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Molecular Diagnostic Techniques of Infectious Diseases: An Overview*

Michael Baclig

College of Medical Technology, Trinity University of Asia, Quezon City, Philippines

ABSTRACT

Recent advancements in molecular techniques such as real-time PCR, isothermal amplification, next-generation sequencing, metagenomics, microarray, and CRISPR-infectious disease diagnostics have significantly evolved and improved over the past years. This overview will explore the innovations that have shaped the molecular diagnostics workflow, as well as the progress made in these innovative techniques. Additionally, it will address existing gaps, unmet needs, and the potential future directions for further enhancing diagnostic capabilities in the field.

Key words: molecular techniques, real-time PCR, isothermal amplification, next-generation sequencing, metagenomics, microarray, CRISPR-infectious-disease diagnostics

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Corresponding author: Michael O. Baclig, PhD
E-mail: mobaclig@tua.edu.ph
ORCiD: https://orcid.org/0000-0001-6733-3916

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INTRODUCTION

The World Health Organization (WHO) has reported that infectious diseases are emerging and spreading at an unprecedented rate, and that new infectious diseases are being discovered more frequently than ever before. In 2002, severe acute respiratory syndrome (SARS) emerged in China and spread to nearly 30 countries. SARS is caused by SARS-CoV-1, a strain of coronavirus that spreads through tiny infectious respiratory particles. In 2012, Middle East Respiratory Syndrome (MERS) emerged, causing a serious viral respiratory disease known as MERS-CoV. In 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which started as an outbreak in Wuhan, China, subsequently resulted in the COVID-19 pandemic.[1,2]

Infectious disease diagnostics is based on clinical presentation and laboratory findings using various techniques such as microscopy, culture, biochemical tests, to name a few. Over the past decades, we have witnessed the explosive growth of molecular diagnostics, as well as the availability of new assays to rapidly detect and characterize clinically important pathogens with a high degree of accuracy.[3] Indeed, molecular diagnostics of infectious diseases have evolved through the years and significantly advanced disease diagnostics by enabling the rapid identification of infectious disease pathogens.[4]

It is well recognized that the choice of molecular diagnostic techniques for infectious diseases depends on several factors including but are not limited to the target pathogen, turnaround time, clinical setting, and availability of resources. It is anticipated that as technology advances, newer techniques will emerge that are more suitable for the rapid identification of infectious disease pathogens, especially in resource-limited settings. Currently, the commonly used molecular diagnostic techniques for infectious disease include real-time PCR, isothermal amplification, next-generation sequencing, metagenomics, microarray, and CRISPR-infectious-disease diagnostics. A timeline of methodological discoveries is shown in Figure 1.

