

DNA-Based Technology for Herpesvirus Detection

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Abstract: The detection of viral DNA is considered crucial in both diagnosis and prognosis. Nowadays, molecular diagnostic approaches represent the most promising tools for the clinical detection of viral infections. This review aims to investigate the most used and promising DNA-based technologies for viral detection, focusing on herpesviruses because of their ability to undergo latent and reactivation cycles, persisting lifelong in the host in association with several diseases. Molecular technologies, such as PCR-based assays, enhance sensitivity and specificity in identifying viral DNA from clinical samples such as blood, cerebrospinal fluid and saliva, indicating PCR and its derivatives as the gold standard methods for herpesvirus detection. In conclusion, this review underscores the need for continuous innovation in diagnostic methodologies to address the complexities of herpesvirus identification in different clinical samples.

Keywords: viral DNA detection; herpesvirus; clinical samples



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1. Introduction

Viruses pose a serious threat to humans and are a significant global concern [1]. Given the huge impact of viral infections on human health, virologists and clinical biologists are increasingly focused on designing effective and precise detection methods, primarily based on molecular biology techniques. Herpesviruses represent a large family of DNA viruses characterized by their ability to persist lifelong within the host, undergoing latency/reactivation cycles [2] which make diagnosis a considerable challenge.

During primary infection, herpesviruses typically cause active lytic infection, characterized by viral replication, cell lysis, and occasionally clinically evident symptoms [3].

Following the primary infection, the virus enters a latent phase in specific cell types. During the latency phase, the viral genome persists in infected cells with minimal to no gene expression, allowing the virus to evade the immune response and persist for the lifetime of the host. The ability of herpesviruses to periodically reactivate leads to recurrent infection that results in severe dissemination and complication, especially in immunocompromised subjects.

In light of recent evidence suggesting a potential role for the herpesvirus latency/reactivation cycle in the development or worsening of several diseases and clinical conditions—including cancer, autoimmunity, and neurological diseases [4]—the use of specific and rapid DNA-based detection methods has become extremely relevant.

In this review, we explore the main DNA-based molecular diagnostic methods currently employed for the detection of human herpesviruses.

2. Diagnostic Method for Virus Detection

Virus detection methods primarily rely on molecular-based techniques to improve efficiency and reduce diagnostic turnaround time. These approaches use immunological detection techniques or specific oligonucleotide probes designed to bind selectively to

viral nucleic acids [5,6] (Table 1). This section provides an overview of the main molecular techniques used for virus detection.

Table 1. Main molecular techniques currently used for viral diagnostics.

Technique	Principle	Samples	Advantages	Disadvantages
PCR	Amplifies specific DNA sequences to detect viral presence.	Swabs, CSF, blood, mucosal samples	High sensitivity and specificity; rapid results	Single-pathogen detection per reaction (conventional PCR)
Real-Time PCR (qPCR)	A variant of PCR that quantifies DNA in real time during amplification	Swabs, CSF, blood, mucosal samples	Rapid, quantitative; widely applicable	Low throughput, single-pathogen detection
Multiplex qPCR	Detects multiple pathogens using distinct primers	Swabs, CSF, blood, biopsies	Simultaneous detection; cost-effective	Risk of cross-reactivity, complex setup
Droplet Digital PCR (ddPCR)	Absolute quantification of DNA via droplet partitioning	Swabs, CSF, blood, saliva	High precision and sensitivity; ideal for low viral loads	Expensive, prone to contamination, requires specialized equipment
Loop-Mediated Isothermal Amplification (LAMP)	Amplifies DNA at a constant temperature, eliminating thermal cycling	Swabs, CSF, blood, ocular fluids	Rapid, field-friendly; no complex equipment needed	Less specific than PCR; primer design is critical
Microarrays	Hybridization-based detection of thousands of DNA fragments simultaneously	Blood, CSF, tissue samples	High throughput; can detect multiple pathogens at once	High cost; requires complex data interpretation
Biosensors	Converts DNA-binding events into electrical, optical, or acoustic signals	Saliva, blood, ocular fluids	Rapid, portable; cost-effective	Limited availability; requires advanced transducer technologies
CRISPR Technology	CRISPR-Cas systems target and detect viral DNA/RNA	Swabs, CSF, blood, saliva	High sensitivity and specificity; potential for multiplexing	Pre-amplification often required; contamination risk
Next-Generation Sequencing (NGS)	High-throughput sequencing for comprehensive pathogen analysis	Blood, CSF, biopsies, respiratory samples	Detects co-infections and mutations; ideal for unknown pathogens	Expensive; requires bioinformatics expertise

2.1. Viral Molecular Detection Techniques

Molecular techniques are increasingly employed in clinical settings due to their rapid and precise identification of viral agents [2]. These methods mainly consist in the identification of viral genomes or specific genes by nucleic acid amplification techniques (NAATs), hybridization, and sequencing.

2.1.1. PCR-Based Methods

Polymerase Chain Reaction (PCR)

PCR is the most widely used nucleic acid-based technique for viral detection. It is fast, sensitive, and specific, enabling the amplification of target DNA sequences via DNA polymerase. This exponential amplification ensures high sensitivity, with amplicons visualized through gel electrophoresis [7]. PCR can detect both DNA and RNA viruses, with the latter requiring reverse transcription prior to amplification.

Real-Time PCR (qPCR)

Although conventional PCR provides qualitative or semi-quantitative results, real-time quantitative PCR (rtPCR) addresses the need for accurate viral load quantification

in clinical diagnostics. This technique uses fluorescent dyes or specific probes to monitor DNA amplification in real time. Fluorescence intensity is plotted as a function of amplification cycles, providing quantitative data [8]. Common dyes include SYBR Green, which binds to all double-stranded DNA (dsDNA), though it may produce non-specific signals. To overcome this, fluorophore-bound probes are used, emitting fluorescence only when hybridized with complementary sequences, enhancing specificity.

Multiplex PCR

Multiplex PCR employs multiple primers and probes to detect different viral targets within the same reaction. This method is especially valuable in respiratory infections where co-infections are common. Some assays can identify up to eight different viruses in a single test, reducing time and costs [9–11].

Droplet Digital PCR (ddPCR)

A significant advancement in PCR technology is droplet digital PCR (ddPCR). This method partitions DNA into millions of microdroplets, amplifying each droplet independently. Absolute quantification is achieved without standard curves by applying the Poisson distribution to the fluorescence signal [12,13]. ddPCR is particularly suited for detecting low viral loads and provides high sensitivity and precision. However, limitations include its cost, sensitivity to contamination, and the need for specialized equipment [14].

2.1.2. Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) amplifies DNA at a constant temperature, eliminating the need for thermal cycling. This method uses primers designed to form stem-loop structures, enabling rapid and efficient amplification [15,16]. LAMP is highly sensitive and specific and is widely used in field settings due to its simplicity and minimal equipment requirements. However, its specificity may be lower than PCR, as primer design is critical to prevent non-specific amplifications [17,18].

2.1.3. Hybridization Techniques: Microarrays and Biosensors

Microarrays

Microarray technology allows for the simultaneous hybridization of target DNA to thousands of immobilized probes on a solid surface. This approach is ideal for detecting multiple pathogens or identifying novel variants through conserved gene sequences [19,20]. Techniques like using clinical array technology (CLART) enable the sensitive and automated detection of various viruses, including HPV and herpesviruses, offering cost-effective solutions for high-throughput diagnostics [20–22].

