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REVIEW

Current status of diagnostic assays for emerging zoonotic viruses: Nipah and Hendra

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ABSTRACT

Introduction: Nipahand Hendra viruses belong to the Paramyxoviridae family, which pose a significantthreat to human health, with sporadic outbreaks causing severe morbidity andmortality. Early symptoms include fever, cough, sore throat, and headache,which offer little in terms of differential diagnosis. There are no specifictherapeutics and vaccines for these viruses.

Areas covered: Thisreview comprehensively covers a spectrum of diagnostic techniques for Nipah andHendra virus infections, discussed in conjunction with appropriate type ofsamples during the progression of infection. Serological assays, reversetranscriptase Real-Time PCR assays, and isothermal amplification assays arediscussed in detail, along with a listing of few commercially availabledetection kits. Patents protecting inventions in Nipah and Hendra virusdetection are also covered.

Expert opinion: Despiteseveral outbreaks of Nipah and Hendra infections in the past decade, in-depth researchinto their pathogenesis, Point-of-Care diagnostics, specific therapies, andhuman vaccines is lacking. A prompt and accurate diagnosis is pivotal forefficient outbreak management, patient treatment, and the adoption ofpreventative measures. The emergence of rapid point-of-care tests holds promisein enhancing diagnostic capabilities in real-world settings. The patent landscapeemphasizes the importance of innovation and collaboration within the legal andbusiness realms.

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Isothermal amplification; lateral flow assays; molecular diagnosis; re-emerging infectious diseases; RPA; RT-LAMP; serological diagnosis

1. Introduction

Nipah Virus (NiV) and Hendra Virus (HeV) are closely related zoonotic viruses that belong to the Henipavirus genus of Paramyxoviridae family. Both NiV and HeV have negative-sense, single-stranded RNA and differ from other paramyxoviruses due to their genetic make-up, high virulence, and broad host range. These paramyxoviruses have been classified as biosafety level 4 (BSL 4) pathogens. Fruit bats belonging to the genus *Pteropus* serve as reservoir host for the NiV and HeV [1]. The HeV and NiV have comparable pathophysiology of infection, and large genetic similarities [2]. NiV and HeV vary in disease progression, with severe symptoms potentially leading to fatality [3]. There is no specific medication available in the market for these viruses; however, a broad-spectrum nucleoside antimetabolite antiviral drug ribavirin and/or chloroquine have been suggested as a treatment option for henipaviruses [4].

In 1994, HeV was found in Hendra, Australia, during an outbreak of horse and human respiratory and neurological illness. Within 2 weeks, 14 horses perished from this disease [5]. NiV infection has caused fatal encephalitis in Malaysia, Singapore, Bangladesh, Philippines, and India. NiV infection in humans was initially found in Malaysia and Singapore pig farmers between September 1998 and May 1999, with 276 cases and 105 deaths. The Nipah virus (NiV) is named after Sungai Nipah in Negeri Sembilan, Malaysia. In 1999, blood samples from encephalitis patients in this village showed NiV [2,4,6]. Between 2001 and 2013, Bangladesh saw annual NiV outbreaks with a 70% death rate [7]. Since 2001, Bangladesh has reported more than 300 NiV infections and several deaths [8]. Raw date palm sap was the main transmission route from bats to humans in Bangladesh [8]. The last NiV outbreak in Bangladesh was in February 2023 with 11 cases [8]. Philippines had 17 NiV infections and 52.9% death rate in 2014 [9]. The first incident in India occurred in Siliguri, West Bengal, in January and February 2001. This outbreak killed 45 of 66 verified victims, a 68% fatality rate [10]. NiV infection occurred again in Nadia, West Bengal, in 2007, with five cases and 100% fatality [7]. NiV infection was reported in Kerala in 2018, with 23 cases with 91% fatality [11,12]. In India, NiV infection outbreaks were again recorded in 2001, 2007, 2019, 2021, with most recent one in 2023 (Figure 1) [13–15].

Genomes of NiV and HeV viruses have been sequenced. NiV's gene start, and end sequences and intergenic regions were highly similar to HeV's. NiV has six genes that code for the RNA-dependent RNA polymerase protein (L), fusion protein (F),

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Article highlights

- In the absence of specific medication and vaccines, resurgent occurrences of lethal infections caused by Nipah and Hendra viruses in Southeast Asia pose a significant threat to human life not only in this region but also in other parts of the world.
- Quick, cheap, sensitive, specific, and readily available diagnostic assays are crucial to identifying and isolating infected people, performing contact tracing, and preventing the spread of the infection.
- Heating, organic solvents, fixatives, detergents or guanidinium isothiocyanate treatment may be employed for inactivation of viruses in infected samples, permitting further diagnostic processing in a low containment facility.
- Western Blotting, Virus Neutralization Assay, and Enzyme-Linked Immunosorbent Assay are some of the serological assays that have been used for detection of Nipah and Hendra Viruses.
- Reverse Transcription Real-Time PCR, TruNAT, Reverse Transcription Loop-Mediated Isothermal Amplification, and Recombinase Polymerase Amplification assays are among the molecular methods for detection of Nipah and Hendra Viruses.
- Analysis of the patent landscape revealed technological gaps as well as opportunities for innovation in the development of point-of-care devices for Nipah and Hendra Virus detection in low-resource settings.

Figure 1. Timeline of infections from Nipah and Hendra Viruses.

phosphoprotein (P), matrix protein (M), and nucleocapsid (N) [16]. Six significant proteins are also encoded by the HeV genome. A ribonucleoprotein complex (RNP) is formed when the freshly synthesized RNA genome interacts with the nucleoprotein (N), phosphoprotein (P), and polymerase (L) proteins [17]. The NiV P gene contained the ORFs for the V and C proteins. Projected amino acid homologies between NiV and HeV range from 92% to 67%, whereas nucleotide homologies range from 88% to 70% [16]. The genomic sections with less homology to other viruses can be employed as biomarkers for molecular and serological identification. Ephrin B2 and B3 cell surface receptors, found on neurons, artery endothelium, and smooth muscle cells, bind to the virus G glycoprotein and were employed as a capture ligand for ELISA-based Henipavirus serological detection [18,19]. Most of the serological studies targeted N, P and G protein for detection of NiV and HeV [20–22]. Clinical evaluation (symptoms, travel, and exposure history) and laboratory testing for viruses using ELISA or RT-PCR are the main methods for diagnosing NiV and HeV infections. NiV and HeV RNA can be detected in blood, serum, or other bodily fluids using PCR [23]. Serological tests can identify HeV and NiV-specific IgM and IgG antibodies using enzyme-linked immunosorbent assay (ELISA) [21]. Imaging studies, which include chest X-ray, Computed Tomography (CT) scan, Magnetic Resonance Imaging (MRI), Transmission Electron Microscope (TEM) and Super Resolution Microscopy (SRM), also aid in diagnosis of NiV and HeV. Infected vero cells displayed pleomorphic virus-like structures, identified as round HeV virions using TEM and SRM [19,24].