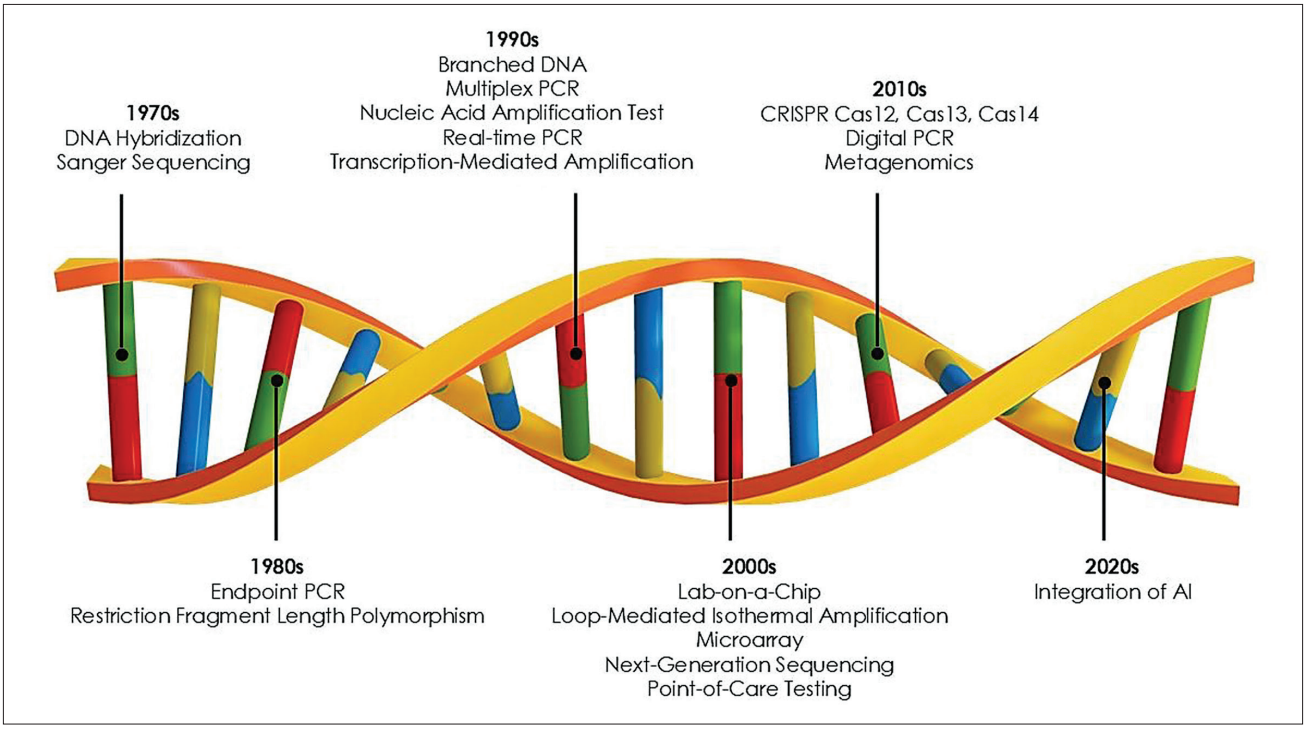

Figure 1. Timeline of major molecular diagnostic techniques for infectious diseases.

The advent of automation, integration, and consolidation in laboratory workflow has ushered in a new era for molecular diagnostics. Automation has significantly reduced handling time and turnaround times while enhancing both efficiency and productivity. Integration, on the other hand, involves the seamless combination of various analytical technologies and instruments to optimize the diagnostic process. Consolidation refers to the merging of multiple sample types and processes into a unified system using one or more equipment.[5] A notable advancement is the development of closed systems, where all phases of the molecular diagnostic workflow (e.g., detection, identification, characterization) occur within sealed containers, minimizing the risk of contamination. In particular, the sample-to-result approach, which integrates nucleic acid extraction, target amplification, and analysis into a single instrument, streamlines the diagnostic process, making infectious disease diagnostics more efficient and productive.

This article provides an overview of various molecular platforms and devices for point-of-care (POC) testing, lab-on-a-chip (LOC), and CRISPR-based diagnostics, particularly in resource-limited settings. In addition, it highlights the gaps and unmet needs in the field. The literature included in this overview was selected based on publications indexed in PubMed, Scopus, and Clarivate Analytics (formerly ISI and Thomson Reuters), ensuring the inclusion of high-quality, peer-reviewed research. This review will cover the most used techniques in molecular diagnostics of infectious diseases, as well as next-generation tools such as POC testing, LOC, and CRISPR-based assays. The future of diagnostics lies in rapid, accurate, and accessible platforms that support global surveillance, outbreak response, and personalized treatment strategies.

INNOVATIONS IN MOLECULAR DIAGNOSTICS WORKFLOW

The molecular diagnostics workflow has rapidly evolved and significantly improved through the years from sample preparation (e.g., nucleic acid extraction) to target amplification (e.g., endpoint PCR, real-time PCR, digital PCR, isothermal amplification), as well as identification of the target pathogen (e.g., colorimetric, spectrometric, fluorescence, chemiluminescence, electrochemical). Taken together, these developments are revolutionizing clinical practice by providing more accurate diagnosis and management of infectious diseases.[6]

There are several ways of extracting nucleic acids (e.g., DNA, RNA) from various clinical samples such as the use of inorganic and organic solvents (e.g., saturated NaCl, phenol-chloroform extraction), solid-phase extraction methods (e.g., spin column with a silica-based membrane) and magnetic beads. The spin column and magnetic bead-based extraction are readily scalable and amenable to automation.[7]

For target amplification, real-time PCR uses fluorescently labeled probes to quantitatively analyze the fluorescence signal of the amplicons without the need for post-PCR analysis such as gel electrophoresis. On the other hand, digital PCR uses the same primers and probes, except that the samples are partitioned into thousands of chambers, thereby increasing its analytical sensitivity. One of the advantages of digital PCR over real-time PCR is that the quantification of nucleic acids is absolute. An example of this application is the absolute quantitation of cytomegalovirus (CMV).[8]

