Vol-1, Issue -3, 68-75.
Website: www.farmerstoday.org
e-magazine
ID: 01-03, 68-75 Oct-Nov-Dec 2024

FARMERS TODAY

Vol:1, Issue: 3 Oct-Nov-Dec 2024

OPEN ACCESS

Revolutionizing Pathogen Detection: Molecular Techniques and Their Applications

Imran Ali[1], Kaushal Kumar[1], Ritesh Patel[1], Alok Bharti[2] and Manoj Kumar Singh[3]
Department of Veterinary Pathology[1], Department of Animal Genetics and Breeding[2],
[3]Department of Veterinary Anatomy
Bihar Veterinary College, BASU, Patna

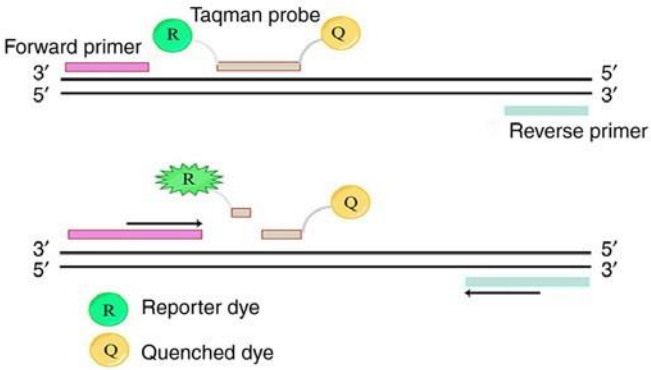
Introduction

Infectious diseases resulting from bacteria, viruses, parasites, and fungi account for the greatest proportion of death and illness globally. Data shows nearly 9.6 million people died in India every year, about 25 percent die from infections. Infectious diseases that break out are a major challenge to public health and the socio-economic structure of society. Infectious diseases can be reduced through vaccines and by the use of antimicrobial and antiviral medications but their efficacy decreases as new pathogens emerge or resistance to medications develops. Vaccines and medicines, as a rule, cannot be produced as fast as needed, due to the lengthy clinical trials, which slow down the effective suppression of infectious diseases. Since there have been no special vaccines and drugs created to cure these diseases, the fast and accurate identification of pathogens by proper detection methods is actually the best approach to the disease. It may also improve the treatment outcomes, slow the transmission of diseases and foster effective response regarding severe health emergencies. Some of the past diagnostic practices involve microbial culture, HI test and ELISA. Microbial culture is undoubtedly very time-consuming and morphological characteristics are used for identification of pathogen which reduces specificity and sensitivity of test. Immunological methods including HI and ELISA are easy to perform but have drawback like high false positive result, expensive and not heat stable. Modern molecular methods of diagnostics

involve the identification of nucleic acids, due to the progress in the field of genetics and genomics. These techniques provide relatively short time needed for sample analysis and high sensitivity – disease agents' detection and the presence of drug resistance genes and homology between different pathogens. Therefore, increased attention has been paid to molecular diagnostic techniques used for early diagnosis of infectious disease. PCR and Gene Chip Technology are the most recognized molecular diagnostics techniques in use today.

A. POLYMERASE CHAIN REACTION (PCR)

PCR discovered in 1985 and it has emerged as the most popular technique for amplification of nucleic acids for pathogens identification. PCR is very important in diagnosing infectious diseases in the initial stages. The applications of PCR have developed subtypes like quantitative PCR (qPCR) and digital PCR (dPCR) that work on the basic principle of conventional PCR (cPCR).



