNA-based molecular methods.

Molecular methods are an improvement over conventional phenotypic methods for testing microbial relatedness in many ways. Currently, their most practical and useful application is in identifying outbreaks of infection, determining the mode and source of acquisition of a pathogen, recognition of a particularly virulent strain of microorganism, identifying relapse of infection due to a single strain versus separate episodes of infections, and in defining effective preventive and therapeutic measures.

The most commonly used molecular methods for epidemiological analysis of infectious agents are Pulsed-field gel electrophoresis (PFGE), ribotyping, plasmid profiling, and Polymerase Chain Reaction (PCR). These molecular methods allow comprehensive discrimination of the chromosomal DNA and have been applied to a wide range of organisms. Recently, PFGE which separates large DNA fragments after digestion with infrequently cutting restriction enzymes has proven to be a good approach for the molecular differentiation of individual strains of a variety of bacterial pathogens.

The data obtained from these molecular methods can be complimented with robust clinical and epidemiological data to create a database of characterized infectious agents for present and future epidemiological reference. These in turn will contribute to the global epidemiological surveillance database of infectious organisms.

### Key words

Epidemiological analysis, Infectious diseases, Molecular techniques

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### Introduction

Molecular techniques for identifying and detecting microorganisms in the clinical microbiology laboratory have become routine practice in most clinical settings. Molecular diagnosis is most appropriate for infectious agents that are difficult to detect, identify, or test for susceptibility by using conventional phenotypic methods. Phenotypic methods have occasionally been useful in describing the epidemiology of infectious diseases. However, they are too variable, slow, and some times labor-intensive. DNA-based typing methods have eliminated most of these limitations and are now commonly used for epidemiological studies<sup>1</sup>. Although technical issues such as ease of performance, reproducibility, sensitivity, and specificity of molecular tests are important, cost and potential contribution to patient care are also of concern<sup>3</sup>. Nucleic acid-based tests employed in diagnosing infectious diseases use standard methods for isolating nucleic acids from organisms and clinical material and restriction endonuclease enzymes, gel electrophoresis, and nucleic acid hybridization techniques to analyze DNA or RNA<sup>2</sup>. Because the target DNA or RNA may be present in very small amounts in clinical specimens, various target amplification techniques have been used to detect infectious agents in clinical diagnostic laboratories.

Commercial kits for the molecular detection and identification of infectious pathogens may also play an important role in molecular diagnostics in the clinical microbiology laboratory, but some clinically important pathogens require investigator-designed methods. The most commonly used molecular typing methods include plasmid profiling, restriction endonuclease analysis of plasmid and genomic DNA, Southern hybridization analysis using specific DNA probes, and chromosomal DNA profiling using either pulsed-field gel electrophoresis (PFGE) or PCR-based methods<sup>9</sup>.

Molecular typing is performed to determine whether different isolates give the same or different results for one or more tests. Epidemiologically related isolates share the same DNA profile or fingerprint, whereas sporadic or epidemiologically unrelated isolates have distinctly different patterns. If isolates from different patients share the same fingerprint, they probably originated from the same clone and were transmitted from patient to patient by a common source or mechanism. Molecular typing methods are also used as an approach for determining the transmission of multidrug-resistant infectious agents by analyzing the genetic diversity and by determining molecular basis of drug resistance among the Multi-Drug Resistant (MDR) isolates circulating in different regions of the world<sup>7</sup>. In addition, genotyping has

proven useful in guiding therapeutic decisions for certain viral pathogens and for epidemiologic investigation and infection control<sup>4</sup>.

### Molecular Techniques for Epidemiological Analysis of Infectious diseases

Genotypic techniques are those that involve direct DNA-based analysis of chromosomal or extra chromosomal genetic elements. Molecular typing methods are those that mainly focus on the identification of unique genotypes among bacterial strains which determine the strain diversity and monitor infectious outbreaks. There is no dominant method available for all of the pathogen categories of microorganisms like bacteria, fungi, and parasites. For example, Restriction Fragment Length Polymorphism (RFLP)-PFGE is the most commonly used method for *Streptococcus pneumoniae* and *Staphylococcus aureus*. RFLP with probe is the most common method for *Mycobacterium tuberculosis*. The same variability exists among fungal and parasite species. The reason for this variability may be the differences between the genomes of different species and different methods are effective for measuring different levels of relatedness<sup>38</sup>. Table 1 illustrates the examples of available methods and applications.