Recurrent NiV and HeV infections are mostly found in Southeast Asia and have little influence on the Western world. Western markets are relatively unconcerned about diagnosing and treating these diseases, but no part of the world is isolated from these kinds of infections. NiV and HeV have a high Case Fatality Rate (CFR) and no vaccine for human use, although there have been reports of viable candidates and a vaccine is available for horses [25,26]. Thus, diagnosing NiV and HeV infections is crucial to medical treatment, public health, and global health security to prevent new epidemics. Early diagnosis helps identify infected people and others who came into close contact with patients. This diagnosis helps monitor and isolate at-risk persons, preventing transmission. A variety of assays and diagnostic kits are available to quickly identify NiV and HeV. In this comprehensive review, we discuss disease prognosis with various symptoms, suitable sample types during disease progression, molecular diagnostic techniques like serological, reverse transcriptase-based, and isothermal amplification-based tests, commercially available kits, lateral flow devices, and intellectual property (IP) status for NiV and HeV detection methods. Knowledge of IP status and availability of NiV and HeV diagnostic kits play an important role in stimulating innovation, upholding quality standards, enhancing accessibility, and encouraging healthy competition within the realm of NiV and HeV detection kit development.

2. Disease progression and sample types

NiV and HeV can cause serious infections with high CFR, thus they require specialized laboratory testing. NiV and HeV assays require specific sample-types for accurate detection. The stage of infection, host species, and sample type may affect the diagnostic method. Direct contact with body fluids like saliva, urine, or droplets from infected bats, pigs, or humans can expose people to NiV and HeV. Contact with body fluids is more contagious than aerosol and only physical contact with the patient [27]. The course and severity of NiV and HeV infections differ by patient. There is usually an incubation period between virus exposure and symptom onset. Incubation time for NiV and HeV is 4–14 and 5–21 days, respectively. The illness generally begins with 3–14 days of fever, painful headache, and respiratory illness symptoms including cough, sore throat, dyspnea, and diarrhea [28], whereas early HeV symptoms may show within 5–8 days with fever, cough, sore throat, headaches, and muscle pains like the flu. Some patients may experience breathing issues, tiredness, and confusion. Severe NiV infections can cause acute respiratory syndrome and CNS involvement 7 days after infection. This infection may also induce acute encephalitis, which can cause confusion, disorientation, and coma within 24–48 hours. In 7 days, HeV can cause severe respiratory distress, pneumonia, and encephalitis, causing convulsions, confusion, and disorientation. NiV infection has a 40% to 75% fatality rate and HeV infection 60% to 70%, depending on the pandemic and healthcare availability [28–31].

The reliability of test results may depend on sample selection and timing. It is generally most beneficial to get samples when symptoms are still mild, especially if the patient exhibits flu-like symptoms, including fever, headache, and muscular discomfort. At this point, the virus is actively growing in the body, making it easier to identify in samples. In the early stages of infection, RT-PCR can detect viral RNA in blood or nasopharyngeal samples (Figure 2). Since nasal and throat swabs and blood samples are easy to obtain, these are the most popular sampling methods. Post-mortem lung, liver, and brain samples can confirm NiV and HeV infection. CSF from a lumbar puncture can be tested for the virus, indicating CNS involvement [32–34]. According to the World Health Organization (WHO), NiV and HeV belong to Category A and require triple-layer packaging with a glass, metal, or plastic