Biosensors

Biosensors utilize DNA nanotechnology to detect viral DNA by converting molecular interactions into measurable signals, such as electrical or optical outputs [23,24]. These devices are portable, cost-effective, and rapid, making them suitable for point-of-care diagnostics. Recent advancements include aptamers and DNAzymes, which enhance sensitivity and specificity while offering therapeutic potential. Biosensors hold promise for both diagnostics and therapeutic applications, but further development is needed to expand their clinical utility [25–27].

2.1.4. CRISPR-Based Detection

CRISPR technology has revolutionized viral diagnostics. Systems like CRISPR-Cas12 and CRISPR-Cas13 provide high sensitivity and specificity for DNA or RNA detection [28,29]. Platforms such as DETECTR leverage CRISPR-Cas12 for fluorescence-based or colorimetric detection, making these methods suitable for field diagnostics or point-of-care applications [30,31].

CRISPR-based diagnostics rival PCR in sensitivity but often require the pre-amplification of target nucleic acids, increasing complexity and contamination risks. Future improvements aim to simplify workflows, minimize contamination through closed-tube reactions, and enable multiplex detection for broader clinical adoption [30].

2.1.5. Next-Generation Sequencing (NGS)

NGS is a transformative tool for comprehensive viral genome analysis. In complex clinical cases requiring viral discrimination, additional laboratory procedures, such as restriction enzyme analysis or complete genome sequencing, may be necessary using next-generation sequencing (NGS) technologies [32]. These massively parallel sequencing platforms can sequence highly diverse genetic materials with excellent sensitivity [33]. Furthermore, these platforms are sequence-independent, making them exceptional tools for virus identification. They provide unparalleled sensitivity for detecting low viral loads, co-infections, and unknown pathogens. Metagenomic NGS (mNGS) has proven particularly useful in diagnosing respiratory infections, central nervous system infections, and other complex cases [34,35].

NGS also facilitates detailed virome analysis, enabling the identification of mutations and the monitoring of emerging variants [36]. However, its adoption is limited by high costs, labor-intensive sample preparation, and the need for bioinformatics expertise. Despite these limitations, NGS remains invaluable for uncovering viral interactions and advancing diagnostics in complex clinical scenarios.

Molecular techniques are integral to efficient and accurate herpesvirus detection. PCR-based methods, including qPCR, remain the gold standard due to their reliability and widespread use. However, emerging technologies like CRISPR and NGS are expanding the boundaries of viral diagnostics by offering more comprehensive pathogen detection and insights into viral behavior [37], improving the possibility to discriminate between lytic and latent infections [32]. The integration of these advanced methods into clinical practice will require addressing cost, accessibility, and workflow complexity, ensuring broader applicability across healthcare settings.

In the next chapters of this review, we will describe the main DNA-based methods used for viral identification of herpesviruses.

3. Herpesviruses

The identification of herpesviruses in clinical samples of different origin is critical due to their widespread prevalence [38] and their ability to establish lifelong latent infections in the host, often associated with serious diseases [39].

Herpesviruses infect a wide range of animals, with more than 200 species identified, including nine that infect humans (HHVs) [40]. These viruses are classified into three subfamilies based on their tropism and latency sites, namely *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammapherpesvirinae* [41,42] (Table 2).

The *Alphaherpesvirinae* subfamily include herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) and Varicella-zoster virus (VZV). These viruses are characterized by the ability to replicate in a wide variety of host tissues and then establish a latent infection in the ganglia [43,44] (Table 2).

On the contrary, *Betaherpesvirinae* show a restricted host range, establishing latent infection in lymphocytes, secretory glands, cells of the reticuloendothelial system, and kidneys. The viruses that take part of this subfamily are cytomegalovirus (CMV), human herpesvirus-6 (HHV-6) A and -6B, and human herpesvirus 7 (HHV-7) (Table 2).

Gammapherpesvirinae have the most limited host range. They replicate in lymphoblastoid cells, leading to lytic infections and establishing latent infections in lymphoid tissue. Epstein-Barr virus (EBV) and human herpesvirus 8 (HHV-8) are members of this subfamily (Table 2).

Table 2. Characteristics of herpesvirus subfamilies.

Subfamily	Virus	Primary Sites of Replication	Site of Latency	References
<i>Alphaherpesvirus</i>	HSV-1	Epithelial cells in and around mouth, ocular, genital area	Sensory nerve cells	[44]
	HSV-2	Epithelial cells in and around genital area	Sensory nerve cells	[45]
	VZV	Epithelial cells in skin	Neurons of trigeminal ganglia and dorsal root ganglia	[46,47]
<i>Betaherpesvirus</i>	CMV	Epithelial cells of salivary glands, kidneys, genital tract	CD34 ⁺ myeloid progenitor, CD14 ⁺ monocytes	[48–50]
	HHV-6A/B	Epithelial cells of salivary glands, CD4 ⁺ T lymphocytes	CD34 ⁺ stem cells, monocytes	[51–54]
	HHV-7	Epithelial cells of salivary glands, CD4 ⁺ T lymphocytes	CD4 ⁺ T lymphocytes	[55]
<i>Gammapherpesvirus</i>	HHV-8	Epithelial cells in oropharynx	B cells	[27,56,57]
	EBV	Epithelial cells in oropharynx, genital tract	B cells	[58,59]

Latency and Reactivation

After primary infection, HHVs can establish latency following two main strategies. The viral genome can persist as a circular episome in the cell nucleus due to the expression of latency-associated viral genes [60,61] or, less frequently, viral DNA can integrate into human genomic DNA [62]. Reactivation occurs in response to various stimuli, including stress, hormonal changes, UV light exposure, and immunosuppression [1,7,63,64]. Although most reactivations are clinically asymptomatic, they play a crucial role in maintaining a viral reservoir and can lead to severe diseases, such as Alzheimer’s disease, multiple sclerosis, or lupus, especially in immunocompromised individuals [3].

4. DNA-Based Technology for Herpesviruses Detection

The early and accurate identification of herpesvirus DNA in clinical samples is critical for managing and treating infections. In fact, a prompt detection enables early antiviral therapy, reducing the risk of complications, disease progression, and transmission [5]. Regular monitoring is especially important for immunocompromised patients, as viral reactivation can significantly impact disease outcomes. Molecular tools, such as PCR, qPCR, and next-generation sequencing (NGS), remain the most effective methods for herpesvirus diagnosis.

Given the clinical and genomic peculiarities that characterize each herpesvirus, this review will report the most effective molecular tools currently used for HHV diagnosis (Table 3).

Table 3. DNA-based molecular techniques for herpesvirus infection diagnosis described in this review.

Virus	Molecular Method of Viral Genome Detection	Type of Sample	Clinical Applications	References
HSV-1	qPCR	CSF, saliva, blood, swabs from lesions, BAL	Rapid detection of herpes encephalitis; diagnosing oral/genital herpes; outbreak monitoring	[65,66]
	Multiplex qPCR	CSF, feces, urine, saliva, blood, swabs, corneal scrapes, biopsies	Detects multiple viruses; rapid herpes encephalitis diagnosis; early infection detection in transplant patients	[67,68]
	Droplet Digital PCR	CSF, blood, saliva, urine, swabs	Accurate monitoring of viral replication and treatment response; detects low-concentration samples	[69,70]
	NGS	Blood, NPAs, lung lavage, stomach fluid	Detects unknown or co-infections; suitable for low viral loads	[71]

Table 3. Cont.