**Table 1: Genotypic methods for epidemiologic typing of microorganisms**

Method	Examples	Comments
Pulsed-field gel electrophoresis (PFGE)	<i>Enterobacteriaceae</i> <i>Staphylococci</i> <i>Enterococci</i> <i>Candida spp</i>	Fewer bands Amenable to computer analysis Very broad application
Polymerase Chain Reaction (PCR) based methods	<i>Enterobacteriaceae</i> <i>Acinetobacter spp</i> <i>Staphylococci</i> <i>M.tuberculosis</i> HCV	Crude extracts and small amount of DNA may suffice
Plasmid profile analysis	<i>Staphylococci</i> <i>Enterobacteriaceae</i>	Only useful when organisms have plasmid
Restriction enzyme analysis of DNA	<i>Enterococci</i> <i>Staphylococcus aureus</i> <i>Clostridium difficile</i> <i>Candida spp</i>	Large number of bands; difficult to interpret Not amenable to computer analysis
Restriction fragment length polymorphism (RFLP)analysis possible	<i>Enterobacteriaceae</i> <i>Staphylococci</i>	Produce fewer bands Computer analysis

**Note:** The table contains examples of available methods and applications and is not intended to be all-inclusive.

### Pulsed Field Gel Electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) is an electrophoretic technique designed to separate pieces of DNA up to 10 Mb in length<sup>27</sup>. The DNA is usually in the form of fragments resulting

from enzymatic digestion with diagnostic restriction endonuclease. PFGE, coupled with certain rare cutting restriction enzymes, enables a researcher to scan the entire bacterial chromosome for sequence polymorphisms based on the presence or absence of rare restriction sites<sup>11</sup>. Like RFLP analysis, PFGE relies on genomic digestions as input to be loaded on to the gels. However, these two different analytical procedures possess very different resolving capabilities. PFGE can successfully resolve significantly larger fragments than conventional electrophoresis by requiring the DNA fragments to alter their direction of migration through the gel. This in turn maintains a linear configuration of the DNA, allowing it to pass through the gel matrix in a constant and uniform fashion. This unique property of the technique has made it useful for a number of applications including large scale physical mapping of the bacterial genome, investigations into the size and shape of chromosomes and epidemiological approaches based on chromosomal digests<sup>27</sup>.

### General and technical considerations

The CHEF-DR (contour clamped homogeneous electric field) utilizes multiple electrodes, which are arranged in an hexagonal array and clamped to a series of predetermined electrical potentials. These electrical potentials are equal in strength to two electrodes that are arranged in parallel. Specific electrophoretic conditions will vary depending on the system being used, size fragments being investigated and the gel dimensions. In general and for the CHEF-DR-111 apparatus, field strength of 5-6 V/cm for 16-22 h at a temperature of 14° C has been employed. In addition, ramped pulse time gives optimal resolution of fragments of specific length.

There are a number of factors which can alter the resolution of DNA fragments when performing PFGE. These are:

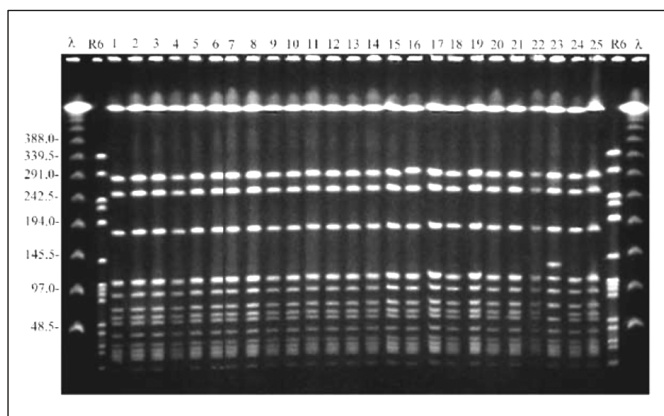
1. Running temperature
2. Angle that the electric field is applied
3. Pulse time
4. Primary and secondary DNA structures
5. Agarose concentration in the gel is one of the most significant extrinsic factor that may affect DNA fragment resolution. As the agarose concentration increases, fragment mobility decreases, but resolution is enhanced. The ability to resolve more lengthy fragments sharply decreases as agarose concentration increases.