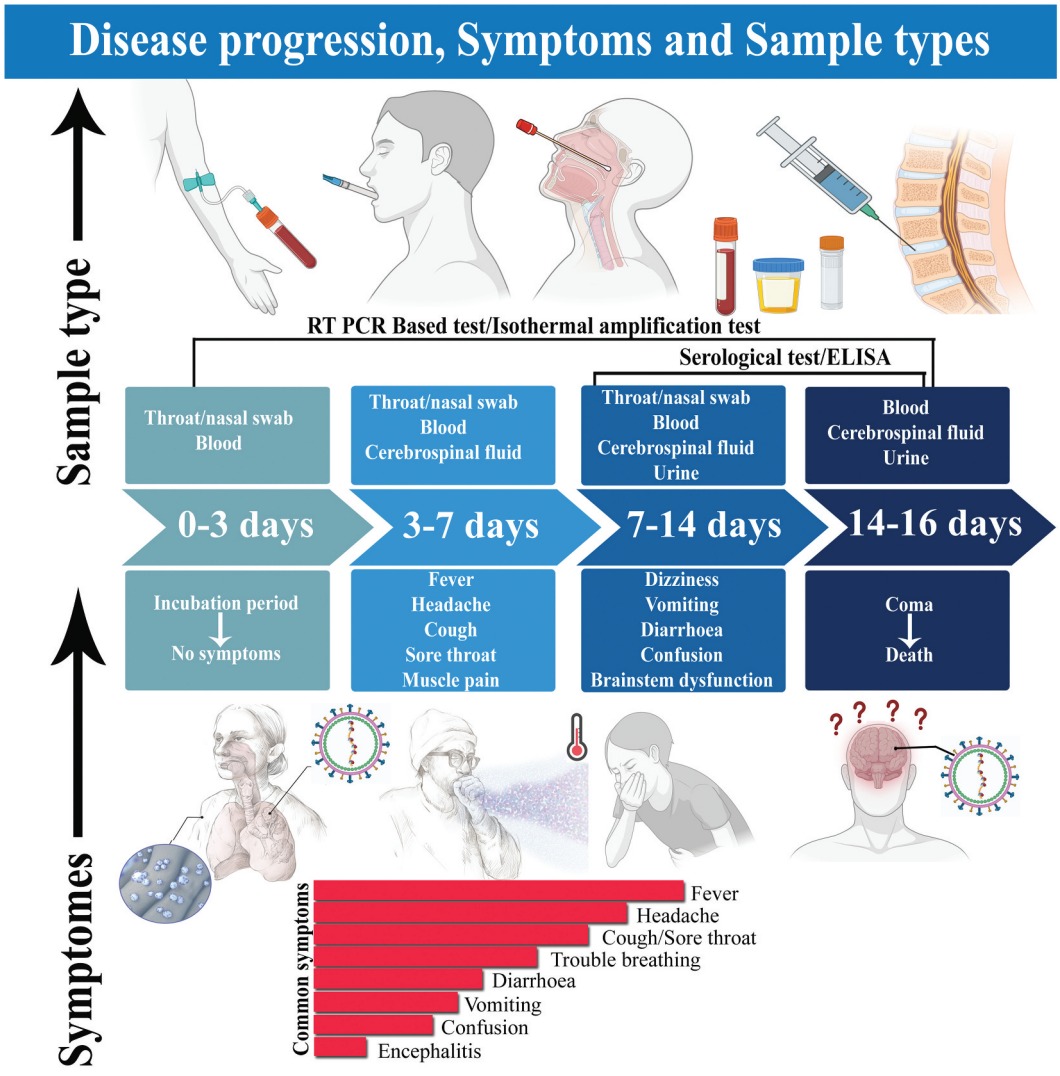

Figure 2. Sample Types during Disease Progression of Nipah and Hendra Infections.

first layer and a hard outer layer [35]. Instructions from National Institute of High Security Animal Diseases (NIHSAD) are followed for shipping and collection of samples from NiV or HeV infected animals [36].

Since NiV and HeV are BSL-4 pathogens, sample inactivation protocols for these viruses have been devised which permit further diagnostic procedures to be conducted in a BSL-2 facility. Acetone and methanol, fluoropolymers/aldehyde fixatives (PFA, C/C, and 10% neutral-buffered formalin), detergents (sodium dodecyl sulfate (SDS) and Triton-X 100), and reagents containing guanidinium isothiocyanate have been used for inactivation of NiV. Among these, samples inactivated using guanidinium isothiocyanate are generally amenable for nucleic acid detection methods. Alternatively, heating the serum samples at 56°C for 30 min inactivated the NiV virus [37]. In another method, 60°C treatment for 30 min inactivated NiV and did not interfere with ELISA titers and only minimally affected the sensitivity in RT-PCR [38]. The Vero cell lines infected with NiV were inactivated by using various methods, including cell lysis, SDS, RLT + EtOH, Triton-X 100, and Trizol. Inactivation of NiV-infected supernatants using Trizol, UV light, and 10% neutral-buffered formalin was performed on virus stocks and infected organs. Samples inactivated with Trizol are amenable for RT-PCR [38]. Nipah and Hendra infection's precise pathogenic mechanism is still not well understood and detailed clinical research is required to determine how long a patient will shed infectious particles in various body fluids. However, it is assumed from studies that the individual may have been infected from the day when symptoms started, until 21 days [39].

3. Types of diagnosis assays

NiV and HeV have the potential to cause serious respiratory and neurological conditions in both humans and animals, making early and precise diagnosis essential. The best tool for controlling disease outbreaks is prompt diagnosis, which allows for early identification of index case, isolation, contact-tracing, and treatment. Different types of molecular diagnostic assays have been developed to detect NiV and HeV infections (Figure 3, Table 1), and they are described in this section.

3.1. Serological test for diagnosis of NiV and HeV

Serological methods refer to diagnostic approaches that encompass the identification and examination of antibodies or antigens present in the serum or other body fluids of a patient. This technique is frequently used in clinical settings for the diagnosis of infectious diseases, autoimmune disorders, and other ailments. Serological assays offer crucial insights about an individual's immune response to a particular pathogen. Serological testing is also used for epidemiological monitoring. Serological

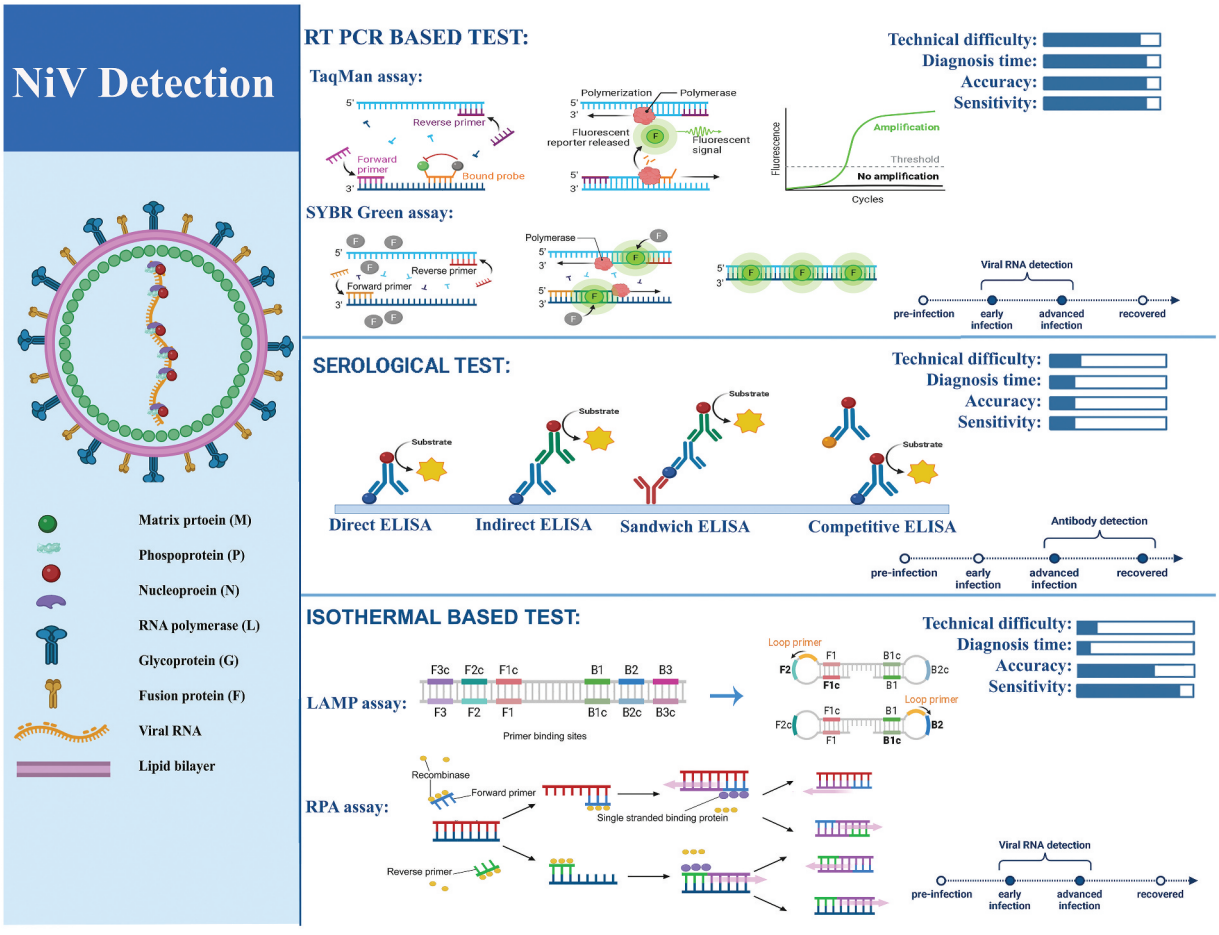

Figure 3. Diagnostic methods for detection of Nipah Virus.

