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Review

Application of Nipah Pseudovirus System for Development of Antibody Neutralization Assay

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Abstract. Nipah virus (NiV) is a type of virus that can make people and many animals very sick. It can cause serious breathing problems and brains welling. Because of how dangerous and deadly it is, the World Health Organization (WHO) sees NiV as a global healthrisk. It needs to be handled in special labs that have the highest safety measures, called Biosafety Level-4 (BSL-4) facilities. Rightnow, there isn't a good vaccine or treatment available for NiV. It could be a health risk for Indonesia since it has been found in nearby countries. Indonesia doesn't have a BSL-4 lab yet. So, we need a way to evaluation NiV vaccine that can be done in a BSL-2 lab. The NiV pseudovirus (PV NiV) has special proteins that help it attach to and enter mammal cells. It is made using a system based on HIV and includes a signal detector. This setup can help create tests to measure how well antibodies work against NiV. It can also be used to monitor infections, check community immunity, develop NiV vaccines, and research new treatments to fight NiV infections.

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1. Introduction

Nipah virus (NiV) has been a deadly zoonotic pathogen for the past 25 years. NiV is an enveloped, negative-sense, single-stranded RNA virus belonging to the genus Henipavirus of the family Paramyxoviridae. NiV was first identified following an outbreak of severe encephalitis in humans exposed to pigs in Singapore and Peninsular Malaysia between 1998 and 1999. Since then, outbreaks of NiV have been documented in five countries in South and Southeast Asia (Bangladesh, India,

Malaysia, the Philippines, and Singapore) [1]. As of May 2024, there have been 754 confirmed human cases of NiV with 435 deaths (CFR: 58%) reported in these five countries. Bangladesh recorded the highest incidence (341 cases and 241 deaths; CFR: 71%) followed by Malaysia (283 cases and 109 deaths; CFR: 39%), India (102 cases and 74 deaths; CFR: 73%), Philippines (17 cases and nine deaths; CFR: 53%), and Singapore (11 cases and one death; CFR: 9%) [2].

NiV has a broad host species tropism, high mortality rate, and human-to-human respiratory transmission that indicate the potential for NiV to pose a major public health threat. The World Health Organization (WHO), the US Centers for Disease Control and Prevention (CDC), and the Coalition for Epidemic Preparedness Innovations (CEPI) have identified NiV as a BSL-4 pathogen or a high priority pathogen with a pandemic threat [3].

NiV can be spread through human respiratory secretions and is one of the main sources of human-to-human transmission [4]. Infected individuals can spread it through coughing, sneezing, urine, saliva, etc. During the early stages of infection, the diagnosis of NiV can be made in epithelial cells, especially in the bronchioles [5]. Viral antigen has been observed in the lungs, especially in the bronchi and in some cases even in the alveoli. Cytokines are secreted as the respiratory epithelium swells due to infection, eventually leading to the development of disorders such as acute respiratory distress syndrome (ARDS) [6]. Inflammatory mediators such as *interleukin granulocyte-colony stimulating factor* are also secreted in the respiratory epithelium during the later stages of infection [7]. From the respiratory epithelium, the virus is distributed to the endothelial cells. As the infection progresses, the virus can gain access to the bloodstream. In addition to the respiratory, digestive, and excretory systems, the brain and other organs can also be targets, leading to multi-organ failure [8].

NiV has a genome that encodes six structural proteins, namely nucleocapsid (N); phosphoprotein (P); matrix protein (M); fusion protein (F); attachment protein (G); and RNA polymerase (L). NiV has two envelope proteins, namely attachment protein G/RBP (receptor-binding protein) and fusion protein (F) that mediate receptor binding and entry into the host cell and are targets of the host antibody response. Entry of NiV into the host cell requires fusion of the viral membrane and the host plasma membrane through the concerted action of protein G and protein F. The NiV entry receptor on the host cell surface is the transmembrane tyrosine protein kinase ephrin-B2 or ephrin-B3. NiV F and G proteins are targets of the humoral immune response and serum neutralization correlates with protection in animal models of infection. Both the F and G proteins present attractive targets for vaccine design, and antibodies generated against F or G have been shown to neutralize the fusion machinery and inhibit NiV infection [1-2].

