Editorial

Molecular methods in medical microbiology: Current and future trends

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Introduction

Microbial diseases are the principal causes of morbidity and mortality in the third world and are presenting new problems in developed countries in the form of nosocomial and opportunistic infections (Hopkin and Wakefield, 1990). Recent development in the field of molecular diagnostic methods made convenient for the diagnosis of the diseases. It is the fast growing segment in diagnostic discipline of clinical, epidemiological, pathological, and pharmaceutical as well as research laboratories. The tools for molecular techniques have proven readily adaptable for use in the clinical diagnostic laboratory and promise to be extremely useful in diagnosis, therapy, and epidemiologic investigations and infection control. These methods may be an improvement over conventional microbiologic testing in many ways.

In molecular methods, primarily nucleic acid-based tests are used in diagnosing infectious diseases. Nucleic acid amplification techniques have come of age. The specific amplification and detection of oligonucleotide sequence have gone from the fictional to the mundane in the span of two decades. Applications, once limited to research laboratories, have shifted to the realm of routine commercially available clinical platforms that can be found in most of the hospital laboratories.

In these methods nucleic acids from organisms and clinical materials are extracted for the purpose. The target DNA or RNA may be present in very small amounts in clinical specimens, various signal amplification and target amplification techniques have been used to detect infectious agents in diagnostic laboratories. Therefore, now most clinicians and microbiologists enthusiastically welcome the new molecular tests for diagnosing infectious disease and research. Fortunately, commercial kits for the molecular detection and identification of infectious pathogens have provided a degree of standardization and ease of use that has facilitated the introduction of molecular diagnostics into the clinical and research laboratory of microbiology. It is well established now that these methods are easier, accurate, less time consuming, dependable and acceptable in most of the countries in the world Therefore, the editorial deals with an outline of recently used molecular techniques and their present applications in Medical Microbiology and future trends.

Commonly used molecular techniques

1. Polymerase Chain Reaction(PCR)
2. Reverse Trancriptase Polymerse Chain Reaction(RT-PCR)
3. Multiplex PCR
4. Real Time PCR

Some other amplification Techniques

1. Strand Displacement Amplification
2. Transcription Mediate Amplification
3. Nucleic acid sequence –based amplification
4. Ligase chain Reaction Nucleic acid amplification

Hybridization Techniques

1. Liquid phase Hybridization
2. Dot Blot and reverse Dot blot
3. Southern blot
4. Northern blot
5. Western blot

Others

1. Plasmid profiling
2. mol% G+C content
3. Restriction fragment length profiling (RFLP)
4. Pulse field Gel electrophoresis (PFGE)

Brief principles and images and of some techniques

Polymerase Chain Reaction (PCR)

It is the enzymatic process, is carried out in cycles. Each repeated cycles consists of DNA denaturation, primer annealing and extension of the prime DNA sequence. Each cycle theoretically doubles the amount of specific DNA sequence present and results in an exponential accumulation of the DNA fragment being amplified.

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Reverse Transcriptase polymerase chain reaction (RT-PCR)

RT-PCR uses a pair of primers that are complementary to a defined sequence on each of the two strands of the cloning DNA. The primers are then extended with DNA polymerase and a copy of the strand is made after each cycle, leading to exponential amplification. RT-PCR has three major steps. The first step is reverse trancription (RT) in which RNA is reverse transcribed to cDNA using enzyme reverse transcriptase. This step is important to perform PCR since DNA polymerase can act only on DNA template.

The RT step can be performed either in the same tube with PCR (one-step PCR) or in a separate one (two-step PCR) using a temperature between 40°C and 50°C, depending on the properties of the reverse transcriptase used. The next step is the denaturation of dsDNA at 95°C, so that the two strands separate and the primers can bind again at lower temperatures and begin a new chain reaction.

The final step of PCR amplification is DNA extension from the primers. This is done with Tag polymerase and PCR product is detected by gel electrophoresis.

