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Recent Molecular Approaches to Clinical Laboratory Diagnosis of Hand, Foot and Mouth Disease (HFMD)

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Introduction

Hand, Foot and Mouth Disease (HFMD) is a highly contagious viral illness that predominantly affects infants and young children. Although historically considered a mild and self-limiting disease, large outbreaks associated with severe neurological and cardiopulmonary complications have elevated HFMD to a significant public health concern, particularly in the Asia-Pacific region. The disease is caused primarily by members of the Enterovirus A species, including Enterovirus A71 (EV-A71), Coxsackievirus A16 (CV-A16), Coxsackievirus A6 (CV-A6), and Coxsackievirus A10 (CV-A10). Because clinical manifestations overlap considerably among enteroviral infections, laboratory confirmation is essential for accurate diagnosis, epidemiological surveillance, outbreak management, and risk stratification. Recent advances in molecular diagnostics have transformed HFMD diagnosis from conventional virus isolation methods to highly sensitive, rapid, and multiplex nucleic acid-based approaches [1, 2].

Hand, Foot and Mouth Disease (HFMD) is a highly contagious viral illness that predominantly affects infants and young children. Although historically considered a mild and self-limiting disease, large outbreaks associated with severe neurological and cardiopulmonary complications have elevated HFMD to a significant public health concern, particularly in the Asia-Pacific region. Historically, viral culture was regarded as the gold standard for enterovirus detection. However, culture-based techniques require specialized cell lines, prolonged incubation periods, and substantial technical expertise. Moreover, some HFMD-associated enteroviruses exhibit poor growth characteristics *in vitro*, reducing diagnostic sensitivity. Consequently, molecular diagnostics have largely replaced conventional methods in clinical laboratories [3, 4].

Real-time reverse transcription polymerase chain reaction (RT-qPCR) remains the cornerstone of molecular HFMD diagnosis. This technology enables direct detection of viral RNA from throat swabs, vesicular fluid, stool samples, and rectal swabs with excellent sensitivity and specificity. RT-qPCR assays targeting conserved genomic regions such as VP1, VP4, and the 5′ untranslated region (5′UTR) can identify enteroviruses rapidly, often within a few hours. Importantly, quantitative amplification provides estimates of viral load, which may correlate with disease severity in certain clinical contexts. Several multiplex RT-qPCR assays have been developed to simultaneously differentiate EV-A71, CV-A16, and pan-enteroviruses in a single reaction tube, significantly improving laboratory efficiency and reducing costs. Clinical evaluations have demonstrated sensitivities approaching 99% with excellent analytical specificity [1, 3, 4].

The evolving epidemiology of HFMD has further stimulated the development of expanded multiplex molecular platforms. While EV-A71 and CV-A16 were once considered the dominant etiological agents, recent outbreaks have increasingly involved CV-A6 and CV-A10. Consequently, newer multiplex RT-PCR assays simultaneously detect four or more HFMD-associated enteroviruses. Such platforms facilitate pathogen-specific surveillance and improve outbreak investigations by identifying shifts in circulating viral genotypes. These assays also support vaccine evaluation studies by monitoring genotype replacement phenomena following EV-A71 vaccination programs [1, 2].

Although RT-qPCR provides excellent performance, its dependence on sophisticated thermal cyclers and centralized laboratory infrastructure limits accessibility in resource-constrained settings. This challenge has accelerated interest in isothermal nucleic acid amplification technologies. Unlike conventional PCR, these methods amplify nucleic acids at a constant temperature, eliminating the need for thermal cycling [5].

Loop-mediated isothermal amplification (LAMP) was among the first alternative amplification technologies applied to HFMD diagnosis. LAMP assays offer rapid amplification, visual readout capabilities, and reduced equipment requirements. More recently, reverse transcription-isothermal multiple-self-matching initiated amplification (RT-IMSA) has demonstrated

improved sensitivity compared with RT-LAMP for detection of EV-A71 and CV-A16. The method can generate results within approximately one hour while maintaining high analytical performance. Studies suggest that RT-IMSA may detect lower viral copy numbers than conventional LAMP assays, potentially enhancing early infection diagnosis [1, 2, 4].

Another promising development is recombinase-aided amplification (RAA) and recombinase polymerase amplification (RPA). These technologies utilize recombinase enzymes to facilitate primer binding at constant temperatures, enabling rapid nucleic acid amplification within 20–30 minutes. Duplex RT-RAA assays have been successfully developed for simultaneous detection of EV-A71 and CV-A16. Integration with lateral flow strip technologies further enables visual interpretation without specialized instrumentation. Such approaches are particularly attractive for decentralized testing, field surveillance, and outbreak response in low-resource settings. Clinical studies have demonstrated diagnostic sensitivities exceeding 90% and specificities approaching 100% when compared with RT-qPCR reference methods [1, 3].