Principle of quantitative PCR.

qPCR: Real-time PCR or quantitative PCR or qPCR employ fluorescently labelled probes or double stranded DNA specific fluorescent dyes to qualify and quantify fluorescence signals of the amplification product without any need of additional gel electrophoresis of PCR products. Compared with the cPCR, this method is more automatically and less contaminated risk, qPCR has been widely used in early diagnose and detection of drug resistance in common clinical pathogens, has higher sensitivity, specificity, simple and fast compared with traditional diagnosis methods. While qPCR procedure is faster, more sensitive and precise over traditional diagnostic approaches, this method has a major drawback of high throughput as it is capable of analysing only a single pathogen per amplification reaction. Given these concerns, to achieve high throughput detection, the research community has advanced multiplex qPCR or (MqPCR). With different sets of primers and probes, MqPCR allows the identification of multiple pathogenic

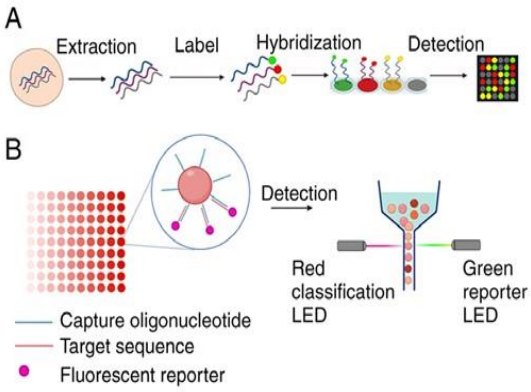
infections in one sample, as well as decreasing detection time, labour and reagent costs, as well as amount of sample consumption. MqPCR exhibits the high sensitivity and specificity comparable to the qPCR techniques. Recently Jiang *et al.* have successfully created an MqPCR assay that assayed nine respiratory contagions simultaneously with a minimum cross interference and an load of detection (LoD) in the range of 250-500 copies/ml or 1.25-2.5 copies/reaction that could be used in the early diagnosis of acute respiratory tract infections. However, owing to its low cost and well-established technology, qPCR continues to be a technique of choice for the quantitative detection of routine organisms in general laboratories. Despite its advantages, qPCR is limited by nucleic acid carryover, primer dimerization, inaccurate baseline determination and other problems that result in false positive results. That is why false negative results can occur due to sample inhibitors, enzyme inactivation, insufficient enzyme concentration, low template amounts or improper annealing temperatures. Moreover, qPCR is time consuming owing to the requirement of accurate ± 0.5˚C and rapid > 10˚C /s thermal cycle that are expensive and available only with specialised trained personnel which makes it difficult to use in areas or hospitals where there is lack of precision instruments.

dPCR: Digital PCR (dPCR) is based on the absolute quantification of target genes in samples due to the compartmentalisation of the amplification reaction into thousands of separate sections using microplates, capillaries, oil emulsions or microarrays. Every target gene is replicated in distinctive sections and the samples are analysed under different categories: negative droplets and positive droplets with respect to a particular fluorescent level. The target gene content is then obtained through dividing negative to positive droplets. This partitioned amplification militates against template competition, boosts the reaction's efficiency and enables dPCR to detect low levels of pathogens, small mutation and low frequency allele targets. There is a specific identification that dPCR can help identify and count number of viruses with sequence variation and low viral load in the sample such as BK polyomavirus, human rhinovirus (HRV) and human immunodeficiency virus (HIV). It can quickly detect smaller alterations in the DNA, for instance drugs resistance gene mutations of the influenza viruses and mutations that are present on the gene that codes for the Hepatitis C virus core protein. Compared to other molecular diagnostic methods, it can identify less than 0.1% of low prevalence mutation in background of wild-type virus. The primary strength of dPCR over qPCR is that it can obtain absolute quantification without utilising the standard

curve. That gives a specific advantage for quantitative studies associated with low-level pathogen detection during disease onset or after medication intake in the case of dPCR. However, the absolute count with the dPCR depends on the properly calibrated threshold that separates positive and negative droplets and this discrimination depends on factors such as sample quality and quantity, melting temperature and length of primers and probes. The main problem associated with dPCR is its high cost of the instruments and reagents required for its operations that result in expensive detection. Aditionally, the condition of droplet preparation in this equipment is open, which makes their contamination probable and their further influence may lead to the provision of false-active cells.