Table 1. Different diagnosis assays for Nipah and Hendra detection.

Assays	Diagnostic test	Virus Detection	Specificity	Sensitivity	Sample types	References
Serological Assays	ELISA	HeV	98%	100%	Serum	[52]
	SYBR Green RT PCR	NiV	-	1 pfu/ml	African green monkey kidney cells	[59]
PCR based assay	TaqMan RT PCR	NiV	No cross reactivity	1 pfu		[23]
	LAMP	NiV	No cross reactivity	100 pg of pseudo virus RNA	Pseudo viruses Plasmids and viruses	[70]
Isothermal Amplification Assay	RPA	NiV	No cross reactivity	10^3 copies/ml	Synthetic RNA	[85]
	RPA	HeV	-	10000 $TCID_{50}$/ml	Vero cell lines	[82]

pfu: plaque-forming unit; TCID: tissue culture infectious dose.

tests which include western blotting, neutralization assay and Enzyme-Linked Immunosorbent Assay (ELISA) are used for the detection of particular proteins in a sample.

3.1.1. Western blotting

A study conducted for the detection of Malaysia and Bangladesh strains of NiV employed western blotting using monoclonal antibody against NiV N protein [40].

3.1.2. Virus neutralization assay

A virus neutralization assay is used to detect the presence and evaluate the efficacy of antibodies to counter the infectivity of the virus. Some studies have shown the use of neutralization assay for the detection of viruses like rabies [41], SARS-CoV 2 [42], Adeno-associated viruses [43], etc.

3.1.3. ELISA

This technique is used in most of the serological tests for the diagnosis of viruses. The ELISA is based on the principle wherein an antigen selectively binds to its corresponding antibody and can be detected using an enzyme-conjugated secondary antibody and a suitable substrate. There are different types of ELISA such as direct, indirect, competitive, and sandwich [44,45]. Different types of antibodies are used in ELISA such as monoclonal, polyclonal, and conjugated [46].

There are several reports where ELISA is used for the detection of different viruses such as rabies [47], measles [48], SARS-CoV 2 [49], African swine fever virus [50], etc. Indirect ELISA has been developed for discrimination between NiV-infected and uninfected pigs and detection of henipavirus-specific antibodies in sera samples of pigs [20]. In another study, recombinant ephrin B2 was utilized as a capture ligand with mAb F20NiV-65 (monoclonal antibody) for NiV and mAb F27NiV-34 (monoclonal antibody) for HeV for the detection of NiV and HeV infection using ELISA-based assay [51]. Another ELISA assay was developed by using IgM antibody against N protein for early detection of HeV infection. Further, this assay could also help to differentiate between pathogen Vs vaccine-induced antibodies [52]. Rubbo et al. developed a blocking format ELISA for the detection HeV G protein-specific antibodies in the serum of several animals. They used two previously characterized neutralizing monoclonal antibodies. The results of the analytical specificity testing of this assay demonstrated significant cross-reactivity with the closely related Nipah virus, but no cross-reactions were observed with other paramyxoviruses [53]. Zhu et al. developed a competitive ELISA assay using monoclonal antibody (mAb) and recombinant NiV glycoprotein for the detection of NiV and HeV. Since, NiV and HeV glycoproteins are highly conserved and share 80% of their amino acid composition, thus it can be used for the detection of both viruses [21].

3.1.4. Commercially available serological assay

Human Hendra Virus (HV) ELISA Kit available from MyBiosource (San Diego, United States of America), is a ready-to-use microwell, strip plate ELISA kit which costs approximately 11–12 USD and has a storage temperature of 2°C to 8°C. This kit is based on HeV antibody-HeV antigen interactions (immunosorbency) and an HRP colorimetric detection system to detect HeV antigen targets in samples like serum, plasma and other biological samples (Table 2). Secretions, tissue homogenates, and/or undiluted body fluids are examples of appropriate sample types used for detection through this kit. The percentage coefficient of variation (CV %) of this kit is less than 15% for both intra-assay and inter-assay. Horse Hendra Virus (HeV) ELISA Kit is available for detection of Horse Hendra virus.

3.2. Reverse transcription polymerase chain reaction assay

RT-PCR is a powerful molecular biology technique used for the diagnosis of viral infections, including those caused by NiV and HeV. RT-PCR is one of the most sensitive methods for the detection and quantification of mRNA. Several body fluids and tissues can be screened clinically for the expression of low copy transcripts using RT-PCR.

3.2.1. Gel based RT-PCR assay

In gel-based RT-PCR assay, viral RNA is isolated and reverse transcribed into cDNA followed by polymerase-chain reaction (PCR) and detected through agarose gel electrophoresis. This method is generally not used for routine clinical diagnosis. A duplex nested one-step RT-PCR method with internal control (IC) was developed for detecting NiV RNA in fruit bat urine samples. For second stage (nested) PCR, 1 µL of the primary amplification products were added to a new PCR mixture. The method could differentiate between false and true negative results, and the presence of PCR inhibitors minimally affected the results [54]. It is more labor intensive, cumbersome and requires longer time from sample to results. It can be customized

Table 2. Commercially available kits for the detection of Nipah and Hendra Virus.