Virus	Molecular Method of Viral Genome Detection	Type of Sample	Clinical Applications	References
HSV-2	qPCR	CSF, swabs from genital lesions, blood	Diagnoses genital herpes and HSV-related meningitis; rapid detection of genital herpes	[72,73]
	Multiplex qPCR	CSF, blood, cervical, endocervical, vaginal samples	Rapid detection of multiple viruses; comprehensive diagnostics for genital infections	[74,75]
	Droplet Digital PCR	CSF, blood, genital lesion swabs	Monitors viral load; co-detection of HSV-1 and HSV-2	[76,77]
	NGS	CSF, blood, lesion swabs	High sensitivity; useful for early-stage infection or co-infection detection	[78]
VZV	qPCR	Vesicle fluids, swabs, crusts, CSF, amniotic fluid	Diagnosis of central nervous system infections, prenatal congenital varicella syndrome	[74,75]
	Nested PCR	Vesicle fluids, swabs, CSF, amniotic fluid	Detects VZV in skin lesions, encephalitis, meningitis	[79]
	NGS	CSF	High sensitivity; identifies multiple pathogens in CNS samples	[80,81]
EBV	qPCR	CSF, peripheral blood, gingival/salivary swabs	Mononucleosis diagnosis; monitoring EBV in cancers; oral hairy leukoplakia detection	[82–84]
	NGS	Biopsy samples	Identifies cancer associations (e.g., NPC, Burkitt's lymphoma)	[85]
CMV	qPCR	Blood, saliva, urine, amniotic fluid	Diagnoses congenital infections; monitors immunocompromised patients	[86]
	NGS	Aqueous humor	Detects ocular infections and antiviral resistance	[87]
HHV-6	qPCR	Oral samples, CSF, blood, tissues	Identifies active infection; monitors transplant patients for graft-related complications	[88,89]
	Multiplex qPCR	CSF, oral samples, blood, tissues	Rapid encephalitis detection; post-transplant viral monitoring; classification of HHV-6 A and B	[90,91]
	Droplet Digital PCR	Oral samples, CSF, blood	Detects chromosomally integrated HHV-6 (ciHHV-6); quantifies low-grade infections	[92]
	Conventional PCR	CSF, oral samples, blood, serum	Differentiates between latent and active infections and HHV-6 A and B; standardized methods and rapid results	[89]
	NGS	CSF	Comprehensive virome analysis; tracks neurotropic impacts	[93]
HHV-7	qPCR	Blood, CSF, saliva	Rapid detection of reactivations; diagnoses conditions like pityriasis rosea	[94]
	Multiplex qPCR	Blood, CSF, saliva	Screens blood donors; detects ocular infections	[95,96]
	Droplet Digital PCR	Blood, CSF, saliva	Monitors viral loads in immunocompromised patients	[97]
HHV-8	qPCR	Saliva, blood, semen	Detects Kaposi's sarcoma-associated virus; monitors immune-suppressed patients	[98,99]
	Nested PCR	Saliva, blood, semen	Identifies early lesions; frequent monitoring for disease progression	[100]
	Droplet Digital PCR	Saliva, blood, semen	Monitors therapy response; tracks epidemiological transmission patterns	[101]

4.1. Herpes Simplex Virus (HSV)

HSV is a neurotropic virus which consists of HSV-1 and HSV-2 that cause a spectrum of diseases ranging from asymptomatic infections to life-threatening conditions like herpes simplex encephalitis and neonatal herpes [102]. HSV-1 is typically transmitted via the oral route and is associated with upper body infections, including oral and ocular herpes, while HSV-2 is a sexually transmitted infection that primarily causes genital herpes [103]. The choice of clinical sample for HSV detection depends on the infection site (Table 3), such as saliva for oral herpes, genital swabs for genital herpes, or cerebrospinal fluid for suspected encephalitis [104].

Additional diagnostic tests for HSV include the collection of sore fluid from oral, genital, or ocular lesions for direct detection in cell cultures. However, this labor-intensive procedure has been largely replaced by PCR analysis of genetic material extracted from the sample. PCR provides 100% sensitivity and specificity compared to viral culture methods, which achieve only 50% sensitivity [67].

HSV can also infect ocular cells, leading to complications in the retina, cornea, and other parts of the eye [105]. In cases of HSV-associated ocular infections, such as uveitis—a serious sight-threatening condition commonly caused by HSV—samples from intraocular specimens must be collected and analyzed for the presence of the virus.

For cases of herpes simplex encephalitis (HSE), HSV detection can be performed on CSF samples using molecular assays. In more complex clinical cases, metagenomic next-generation sequencing (mNGS) can also be employed to identify the virus [71,106].

4.1.1. Detection of HSVs in Oral and Vaginal Samples

As mentioned above, HSV-1 is commonly associated with oral herpes and only in rare cases, genital herpes, which is most commonly due to HSV-2 infection [107,108]. The most frequent complications associated with HSV mucosal infection include the appearance of highly painful sores and scabs, which resolve within few weeks. For proper diagnosis, it is important to take a swab sample from these lesions before they resolve in order to collect intact virions [103]. Molecular techniques like PCR have replaced traditional methods such as viral culture [105,109], providing higher sensitivity and specificity for detecting HSV in oral and genital lesions [110]. The gold standard molecular technique for HSV diagnosis is PCR [5], particularly qPCR [111]. The fluorescence-based real-time PCR technique has completely revolutionized PCR-based systems for HSV detection in various types of clinical samples. This method also allows for the discrimination between HSV-1 and HSV-2 using multiplex PCR assays [112]. The enhanced sensitivity of multiplex qPCR for HSV detection was confirmed in a study [75], which reported successful HSV detection in genital samples from four patients who had tested negative using the viral culture method. HSV typing was performed using multiplex qPCR with specific primers targeting the TK3 and POL genes for HSV-1 and HSV-2, respectively, demonstrating the specificity and reliability of this technique for diagnosing genital herpes [82]. The same method has also been used to detect HSV in oral mucocutaneous samples, further confirming its diagnostic efficacy [113,114].

Other molecular approaches have explored the use of a restriction fragment length polymorphism (RFLP) analysis of HSV PCR products. This technique enables the detection of variations at specific single nucleotide polymorphism (SNP) loci through enzymatic restriction, providing valuable insights into viral populations. Such information is critical for viral classification and epidemiological studies that investigate host–pathogen interactions [115]. The *DNA polymerase I* genes of HSV-1 and HSV-2 exhibit 93% sequence identity; therefore, polymorphisms in this viral gene can be identified using RFLP analysis. This approach utilizes sequence data from public databases to design restriction endonuclease recognition sites for the specific identification of HSV strains.

4.1.2. Detection of HSV from CSF Samples

Although the most accurate method for diagnosing herpes simplex encephalitis (HSE) involves the isolation of HSV or the detection of its viral antigens in brain tissue, the detection of HSV DNA in cerebrospinal fluid (CSF) is critical for diagnosis [116] (Table 3).

Currently, while no FDA-approved assays exist for prenatal HSV meningitis or HSE screening in CSF, many molecular diagnostic tests are in use [117–120]. Detecting HSV DNA by PCR in CSF samples is highly effective, with a sensitivity of 74–98% and a specificity of 100%, making it a valuable tool for diagnosing HSE or neonatal HSV infections [121]. However, the routine clinical use of semi-quantitative PCR assays for HSV detection in CSF remains uncertain, particularly regarding their ability to discriminate between HSV-1 and HSV-2 [122–124].

This limitation has been addressed by employing multiplex droplet digital PCR (ddPCR) for CSF samples. Xunhua Zhu et al. suggested that the detection of HSV DNA using ddPCR may serve as a useful diagnostic method for HSE, correlating disease severity and patient age with the number of HSV DNA copies detected in CSF [69]. In this study, CSF samples from patients with viral central nervous system (CNS) infections were analyzed using quadruplex ddPCR, which proved to be an accurate and specific method for detecting both HSV-1 and HSV-2. The results were comparable to those obtained using qPCR and quantitative reverse transcription PCR (qRT-PCR) assays through commercial kits [69].

