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Molecular Diagnostics and Next-Generation Sequencing in Bleeding Disorders

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ABSTRACT

Molecular diagnostics has become a cornerstone of modern clinical practice, enabling precise detection and characterisation of genetic and infectious diseases. Advances in molecular biology and nucleic acid analysis have transformed traditional diagnostics by allowing identification of disease-associated alterations at the DNA and RNA levels. Polymerase chain reaction (PCR)–based assays have significantly improved sensitivity and specificity in pathogen detection and genetic testing, while non-PCR–based techniques have expanded diagnostic capabilities in point-of-care and resource-limited settings. More recently, next-generation sequencing (NGS) has revolutionised molecular diagnostics by enabling high-throughput, parallel analysis of multiple genetic targets. NGS is valuable in infectious disease surveillance, metagenomic pathogen detection, and the molecular diagnosis of inherited disorders. In bleeding disorders, NGS has improved understanding of the genetic basis of haemophilia A, haemophilia B, and von Willebrand disease, supporting accurate diagnosis, carrier detection, and prenatal screening. Despite these advances, disparities in access to molecular diagnostics persist, especially in low- and middle-income countries, contributing to underdiagnosis and delayed care. This review summarises the evolution of molecular diagnostic technologies, highlights current NGS applications in infectious diseases and inherited bleeding disorders, and addresses challenges and opportunities for improving diagnostic equity. Integration of advanced molecular tools into clinical practice is essential to enhance accuracy, guide therapy, and improve patient outcomes worldwide.

Keywords: Molecular diagnostics, Next-generation sequencing, Bleeding disorders, Infectious disease genomics.

INTRODUCTION

Molecular or nucleic acid–based diagnosis of human disorders involves the identification of disease-associated alterations in DNA and/or RNA to support accurate detection, diagnosis, subclassification, prognosis, and assessment of therapeutic response. By integrating laboratory medicine with molecular genetics, molecular diagnostics has transformed clinical practice over recent decades, largely driven by major advances in molecular biology.[1] Advances in understanding the molecular basis of both rare and common disorders, together with improvements in DNA analysis technologies, have significantly altered the landscape of molecular genetics and genomic testing. High-resolution molecular cytogenetic techniques can now detect submicroscopic deletions or duplications that were previously undetectable by conventional microscopy.[2] Diagnostic testing for single-gene disorders can be achieved through targeted mutation analysis, gene-specific sequencing, or multi-gene testing in disorders with overlapping phenotypes. The advent of massively parallel next-generation sequencing (NGS) has further enabled simultaneous analysis of multiple genes and is now widely used for clinical exome sequencing.[3]

The application of NGS to obtain complete genomes has also emerged as a powerful epidemiological tool for tracking pathogen transmission, identifying outbreaks, and detecting diagnostic mismatches. Unbiased metagenomic sequencing using random priming allows detection of a broad range of pathogens, including viruses, bacteria, parasites, and fungi. However, the presence of abundant host background reads can limit sensitivity, particularly for small, diverse, low-copy, or highly structured viruses such as the hepatitis B virus (HBV) and the hepatitis D virus (HDV).[4] To overcome these limitations, target enrichment strategies have been developed to enhance sensitivity and sequencing depth. Single-stranded DNA probes can be hybridised to metagenomic libraries to selectively capture viral sequences, making this approach particularly useful in samples with low viral loads. Post-library capture methods such as xGen have been successfully applied to blood-borne RNA viruses like HIV and HCV, although their application to smaller viruses such as HBV and HDV remains limited.[5]

Bleeding disorders represent a diverse group of acquired and inherited conditions characterised by defective blood clotting. Globally, bleeding disorders affect more than 1 in 1,000 individuals. Haemophilia A and von Willebrand disease (VWD) are the most commonly diagnosed inherited bleeding disorders worldwide. However, diagnosis rates differ markedly between high-income countries (HICs) and low- and lower-middle-income countries (LMICs). While nearly all individuals with haemophilia A are diagnosed in HICs, only approximately 12% are diagnosed in LMICs. This review discusses the molecular genetics of bleeding disorders, challenges contributing to underdiagnosis in LMICs, and approaches to narrowing disparities in bleeding disorder care.[6]

This review aims to provide an overview of contemporary molecular diagnostic approaches, with particular emphasis on next-generation sequencing technologies and their application in infectious diseases and inherited bleeding disorders. It further seeks to examine the molecular genetic basis of bleeding disorders, discuss challenges contributing to underdiagnosis in low- and middle-income countries, and outline strategies to improve diagnostic equity and patient care.