### Genomic DNA preparation and gel run

Good quality genomic DNA is required for PFGE analysis. The rare cutting restriction enzymes employed with PFGE require DNA integrity substantially greater than for use in other enzymatic assays<sup>27</sup>. Since each enzyme may cut the chromosome in several places, it is imperative that the chromosome be intact and free from impurities prior to digestion. To protect the DNA from shearing of environmental forces, DNA is prepared in

agarose plugs made of the same agarose used to make the gel. The bacterial cells are suspended in agarose, and treated to remove the cell wall and other cellular debris. The DNA remains trapped within the agarose matrix of the plugs and are treated with rare-cutting restriction endonucleases, leaving fragments of chromosomal DNA. The plugs are introduced into the wells of the gel and electrophoresed under the desired conditions. Marker molecules are run alongside the experimental DNA as an indication of individual fragments size. Following electrophoresis the gel is stained with ethidium bromide, and viewed with a transilluminator. The gel is then analyzed using software and database of strain types can be built and stored.

In Pakistan, limited data of genetic diversity of infectious agents utilizing PFGE techniques is available. An example of MRSA study utilizing Pulse-field gel electrophoresis technique showed outbreak situation in Israel in 1997 (figure 1)<sup>20</sup>.



**Figure 1:** The gel includes 25 representative MRSA isolates. All isolates but one showed an indistinguishable banding pattern, thus representing the outbreak strain. Isolate number 23 shows a closely related pattern (one band difference) and is considered to belong to the outbreak strain<sup>20</sup>.

### Advantages

PFGE provides a highly reproducible restriction profile that typically shows distinct, well-resolved fragments representing the entire bacterial chromosome in a single gel. If present, genetic differences between isolates, insertions, deletions, or point mutations may be apparent on the PFGE profile.

### Limitations

Theoretically, all bacterial isolates are typeable by PFGE, while the process is technically more demanding than conventional restriction enzyme analysis and requires more expensive specialized equipment. Another limitation of this method is the time consuming aspect of DNA preparation. However, the discrimination between strains is much increased over other typing methods, both phenotypic and genotypic.

### Polymerase Chain Reaction (PCR) and Nucleic Acid-based Amplification Method

PCR is a technique which provides nucleic acid amplification,

and because of its flexibility and ease of performance it remains the most widely used molecular diagnostic technique in both research and clinical laboratories. PCR is used to amplify specific DNA sequences in a complex mixture when the ends of the sequence are known. Source DNA is denatured into single strands. The genomic DNA remains denatured because the complementary strands are at too low concentration to encounter each other during the period of incubation, but the specific oligonucleotides hybridize with their complementary sequences in the genomic DNA. The hybridized oligos then serve as primers for DNA synthesis, which begins upon addition of a supply of nucleotides and a temperature resistant polymerase such as Taq polymerase. Taq polymerase extends the primers at temperatures up to 72°C. When synthesis is complete, the whole mixture is heated further (to 95°C) to melt the newly formed duplexes. Repeated cycles (25–30) of synthesis (cooling) and melting (heating) quickly provide many DNA copies. Several different amplification-based strategies have been developed and are available commercially. Commercial amplification-based molecular diagnostic systems for infectious diseases have focused largely on systems for detecting *N. gonorrhoeae*, *C. trachomatis*, *M. tuberculosis*, and specific viral infections such as HBV, HCV, HIV, CMV, and Enterovirus. Numerous other infectious pathogens have been detected by investigator-developed or home-brewed PCR assays. In addition to qualitative detection of viruses, quantitation of viral load in clinical specimens is now recognized to be of great importance for the diagnosis, prognosis, and therapeutic monitoring for HCV, HIV, HBV, and CMV. None of the newer methods provides a level of sensitivity greater than that of PCR. Both PCR and nucleic acid strand-based amplification systems are available for quantitation of one or more viruses.