Next-generation sequencing (NGS) has also emerged as a powerful tool for detecting HSV in CSF samples, particularly in cases of suspected viral infections affecting the CNS [71]. NGS provides detailed genomic information that can identify mutations or variations within viral strains. This is particularly valuable when the causative agent is unknown or when multiple infections are suspected. For instance, a study demonstrated that NGS could identify various herpesviruses in patients with neurological symptoms, offering improved diagnostic accuracy compared to conventional PCR methods [125,126].

4.1.3. Detection of HSV in Intraocular Samples

HSV can cause serious ocular complications, such as keratitis and uveitis, which may lead to blindness if left untreated [127,128]. These manifestations can result from either primary ocular HSV infection or recurrent episodes. During herpes simplex keratitis or infectious uveitis, the presence of HSV is typically confirmed using a multiplex qPCR assay, which can differentiate between HSV-1, HSV-2, VZV, and CMV [129]. Although molecular diagnostic tests for HSV infections in ophthalmology are widely available and highly sensitive, recent years have seen the adoption of metagenomic deep sequencing. This advanced technique allows for the detection of infections from less than 50 µL of intraocular fluid, providing an innovative approach to diagnostics [127,130,131].

4.2. Varicella-Zoster Virus (VZV)

VZV, an Alphaherpesvirus, causes primary chickenpox infection and remains latent in dorsal root ganglia. Reactivation results in herpes zoster (shingles), often with complications like post-herpetic neuralgia [132]. Detecting VZV DNA is particularly important during pregnancy and in CNS infections. Approximately 90% of the adult population tests positive for VZV antibodies, and studies on pregnant women report seropositivity rates between 80% and 91% [133–135]. Primary infection with VZV during pregnancy increases the risk of clinical complications for both the mother and fetus. The complications and sequelae of gestational VZV infection include pneumonia, an increased risk of premature abortion, congenital varicella syndrome (CVS), neonatal varicella, and herpes zoster during the first year of life [136,137]. The risk of adverse effects is greater for the mother in the third trimester of pregnancy, whereas it is higher for the fetus in the first and second trimesters [138]. For these reasons, the detection of VZV, especially during pregnancy, is crucial to prevent adverse outcomes, guiding antiviral therapy and preventive measures, ensuring proper prenatal care [137]. VZV can also lead to encephalitis, a relatively rare condition that can cause significant morbidity and mortality, particularly in vulnerable

populations such as the elderly and immunocompromised individuals. Recent studies indicate that the incidence of VZV encephalitis is higher than previously estimated, occurring at a rate of approximately 5.3 cases per million people annually [139], mainly affecting elderly adults [140]. These findings highlight the importance of detecting VZV DNA in cerebrospinal fluid (CSF) samples as a key diagnostic method [141].

In summary, depending on the symptomatology, clinical samples for VZV detection may include vesicle/skin swabs, CSF, the respiratory system or eye swabs, genital swabs, blood, or other body fluids (Table 3). Once collected, these samples are typically analyzed using PCR-based assays, which represent the most sensitive methods for detecting VZV across a variety of specimens in cases of both invasive disease and localized lesions [142].

4.2.1. Detection of VZV in Vesicle Fluids Swabs, Crusts, or Fixed Tissue Samples

Vesicle fluid swabs, dried crusts, skin biopsy, or fixed tissue specimens are the best source of VZV DNA for molecular assays [75,143].

Currently, PCR is the most useful laboratory test to confirm suspected varicella and herpes zoster (Table 3). PCR can detect VZV DNA rapidly and sensitively in skin lesions, vesicles, scabs, and maculopapular lesions [144,145]. Modifications of the basic PCR technique have been used to increase assay sensitivity, including nested PCR, real-time PCR, and multiplex assays.

Among these methods, the Lyra Direct assay (Lyra™ Direct HSV 1 + 2/VZV Assay; Quidel Corp. CA, USA) is a multiplex real-time PCR for the detection and differentiation of HSV-1, HSV-2, and VZV in cutaneous or mucocutaneous lesion samples from symptomatic patients, approved by the FDA in 2014 [76]. This assay identifies and differentiates HSV-1, HSV-2, and VZV using species-specific probes (TaqMan-based probes) under optimized conditions in a single reaction tube, generating distinct amplicons for each virus [76]. To confirm these results, positive amplicons are cloned and validated through sequencing [146].

4.2.2. Detection of VZV in CSF Samples

As with other HHV infections, molecular tests have also rapidly become the gold standard for CSF sample analysis. Numerous studies have confirmed the high sensitivity of PCR-based molecular methods for detecting VZV in various clinical specimens [147], including CSF for diagnosing herpetic encephalitis and meningitis [148,149], as well as amniotic fluid [144,150]. Rapid laboratory diagnosis is particularly crucial when the CNS is involved, especially in cases of clinically ambiguous varicella-related skin manifestations that may suggest VZV infection. Specific molecular analyses for VZV play a critical role in guiding therapeutic decisions and preventing CNS complications as the infection progresses. Real-time PCR assays have proven to be as sensitive as nested PCR for detecting VZV, while being faster, easier to use, and significantly reducing the risk of molecular contamination [151]. In recent years, next-generation sequencing has emerged as a new promising diagnostic tool for VZV-related CNS infections in CSF samples. NGS provides high sensitivity, the capability to detect multiple pathogens simultaneously, and the ability to identify unexpected co-infections. Recent studies have shown that NGS, particularly metagenomic NGS (mNGS), provides higher sensitivity for detecting VZV in CSF compared to conventional methods such as PCR and antibody tests. In one study involving patients with suspected VZV CNS infections, mNGS identified VZV DNA in 16 patients, with some samples showing co-infection with other pathogens like cytomegalovirus and herpes simplex virus. This ability is crucial in clinical settings where rapid and accurate diagnosis is essential for effective clinical management [80,81] (Table 2).

4.2.3. Detection of VZV in Amniotic Fluid Samples

The detection of VZV in amniotic fluid samples is crucial for diagnosing congenital varicella syndrome, particularly when maternal infection occurs during pregnancy [144]. PCR is the most effective method for detection, demonstrating significantly higher sensitiv-

ity compared to traditional cell culture techniques. It represents a vital tool for prenatal diagnosis, enabling the timely intervention and management of potential congenital infections [79]. In a study involving 107 women with clinical varicella before 24 weeks of gestation, PCR detected VZV in 8.4% of samples, whereas only 1.8% tested positive using cell culture methods [144].

In 2021, a case report described a mother diagnosed with varicella at 12 weeks of gestation who underwent amniocentesis. VZV was detected in the amniotic fluid via PCR, confirming congenital varicella syndrome. The newborn exhibited severe abnormalities and, unfortunately, did not survive long after delivery [152]. These findings underscore the importance of early VZV detection in pregnant women, with qPCR remaining the gold-standard diagnostic technique.

4.3. Epstein–Barr Virus (EBV)

EBV infects over 95% of adults worldwide and is linked to diseases like infectious mononucleosis and several cancers, including Burkitt’s lymphoma and nasopharyngeal carcinoma [153–155]. EBV can infect a wide range of cells and tissues, including T and B lymphocytes, nasopharynx and oropharynx squamous epithelial cells, salivary and stomach glands, thyroid glandular epithelial cells, and smooth muscle and follicular dendritic cells [59,60] (Table 2). The quantitative measurement of EBV DNA is necessary to distinguish between healthy carriers and patients with EBV-related diseases [156]. Various molecular techniques have been established and used for the identification of EBV DNA and to measure viral load [156]. To date, in situ hybridization (ISH), protein-based assays, qPCR, and immunoblotting have been utilized in the diagnosis and staging of EBV infection. Although these methods assist in diagnosis, the lack of standardization means that the observed differences in sensitivity and specificity among laboratories should be systematically addressed [157,158].