The emergence of CRISPR-based diagnostics represents one of the most exciting innovations in molecular infectious disease testing. CRISPR-Cas systems exploit programmable nucleic acid recognition mechanisms to achieve highly specific pathogen detection. In HFMD diagnostics, CRISPR-Cas12a and Cas13a platforms have recently been coupled with isothermal amplification techniques such as RPA and multiple cross displacement amplification (MCDA). These integrated systems provide exceptional analytical sensitivity, detecting viral RNA at concentrations as low as a few copies per microliter while maintaining remarkable specificity. Furthermore, CRISPR-based assays can be adapted to lateral flow formats, enabling rapid point-of-care testing. Recent studies have demonstrated simultaneous detection of EV-A71 and CV-A16 using dual-target CRISPR platforms, with total assay times of less than 40 minutes. Such developments may ultimately bridge the gap between laboratory-grade molecular testing and bedside diagnostics [1, 2, 4].

Metagenomic next-generation sequencing (mNGS) has emerged as a transformative technology for comprehensive pathogen identification. Unlike targeted PCR assays, mNGS does not require prior knowledge of the infecting organism. Instead, all nucleic acids present within a clinical specimen are sequenced simultaneously. This unbiased approach is particularly valuable in atypical HFMD cases, severe neurological disease, or situations where conventional assays yield negative results despite strong clinical suspicion. mNGS can identify novel enterovirus variants, characterize mixed infections, and reveal genomic mutations associated with virulence or immune escape. Furthermore, whole-genome sequencing facilitates phylogenetic analyses that support outbreak investigations and molecular epidemiology studies. Although cost, turnaround time, and bioinformatics requirements currently limit widespread clinical implementation, sequencing technologies continue to become faster and more affordable [5].

Targeted next-generation sequencing approaches are also gaining importance in HFMD surveillance. Sequencing of the VP1 region remains the standard method for enterovirus genotyping because VP1 contains serotype-specific neutralization epitopes. Modern sequencing platforms enable rapid characterization of circulating strains and facilitate monitoring of viral evolution. Such surveillance is particularly relevant for EV-A71, which exhibits substantial genetic diversity and varying neurovirulence among subgenogroups. Molecular epidemiological data generated through sequencing can inform vaccine development strategies and support public health interventions [4, 5].

Digital PCR (dPCR) represents another emerging molecular technology with potential applications in HFMD diagnosis. By partitioning nucleic acid templates into thousands of microreactions, dPCR allows absolute quantification of viral RNA without reliance on standard curves. This technology offers superior precision, improved tolerance to inhibitors, and enhanced sensitivity for low-abundance targets. Although currently used primarily in research settings, dPCR may eventually contribute to viral load monitoring and prognostic assessment in severe HFMD cases [5].

The integration of molecular diagnostics with automation and artificial intelligence (AI) is expected to further transform HFMD laboratory testing. Automated sample preparation systems reduce operator-dependent variability, while AI-assisted bioinformatics pipelines facilitate rapid interpretation of sequencing data. Combined with portable molecular devices, these innovations may enable near-patient testing and real-time epidemiological surveillance during outbreaks [5, 6].

Despite remarkable progress, several challenges remain. Genetic variability among enteroviruses can compromise primer and probe binding, potentially leading to false-negative results. Continuous genomic surveillance is therefore necessary to ensure assay performance. Standardization of molecular protocols across laboratories remains another important issue, particularly for multicenter surveillance programs. Additionally, balancing diagnostic accuracy with affordability will be critical for implementation in low- and middle-income countries where HFMD burden is often greatest [7].

Conclusion

Molecular diagnostics have revolutionized the clinical laboratory diagnosis of HFMD. RT-qPCR remains the current diagnostic standard; however, multiplex assays, isothermal amplification technologies, CRISPR-based detection systems, digital PCR, and next-generation sequencing are rapidly expanding the diagnostic arsenal. These approaches offer improvements in sensitivity, specificity, turnaround time, and pathogen characterization. As molecular technologies continue to evolve, future HFMD diagnostics will likely become increasingly decentralized, rapid, and genomically informed, ultimately enhancing patient management, outbreak control, and public health surveillance [8].

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