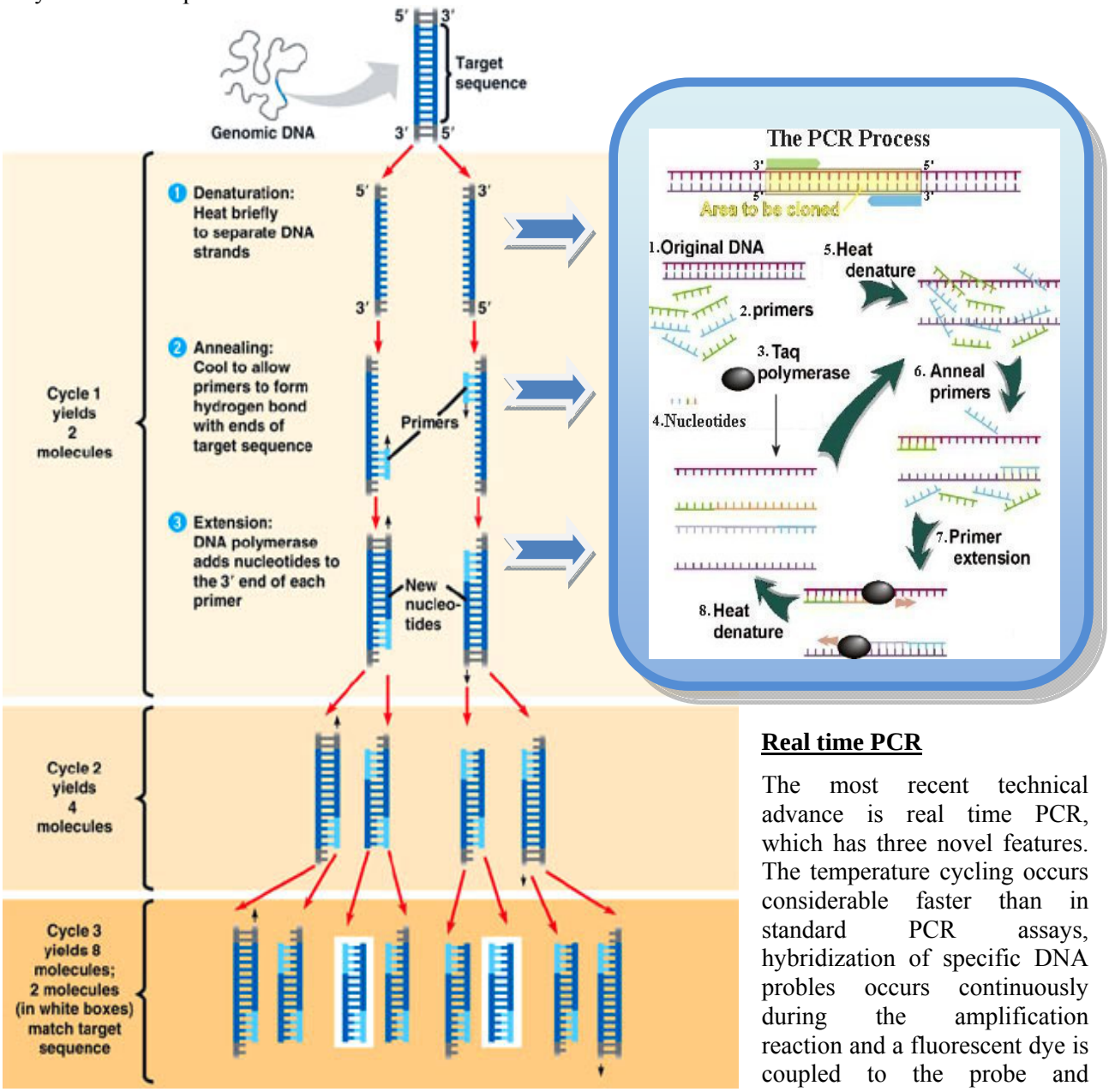


Figure 1: Polymerase Chain Reaction (PCR)

Real time PCR

The most recent technical advance is real time PCR, which has three novel features. The temperature cycling occurs considerable faster than in standard PCR assays, hybridization of specific DNA probles occurs continuously during the amplification reaction and a fluorescent dye is coupled to the probe and fluoresces only when hybridization takes place. No post PCR processing of amplified products is necessary. The production of amplified products is

observed automatically by continuous monitoring of fluorescence. Depending on the amount of target present, a small signal can be produced within 30 to 45 minutes. Since the tubes do not have to be opened during the reaction, the possibility of carryover contamination is considerably reduced.