4.3.1. Detection of EBV from Gingival Swabs and Salivary Swabs

Gingival crevicular fluid (GCF) and saliva have been identified as the primary source of EBV (Table 3). Sampling is achieved by rubbing a swab along the gum line [159]. There are several methods to detect and quantify EBV from these samples, including in situ hybridization, an EBV clonality assay, immunohistochemistry, ELISA, and PCR. In particular, qPCR performed with specific labeled probes provides a precise and sensitive method for EBV quantification in oral samples [83]. Another disease caused by EBV is oral hairy leukoplakia, which is characterized by white patches on the lateral borders of the tongue. It is most common in immunocompromised individuals, particularly those with HIV/AIDS. The detection of EBV in cases of oral hairy leukoplakia can be performed using molecular techniques, such as multiplex PCR to detect EBV DNA in tissue samples from the lesions [82].

4.3.2. Detection of EBV from CSF and Peripheral Blood

The chronic persistence of EBV-infected cells in the peripheral circulation and/or in the CNS is possibly associated with lytic viral reactivation. This clinical aspect has received increasing attention in recent years as a potential cause of MS onset and progression [160]. A real-time PCR assay was shown to be the best technique for the detection and quantification of EBV viral load in this type of sample (Table 3). Furthermore, to establish whether the presence of the virus is associated with latently infected cells or viral lytic reactivation, EBV DNA detection could be performed on both cell-associated CSF fractions and peripheral blood mononuclear cells (PBMCs) to investigate the possible role of EBV systemic infection in the disease. This method allows for the identification of lymphomonocytes acting as carriers of viral particles from the periphery to the CSF or the assessment of the release of a free virus following lytic infection [161].

4.4. Human Cytomegalovirus (CMV)

CMV is a highly prevalent Betaherpesvirus, with seroprevalence rates ranging from 60% to over 90% worldwide [162]. However, most infected individuals remain asymptomatic because of the development of an effective memory immune response [163]. Following initial infection, the virus can remain latent in myeloid lineage cells, such as CD34+ progenitor cells and peripheral blood monocytes. CMV is acquired most commonly early in life, during childhood to early adulthood, through exposure to saliva, tears, urine, stool, breast milk, semen, and other body secretions from infected individuals [164]. It can also be transmitted efficiently via organ and tissue transplantation and blood transfusions [165], making the screening for CMV positivity crucial to avoid graft.

Upon the resolution of primary infection, similar to other herpesviruses, CMV establishes lifelong latency, but in contrast to HSVs, genes expressed during CMV latency are not latency-specific, as their expression was observed also during the lytic cycle [166,167].

A critical aspect of CMV infection is gestational infection. Primary CMV infection occurs in 0.7 to 4.1% of all pregnancies and is asymptomatic in approximately 75 to 95% of mothers. In some cases, it may manifest as a mild mononucleosis or flu-like syndrome with persistent fever and fatigue [49,168]. CMV is the most frequent cause of congenital infections, sensorineural hearing loss, and congenital malformations. Complications due to fetal CMV infection may also involve calcifications around the brain's ventricles, enlargement of the cerebral ventricles, microcephaly, hepatosplenomegaly, cerebellar hypoplasia, restricted fetal growth, pleural effusion, and placental enlargement [166,168]. To prevent these issues, it is crucial to promptly conduct diagnosis. Furthermore, CMV infection continues to be a major concern in post-solid organ transplant (SOT), affecting both the allograft and recipient survival by causing CMV disease, drug-related side effects, bacterial and opportunistic infections, and transplant rejection. Moreover, it may negatively impact the quality of life for recipients of solid organ transplants [169].

In addition to plasma, where viremia and specific antibodies can be detected, CMV is often found in CSF and urine (Table 3). For these reasons, monitoring CMV viral load in these samples is necessary, particularly in critical care patients [170]. Many of the current techniques for CMV detection and quantification are either imprecise, have poor sensitivity, or require long processing times, as seen in ELISA serological analysis [171,172]. However, qPCR with automated DNA extraction has the potential to improve these deficiencies [173,174].

4.4.1. Detection of CMV in Oral, Urine, and Amniotic Fluid Samples

The standard method to diagnose CMV consists of virus isolation from urine or saliva in tissue cultures, but this procedure is labor-intensive, costly and not suitable for high-volume screening purposes. Recent large-scale studies have demonstrated that CMV real-time PCR performed on saliva is a sensitive, acceptable, and feasible method for hospital newborn screening [86,175] (Table 3). In fact, the rapid identification of newborns with CMV congenital infection after birth is crucial to promptly address possible CNS concerns, hearing loss, and developmental delays in order to perform proper diagnosis, monitoring, and intervention [176,177]. Urine samples are commonly utilized for congenital CMV screening infection because in these samples, infected infants often exhibit high virus levels [178]. In fact, Yamamoto et al. demonstrated that urine samples can be as effective as saliva samples in identifying CMV-infected infants through molecular screening programs [176]. Anyway, even if molecular assays are effective in detecting CMV in both saliva and urine during screening, these methods need to be further improved [176]. Another approach for the early identification of congenital CMV infection is the use of amniotic fluid. Amniocentesis is usually performed between 20 and 24 weeks of gestation when there is suspicion of CMV infection, such as when a fetal ultrasound shows signs consistent with infection. Amniotic fluid collected during amniocentesis can be analyzed by qPCR to allow for the early detection of CMV for a better management of affected pregnancies [178,179].

4.4.2. Detection of CMV in Blood Samples and Aqueous Humor

Recent studies have suggested that plasma-based PCR assays result in a delayed detection of CMV DNA due to the lower sensitivity of these assays compared to cell-based and whole-blood assays [179,180]. However, plasma does not require lengthy cell separation procedures and offers a much better opportunity to detect CMV viremia during periods of severe cytopenia when cell-based assays perform poorly. Plasma CMV viremia is associated with overall mortality in stem cell transplant recipients [181] and HIV-infected individuals [182]. Recently, the detection of CMV DNA in EDTA whole-blood samples has been suggested as the most sensitive method for monitoring CMV infection in immunosuppressed patients [183]. A high-sensitivity molecular assay may detect the latent virus, but the clinical relevance of low-level CMV DNA in whole blood is unresolved. Nevertheless, the early detection of CMV DNA has the advantage of warning clinicians of the evolution of infection and carefully monitoring viral load kinetics [184]. Razonable et al. reported the use of an automated qPCR to simultaneously measure the CMV DNA levels in whole blood (WB), plasma (PL), peripheral blood leukocytes (PBLs), and peripheral blood mononucleated cells (PBMCs). Higher levels of CMV DNA were observed in WB than PLs, and CMV DNA levels of PBLs and PBMCs were highly comparable. In spite of the adequacy of all blood compartments for CMV DNA quantification, the higher sensitivity of WB and its yield of higher CMV DNA render it an optimal sample for monitoring CMV DNA load during CMV disease, especially in immunocompromised patients [185]. The analysis of aqueous humor is essential for diagnosing ocular diseases associated with CMV. PCR is the primary method used to detect CMV DNA in aqueous humor. It offers high sensitivity and specificity. For example, studies have reported that PCR can detect CMV DNA in 93% of aqueous humor samples from eyes with active retinitis compared to 100% detection rates with next-generation sequencing [186,187] (Table 3).