### MULTIPLEX PCR

Multiplex PCR is a variant of PCR which enables simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. Since its first description in 1988 by Chamberlain et al<sup>28</sup>, this method has been applied in many areas of DNA testing, including analyses of deletions, mutations, and polymorphisms, or quantitative assays and reverse transcription PCR. Typically, it is used for genotyping applications where simultaneous analysis of multiple markers is required, detection of pathogens or genetically modified organisms (GMOs), or for microsatellite analyses. Multiplex assays can be tedious and time-consuming to establish requiring lengthy optimization procedures.

### Plasmid analysis

Plasmid profile analysis is a useful DNA-based technique applied in epidemiological studies. Plasmids are extrachromosomal genetic elements which autonomously replicate double stranded DNA molecules and are distinct from the cellular chromosome. They carry genes that are not essential for host cell growth while chromosomes carries all the necessary

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genes. Plasmids can be lost spontaneously from or acquired readily by a host strain. Moreover, due to pressure for nosocomial organisms to develop antibiotic resistance and the frequency with which plasmids carry resistance determinants, such plasmids may spread rapidly from one strain to the next and can persist for prolonged periods within an institution.

Plasmids are isolated from clinical bacterial isolates and then separated electrophoretically on an agarose gel to determine their number and size. Further information can be obtained by digesting the plasmids with a restriction endonuclease and then comparing the number and size of the resulting restriction fragments. Technically, plasmid analysis is the simplest of the DNA-based methods and can be efficiently performed with basic electrophoretic equipment and commercially available reagents.

Plasmids may also be involved in the spread of virulence factors and may be selected for on that basis. For example, aerobactin, which encodes for iron uptake and may thereby confer a growth advantage to *E. coli* strains, is typically plasmid-associated among clinical isolates. Overall, plasmid analysis is most effective in studies that are restricted in terms of time and place, for example, those involving acute outbreaks within a single hospital.

The discriminatory power of this technique is poor for those strains that lack plasmids or possess only one or two.

### **Ribotyping**

Ribotyping has become a very common DNA-based technique in many laboratories. It refers to the use of Southern blot analysis to detect polymorphism associated with the ribosomal operons<sup>23</sup>. Ribosomal sequences are highly conserved and a probe derived from the *E. coli* ribosomal operon can be used with a wide range of bacterial species. All bacteria carry these operons and therefore are typeable. For organisms with multiple ribosomal operons, such as *E. coli*, *Klebsiella*, *Haemophilus* and *Staphylococcus*, ribotype patterns typically have 1-15 bands and provide moderate to good discriminatory powers. For *Mycobacteria*, which have only a single ribosomal operon, ribotyping detects only one or two bands and consequently is of limited utility.

In general, ribotypes are a relatively stable characteristic within a species, and epidemiologically unrelated isolates sometimes demonstrate the same pattern, therefore limiting the discriminatory power of the method. However, isolates of an outbreak-associated strain typically have identical ribotypes. Thus, this technique is useful in some respect.

### **Restriction Fragment Length Polymorphism (RFLP)**

Restriction Fragment Length Polymorphism (RFLP) is one of the earliest methods for DNA fingerprinting of infectious

microorganisms using restriction enzymes. This technique involves the generation of fragment patterns with restriction enzymes that cut a limited number of times per genome, combined with PFGE. The bacterial genome is usually composed of a single DNA molecule. These genomes can vary significantly in size and complexity but in almost all cases bacterial genomes are far less complex than lower eukaryotic genomes. Therefore, greater resolution is observed in RFLP pattern generated with a prokaryotic genome. When a frequent cutter is used, the ethidium bromide stained pattern is still crowded. Standard RFLP analysis without probes has therefore not been a popular epidemiological tool for bacteria. Instead, bacterial epidemiologists have used infrequent cutters to generate a limited number of DNA fragments, which are then separated by an electrophoretic method customized for large fragments<sup>11</sup>.

RFLP has been useful in limited epidemiological analysis of lower eukaryotic pathogens, but because of the composition of eukaryotic genomes, RFLP as presently applied has a few severe limitations as a DNA fingerprinting method.