Assays	Company	Diagnostic Principal	Virus Detection	Specificity	Sensitivity	Sample Types
Serological based diagnostic kits	My Biosource (United States of America)	Sandwitch ELIsA	Human HeV	-	-	Serum, Plasma, and other biological fluids
Nucleic acid based diagnostic kits	True Nat Molbio Diagnostics (India)	Truenat® Nipah	NiV	No cross-reactivity with seven different families of viruses	10 copies/ml	Oropharyngeal swab, EDTA blood, and serum
	Creative Biogene (United States of America)	Real Time RT-PCR	NiV	-	-	Serum, plasma, and infected animal tissue
	BioPerfectus (China)	Real Time PCR	HeV	No cross reaction with other similar symptoms viruses	5 copies/reaction	Cerebrospinal fluid, human serum, or throat swabs
	BioPerfectus (China)	Real Time PCR	NiV	No cross reaction with other similar symptoms viruses	5 copies/reaction	Human serum, plasma, whole blood, and urine
	Liferiver™ (China)	Real Time PCR	NiV	-	1×10^4 copies/ml	Plasma, serum, infected animal tissue or secretion
	HELINI Biomolecule (India)	Real Time PCR	NiV	High	0.45 copies/µL or 2 copies/reaction	-
	Life Technologies, India	Real Time-PCR	NiV	-	1×10^4 copies/ml	Plasma, serum, infected animal tissue or secretion

as per the need of the users and may be optimized to give accurate results, albeit the sensitivity might remain low.

3.2.2. Dye based Real-Time PCR assays

Real-time RT-PCR (rt-RT-PCR) is a type of PCR in which amplification is continuously monitored in real-time. In dye-based assay, fluorescent dyes are added to the reaction mixture. These dyes are designed to bind to double-stranded DNA and emit fluorescence when exposed to a specific wavelength of light. As the DNA amplification progresses, the amount of double-stranded DNA increases, leading to an increase in fluorescence intensity. There are several reports in which dye-based rt-RT-PCR assays were used for the detection of several viruses [55–57]. The detection of NiV was also reported by using SYBR Green rt-RT-PCR using one-step as well as two-step [58]. A study conducted in Vero cells evaluated the synthesis of NiV RNA by detection of NiV N gene using SYBR Green I-based one-step rt-RT-PCR [59]. Since in such assays the detection is real-time, they usually have higher sensitivity than gel-based assays. However, there are chances of nonspecific amplifications which may impact the accuracy of the assay. Further, since the step of gel electrophoresis is obviated, the dye-based rt-RT-PCR assays are less cumbersome and require less time to arrive at the results.

3.2.3. TaqMan® probe-based assay

The TaqMan® rt-RT-PCR assay is a widely employed molecular biology technique for the identification and quantification of particular DNA or RNA sequences in a given biological sample. This technique is a modified version of PCR that integrates oligonucleotide TaqMan® probes labeled with a fluorescent reporter dye at the 5′ end and a quencher at the 3′ end, to facilitate the continuous monitoring of the amplification process in real-time. TaqMan® assays are extensively used for gene expression analysis, quantification of viral load, genotyping, and detection of pathogens. For instance, a study was conducted for the detection of N gene of NiV and HeV using one-step as well as two-step TaqMan® assay with FAM labeled probes [58]. Guillaume et al. also used FAM-TAMRA labeled probes in one-step and two-step TaqMan® assays for the detection of N gene in sera of NiV infected hamsters [23]. One-step TaqMan® rt-RT-PCR assay has also been used to detect matrix (M) gene RNA of HeV in tissue and fluid samples of bats and horses. Here again, FAM was used as a fluorophore, while TAMRA was used as the quencher in TaqMan® probe [60]. These assays are very sensitive and accurate and are considered as the gold standard for NiV and HeV detection. The assay cost is higher than the above two methods. However, it offers the advantage of user customization.

The availability and use of commercial kits (Table 2) has revolutionized the detection and analysis of various biological molecules and disease-causing agents. Few RT-PCR-based commercial kits are available in the market for NiV and HeV diagnosis.

The US-based Creative Biogene Real-time RT-PCR kit is used to identify the Nipah Virus (NiV) in serum, plasma, infected animal tissue, or secretions by using viral RNA. However, this kit is available only for research use; not for diagnostic purpose. This kit costs approximately 31 USD per reaction and requires to be stored at −20°C. Repeated freeze-thaw may decrease the sensitivity of Kit.

The BioPerfectus Hendra Virus Real-Time PCR Kit can be used with samples such as cerebrospinal fluid, human serum, or throat swabs. This kit has a run time of 79 min. BioPerfectus Nipah Virus Real-Time PCR Kit is also available for the detection of NiV. The kit has a 12-month shelf life and is stored between −20°C and 5°C. The Nipah virus RNA can be detected with the BioPerfectus Nipah Virus Real-Time PCR Kit, which uses real-time PCR technique. Urine, whole blood, plasma, and serum from humans can all be used as samples. As per the kit literature, both HeV and NiV detection kits do not show any cross reactivity with other viruses with similar symptoms. Limit of detection (LOD) of both these kits is 5 copies/reaction.

The Nipah Virus real-time RT-PCR kit (Liferiver™) can also be used with different samples such as serum, plasma,

infected animal tissue, and other body fluids. Reagent storage is suggested at −20°C. This kit costs approximately 15 USD per test. The sensitivity of the kit is 1×10^4 copies/ml. However, the sample volume, elution volume, nucleic acid extraction technologies, and other variables may affect the sensitivity of the analysis.

YouSeq NiV diagnostic kit is qPCR-based kit with run time of 1 h 30 min. This kit costs approximately 6 USD per reaction. Target Species MONODOSE dtec-RT-qPCR kit is another commercially available real time PCR kit for NiV diagnosis with an approximate cost of 6.5 USD per reaction and a storage temperature of −20 to −15°C.

HELINI Nipah virus Real-time PCR Kit is a ready-to use system for the detection NiV RNA in FAM channel using PCR. This kit costs approximately 5–6 USD per reaction and the run time is 1 h 30 min. The target gene sequence, nucleoprotein, is highly conserved, and its sequences have 100% homology with a wide range of reference sequences that are clinically important, according to a comprehensive bioinformatics analysis. The analytical sensitivity of PCR is 0.45 copies/µL or two copies per reaction under optimal conditions.

Real-time PCR kits for NiV diagnosis are also available from Biopremier and LIFE TECH Life Technologies. The LIFE TECH Life Technologies Nipah Virus (NiV) Real-Time Rt-PCR Kit is used to detect the presence of the Nipah virus in serum, plasma, or infected animal tissue or secretions. Sensitivity of kit is 1×10^4 copies/ml, but repeated freeze-thawing may reduce its sensitivity.