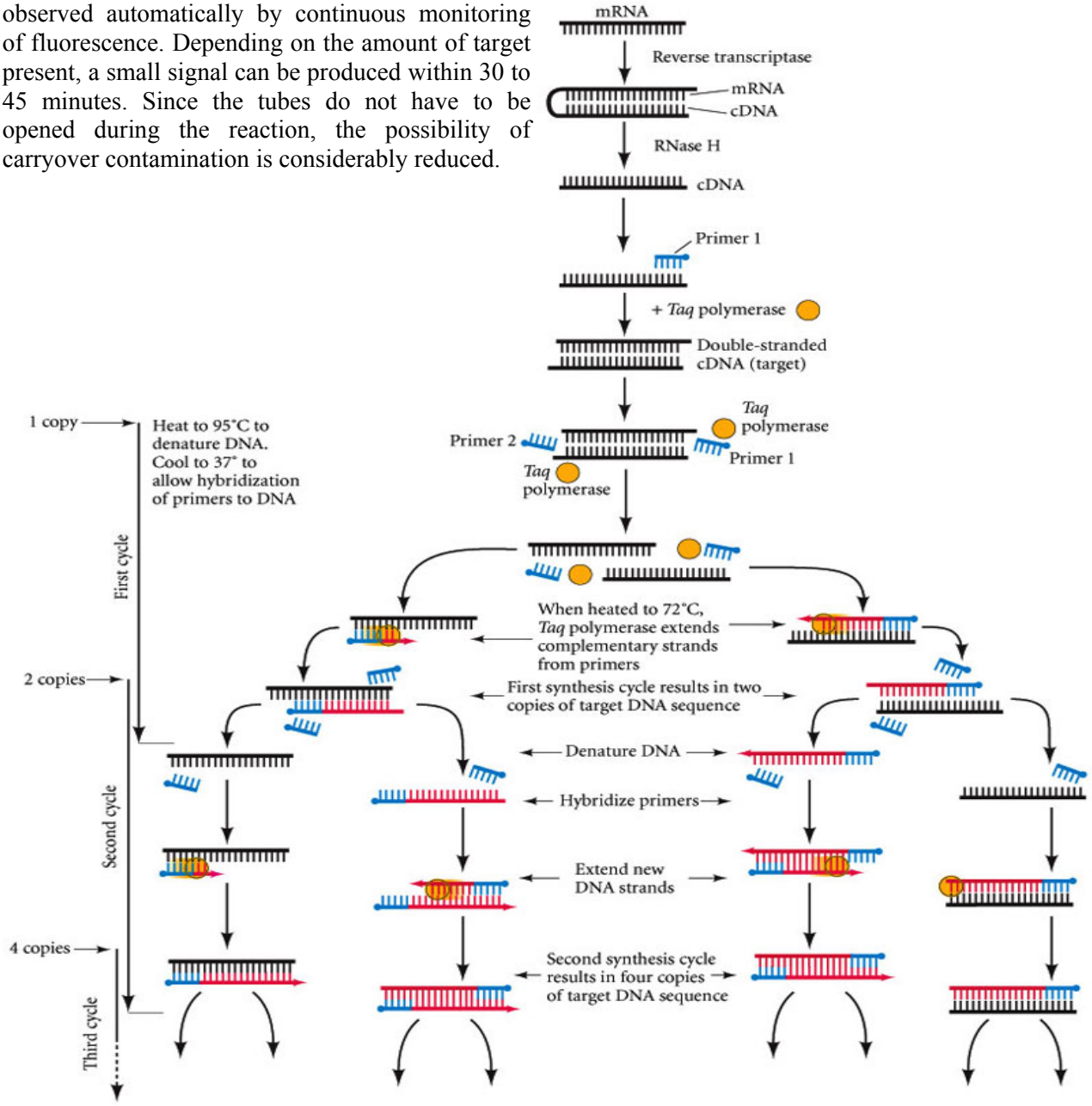


Figure 2: Reverse Transcriptase Polymerase Chain Reaction (RT- PCR)