### **Southern Blots**

Edwin Southern invented this method and optimized the procedure of transferring DNA fragments from agarose gels to nitrocellulose paper. Southern blot hybridization of endonuclease-digested DNA with a fingerprinting probe has been used to analyze both prokaryotic and eukaryotic pathogens. The success of this fingerprinting strategy has been mixed and detailed analysis of this strategy has revealed that the power and efficacy of the method depends on the selected hybridization probe. Some of the first probes used to DNA fingerprint the infectious fungi included single gene sequence, or mitochondrial DNA. It should be evident that a single gene probe will usually hybridize to only one band in a haploid organism and to one or two bands in a diploid organism. Although differences in size of the fragment carrying the gene may exist between isolates, the data are never sufficient for epidemiological studies that require measurements of moderate relatedness<sup>24</sup>.

### **Random Amplification of Polymorphic DNA (RAPD)**

RAPD represents one of the most frequently used methods for DNA finger printing of eukaryotic organism<sup>26</sup>. Earlier this method was thought to be highly effective in assessing relatedness at all levels of resolution. However, in contrast to RFLP, RFLP-PFGE, and RFLP with hybridization probe, the RAPD method is compromised by problems of reproducibility among laboratories. Using random primers of approximately 10 bases amplicons throughout the genome are amplified by PCR. The amplification products are separated on an agarose gel and visualized by ethidium bromide staining. Polymorphisms arise when the distance between primer hybridization sites change or when primer sites appear, disappear, or change location due to insertion, deletion or recombination. If a primer hybridizes to a large number of sites on opposing Crick-Watson

strands, that primer will generate a complex pattern. Because some minor bands may not be highly reproducible in repeat experiments in the same laboratory, only the major bands are usually used for analysis. This irreproducibility is probably the result of low annealing temperature and the slot primer sequences, which result in mispairing between primer and template. This produces a pattern with too low a complexity for many of the demands made in epidemiological studies. For that reason, several primers must often be used and the data are probed to obtain the necessary level of complexity for assessing genetic relatedness.

### Criteria for evaluating typing technique

A number of protocols now provide efficient, economical and safe identification and discrimination of bacterial strains in the molecular microbiology laboratory. With the recent advancement in DNA sequence analysis and hybridization techniques, most approaches are now performed completely free of radioactive labels. Several criteria should be considered in evaluating typing and identification systems. Table 2 summarizes the comparison of different commonly used molecular typing methods<sup>39</sup>.

**Table 2: Comparison of molecular typing methods considering evaluating criteria**

	PLASMID TYPING	RE ANALYSIS	PFGE	RIBO-TYPING	PCR TYPING
Strains Typeability	Most	All	All	All	All
Reproducibility	Good	Good	Excellent	Excellent	Good
Discriminatory Power	Good	Good	Excellent	Moderate	Good
Ease of Interpretation	Moderate	Difficult	Good	Moderate	Moderate
Ease of Performance	Moderate	Difficult	Good	Difficult	Moderate

The evaluating criteria are defined below:

### Typeability

Typeability refers to the ability to obtain an unambiguous, positive results for each isolate analyzed. Non-typeable isolates are those for which typing yields either a null or non-interpretable result.

### Reproducibility

Reproducibility refers to the ability of a technique to yield the same result when the same strain is tested repeatedly. This criterion may be affected either by day-to-day variation in the results of the particular method or by variation in the stability of the bacterial characteristic being examined.

### Discriminatory power

Discriminatory power refers to the ability to differentiate among unrelated strains.

### Stability

Stability is the ability of a typing system to recognize the colonial nature of strains despite the phenotypic or genomic variation that may occur. Stability is calculated as the number of tests in which the same strains were correctly identified as the same type on repeat testing/total number of tests.

There is currently no “gold standard” or defined typing system for epidemiological and molecular analysis of microorganisms. Almost any system will have sufficient reproducibility and discriminatory power to indicate that the outbreak stains are more similar to each other than to a few other random isolates. Rigorous evaluation of a method involves both analyzing adequate numbers of epidemic and sporadic isolates and comparing the results directly with those of previously well-studied approaches. In determining discriminatory power, it is particularly informative to test epidemiologically unrelated isolates that have proved to be indistinguishable by other techniques. Interpretations are most reliable if they are based on logical, objective, readily applied criteria. The ideal typing system should be rapid, inexpensive and simple<sup>27</sup>.