History and Evolution of Molecular Diagnosis

In 1949, Pauling and his colleagues introduced the concept of a molecular disease into medical terminology, following their discovery that a single amino acid substitution in the β-globin chain causes sickle cell anaemia, which is primarily characterised by recurrent painful episodes due to vessel occlusion. This work laid the foundation for molecular diagnostics, although the widespread clinical impact emerged much later. At the time, with molecular biology still in its early stages, providing molecular diagnostic services was technically unfeasible and beyond practical implementation.[7] During the 1990s, the discovery of new genes and advances in DNA sequencing technologies gave rise to molecular and genomic laboratory medicine as a distinct field. Beginning in 2002, the HapMap Project catalogued single-nucleotide polymorphisms (SNPs) across human populations and explored their relationship with disease. By 2012, molecular diagnostic approaches for thalassemia utilised genetic hybridisation techniques to identify the specific SNPs responsible for individual disease phenotypes.[8]

Current Molecular Diagnostic Approaches

Clinical microbiology laboratories employ a variety of techniques to aid clinical decision-making related to the diagnosis, management, and prognosis of infectious diseases. Diagnostic strategies broadly fall into two categories, with direct identification of the causative pathogen in clinical specimens considered the gold standard. Methods commonly used for this purpose include microbial culture, electron microscopy, and detection of pathogen genetic material through polymerase chain reaction (PCR).[9]

Infectious diseases caused by bacteria, viruses, parasites, and fungi continue to be a major contributor to global morbidity and mortality. Current estimates indicate that approximately 60 million deaths occur worldwide each year, with infectious diseases accounting for nearly one-quarter of these cases. The continued emergence and re-emergence of infectious agents pose significant challenges to public health systems and socioeconomic stability. Although vaccines and antimicrobial or antiviral therapies have helped reduce disease burden, their effectiveness may be compromised by the appearance of novel pathogens and increasing antimicrobial resistance. Furthermore, the prolonged timelines required for vaccine and drug development limit the ability to rapidly contain and control infectious disease outbreaks.[10]

PCR-Based Molecular Diagnostic Techniques

Allele-specific amplification, also known as allelic discrimination, can be achieved using two different TaqMan probes in combination with the 5′ nuclease activity of Taq DNA polymerase, allowing rapid genotyping of individuals. This approach relies on endpoint fluorescence measurement rather than continuous real-time analysis. Polymorphism studies using this method have been carried out for several enzymes, including aldehyde dehydrogenase, N-acetyltransferase, thiopurine methyltransferase, glutathione S-transferases (GSTM1, GSTT1, GSTP1), and cytochrome P450 isoforms such as CYP2C and CYP2D.[11] Chip-based PCR platforms have also been developed to simplify molecular testing. VereChip™ is a silicon-based system that combines a miniaturised PCR amplification unit with a customizable microarray, enabling efficient nucleic acid analysis with reduced cost, time, and operational demands. The integrated optical reader allows rapid data acquisition, making the platform suitable for point-of-care (POC) diagnostic use.[12]

Non-PCR–Based Molecular Diagnostic Techniques

Recent developments in molecular diagnostics have introduced alternative approaches that function independently of conventional PCR amplification. Isothermal amplification techniques, such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA), allow nucleic acid detection at a constant temperature and have been increasingly applied for rapid and point-of-care diagnostic testing in infectious diseases.[13] These methods reduce reliance on complex instrumentation and shorten turnaround time. In parallel, CRISPR-based diagnostic systems have emerged as highly specific tools that use programmable Cas enzymes to identify target nucleic acid sequences, enabling rapid and sensitive detection without thermal cycling.[14]

Next-Generation Sequencing (NGS)

Next-generation sequencing has become an essential tool in both clinical diagnostics and research due to its ability to generate high-throughput genomic data with high sensitivity. In clinical applications such as aneuploidy screening, analysis of cell-free DNA (cfDNA) commonly relies on either whole-genome (random) sequencing or targeted sequencing strategies, each offering distinct advantages in terms of coverage and analytical depth.[15] Recent advances in blood collection and cfDNA stabilisation methods have improved sample integrity by minimising leukocyte lysis and background genomic DNA contamination, thereby enhancing analytical accuracy.[16]

NGS is also widely applied in viral genomics, where multiplexed and barcoded libraries enable simultaneous sequencing of multiple samples while maintaining sufficient read depth for genotype determination. Sequencing data are processed through bioinformatics pipelines that map reads to reference genomes to identify viral genotypes and sequence variation. These approaches support accurate molecular characterisation and continue to play a significant role in pathogen surveillance and clinical decision-making.[17]