4.5. Human Herpesvirus 6 (HHV-6)

HHV-6 belongs to the Betaherpesvirus subfamily (Table 2) [188]. HHV-6 is one of the most prevalent herpesviruses in humans. It spreads primarily through saliva, making close contact a common way of transmission. The diagnosis of HHV-6 infection can be performed by serology (indirect method) or real-time PCR, viral culture, in situ hybridization, and immunohistochemistry (direct methods). However, the most prominent technique is the quantification of viral DNA in blood, other body fluids, and organs using real-time PCR (Table 3). Although qPCR is useful for HHV-6 diagnosis and in determining viral load, serological tests, such as immunofluorescence and ELISA assays, have the potential to differentiate latent from lytic infection and can detect past exposure [88]. PCR-based methods are financially accessible, quick, safe, and currently widespread. In addition, these approaches readily permit the differentiation of the two species of HHV-6, even in cases of mixed infection [89].

Recently, HHV-6 has been subdivided into two different viruses, namely HHV-6A and -6B [189,190]. Even if the two viruses share about 90% of the genome, they have been differentiated based on their epidemiology, pathology association, in vitro growth properties, reactivity with monoclonal antibodies, and restriction endonuclease mapping [188,191]. For this reason, discrimination among the two HHV-6 viruses also represents a crucial clue in diagnosis.

4.5.1. HHV-6A and HHV-6B

HHV-6A and HHV-6B have distinct prevalence and clinical associations. HHV-6A is more common in specific geographic regions, such as sub-Saharan Africa [192], while HHV-6B, which is primarily linked to roseola infantum, a common childhood illness characterized by high fever followed by a rash [193], is diffused worldwide.

While HHV-6B is predominantly associated with childhood illnesses and is better understood, HHV-6A's knowledge is more limited, even if its emerging link to serious neurological conditions highlights its significance in medical research [194]. HHV-6A has

been described in association with neurological disorders, such as MS, systemic sclerosis (SSc), and Alzheimer's disease [89,195,196]. Moreover, HHV-6A endometrial infection has also been described as potentially involved in women with unexplained infertility [197], which seems to be due to the modulation of natural killer cells by the virus [198]. Although both viruses can cause mild infections, HHV-6B may lead to more severe complications in immunocompromised patients, including encephalitis and organ inflammation [199]. Following initial infection, both viruses can enter a latent phase within the body via episome formation. Since both HHV-6A and HHV-6B possess telomere-like repeats at the terminal regions of their genomes, latency may also occur by integration into the host telomeres [51,199]. HHV-6B often integrates into the DNA of immune cells, particularly T-cells, which can subsequently reactivate, leading to symptoms such as a fever and rash. Understanding the behavior of these viruses, especially their latency and reactivation mechanisms, is crucial for managing their health impacts.

To date, several molecular assays are available to distinguish HHV-6A and HHV-6B in different clinical samples.

4.5.2. Chromosomally Integrated HHV-6

Chromosomally integrated HHV-6 (ciHHV-6) occurs when HHV-6 integrates its genetic material into the human genome, typically at chromosome telomeres. This condition differs from standard latent or active infections, as the viral DNA becomes a permanent part of the host's chromosomes and can be inherited [199].

In fact, HHV-6 can integrate into the chromosomes of germline cells and be vertically transmitted [200]. The integrated viral genome is inherited in a Mendelian manner, meaning there is a 50% chance of passing it on to offspring. Consequently, individuals with ciHHV-6 may have one or more copies of the viral genome present in every nucleated cell in their body [201]. About 1% of the population carries ciHHV-6 [200], which results in high viral loads in blood tests, not due to active infection but due to the presence of integrated viral DNA in every cell.

Despite the majority of subjects carrying ciHHV-6 remaining asymptomatic, ongoing research is investigating the implications of its presence on health to improve clinical diagnostics [202,203]. ciHHV-6 diagnosis is primarily performed through PCR, where a persistently high viral load without symptoms may indicate ciHHV-6 [201]. There are concerns about the potential reactivation of the virus, especially in immunocompromised individuals. For instance, considering organ transplant patients, distinguishing ciHHV-6 from reactivated infections is crucial to avoid unnecessary treatments. The detection of ciHHV-6 poses challenges due to the presence of viral DNA in various body fluids, which does not reliably indicate active infection [199]. Reverse transcription PCR (RT-PCR) is effective for identifying active replication by detecting HHV-6 mRNA encoding structural proteins transcribed during the late phase of viral replication [204]. This qualitative assay is informative only during active viral replication, with a sensitivity of 90% and a specificity of 98% [194,205]. Real-time qPCR helps in measuring viral load to differentiate between primary and latent infections, although overlapping values can complicate interpretations [206]. A comprehensive diagnostic approach based on multiple assays has been developed to identify ciHHV-6 with high sensitivity [92,207–211]. Specifically, this is composed by qPCR to categorize viral load and, if necessary, RT-PCR to confirm active replication. Additionally, ddPCR has been created to detect ciHHV-6 with high sensitivity [92,207–211].

4.5.3. Detection of HHV-6 in Oral and Ocular Samples

A practical method to detect HHV-6 involves saliva sample collection using small filter paper strips followed by qPCR. The sensitivity and specificity of this technique are comparable to those of the standard method of saliva collection, even after prolonged drying of the specimens, without affecting the results [212]. Recent studies have shown that qualitative nested PCR of the HHV-6 genome derived from saliva is useful to distinguish among HHV-6A, HHV-6B, and chromosomally integrated viral DNA [213]. Besides

nested PCR, other more advanced assays have been developed to differentiate between HHV-6A and HHV-6B in several fluids, including saliva. These specific PCR techniques can accurately identify both viruses even in mixed infections, with sensitivities as low as one copy per well for HHV-6B [93]. PCR-based assays, particularly multiplex PCR and nested PCR, have proven to be successful to detect HHV-6 also in ocular samples from patients with inflammatory disorders, suggesting its possible involvement in the development of inflammation in the eye [214].

4.5.4. Detection of HHV-6 in CSF Samples

As other HHVs, HHV-6 can also invade the CNS and persist longitudinally in the brain [215]. HHV-6 establishes latency in the nervous tissue, in particular, in several cell types including glial cells, oligodendrocytes, and astrocytes [52–55,188]. The detection of herpetic DNA, including the HHV-6 genome, in CSF by PCR is considered as a diagnostic marker of active infection in the CNS (Table 3) [216]. Recently, the use of multiplex PCR panels for HHV-6 detection in CSF was evaluated. Despite its advantages, this method can be challenging, as the detection of latency or subclinical HHV-6 reactivation may lead to incorrect assumptions of the cause of CNS disease, potentially harmful therapies, and delayed true diagnosis [217]. The interpretation of HHV-6 detection from CSF is further complicated by the fact that HHV-6 can exist as ciHHV-6. Recently, to improve the accuracy of identifying viral infections such as acute encephalitis and encephalopathy caused by HHV-6, next-generation sequencing has been implemented. NGS has been utilized for identifying viruses and finding new viruses in clinical specimens (Table 3). While it has been difficult to identify viral pathogens in patients with encephalitis/encephalopathy, multiple studies have demonstrated the effectiveness of NGS in identifying viral pathogens through the detection of virus-induced DNA or RNA sequences in CSF [218,219].