Most zoonotic viruses that cause disease in humans, such as Ebola virus, Marburg virus, Nipah virus, Chikungunya virus, Smallpox virus, Monkeypox virus, Corona virus, Rabies virus, and Influenza virus are enveloped viruses [9]. Cultivating native viruses can be dangerous and gaining access to these virus strains is often a complex and time-consuming process, requiring Biosafety Level-3 (BSL-3) or Biosafety Level-4 (BSL-4) laboratories. These limitations further limit the number of laboratories studying native viruses, thus hampering the progress of antiviral and vaccine research. The unavailability of drug and vaccine development requires infection prevention. The development of vaccines needs to be tested for effectiveness with neutralization antibody tests so that antibodies can inhibit the virus from entering cells. Clearly, developing alternative measures applicable in most basic and clinical laboratories would facilitate the evaluation of NiV vaccine data, and the identification of correlates of protection. For pseudovirus (PV) NiV is made on the HIV platform with a recombinant engineered system.

Pseudoviruses (PVs) of enveloped viral pathogens serve as valuable surrogates for studying their structural and functional interactions with host organisms and provide an excellent platform for testing vaccine efficacy [2]. PVs are 'defective nonpathogenic enveloped viruses that express desired heterologous viral envelope proteins,' can enter cells, and mimic early stages of infection, but cannot complete a full replication cycle to produce new infectious particles. To render them replication

defective and to facilitate incorporation of heterologous viral envelope proteins, the native viral envelope gene is deleted, and other genes involved in pathogenicity are also removed. What remains are the structural and enzymatic proteins and genomic elements required for virion assembly, host entry, and gene expression [10].

PVs can be produced *on a large scale in vitro*, by co-transfecting producer cells with plasmids expressing the envelope proteins of the desired viral pathogen, viral packaging plasmids to express structural proteins, and defective viral genomes that often include reporter genes to facilitate quantification of infection. The incorporation of heterologous viral envelope proteins allows PVs to use the same pathways that the native pathogenic virus uses to enter cells, and through selective expression of envelope proteins [11].

PVs can facilitate systematic studies of the mechanisms of virus entry into the cell and enable research on entry inhibitors as antivirals. The PV platform can serve as a well-controlled model to evaluate the efficacy of antibodies in neutralizing viruses, making it a valuable tool for the preclinical phase of vaccine development. PVs can be prepared using a variety of enveloped virus genomes as packaging backbones to tailor the design to a specific viral target of interest. In addition, they can express multiple viral proteins, including proteins from different viruses or variants of the same virus, making them a versatile platform to study how viral proteins interact with the cell. Thus, PVs help model the interactions between viruses and their cellular receptors [12].

PV can be concentrated to high titers and used as a substitute for native viruses that are difficult to culture. PV can also be engineered to have a wider host range, which helps in studying cell, tissue, or organ tropism. However, in making PV it is important to maintain the protein in a state that is most representative of the native virus for use in neutralization assays. This can be achieved by using vector platforms that are structurally similar to the native virus. Although PV envelope proteins can largely mediate virus entry in a manner similar to native viruses, they can only replicate for one round and may not always induce pathogenesis as native viruses do. Therefore, results from assays using PV must be compared and validated against native virus-based assays, which remain the gold standard [13].

Over the past two decades, several PV systems have been tested, covering a wide range of zoonotic viruses. Examples of viruses belonging to several virus families include *Arenaviridae* (e.g. Lassa virus), *Coronaviridae* (e.g. Severe Acute Respiratory Syndrome Coronavirus SARS-CoV-1 and SARS-CoV-2), *Filoviridae* (e.g. Ebola and Marbur viruses), *Flaviviridae* (e.g. Dengue virus and Zika virus), *Hantaviridae* (e.g. Hantavirus), *Hepeviridae* (e.g. Hepatitis E virus), *Nairoviridae* (e.g. Crimean Congo Hemorrhagic Fever Virus), *Orthomyxoviridae (e.g. Influenza A virus*), *Paramyxoviridae* (e.g. Nipah virus), *Peribunyaviridae* (e.g. La Crosse Encephalitis virus and Rift Valley fever virus), *Pneumoviridae* (Human Respiratory Syncytial Virus RSV), *Retroviridae* (e.g. Human Immunodeficiency Virus), Rhabdoviridae (e.g. Rabies virus) and *Togaviridae (e.g.* Chikungunya virus and Ross River virus). PV has been used to study virus entry, and in some cases, has been applied to evaluate antibody-mediated neutralization. In particular, for many viruses that still lack effective vaccines, the PV platform can be used to accelerate further research [14].