B. GENE CHIP TECHNOLOGY

Solid-phase and liquid-phase gene chips belong to traditional gene chip technology, which is based on the molecular hybridization of nucleic acids. It detect gene sequences in samples by using molecular hybridization between the target sequences and probes attach on different materials. This enables the identification of different pathogens at once and delivers to the clinician a rich pathogen data set in a timely manner. However, this technology demands large amounts of known pathogen genetic information and it can only be used for intentional screening of known pathogen genomes; it cannot identify new pathogens.



Principle of gene chip (A) Solid-phase chip (B) Liquid-phase chip

Solid-Phase Chip: In solid-phase microarrays, a set of specific probes immobilised on a solid phase interact with labelled target molecules in solution. This method can identify and quantify a large number pathogens at the same time, which means this method can shorten the time taken. It is a high throughput molecular diagnostic tool, ideal for detecting multiple pathogens of infectious diseases and for analysing drug resistance genes. Recent studies have shown the possibility to

detect resistance to rifampicin and isoniazid of MTB, as well as the presence of corresponding genes in 6 hours using the CapitalBio DNA chip, while more time-consuming bacterial culture and drug susceptibility tests are used currently. A number of automatic detection tools using solid-phase chips are available today. It consists of a fully automated sample preparation, nucleic acid extraction, PCR and automatic detection that is capable of detecting over 100 different nucleic acid targets at a time and that can identify several common respiratory pathogens from the same sample at an hour. This technique is particularly valuable for setting clinical diagnosis in the identification of the pathogen spectrum of a polymicrobial infection and also in epidemiological surveys of common infectious diseases. Despite its advantages, solid-phase chip technology have its its drawbacks: fluorescent oligonucleotides are rather expensive; and depending on the high variability of the pathogen, false negative results may be obtained by virtue of the fact that array design relies on reference sequence data.

Liquid-Phase Chip: Liquid phase suspension chip technology involves attaching of oligonucleotide probes to fluorescent microsphere with varying color patterns and uses laser detection and flow cytometry to automate the identification of sample internal colors. Applicable in gene expression analysis, microRNA analysis, single nucleotide polymorphism analysis, specific sequence analysis and microbial detection. In the diagnosis and genotyping of multiple pathogens in a single complicated sample, liquid chip technology is very sensitive, highly through and highly automated. This type of screening is ideal for big and widespread infection disease tests at entry and exit points. There is technology that has been accredited by the FDA for multiplexed detection of pathogens comprising of viruses, bacteria, parasites and fungi and that is Luminex xMAP® and using it, the diagnosis of diseases for instance through stools samples in this case can be rapidly made.

Table 1: Summery of application characteristics of molecular diagnostic techniques.

S. No.	Method	Principle	Applicability	Advantages	Limitations
1.	qPCR	Real-time monitoring of PCR progression was performed using	Routine quantitative detection of common pathogens.	Good specificity, high sensitivity, high degree of	Numerous interference factors, time consuming and

		fluorescence signal accumulation.		automation and low cost	instrument are expensive.
2.	dPCR	Amplification reactions were performed for individual nucleic acid molecules in a separate space.	Absolute quantification of low content pathogens.	Absolute quantification, low sample requirement and high tolerability.	High cost, limited through and complex operation.
3.	Solid-phase chip	Nucleic acid hybridization combined with fluorescence detection.	Large scale screening is required to determine the pathogen composition of mixed infections.	High throughput, high efficiency and fast integration	High cost and low sensitivity
4.	Liquid-phase chip	Nucleic acid hybridization combined with fluorescence detection and flow cytometry.	Large scale screening for influenza virus, respiratory syncytical virus and novel corona virus was carried out at entry and exit ports.	High through, high sensitivity, fast reaction and small amount of samples.	Cross reactivity

Table 2: Summery of Sample characterization of molecular diagnostic techniques.