Genetic Basis of Bleeding Disorders

Inherited bleeding disorders arise from pathogenic variants in genes encoding coagulation factors or proteins involved in platelet adhesion and stabilisation. Haemophilia A and haemophilia B are X-linked recessive disorders caused by mutations in the *F8* and *F9* genes, respectively, resulting in deficient factor VIII or factor IX activity. Disease severity correlates with residual clotting factor levels and directly influences bleeding phenotype, ranging from spontaneous haemorrhage in severe cases to trauma-associated bleeding in milder forms.[17] Von Willebrand disease is caused by quantitative or qualitative defects in von Willebrand factor, a multimeric glycoprotein essential for platelet adhesion and protection of factor VIII. Genetic variation within the *VWF* gene contributes to heterogeneous clinical manifestations and complicates molecular diagnosis due to allelic diversity and structural complexity.[17]

SNPs and Polymorphism Analysis

Single-nucleotide polymorphisms (SNPs) represent one of the most common forms of genetic variation and play a critical role in disease susceptibility, drug response, and molecular diagnosis. Advances in sequencing-based diagnostics have enabled accurate detection of SNPs across coding and non-coding regions, facilitating diagnosis of Mendelian and complex disorders. Clinical RNA sequencing and targeted DNA sequencing approaches have improved variant interpretation by correlating polymorphisms with functional and phenotypic consequences in affected individuals.[1] High-throughput exome and targeted sequencing strategies have further demonstrated high positive predictive value for clinically relevant copy number variations and single-nucleotide variants in suspected genetic diseases, supporting their utility in polymorphism analysis in diagnostic workflows.[3]

Biochips and Microarrays

Biochip- and microarray-based platforms have contributed significantly to molecular diagnostics by enabling parallel analysis of multiple genetic targets. Probe-based enrichment and hybridisation strategies allow simultaneous detection of multiple viral or genetic sequences, improving throughput and diagnostic efficiency. Advanced molecular surveillance studies using probe capture technologies demonstrate the effectiveness of multiplexed detection in blood-borne viral infections, highlighting the continued relevance of array-based concepts in modern diagnostics.[4] Microfluidic integration has further enhanced biochip performance by reducing assay time and sample volume while maintaining analytical sensitivity.[12]

Proteomics and Protein-Based Diagnostics

Protein-based diagnostics focus on detecting disease-associated proteins or functional consequences of genetic variation, complementing nucleic acid–based methods. Advances in precision medicine emphasise integrating molecular and biochemical data to improve disease classification and clinical decision-making. Molecular diagnostic approaches increasingly recognise the importance of protein-level validation to support genetic findings and therapeutic monitoring, particularly in inherited and metabolic disorders.[2] However, large-scale clinical proteomics platforms and biomarker discovery pipelines are not extensively covered in the provided references.

Diagnosis in LMIC Settings

Diagnosis of genetic and bleeding disorders in low- and middle-income countries (LMICs) remains challenging due to limited laboratory infrastructure, restricted access to advanced molecular testing, and delayed clinical recognition. Recent studies highlight significant underdiagnosis of bleeding disorders in LMICs despite their substantial disease burden. Barriers include a lack of specialised coagulation testing, a shortage of trained personnel, and limited availability of molecular diagnostic facilities. Strengthening diagnostic capacity through targeted molecular testing and improved healthcare infrastructure is essential to reducing disparities in care.[6]

Prenatal and Carrier Screening

Prenatal and carrier screening have evolved with the adoption of targeted sequencing and next-generation sequencing technologies, enabling early detection of pathogenic variants associated with inherited disorders. Targeted sequencing approaches have demonstrated clinical utility in identifying disease-causing mutations and informing reproductive decision-making. Improvements in pre-analytical workflows, including optimised sample handling and sequencing strategies, have enhanced the reliability of molecular results in prenatal and carrier screening contexts.[15] Integration of high-throughput sequencing into clinical practice continues to expand the scope of genetic screening while improving diagnostic accuracy.[1]

CONCLUSION

Molecular diagnostic technologies have evolved from conventional laboratory methods to highly sophisticated genomic approaches, with next-generation sequencing now playing a central role in clinical diagnostics. The ability of NGS to provide comprehensive genetic information has significantly improved the diagnosis of infectious diseases and inherited bleeding disorders, enabling precise molecular characterisation and informed clinical management. While these advances have strengthened diagnostic accuracy and expanded screening capabilities, disparities in access to molecular diagnostics remain a major challenge, particularly in low- and middle-income countries. Addressing these gaps through improved infrastructure, targeted testing strategies, and equitable implementation of advanced technologies is critical. As molecular diagnostics continues to advance, its integration into routine clinical practice will be key to achieving timely diagnosis, personalised treatment, and improved health outcomes across diverse populations.

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