Western blot

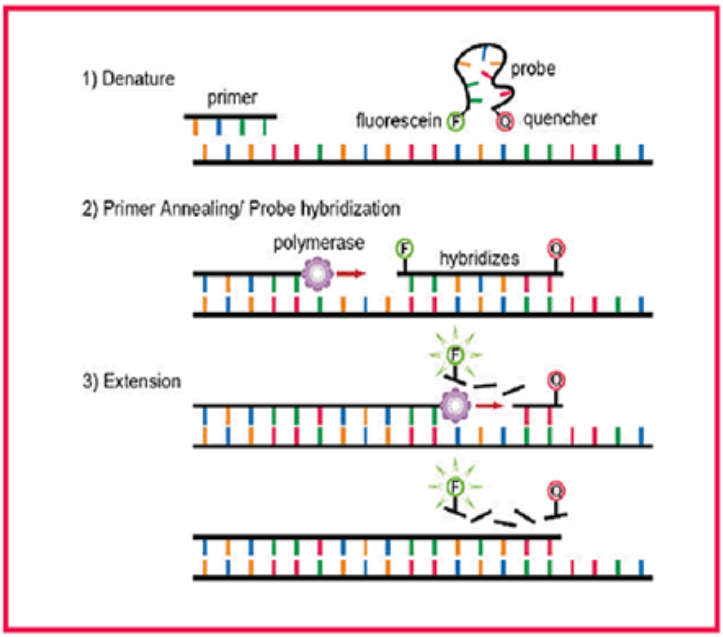
Western blot detects antibodies to specific epitopes of antigen subspecies. Electrophoresis of antigenic materials results in separation of the antigen components by molecular weight. Blotting the separated antigen to nitrocellulose, retaining the electrophoretic position and causing it to react with the patient specimen will result in the binding of specific antibodies, if present, to each antigenic band. Electrophoresis of known molecular weight allows for the determination of each antigenic band to which antibodies may be produced.

Micro-arrays

The latest evolution of molecular technique holding much promise in future is the detection of microorganisms through microarrays on DNA chips. Oligonucleotides are immobilized on flat surface, and genome fragments from an isolate or a clinical specimen after hybridization is detected by fluorescence. Diagnostic kits may be imagined for the detection of bacteria, viruses, fungi and drug resistant phenotypes.

Major application of molecular techniques in clinical microbiology (Rao, www.microrao.com)