3.2.4. TrueNAT® assay

A molecular diagnostic platform called TrueNat® is largely employed for the diagnosis of infectious diseases like tuberculosis (TB) and COVID-19 [61,62]. The Molbio Diagnostics-created TrueNat® platform may be used for a variety of purposes, including the identification of numerous infectious agents. To perform an rt-RT-PCR test for the detection and diagnosis of Nipah, the Truelab® Real Time Quantitative Micro PCR Analyzer is used with the disposable, room temperature stable, and Chip-based TrueNat® Nipah test. In this technique, internal positive control (IPC) is used along with test samples which undergo the whole process from the extraction to amplification. The test includes dried $MgCl_2$ in the reaction well and freeze-dried RT-PCR reagents in microtubes. For the analysis 6 µL of pure RNA is required to be added in the reaction well. At the end of the run, the results are displayed as detected and not-detected, along with the Ct values [63]. It is also very fast, sensitive and accurate; however, it is pertinent to note that it is a closed system and users cannot design and use their own assays.

N gene is used as the target by combining the room-temperature stable TrueNat® micro-PCR chips with battery-powered Truelab® Real Time Quantitative micro – PCR Analyzer and Trueprep® AUTO/AUTO v2 Universal Cartridge-Based Sample Prep Device. The duration of test is 35 min and each test costs approximately 15 USD. TrueNat® Nipah chip is stable for 6 months from the date of manufacture if stored between 2°C and 30°C Trueprep AUTO Universal Sample Pre-Treatment Pack is stable for 2 years from the date of manufacture if stored between 2°C and 40°C and for 1 month at ≥45°C, hence making it a suitable platform for use in tropical and subtropical regions. The specimens used for this test include human oropharyngeal swab sample, serum and EDTA blood. TrueNat® Nipah shows no cross reactivity with Measles virus (Paramyxoviridae), Influenza virus (Orthomyxoviridae family), SARS CoV-2 (coronavirus), Dengue virus (Flaviviridae), Chikungunya and Rubella viruses (Togaviridae), Herpes Simples virus (HSV) and Varicella Zoster virus (VZV). Sensitivity of the kit was 100% when compared with the gold standard TaqMan Nipah Real-Time RT-PCR, while the calculated analytical sensitivity of the TrueNat® Nipah is 10 copies/ml.

3.3. Isothermal amplification-based tests for diagnosis of Nipah and Hendra virus

3.3.1. LAMP assay

Loop-Mediated Isothermal Amplification (LAMP) employs a set of 4 to 6 primers to amplify target genes at constant temperature using a DNA polymerase with strand displacement activity. Agarose gel electrophoresis, fluorescence, or colorimetric detection can all be used to analyze the test results [64]. Several reports have documented the development of diagnostic assays using Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) techniques. Additionally, commercial kits have been made accessible for several diseases caused by RNA viruses [65–69]. An RT-LAMP assay employing primers for the N gene of NiV was developed for detection of all known strains of NiV. For this assay, a grain of sealant (low-melting-point wax, melting point: 48–50°C) was placed on top of the LAMP mixture, and it was then incubated in a Realtime Turbidimeter LA-32°C for 60 min to capture turbidity data at 61–65°C before being heated at 75°C for 5 min to inactivate the enzymes. A peak time higher than the negative controls and an increase in turbidity of >0.1 were indicators of a positive sample. The RT-LAMP reaction was incubated in a water bath at 61–65°C for 50 min for visual interpretation of results [70]. Foord et al. detected all known HeV strains in horses using LAMP assay that targeted a conserved region of the HeV P-gene [71]. This assay could detect HeV in horses before any clinical symptoms appeared and its specificity and sensitivity were found to be equal to TaqMan® assay. However, there also have been reports of nonspecific amplifications and inaccurate results with LAMP assays when used for the diagnosis of other infectious diseases. The advantages of RT-LAMP are faster run times, ideal for field settings, less labor intensive, low cost, and easy to interpret. Further, these tests can be done with minimal instrumentation requirements.

3.3.2. RPA assay

Recombinase Polymerase Amplification (RPA) is an isothermal nucleic acid amplification technique that utilizes recombinase enzyme, single-stranded DNA-binding proteins, and a strand-displacing DNA polymerase. Recombinase enzymes facilitate the formation of D-loop structures, which serve as templates for amplification [72]. It has been used to amplify several targets from a wide range of species, including RNA, miRNA, ssDNA, and dsDNA [73,74]. RPA assay has been developed for the detection of various viruses [75–80]. Pollak et al. performed RT-RPA assay for NiV diagnosis using TwistAmp™ info kit (TwistDX, Cambridge, United Kingdom) and RAA (Recombinase Aided Amplification)

kit (Qitian, Jiangsu, China) at 39°C for 20 min [81]. HeV RT-RPA-LFD assay was developed for HeV detection using the TwistAmp™ exo kit (TwistDX, Cambridge, United Kingdom) with N gene as the target [82]. To improve the accuracy of RPA, CRISPR/Cas system [83,84] has also been incorporated in the assay. Miao J et al. performed a one-pot RPA-CRISPR/Cas13a assay at 37°C for 2 h in QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific, Shanghai, China) for NiV detection [85]. This assay is fast, user user-friendly with minimal instrument requirement, making it ideal for use in field settings. End users can customize assays for their targets of interest. Compared to LAMP assay, RPA is reported to be more accurate [86], though it could be more expensive.

4. Later flow devices for NiV and HeV detection

Lateral flow devices (LFDs) represent a critical advancement in viral detection. These are generally paper-based devices, wherein the sample is loaded onto the sample pad. The analyte migrates toward the conjugate pad through capillary action and binds with the reporter (usually conjugated gold nanoparticle) molecules. As it moves ahead on the working membrane (usually nitrocellulose membrane), if the sample contains the appropriate ligand (antigen) as the analyte, it gets captured by immobilized specific antibodies on the test line and a positive signal appears due to higher localized concentration of gold nanoparticles. The unbound analyte, devoid of the antigen/ligand of interest moves further ahead and is captured by another affinity-agent (usually biotin), leading to a signal at the control line, which indicates the correct functioning of LFD (Figure 4). LFD-based rapid-tests are commercially available for the detection of several viruses [87–94]. Assays for NiV and HeV have been combined with LFD for easy visual interpretation. For instance, a Nucleic acid lateral flow immunoassay (NALFIA) device was developed with three test lines and one control line for the detection of NiV, MERS and Reston Ebolavirus [95]. Pollak et al. developed two different