MOLECULAR TECHNIQUES OF INFECTIOUS DISEASES

PCR-based diagnostics

Endpoint or traditional PCR amplifies specific DNA or RNA to detect the presence or absence of the target pathogen. This technique utilizes agarose-stained gel for the detection of the amplified product at the final phase of the PCR reaction. Traditionally, ethidium bromide is used to stain the gels; however, its use has been discontinued for safety reasons. Newer intercalating dyes have been developed and offer improved sensitivity for nucleic acid visualization. Real-time PCR (qPCR) is a variation of endpoint PCR wherein the amount of amplification product is measured at each reaction cycle via fluorescent dyes or probes. Real-time PCR allows for quantitative measurement of the target pathogen in each sample through real-time monitoring of DNA or RNA amplification. Sexually transmitted infections (STIs) such as *Chlamydia*, *Neisseria*, *Trichomonas*, and *Mycoplasma* can be detected using a qPCR platform. It is worthwhile to mention that a real-time PCR for the detection and identification of *Plasmodium* spp. to confirm microscopic findings has been developed.[9]

A more sensitive version of real-time PCR is digital PCR (dPCR). This technique can detect low-abundance pathogens in various clinical samples. It can be used for the detection of cell-free human papillomavirus (HPV) in plasma; identification of hetero-resistance in *Mycobacterium tuberculosis* (Mtb) and detection of viral genome integration in the host genome such as human herpes virus 6 (HHV-6).[8]

Reverse transcription PCR (RT-PCR) allows for the detection of RNA viruses such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), dengue virus, and SARS-CoV-2 by converting RNA to complementary DNA (cDNA) before amplification.

Molecular infectious disease diagnostic tests have proven to be faster and often more sensitive than their traditional counterparts.[10] In particular, a sample-to-result molecular testing based on a multiplex real-time PCR platform has been developed and validated for clinical use such as the bioMérieux BioFire® FilmArray® and the QIAGEN QIAstat-Dx panel for the detection of gastrointestinal pathogens.[11]

It is noteworthy to mention that advances in bioinformatics enabled the development of oligonucleotide primers for multiplex PCR. This enables the detection of multiple pathogens from a single sample in a single test. Bispo and colleagues (2018) developed a multiplex PCR coupled with high-resolution melting (HRM) for the rapid detection and identification of uveitis pathogens such as herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), CMV, and *Toxoplasma gondii*. This technology is important especially in cases where the infections have similar clinical signs or symptoms but require different treatment and management.[12]

Isothermal amplification

Isothermal amplification, most notably the loop-mediated isothermal amplification (LAMP), has attracted a lot of attention as a potential rapid and accurate infectious disease diagnostic platform. LAMP uses 4 to 6 primers and is a simplified version of nucleic acid amplification because it does not require the traditional thermal cycling (e.g., denaturation, annealing, extension) and thus makes it suitable for POC molecular diagnostics, especially in low-income countries and resource-constrained environments.[13-14]

The LAMP assay has various formats such as strip, paper and coated tubes. Recently, a LAMP assay for the detection of dengue, Ebola, hepatitis B virus (HBV), HIV, H1N1, HPV, influenza, MERS-CoV, and Zika has been developed.[15]

The other amplification techniques include branched DNA (bDNA), hybrid capture and transcription-mediated amplification (TMA). For bDNA, the capture probe binds to the microwell and the hybridized probe is detected by chemiluminescence reaction. Blood-borne pathogens such as HBV, HCV, and HIV can be detected using bDNA. Human CMV, HPV, *C. trachomatis*, *and N. gonorrhea* can be detected by hybrid capture, while West Nile virus (WNV), *T. vaginalis*, and *Mycoplasma* can be detected by the TMA technique.