1. Detection of pathogenic microorganisms in a mixture



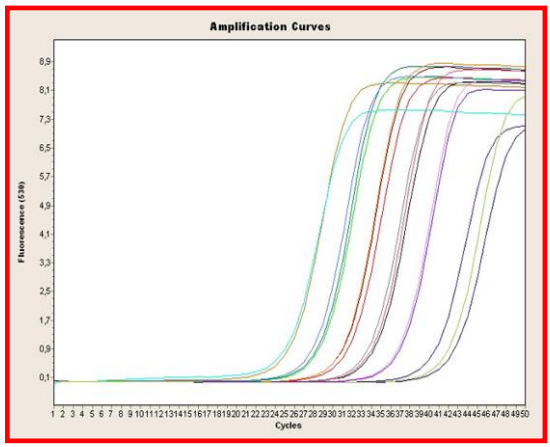


Figure 3: Real Time Polymerase Chain Reaction

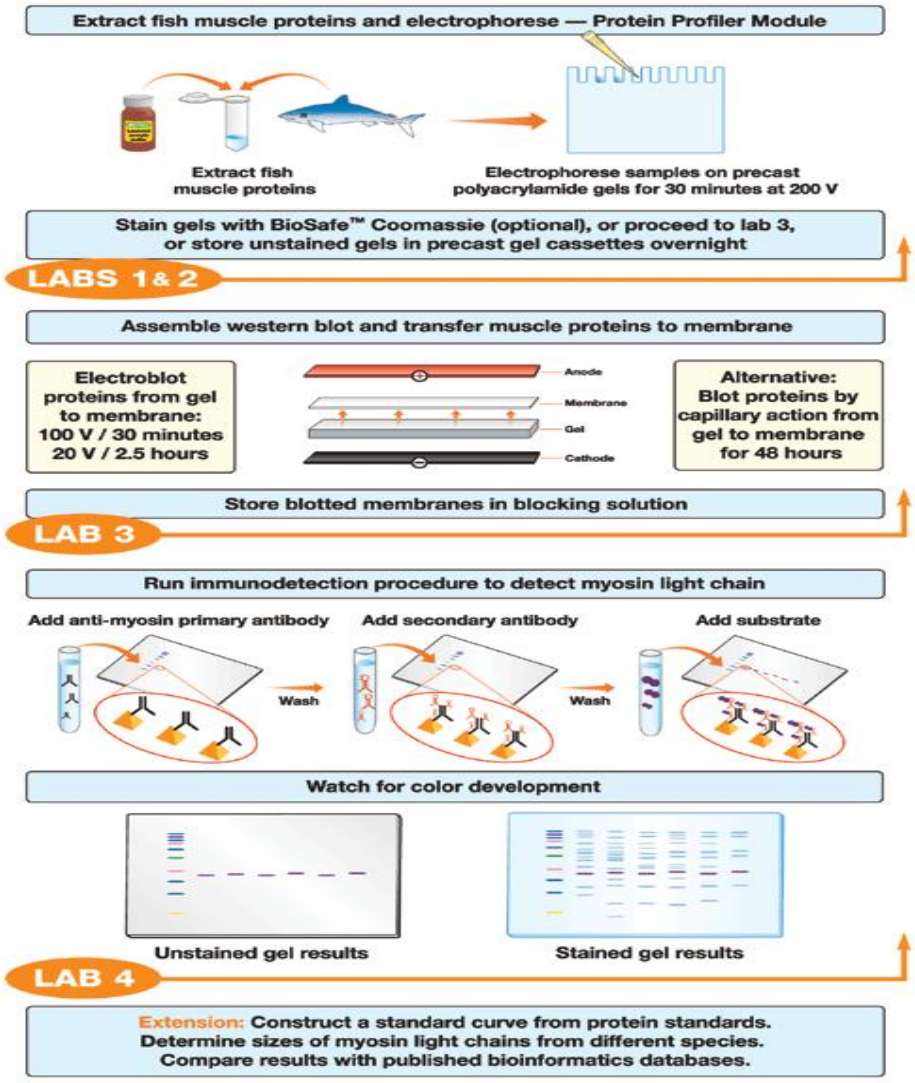


Figure 4: Western blot

2. Detection of organisms that have become non-viable
3. Detection of organisms that cannot be cultured or difficult to grow
4. Rapid detection of organisms that grow slowly
5. Detection of previously unknown (novel) organisms
6. Identification and classification of novel isolates
7. Quantitation of infectious agent burden; of significance in monitoring disease
8. Detection of antimicrobial resistance
9. Detection of organisms for which reliable diagnostic methods are not available
10. Characterization of microorganisms beyond identification
11. Investigation of strain relatedness for epidemiological typing