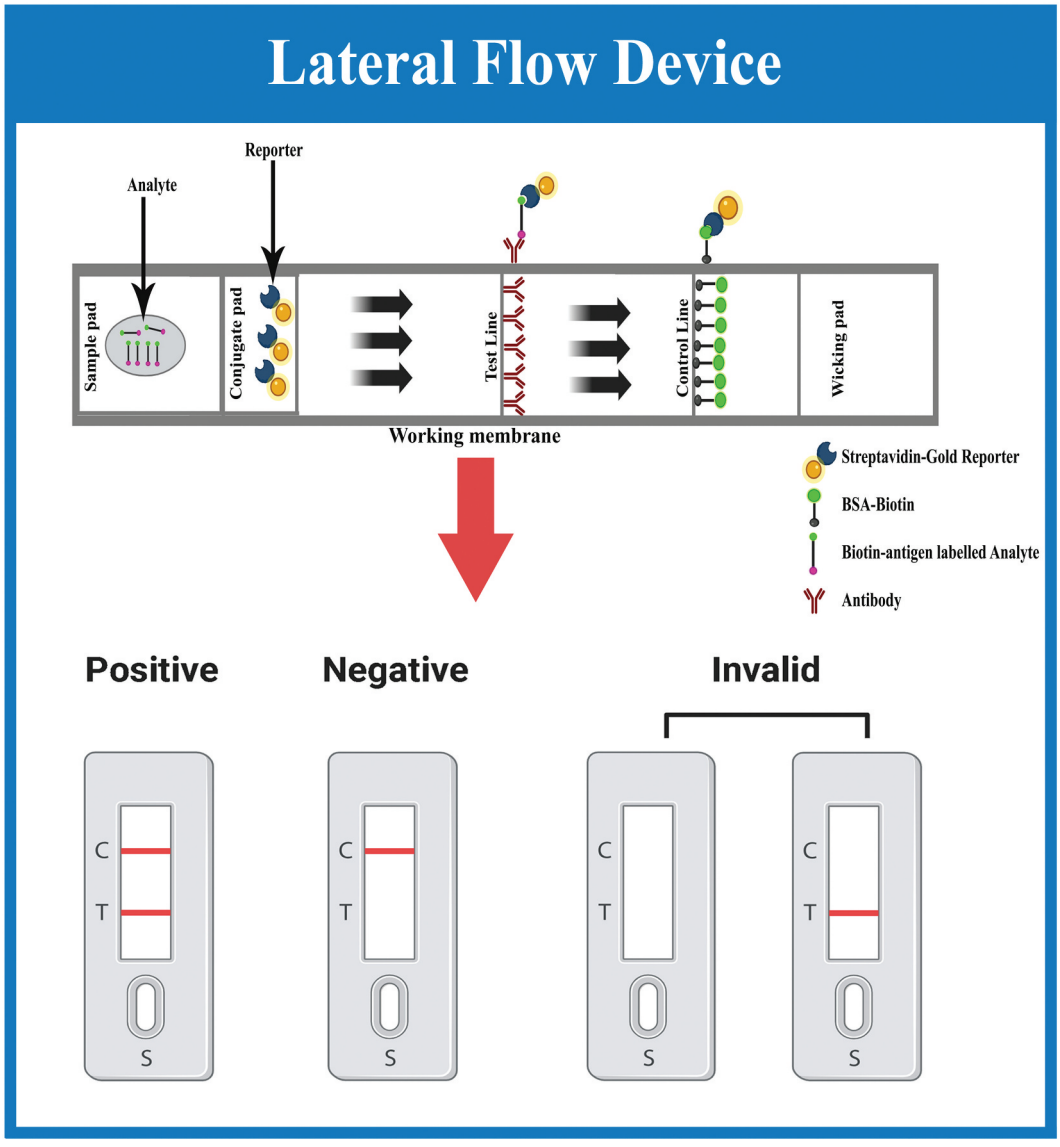

Figure 4. Schematic representation of a lateral flow device.

kits of RT-RPA assay for NiV and HeV diagnosis and combined them with lateral flow detection using HybriDetect lateral flow strips (Milenia Biotec, Giessen, Germany) [81,82]. An RPA-CRISPR/Cas13a assay was developed for NiV detection and combined with lateral-flow paper strip (Tiosbio, Beijing, China) for visualization of results after reaction [85]. These rapid tests offer swift results, in a user-friendly manner with minimal training, making them invaluable for timely diagnosis at the point-of-care. LFDs do not require specialized laboratory equipment, enhancing accessibility, especially in resource-limited areas, while their portability further extends their reach to remote locations. The cost-effectiveness of LFDs plays a pivotal role in healthcare systems with budget constraints. Early viral detection, made possible by LFDs, is essential for prompt treatment and outbreak containment, ultimately improving patient outcomes and reducing transmission rates. Moreover, their use in disease surveillance, emergency response, and various applications underscores their versatility and significant contribution to healthcare delivery, public health, and infectious disease control.

5. Patent landscape

Understanding the patent landscape is of paramount importance in the realms of innovation, business, and research. It serves as a comprehensive view of patents associated with a specific technology or industry. Knowing the existing intellectual property helps in preventing unintended and inadvertent infringement, thus avoiding potentially expensive lawsuits. More importantly, it fosters innovation and research by inspiring new ideas and revealing technological gaps. Further, it provides valuable insights for market assessment, enabling businesses to understand industry trends, competitors, and growth opportunities. The paragraphs below outline a few patents on NiV and HeV detection, also tabulated in Table 3.

An indirect ELISA detection technique for NiV G protein antibodies was patented by a Chinese group in 2021. The procedure entails coating an ELISA plate with NiV G protein, using square titration to establish the ideal conditions, and assessing the specificity, sensitivity, and stability of the results. The anti-NiV G protein antibodies in mouse serum may be found using this technique, which can also be used to assess the particular antibody titers induced by vaccinations **(Patent Number: CN113391067).**

Another patent was filed by a Chinese group in 2006 which disclosed the N gene-specific primer and probes for fluorescent quantitative RT-PCR to detect NiV. The primers may be used to amplify the target fragment, which is 76 bp long and able to amplify the N gene across several NiV strains enabling quick detection **(Patent Number: CN1840701)**. The procedure for finding the NiV in 2012 involved primers and a Taqman-MGB probe for RT-PCR **(Patent Number CN102559935)**. A patent was filed in 2015 by a Chinese group, which disclosed the reagents used for NiV along with porcine reproductive and respiratory syndrome virus using duplex fluorescent RT-PCR. The reagents in this invention include specific primers, TaqMan® probes, and controls (**Patent Number: CN104745578**). TaqMan® probe and two pairs of specific primers for NiV M gene and swine influenza virus M gene for duplex fluorescent RT-PCR-based detection were patented in 2015, for simultaneous detection of Nipah and swine influenza **(Patent Number: CN104745713)**. A method for detecting NiV using an RAA-based fluorescence detection kit was patented by a Chinese group in 2019, which includes the removal of the sample's nucleic acid, mixing in the upstream and downstream primers, probes, and magnesium, and then putting it into the thermostatic fluorescent gene tester for detection (**Patent Number: CN110592285**). The primers and probes for the detection of NiV were patented by a Korean group in 2020 **(Patent Number: KR2284248)**. Another invention disclosed in 2020 comprised of primers and probe sequences used for detection of NiV **(Patent Number: CN105567870)**.