Next-generation sequencing (NGS)

Next-generation sequencing allowed for massive parallel sequencing of DNA and RNA by enabling high-throughput sequencing of genomes of infectious disease pathogens. This technique has paved the way for various sequencing approaches including whole genome sequencing (WGS), targeted sequencing, and shotgun sequencing of clinically important pathogens. These advances have transformed the rapid identification of emerging and re-emerging pathogens. NGS is also widely applied in antimicrobial resistance profiling, disease surveillance, and outbreak investigation.[16-17] Furthermore, it has revolutionized microbiome research by elucidating the role of microorganisms in chronic diseases such as diabetes and inflammatory bowel disease (IBD).[18] Today, several NGS platforms are available, each offering distinct features and tailored applications depending on research or clinical needs (Table 1).[19-26]

Table 1. Major sequencing platforms and their various applications

Technology (Generation)	Timeline	Key Features (Company)	Selected Pathogens	Applications
Sanger Sequencing (First)	1970s to present	Chain termination method using fluorescently labeled ddNTPs (ThermoFisher ABI)	HBV, HCV, HIV, Mtb	16s rRNA, 18s rRNA, 23s rRNA, ITS sequencing, drug resistance mutations, targeted sequencing
Next-Generation Sequencing (Second)	Mid 2000s to present	Sequencing by synthesis (Illumina) Sequencing by ligation (ThermoFisher SOLiD) Semiconductor sequencing (ThermoFisher Ion Torrent)	*E. coli* O104:H4, Ebola, HCV, HIV, HPV, MRSA, Mtb, *P. falciparum*	Drug resistance, metagenomics, outbreak investigation, WGS
Single-Molecule Sequencing (Third)	2010s to present	Real-time sequencing (PacBio, Pacific Biosystems; MinION, Oxford Nanopore)	*C. difficile*, SARS-Cov2	Drug resistance, mutations, genotyping

ddNTPs: dideoxynucleotide triphosphates; ITS: internal transcribed spacer; rRNA: ribosomal RNA; WGS: whole genome sequencing

Metagenomics

Next-generation sequencing (NGS)-based metagenomic approaches enabled the detection and characterization of entire microbial communities without relying on traditional microbiological methods. This technique facilitates the identification of unknown pathogens in diverse clinical samples, even without prior knowledge of the organism. Metagenomic sequencing is particularly valuable for detecting rare and co-infecting pathogens in complex, mixed samples.[27-29] Recently, a rapid metagenomic NGS (mNGS) technique has been developed to detect pathogen cell-free DNA in various body fluids. This platform integrates automated library preparation, a hybrid Illumina and nanopore sequencing protocol, and advanced bioinformatics pipelines for comprehensive metagenomic analysis.[30]

Microarray

Microarrays or chip-based diagnostics (e.g., DNA, RNA, protein) are used as diagnostic tools for the rapid identification of pathogens. It utilizes a set of probes to detect and characterize a broad spectrum of pathogens (e.g., bacteria, viruses, parasites, fungi), as well as to determine antimicrobial resistance profiles.[31] In 2020, Ma and colleagues developed a DNA microarray assay for rapid detection of fifteen bacterial pathogens in pneumonia. This approach offers the potential to provide a faster diagnostic tool than the current standard methods.[32]

As microarrays are increasingly used in clinical practice and research, efforts are being made to validate and standardize these techniques to ensure reproducibility and accuracy. Recently, a multiplex protein microarray for antibody testing for tickborne and other infectious diseases has been developed.[33]

Point-of-care molecular diagnostics

Point-of-care testing refers to diagnostic testing performed near the site of patient care, including but are not limited to doctors' clinics and emergency rooms. The goal of POC is to provide fast results and thus enabling rapid diagnosis and treatment. The WHO ASSURED framework (e.g., affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to the end-users) has provided the roadmap for creating molecular diagnostics that meet the needs of low-income countries and are impactful in resource-limited settings.[34] This framework has evolved and been updated to REASSURED with the addition of real-time connectivity and ease of specimen collection.

Microfluidics and lab-on-a-chip

The lab-on-a-chip is a technology that integrates laboratory functions onto a small chip. The integration of microfluids and miniaturized molecular diagnostics systems has revolutionized the field as it offers a rapid, accurate, and portable platform, especially for remote areas and field settings. These innovations can extract the genetic material from a variety of clinical samples such as blood, saliva, and urine in a closed system, thus making it easier and faster to process samples with minimal hands-on time. The LOC devices can be used for various applications, including POC molecular diagnostics. Recently, a LOC for the simultaneous detection of SARS-CoV-2 RNA and SARS-CoV-2 antibodies in saliva and plasma has been developed. It utilizes an electrochemical sensor that detects the presence of nucleic acid and protein antibodies.[35]

Several LOC devices now integrate with smartphones. This allows for the remote analysis of the results, and this can be immediately shared with the requesting healthcare provider. This in turn, will significantly improve access to care, especially in low-resource settings.