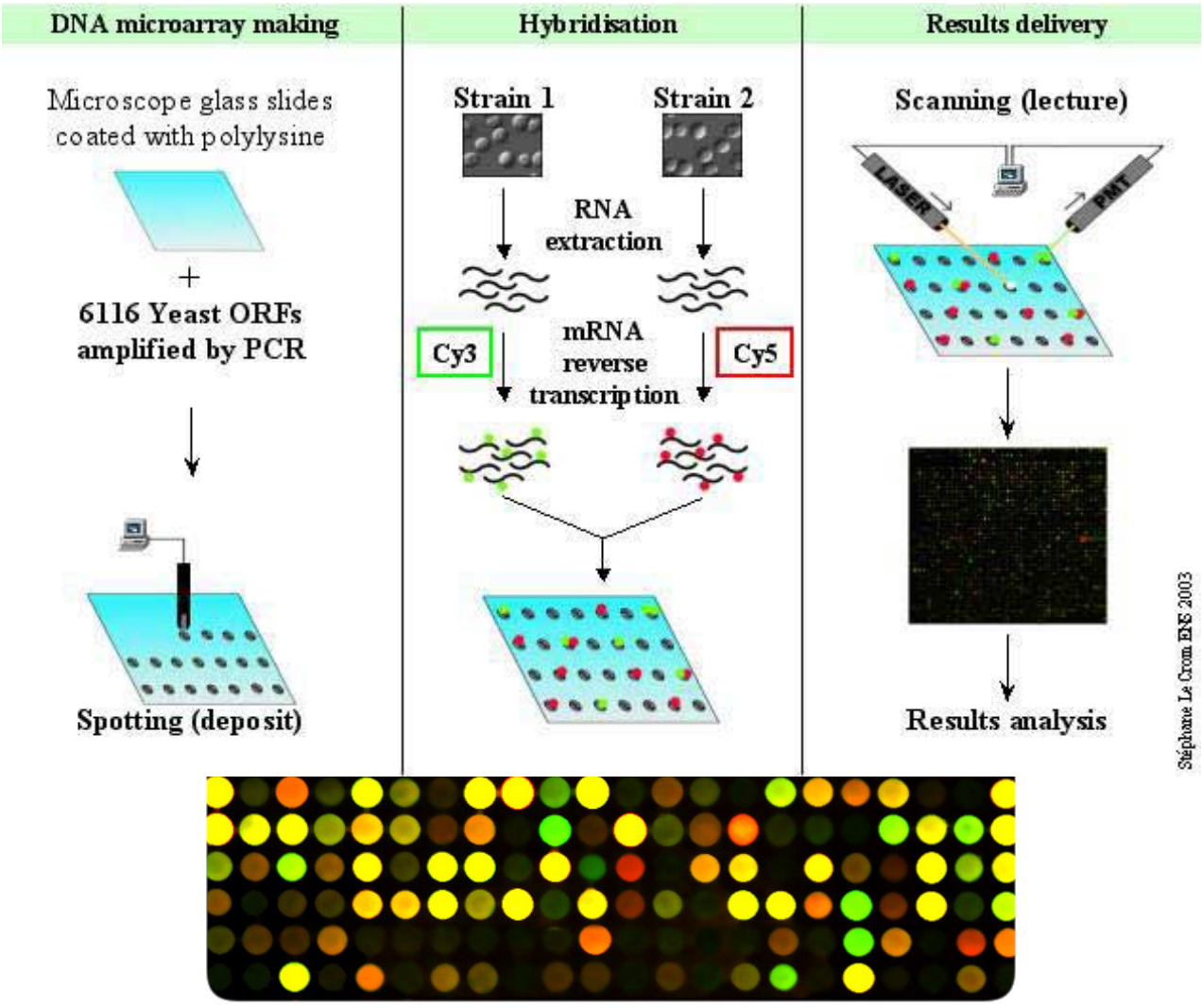


Figure 5: Micro array

Note: The figures above with the courtesy of different published articles and websites

12. Differentiation of toxigenic from non-toxigenic strains.
13. Detection of microbial virulence factors
14. Diagnosis in patients where serological markers are unreliable
15. Differentiation of pathogenic from non-pathogenic isolates
16. Detection of contaminating viruses in tissue culture
17. Synthesis of oligonucleotide probes in large numbers by PCR
18. Exact localization of virus infection or tumors in tissue by in-situ hybridization
19. Identification of etiology where multiple organisms can cause similar conditions
20. Detection of mutations and base pair changes
21. Differentiation of wild-type from vaccine strains
22. Diagnosis of congenital infections
23. Applications in HLA typing, anthropology, disputed paternity etc are some of the other applications.