4.5.5. Detection of HHV-6 in Blood and Tissue Samples

The diagnosis of HHV-6 infection in blood samples poses a significant challenge. Conventional PCR assays for detecting viral DNA in peripheral blood leukocytes cannot distinguish between latent infection—present in the vast majority of healthy individuals—and active infection [220]. Only qPCR assays capable of detecting free HHV-6 DNA in biological fluids offer reliable tools for tracking active HHV-6 infection [194] (Table 3). However, the routine use of these assays in large epidemiological studies has been limited by their technical complexity and labor-intensive nature.

A novel highly sensitive qPCR test has been developed to precisely measure the levels of HHV-6 DNA in cell suspensions from tissues and body fluids. This assay employs a 5′ nuclease fluorogenic test with real-time monitoring of PCR amplification products using the ABI PRISM 7700 sequence detector system [206]. The sensitivity of this method matches that of a nested PCR protocol for both A and B subgroups of HHV-6. Additionally, the assay demonstrated a broader detection range (1 to 10⁶ viral genome equivalents per test) and greater accuracy, repeatability, and reproducibility compared to a quantitative competitive PCR assay using the same reference DNA. This method is flexible and exhibits equal sensitivity and dynamic range when analyzing viral DNA from various fluids (e.g., culture medium or plasma) or cell suspensions obtained from tissues [201]. Nested PCR assays remain available for detecting HHV-6 viral DNA in a variety of body fluids and tissues, including plasma, peripheral blood mononuclear cells (PBMCs), and whole blood [213]. Recently, the ability to differentiate between HHV-6A and HHV-6B has been enhanced by ddPCR assays [196]. Furthermore, PCR-based techniques are also used to detect chromosomally integrated HHV-6 (ciHHV-6) in blood or other tissues (Table 3).

4.6. Human Herpesvirus 7 (HHV-7)

Human herpesvirus 7 (HHV-7) belongs to the Betaherpesvirus subfamily. It is genetically, epidemiologically, and clinically closely related to the more extensively studied HHV-6 [221]. HHV-7 primarily infects CD4⁺ T-lymphocytes, with CD8⁺ and immature

T-cells being infected less commonly [55]. Upon the resolution of primary infection, HHV-7 establishes long-life latency in CD34⁺ hematopoietic progenitor stem cells (HPCs) [55]. HHV-7 is widely spread, and the initial infection typically occurs in young children, reaching its highest incidence between 18 and 36 months of age. In children, HHV-7 infection can manifest in various ways, including exanthema subitum, fever without rash, febrile seizures, and prolonged febrile seizures [222,223].

HHV-7 has also been reported to enter the CNS, although the mechanism by which it crosses the blood–brain barrier (BBB) remains unclear [224]. In the past, there have been reports of individual cases and groups of cases of encephalitis or encephalopathy related to HHV-7 in both immune-competent and immune-compromised children and adults [225]. The range of symptoms related to involving the CNS is varied and distinct from other neurological conditions. Symptoms can include febrile seizures, encephalitis, meningoencephalitis, facial palsy, vestibular neuritis, severe headache, drowsiness, fatigue, nausea, vomiting, sensitivity to light, loss of coordination, and unconsciousness [225–228].

HHV-7 is frequently linked to myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), an illness characterized by long-lasting or repeated disabling fatigue [229]. Even though research has not identified one specific pathogen as the direct cause of ME/CFS [230], HHV-7 infection is commonly found in subjects with the condition. However, the HHV-7 DNA detection rate is similar in ME/CFS and healthy individuals [231]. Therefore, the connection between HHV-7 infection/reactivation and ME/CFS requires further investigation. While large-scale evidence is lacking, a recent study suggests that HHV-7 may also have the ability for chromosomal integration, sharing similar genomic regions to HHV-6, including direct repeats [229,231]. Prusty et al. explored the possibility of germ-line transmission of HHV-7 by developing a qPCR capable of detecting as few as 10 viral genome copies within complex human genomic DNA samples.

4.6.1. Detection of HHV-7 from Saliva

The detection of HHV-7 from saliva specimens has emerged as a promising approach to understand the virus's role in various conditions, including ME/CFS [229] (Table 3). In a study involving participants with ME/CFS, saliva samples were collected monthly over six months, as well as during episodes of symptom exacerbation [229]. The viral load of HHV-7, along with other herpesviruses, was quantified using ddPCR, which allows for the highly sensitive detection of viral DNA [97]. The outcomes revealed that HHV-7 was detectable in saliva, with higher viral loads observed in patients with ME/CFS compared to healthy controls [97]. This correlation suggests that fluctuations in HHV-7 DNA levels may be associated with the severity of symptoms experienced by patients, highlighting the potential significance of saliva as a non-invasive diagnostic sample for monitoring herpesvirus reactivation and its implications in chronic illnesses.

4.6.2. Detection of HHV-7 from CSF

As mentioned above, HHV-7 can enter and infect the CNS. HHV-7 DNA was found in post-mortem brain tissue samples [224] and in the CSF of children with acute/subacute neurological symptoms using real-time PCR [221]. Yet, this approach fails to differentiate between the primary infection and subsequent viral reactivation or reinfection.

Primary HHV-7 infection typically happens in kids, often without or with mild symptoms, and is commonly linked to neurological issues like febrile seizures in children [223,232]. The presence of HHV-7 DNA in CSF has been observed in children with CNS diseases, indicating that neuroinvasion possibly occurs during initial infection [233,234]. In clinical settings, diagnosing a potential CNS disease due to HHV-7 infection typically relies on detecting HHV-7 DNA in CSF by qPCR. However, proving the diagnostic role of this amplification could be challenging, since HHV-7 reactivation in the CSF could be a side effect linked to other inflammatory or non-inflammatory CNS diseases. To overcome the limitations of conventional PCR, multiplex PCR can be employed for a more precise detection of HHV-7. Research by Leveque et al. showed that multiplex PCR is an accurate

and specific method, enabling the rapid and simultaneous identification of HHV-7 from small volumes of CSF samples. This molecular diagnostic tool has the potential to enhance the standard virological diagnosis and treatment of CNS infections by identifying multiple viral infections that could lead to more severe cases of meningitis or encephalitis [235].

4.6.3. Detection of HHV-7 from Blood Samples

HHV-7 can be found in peripheral blood during initial infections and reactivations from latency [236]. Although various studies have confirmed the presence of HHV-7 DNA in peripheral blood lymphocytes (PBLs) using PCR, its clinical relevance remains uncertain because it is frequently detected in healthy individuals [236]. This highlights the need for improved molecular methods to detect HHV-7 in blood and plasma samples. While transmission through blood or blood products may not pose a significant risk to the general population due to widespread immunity, it could present potential dangers for young children, non-immune individuals, or immunocompromised patients [237]. Conventional PCR techniques, however, have limitations regarding sensitivity. Multiplex PCR offers a valuable alternative for screening blood donations for viral pathogens, including HHV-7. For example, a study by Zhang et al. demonstrated the effectiveness of a multiplex qPCR assay in detecting HHV-7 in blood samples from donors and patients with pityriasis rosea. In this study, 55% of blood donor samples tested positive for HHV-7, indicating a significant presence of the virus in the population. This method exhibited high specificity and sensitivity, establishing it as a reliable approach to ensuring blood safety [95].

4.7. Human Herpesvirus 8 (HHV-8)

HHV-8 belongs to the Alphaherpesvirus subfamily and is the first known member of the genus Rhadinovirus (Table 2) [238]. Through the use of classical epidemiological studies and laboratory techniques, scientists were able to associate this virus with Kaposi's sarcoma (KS) [238,239], multicentric Castleman's disease [240], primary effusion lymphoma (PEL) [241], and human immunodeficiency virus (HIV) [242]. HHV-8 establishes latency in B cells, and after primary infection, it can trigger symptomatic reactivations in immunocompromised patients [243,244]. The virus's genetic material has been detected in various proportions in the oropharyngeal epithelium, saliva, semen, and blood, although its precise transmission mechanisms remain unclear [245–248].