PVs can be used to study mutations in novel viral envelope genes that increase infectivity, transmissibility, or disease. Once the mutated envelope sequence of a pathogenic virus is identified, DNA plasmids expressing the viral envelope proteins can be synthesized, facilitating the rapid development of PVs. This makes it easier and faster for research groups around the world to participate in virus research without having to invest in expensive laboratory infrastructure. Therefore, the PV platform has the potential to significantly accelerate research efforts during viral outbreaks and accelerate the development of effective countermeasures such as antivirals and vaccines [15].

PV NiV can be obtained by making 3 types of plasmid designs consisting of plasmids that encode the HIV genome and do not have envelope protein genes, plasmids containing NiV envelope protein genes, and plasmids expressing RNA containing marker genes and can be assembled into virus

particles. When these three types of plasmids are transfected into cells, the PV particles contain the NiV envelope on their surface, with the HIV capsid structure assembling the RNA marker gene and HIV polymerase protein. When these PV particles are infected into mammalian cells, the infected cells will express the marker protein. Because PV particles do not have a complete genome, these virus particles cannot replicate, so that various laboratory tests using PV can be carried out in BSL-2 facilities [3].

In the pseudovirus luciferase assay (PVLA), inhibition of virus entry into cells by NtAb correlates with a decrease in the level of luciferase signal in the cells. This method is superior to the Focus Reduction Neutralization Test (FRNT) due to its simplicity, higher sensitivity and accuracy, and suitability for high-throughput experiments. In addition, no real virus is used during the assay. Therefore, this method can be used as an alternative to safely conduct serological studies in a rapid response to assess the threat posed by NiV. PV-based assays have been widely used to study cellular tropism, receptor recognition, and virus inhibition as well as to evaluate neutralizing antibodies. This easy-to-use PV-based inhibition assay will simplify the detection of neutralizing antibodies against NiV [16].

2. Experimental Section

This study used a literature review by checking PubMed, ScienceDirect, Google Scholar, and other databases containing research findings or scientific articles. Only studies that met the above search criteria were included in the systematic review. Articles were grouped based on the search topics used in this case, namely NiV, PV, NiV-enveloped protein, and neutralizing antibody. There was a limitation on the year of study or publication, which was the last 5 years. We looked for recent studies in an attempt to learn more about PV when vaccines were developed.