Examples

Application of Molecular techniques in Clinical Microbiology

Viral agents	Molecular technique
Influenza A	Multiplex PCR
Influenza B	Multiplex PCR
Respiratory syncytial Virus	Multiplex PCR
Adenovirus	Multiplex PCR
Parainfluenza	Multiplex PCR
HIV	PCR / Real-Time PCR, Western blot
Hand foot and mouth disease virus	PCR

Viral agents	Molecular technique
Epstein Burr Virus	PCR
Dengue	RT-PCR
Chikungunya	RT-PCR
Varicella-zoster virus (VZV)	PCR
Herpes simplex	PCR

Bacterial agents	Molecular technique
Tuberculosis bacillus	Nested PCR
Bordetella pertussis	PCR
Legionella pneumophila	PCR
Chlamydia pneumonia	PCR
Mycoplasma pneumonia	PCR
Helicobacter pylori	PCR

Detecting antimicrobial resistance genes

Organism(s)	Antimicrobial agents	Gene	Detection method
Staphylococci	Methicillin, Oxacillin	*mec A*	PCR
Enterococci	Vancomycin	*Van A, B, C, D*	PCR

Future

Effective vaccine strain selection

It is well known to the clinicians that effective vaccine must have similar genomic nature against which the casual agent it is being vaccinated. This can only be achieved by molecular analysis of the vaccine strain as well as infecting stains. Sequence analysis must be performed before preparation of effective vaccine against which the vaccine is developed. We find many imported vaccines do not induce satisfactory immune response. It might be due to vaccine producing companies try to push sell of their vaccines without performing molecular analysis of the microbial agents against which vaccines are exported. It should be performed to have perfect potent vaccines. It has been observed from observations (author's personal experiences) that if vaccine strains sequence differs more than 20% from the circulating microbial agents it rarely protects the disease. In future it will be mandatory for the production of vaccine to show the results of sequence analysis in addition to potency of vaccine.

Combine amplification

A unified approach to amplification and detection is now emerging in the field molecular diagnostic techniques. The majority of new commercial and in-house diagnostic tests are going to do combine amplification with detection in the form of real-time PCR technology. This approach provides users with the benefit of increased sensitivity and specificity for the detection of several different pathogens or genetic variants through use of a single technology platform.

Fingerprinting

In future the use of these new molecular technologies will not be restricted to only detection and identification of microbial pathogens but also be used routinely for genotyping, allowing one to determine antibiotic resistance or to perform microbial fingerprinting.

Molecular Epidemiology

It has been used for the last decades in determining some epidemiological parameters instead of traditional epidemiology study where main parameters are based on agent, hosts and environments. Nucleic acid based molecular epidemiology study includes detection of agents based on PCR and then sequenced and finally alignments are made to draw phylogentic tree to determine origin, geographical distribution and migration of causal agents as that appropriate prevention measure may be undertaken.

Conclusions

Molecular methods are now included in "National Diagnostic Recommendation" in many countries. This explosion has been driven by the need for faster, accurate diagnostic tests, fuelled by the inception of the PCR twenty five years ago. The techniques are now well recognized as an essential component for the diagnosis of microbial diseases, research and infection control program. They play vital role for effective tracking the spread of nosocomial infections and streamlining the activities too. Effective use of the tools in molecular epidemiology study, vaccine strain determination and confirmatory diagnosis of bacterial, viral, fungal and parasitic diseases proved their importance for further development. Therefore impact of these methods on medical microbiology is undeniable. Indeed, microbiologists have been at the forefront of the molecular biology revolution that has had such a dramatic effect on understanding of science, clinical microbiology, epidemiology, drug resistance gene detection and characterization and over all infection control program.

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