4.7.1. Detection of HHV-8 from Oral Swabs and Saliva

The exact mechanism underlying the progression of HHV-8 infection to HHV-8-associated diseases remains unclear.

HHV-8 is most commonly detected in the oropharynx, with studies identifying its DNA in 15–57% of salivary samples from asymptomatic seropositive individuals [249]. These findings suggest that salivary transmission could represent a significant infection route and that oral replication likely serves as a critical reservoir contributing to systemic dissemination and, ultimately, the development of KS [249]. Taylor et al. found that 40% of the 1074 HHV-8 seropositive women enrolled in their study had HHV-8 DNA detected by PCR [250]. Specifically, the virus was found more frequently in samples taken from the mouth compared to samples taken from the genitals. Detection rates were 34% in oral specimens and 6% in genital specimens, with 32% of saliva samples and 28% of mouth swab samples testing positive for HHV-8 [250]. Furthermore, Derafshi et al. demonstrated that nested PCR or qPCR techniques can effectively amplify the highly conserved ORF73 gene in saliva samples to identify HHV-8 [100]. These findings indicate that oral specimens are effective in detecting HHV-8. However, further advancements in molecular methods are necessary to achieve a faster and more precise detection of HHV-8 infections.

4.7.2. Detection of HHV-8 from Semen and Blood Samples

HHV-8 is considered a significant risk factor for disease development only in the presence of additional cofactors, such as sexual transmission (e.g., concurrent HIV infection)

or nonsexual routes [250,251]. In particular, HHV-8 DNA has been identified in sperm as well as in normal and malignant prostate tissues, reinforcing the sexual route as a critical mechanism for viral spread [252]. Recently, nested PCR demonstrated its potential for assessing HHV-8 DNA prevalence in semen samples (Table 2). Conversely, when analyzing blood samples, the SYBR Green qPCR method is preferred for detecting and quantifying HHV-8 DNA [100,251].

5. Emerging Molecular Approaches and Future Perspectives

To date, several DNA-based methods are already used in the diagnosis of viral infections, such as the detection and genotyping of human papillomaviruses (HPVs) and members of the Herpesviridae family, including HHV-6, HHV-8, VZV, and CMV [253–257].

Recent advancements in molecular biology have significantly enhanced the design of new assays for detecting and diagnosing herpesviruses. This review explores the latest DNA-based technologies shaping the future of herpesvirus detection.

Currently, many molecular assays for virus detection rely on PCR [258,259]. They provide sample-to-result workflows, requiring minimal sample preparation, offering fast and simple reports, and demanding limited interpretation by laboratory personnel with minimal technical expertise [260].

However, existing methods for detecting viral nucleic acids, such as droplet digital PCR and other digital PCR technologies, face limitations that hinder their broad application in clinical settings. While ddPCR offers significant advantages in sensitivity and specificity, the high cost of equipment and reagents can be a substantial barrier [14]. This high cost may limit access for smaller laboratories, especially in developing countries or those in resource-limited settings [261], where ddPCR is less feasible for routine use compared to conventional PCR methods [262]. Although ddPCR simplifies some aspects of viral nucleic acid quantification by eliminating the need for standard curves, the overall workflow remains complex, introducing operational challenges, such as errors during sample preparation and the need for specialized training [14,263]. To address these limitations, ongoing research and recent innovations focus on enhancing the accessibility and efficiency of ddPCR and related technologies. Efforts include developing more affordable reagents, optimizing protocols to streamline workflows, creating portable ddPCR systems for diverse settings like point-of-care diagnostics, and integrating microfluidics and automation to simplify procedures [14].

Among the most promising technologies for improving infection diagnosis and control is NGS. When applied to whole-genome sequencing (WGS), NGS has demonstrated significant advancements in epidemiology, phylogenetics, virulence determinants, and the evolution of antimicrobial resistance [264]. However, barriers to the widespread adoption of NGS in infection control include concerns over data quality and the effective implementation of bioinformatics in clinical laboratories [265,266].

Emerging technologies like biosensors are transforming viral diagnostics. These devices detect biological analytes, including viruses, by converting biological interactions into measurable electrical signals [267]. Their rapid response times, sensitivity, and portability make them ideal for point-of-care diagnostics. Innovations in DNA nanotechnology and aptamer-based detection further enhance their specificity and versatility, promising significant advancements in viral diagnostics. Biosensors offer substantial advantages over traditional diagnostic methods, including rapid results, high sensitivity, and the potential for point-of-care testing.

Different biosensor types have been utilized for virus detection. For example, electrochemical biosensors exploit electrochemical reactions to detect viral components like proteins or nucleic acids. These sensors can achieve extremely low detection limits (femtomolar concentrations) and are frequently used for viruses such as HIV and SARS-CoV-2 [267]. Moreover, the recent integration of nanomaterials enhances their sensitivity and specificity [268]. Optical biosensors, which employ mechanisms such as surface plasmon resonance (SPR) or surface-enhanced Raman scattering (SERS), use light interactions with viral

particles for real-time detection [269]. These methods are highly sensitive and can detect viruses without the need for specific labeling. For instance, plasmonic biosensors provide cost-effective and rapid virus identification due to their tunable properties [270].

The most innovative biosensor-based devices available are nanobiosensors. These biosensors employ nanomaterials like gold nanoparticles and graphene quantum dots to improve performance [271]. These materials improve sensitivity and specificity by providing a high surface area for interactions with viral targets. Nanobiosensors are also highly miniaturizable, making them suitable for diverse settings [23,270].

Although DNA nanotechnology-based biosensors show significant potential for virus identification, further studies are required in this emerging area of research [272,273].

For example, CRISPR-based diagnostics are evolving, with ongoing research addressing detection limitations. Innovations include developing PAM-independent systems [274], exploring alternative Cas proteins [274], exploring other alternative Cas proteins (e.g., Cas14) [275,276], and integrating metagenomics [277]. These advancements aim to simplify workflows, enable the multiplex detection of multiple pathogens, and reduce contamination risks, thereby improving the robustness of CRISPR in diagnostics.

Despite these advancements, universal detection across diverse herpesvirus species remains challenging. The need for high specificity and sensitivity, reducing direct and indirect associated costs, and the importance to improve consistency in clinical diagnostics by standardizing DNA-based herpesvirus detection methods across laboratories continue to drive research on novel molecular assays capable of adapting to emerging viral strains and facilitate point-of-care testing.

6. Conclusions

In this review, we reported how DNA-based molecular technologies have evolved for the identification of viruses, focusing on herpesvirus detection. Methods for identifying herpesviruses primarily rely on PCR-based or related techniques, which enable the amplification of viral DNA fragments from various samples with high specificity and sensitivity.

Advanced techniques, such as ddPCR and biosensors, have significantly improved the management of herpesvirus-related diseases, enabling faster interventions and delivering more accurate prognoses. These new laboratory tools offer the ability to detect latent infections, promising not only faster and more precise diagnostics but also deeper insights into herpesvirus epidemiology and pathogenesis.

Despite the high diagnostic performance achieved, challenges remain. Enhancing sensitivity, reducing costs, and enabling broader accessibility—particularly in low-resource settings—remain critical areas of focus in clinical research. Addressing these challenges will be essential to expanding the global impact of these molecular diagnostic tools on public health.

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