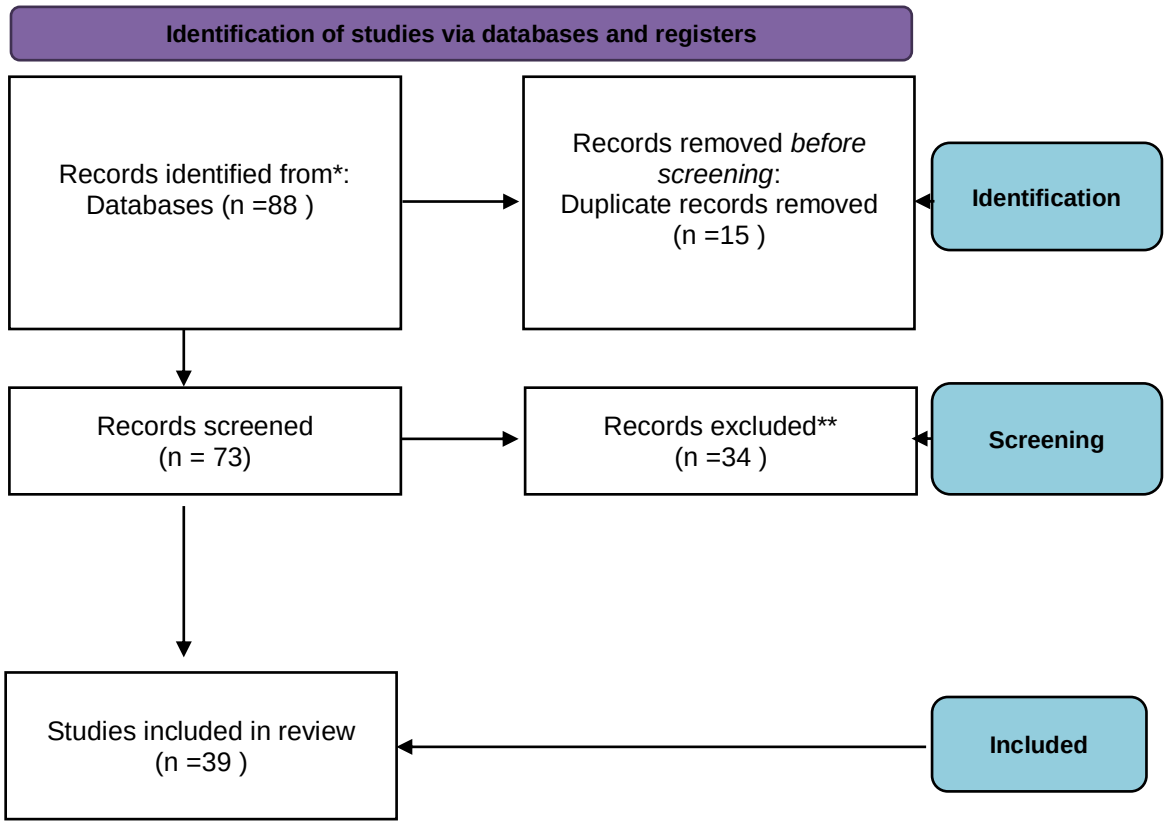

Figure 1 Research scheme

This study used a quantitative meta-analysis that took additional information from previous research reviews. There is a PRISMA diagram to show the rationale for each stage of the screening. A total of 88 articles were found when these keywords were searched across various databases. There were fifteen duplicate articles out of 88 articles that need to be removed at this time. Some publications from abstracts to full-text articles may undergo screening. Due to the impossibility of meeting the requirements, 34 of the 73 products intended for use were removed. As a result, 39 articles met the requirements for inclusion and are currently under review.

3. Results and Discussion

3.1. Nipah Virus (NiV)

Nipah virus (NiV) is a negative-stranded, single-stranded RNA virus that was first isolated and identified during an outbreak in Malaysia and Singapore in 1998-1999 [17]. Since the first outbreak, there have been few cases of NiV in Malaysia or Singapore until 2001, after which NiV outbreaks became an annual occurrence in Bangladesh. In 2023, an outbreak of NiV occurred in Kerala, India, and in 2024, there were 6 cases of NiV infection resulting in 2 deaths [18].

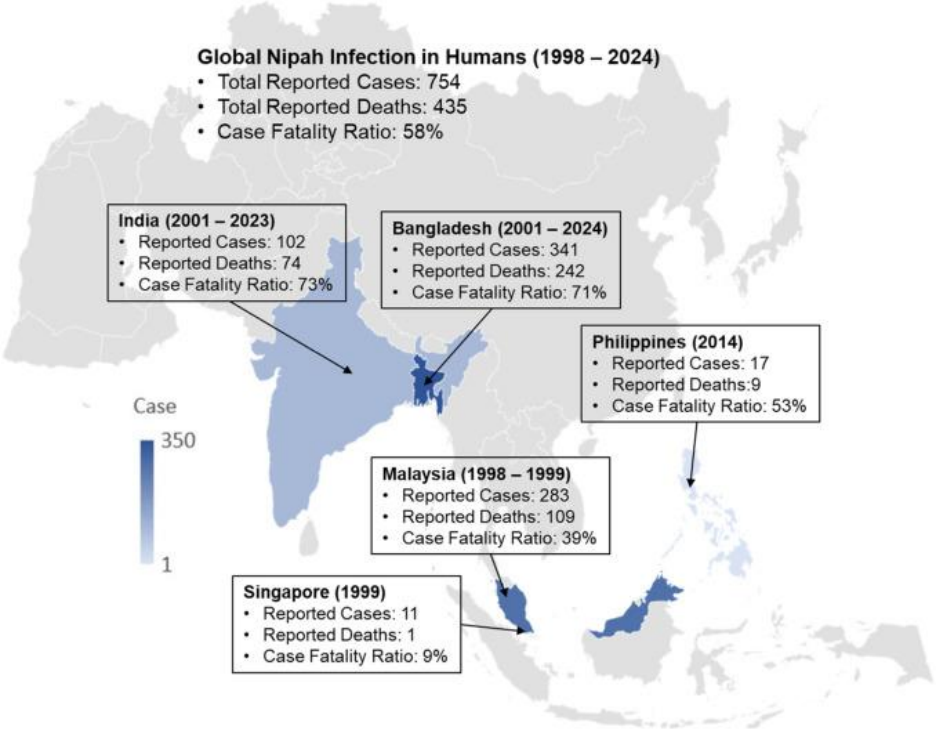

Figure 2. Geographic distribution of human NiV outbreaks during 1998–2024 [18].