6. Challenges and improvements of Nipah and Hendra virus diagnosis kits

NiV and HeV infections pose a significant global health threat due to their high fatality rates, absence of available vaccines, and lack of therapeutic armamentarium. There have been repeated sporadic occurrences of NiV and HeV infections, though mostly limited in Southeast Asia, which perhaps has led the Western markets to remain relatively under-motivated regarding innovations in the diagnosis

Table 3. Patents on NiV and HiV detection.

S.NO.	Patent No.	Technology	Country	Publication date
1	CN1840701	TaqMan® RT PCR	China	04.10.2006
2	CN101195846	TaqMan® RT PCR	China	11.06.2008
3	CN102031311	Microarray	China	27.04.2011
4	CN102559935	TaqMan® RT PCR	China	01.07.2012
5	CN104745713	Duplex TaqMan® RT PCR	China	01.07.2015
6	CN104745578	Duplex TaqMan® RT PCR	China	01.07.2015
7	CN104762404	Duplex TaqMan® RT PCR	China	08.07.2015
8	CN105567870	TaqMan® RT PCR	China	11.05.2016
9	CN109022621	RT LAMP	China	18.12.2018
10	CN110592285	RAA	China	20.12.2019
11	CN112501351	TaqMan® RT PCR	China	16.03.2021
12	KR2284248	TaqMan® RT PCR	Korea	02.08.2021
13	CN113391067	Indirect ELISA	China	14.09.2021
14	WO2022017124	Neutralization Activity	United States	27.01.2022
15	CN114317837	Multiplex TaqMan® RT PCR	China	12.04.2022
16	CN115747380	TaqMan® RT PCR	China	07.03.2023

and management of these infectious diseases. However, it is crucial to emphasize that these viruses are not confined to specific regions, and their potential to cause widespread outbreaks necessitates a proactive approach.

Commercially available NiV and HeV diagnostic kits are mostly based on TaqMan® assays which require sophisticated equipment and trained technicians. An ELISA Kit (MyBioSource) for detection of Hendra is commercially available, though the disclaimer says that it is only for research use and not for diagnostics.

The unavailability of rapid, on-site diagnostic tools hampers the timely identification of infected individuals, delaying critical interventions and increasing the risk of transmission. Furthermore, the storage-temperature requirements for most of the currently available diagnostic kits pose logistical challenges, particularly in resource-constrained settings. Further, most of the commercially available kits employ reagents that may lose efficiency after repeated freeze-thaw cycles.

There is a need for continual improvement in terms of having kits or devices with more accuracy, availability, cost-efficiency and user-friendliness. One of the foremost challenges in NiV and HeV diagnosis is the absence of commercially available point-of-care devices. However, there have been reports of LFD coupled with RPA or RAA for the detection of NiV and HeV. This area can be further explored and may offer opportunities for researchers to innovate cheap and scalable technologies.

A comprehensive understanding of the pathogenesis of these viruses would also help in the development of improved diagnostic methods for their early detection facilitating risk mitigation and improved public health preparedness. Furthermore, rapid serological diagnostics coupled with LFD that may differentiate between vaccine acquired or natural antibodies, need to be developed. A proactive approach to diagnostics, therapeutics, and vaccine development is essential to ensure global health security and prevent potential outbreaks of HeV and NiV in regions beyond Southeast Asia.

7. Conclusion

During outbreaks or pandemics such as MERS, COVID-19, it is imperative to acknowledge the potential risks associated with zoonotic viruses, particularly those capable of human-to-human transmission, as they can pose significant risks and contribute to the occurrence of a pandemic. One significant rationale for expressing apprehension regarding the occurrence of HeV and NiV in the human population is the absence of vaccines, therapies, drugs, and point-of-care devices that have been established to be efficacious. Also, zoonotic virus epidemics present a valuable opportunity for global researchers for the improvement in diverse diagnostic techniques and methodologies as well as in the development of vaccination.

8. Expert opinion

As we project into the future, advancements in diagnostic assays for NiV and HeV viruses are set to undergo a transformative phase, addressing the existing challenges outlined in the current status of diagnostic assays for these zoonotic diseases. The landscape of diagnostic tools for NiV and HeV is anticipated to evolve significantly to meet the growing need for accurate, rapid, and accessible testing methodologies, primarily in response to the identified limitations and gaps.

A major focus of innovation will revolve around the creation of rapid, on-site diagnostic tools, such as POCDs, which are currently absent from the market. The exploration of technologies like LAMP or RPA coupled with LFDs for NiV and HeV detection is anticipated to gain traction. These innovations could offer scalable and cost-effective solutions, particularly beneficial in resource-constrained settings and during outbreaks.

Eventually, if the outbreaks of NiV and HeV become more frequent and prominent, vaccine development would likely pick-up. In such scenario, the future direction of diagnostic enhancements will not only concentrate on improving the accuracy of identifying NiV and HeV but also on advancing serological diagnostics. The development of rapid serological diagnostics coupled with LFDs, capable of distinguishing between vaccine-induced and naturally acquired antibodies would be expected.

Concurrent with diagnostic advancements, a comprehensive understanding of the viruses' pathogenesis will play a pivotal role in the development of improved detection methods, thereby facilitating early identification, better risk mitigation, and enhanced public health preparedness.

The future trajectory of diagnostic assays for NiV and HeV will witness a concerted effort from global researchers and manufacturers, spurred by the necessity to fortify global health security. The advancements in diagnostic techniques and methodologies, alongside the development of effective vaccinations, will serve as crucial pillars in mitigating the risks posed by emerging zoonotic viruses, ensuring a more prepared and resilient global health landscape.

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