CRISPR-based diagnostics

CRISPR-based diagnostics utilize enzymes to cleave DNA or RNA from target pathogens and provide a colorimetric or visual readout. This approach makes infectious disease diagnostics faster and accessible, especially in resource-constrained environments.[36] The CRISPR-based diagnostics have provided new ways of detecting and characterizing nucleic acids with a high degree of accuracy and precision.

Cas12a targets DNA with collateral cleavage of single-stranded DNA. DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) allowed for fast, accurate, and cost-effective molecular diagnostics of infectious diseases such as COVID-19.[37] Cas13a targets single-stranded RNA with collateral cleavage of ssRNA. The Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) has been used to diagnose flaviviruses such as dengue, Zika, WNV, and yellow fever.[38] When Cas12a, Cas13a, or Cas14 binds to target nucleic acid, it releases a fluorescent molecule, which in turn produces a fluorescent signal. Currently, CRISPR Cas-based detection methods include lateral flow device, fluorescence, colorimeter, and quantitative real-time PCR. Some of the advantages of the CRISPR-based diagnostics are as follows: (1) rapid onsite detection; (2) simple and portable; (3) high sensitivity and specificity; (4) timesaving; and (5) multiple detection.[39]

CRISPR-based diagnostics for infectious diseases have demonstrated acceptable technical performance in vitro; however, their real-world field performance has shown inconsistent results. This variability is often attributed to factors such as sample complexity, reagent stability, environmental conditions (e.g., temperature, humidity), and operator variability. Efforts to improve performance in field settings include the following: (1) integrated sample preparation methods such as microfluidics and paper-based extraction; (2) the use of lyophilized reagents to enhance stability and eliminate cold chain requirements; (3) smartphone-based readers for improved result interpretation; (4) standardized protocols; and (5) staff training.[37-41]

Taken together, POC, LOC, and CRISPR-based diagnostics represent interconnected technologies that are driving the next-generation of rapid, accurate, and accessible molecular diagnostics for infectious diseases.

GAPS, UNMET NEEDS AND FUTURE DIRECTIONS

Molecular diagnostics of infectious diseases have advanced significantly, but several gaps and unmet needs persist across clinical, technological, and socioeconomic dimensions. First, the detection of nucleic acids does not always mean

active infection. Thus, there is a need to distinguish between active and latent infections. Second, standardization of viral loads across nucleic acid amplification tests and sample types remains to be seen.[42] Third, there is a need to develop cost-effective molecular diagnostics in resource-limited settings.

The future directions of molecular diagnostic techniques of infectious diseases are centered around making diagnostics faster, more accurate, and smarter. First, integrate omics data (e.g., genomics, transcriptomics, proteomics) and artificial intelligence (AI) to develop innovative strategies for prevention, rapid diagnosis, and treatment. The integration of AI in molecular diagnostics of infectious diseases could significantly play a critical role in interpreting and analyzing data, which in turn will further improve the turnaround time and diagnostic accuracy.[43] Second, expand the test menu for infectious diseases (e.g., bacterial, viral, parasitic, fungal). Third, establish transdisciplinary and international collaborations to address global challenges, as well as emerging and re-emerging infections.

CONCLUSION

The molecular diagnostics workflow has been evolving, and the applications in the field of infectious disease diagnostics have seen significant advancements. Various molecular techniques have significantly improved the accuracy of infectious disease detection, identification, and characterization. Molecular techniques have transformed the landscape of infectious disease diagnostics. Addressing the gaps and unmet needs is key in advancing the ability to diagnose and treat infectious diseases in the future.

Overall, this overview contributes to the existing literature by providing an up-to-date synthesis of next-generation tools for infectious disease diagnostics, with a particular focus on POC, LOC, and CRISPR-based assays, which are especially valuable in resource-limited settings. By integrating technological advances with clinical applicability, this overview offers valuable insights for scientists, clinicians, policymakers, and decision makers seeking to improve diagnostic strategies, clinical outcomes, and healthcare delivery.

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The author certified fulfillment of ICMJE authorship criteria.

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Datasets generated and analyzed are included in the published article.

AUTHOR DISCLOSURE

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