### Discussion

Infectious diseases are one of the leading causes of death worldwide. Five of the 10 leading causes of deaths are related to infectious diseases; Pneumonia, Chronic liver disease, Chronic obstructive lung disease, cancer and AIDS<sup>29</sup>.

The reasons for the increase in incidence of infectious diseases are not fully understood. Changes in human demographics and behavior such as migration and increasing use of day care facilities; technology and industry; economic development and land use; international travel and commerce; and break-down of public health measures, are thought to contribute to new and reemerging infectious diseases which were considered to have been controlled. For example, tuberculosis, malaria, and cholera have reemerged to spread geographically since 1973 in more virulent forms<sup>30</sup>. More than 30 new causative agents have been identified since 1973. *Escherichia coli* 0157, first identified in humans in the 1980s, have caused numerous out breaks and deaths associated with contaminated food and water. In 1993, a new Hantavirus caused deaths, associated with a pulmonary syndrome in the South Western United States<sup>31</sup>. In 1997, an avian strain of influenza began to kill previously healthy people in Hong Kong in 1999. The first known human case of West Nile Virus infection was recorded in the Western hemisphere. In 2000, a new hemorrhagic fever virus, the White water Arroyo virus, caused deaths in California<sup>32</sup>.

To control this situation an improved epidemiological surveillance

is needed especially in regions where periodic outbreaks of infectious diseases happen frequently. The development of rational control strategies for important human infectious disease, as well as the availability of detailed and accurate data related to molecular epidemiology of infectious agents is important for effective surveillance of infectious agents and in identifying the presence of virulent clones. This has led to the development of genotypically based molecular typing methods. Before the development of DNA-based techniques for assessing genetic relatedness, scientists relied on phenotypic techniques which only measure phenotypic characters rather than genetic differences. In discriminating between genera and species in both bacteria and fungi, biotyping still provides fast and reliable diagnostic methods. For example, kits measuring biochemical profiles are still proved to be useful for discriminating among a variety of bacterial and fungal species. In order to accurately identify the origin of a nosocomial infection, transmission of a disease between individuals, the emergence of a new drug resistant strain, the microevolution of an infecting strain, and the general population structure of a pathogen, one must move from species typing to strain and sub strain typing. In recent years, the choice of molecular methods for discriminating infectious agents in defined epidemiological settings has been well proven in numerous studies. For example, (1) PFGE, RAPD-PCR and plasmid profiling was able to discriminate multidrug-resistant *S. typhi* isolates from sensitive strains<sup>34,35</sup>. (2) PFGE, ribotyping and RAPD-PCR used in concordance were able to trace the source of a typhoid outbreak in Zurich to a restaurant worker<sup>33</sup>. (3) Analysis of *S. typhi* strains from sequential blood samples by PFGE, plasmid profiling and ribotyping was able to distinguish relapse from reinfection cases in individuals suffering from typhoid fever<sup>36</sup> (4) Shangkuan and Lin used RAPD-PCR to differentiate between vaccine strain *S. typhi* Ty 21a from the rest of the *S. typhi* strains studied<sup>37</sup>.

Finally, It has become apparent that molecular genetic techniques such as plasmid profile analysis, polymerase chain reaction (PCR), nucleic acid fingerprinting, PFGE and DNA sequence analysis of the chromosomes have allowed remarkable advantages in epidemiological analysis of infectious diseases and in many other areas of microbiology. In addition, sequencing of genes is a rapidly emerging tool for assessing epidemiological and molecular analysis.

### Conclusion

Epidemiological surveillance of infectious agents is needed for effective treatment strategies and for maintaining the useful life of the few remaining antimicrobials. Molecular methods are important in creating such effective epidemiological surveillance data which in turn could contribute in the prevention and control of infectious diseases. The challenge is to limit the spread of existing infectious diseases and to detect novel resistance phenotypes to prevent the spread of new resistant genes. To achieve this, appropriate diagnosis and therapeutic approach

are needed in regions where infectious diseases such as TB, pneumonia, hepatitis, and HIV are most common. The application of molecular techniques such as PCR and multiplex PCR are already possible. Modern technology such as DNA micro arrays which can simultaneously screen hundreds of genes needs to be introduced in the microbiological diagnostic labs.

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