S. No.	Method	Sample type	Sample Preparation	Sample Volume (µL)	Analysis time
1.	qPCR	Blood, Urine, Tissue samples and Faeces	Nucleic acid extraction and purification	200-300	2-3 Hours
2.	dPCR	Blood, Urine, Faeces, Tissue samples and Paraffin-embedded samples	Nucleic acid extraction, purification and microtitration	200-300	2-3 Hours
3.	Solid-phage chip	Blood, Urine, Tissue, Secretion and Faeces	Nucleic acid extraction and purification, PCR amplification and fluorescent labeling	200-300	4-6 Hours
4.	Liquid-phage chip	Blood, Urine, Tissue, Secretion and Faeces	Nucleic acid extraction and purification, PCR amplification and bead hybridization	200-300	35-60 minutes

CONCLUSION

The molecular diagnostic technologies are found to be more effective than the conventional techniques in diagnosing infectious diseases such as microbial culture, hemagglutination inhibition tests and ELISA. These molecular technologies when properly adopted for their specific requirements, among all can give precise information regarding the pathogen detection, leading to proper treatment and disease control. There are multiple proven techniques for detecting pathogens including conventional culture, PCR and more recently, qPCR, which is affordable for general use in standard laboratories to test for the presence or absence of the organisms along with others. Therefore, dPCR outperforms other PCR techniques in accurate quantification of target genes since it does not require the use of standard curves and can be used to quantify samples with low pathogen load or to detect small mutations and rare alleles. Gene chip technology can detect and identify the presence of more than one pathogen at a time, though very important in clinical practice for identification of the type of bacteria in polymicrobial infection. However, gene chips can test only known pathogens and cannot recognize the new and unidentified pathogens. Gene sequencing technology on the other hand offers integrated identification of both pathogen kinds as well as sequences. However, the molecular diagnostic techniques continue to encounter some difficulties even today. The nucleic acid extraction and purification typically laborious, hence there is a constant search for efficient protocols or methods that do not require extraction. Second, most molecular diagnostic reagents have shelf-lives that recommend low-temperature transport and storage, which adds costs and ultimately constrains molecular diagnostic availability to remote and often resourced areas. Further work on preparing ready to use reaction mixtures that are stable at room temperature is likely to help cut these costs and extend the availability of such reagents. Last but not least, the use of complex instruments for such methods as qPCR, dPCR and sequencing hinders provision of fast on-site pathogen detection in may be limited resources environments. Progress in molecular diagnostic tool is required in terms of throughput, automation, portability and sensitivity and specificity with which they can diagnose and treat infectious disease across the globe.

References

Gao Y. P., Huang K. J., Wang F. T., Hou Y. Y., Xu J. and Li G. (2022). Recent advances in biological detection with rolling circle amplification: Design strategy, biosensing mechanism, and practical applications. *Analyst.* **147:** 3396-3414.

Dien Bard J. and McElvania E. (2020). Panels and syndromic testing in clinical microbiology. *Clin. Lab. Med*. **40:** 393-420.

Zhu L., Ling J., Zhu Z., Tian T., Song Y. and Yang C. (2021). Selection and applications of functional nucleic acids for infectious disease detection and prevention. *Anal. Bioanal. Chem.* **413:** 4563-4579.

Huang H. S., Tsai C. L., Chang J., Hsu T. C., Lin S. and Lee C. C. (2018). Multiplex PCR system for the rapid diagnosis of respiratory virus infection: Systematic review and meta-analysis. *Clin. Microbiol. Infect.* **24:** 1055-1063.

Lv C., Deng W., Wang L., Qin Z., Zhou X. and Xu J. (2022). Molecular techniques as alternatives of diagnostic tools in china as schisto somiasis moving towards elimination. *Pathogens*. **11:** 287.

Lei S., Chen S. and Zhong Q. (2021). Digital PCR for accurate quantifica tion of pathogens: Principles, applications, challenges and future prospects. *Int. J. Biol. Macromol.* **184:** 750-759.