NiV belongs to the genus Henipavirus (HNV) in the family Paramyxoviridae, which also includes another highly pathogenic virus, Hendra virus (HenV). In addition, several new henipaviruses have been reported recently, such as Cedar virus (CedV), Ghana virus (GhV), Mojiang virus (MojV), and Langya virus (LayV) [19]. Two strains of NiV have been identified based on the geographic distribution of the virus and genetic diversity, namely the Malaysian NiV strain (NiV-M) and the Bangladesh NiV strain (NiV-B). The Malaysian strain (NiV-M) caused an initial outbreak in Malaysia and Singapore with a CFR of approximately 40%, and then caused an additional outbreak in the Philippines in 2014, with a CFR of approximately 52%. The Bangladesh strain (NiV-B) outbreak has shown a higher CFR of approximately 75%, with human-to-human transmission also observed [7]. Previous studies have shown that NiV-M and NiV-B exhibit very different pathogenicity in African

green monkeys (AGM) and Syrian hamsters. These differences in pathogenicity and transmissibility in humans between NiV-M and NiV-B underscore the need for medical precautions that protect against the Bangladeshi and Malaysian lineages [20].

NiV has a broad host tropism, including bats, pigs, dogs, cats, horses, guinea pigs, and hamsters. Humans are primarily infected through contact with infected animals (such as bats and pigs) and consumption of contaminated food, although direct human-to-human transmission has also been reported periodically, highlighting concerns about the potential global health risk of NiV viruses. NiV infection can cause severe encephalitis and respiratory disease with a mortality rate of up to 75% [21]. In addition, long-term neurological conditions and relapses of NiV infection have been observed in survivors. There is currently no approved vaccine available for humans. The World Health Organization (WHO) has listed NiV infection as a priority disease requiring extensive and urgent research to develop control measures [6].

3.2 Protein Envelope

Nipah virus (NiV) belongs to the genus Henipavirus in the family Paramyxoviridae [22]. The Paramyxoviridae family consists of enveloped, non-segmented, negative-sense RNA viruses. Its genome encodes six structural proteins, namely nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), attachment protein (G), and large protein or RNA polymerase (L). NiV has two surface proteins, namely receptor-binding protein (RBP or G protein) and fusion protein (F protein), which mediate virus entry into host cells and its spread from cell to cell within the infected host [5]. After binding to the host cell receptor, the transmembrane protein tyrosine kinase ephrinB2 or ephrinB3, RBP undergoes a conformational change, which triggers the F protein to shift from a metastable prefusion form to a highly stable postfusion form [23]. This process involves extensive structural rearrangements resulting in virus-cell or cell-cell membrane fusion.

Protein G is a type II membrane protein that has a short N-terminus cytoplasmic tail, a single transmembrane domain, and a fairly large ectodomain. The ectodomain is composed of a stalk region and a C-terminus receptor-binding globular head domain, which are connected by a flexible neck to a helical stalk. Protein G is organized as a homotetramer in which each protomer is linked by disulfide bonds in the neck and stalk regions. The structure of the stalk region includes a collection of four parallel helices. Biochemical studies have shown that the stalk region is important for oligomeric stability, F protein interaction, and specificity. The structure of the globular head domain of protein G is related to the propeller structure of other paramyxovirus attachment proteins with a six- β- sheet propeller structure [24].

Previous studies have shown that ephrin-B2 and ephrin-B3 tyrosine kinases are NiV and HeV receptors, and G protein is responsible for receptor recognition and binding. After receptor binding, G protein undergoes a series of conformational changes, which can activate F protein to initiate and complete membrane fusion with target cells. In addition, many neutralizing and protective antibodies mainly target G and F proteins, indicating that these two viral proteins are key antigens for vaccine development [25]. Several vaccine platforms against NiV infection have been developed recently, including subunit vaccines, mRNA, virus-like particles (VLPs), viral vector vaccines, and DNA vaccines, while no self-assembling nanoparticle platform has been evaluated for NiV infection. Because of their important roles in viral invasion, G and F proteins are prime targets for developing therapeutic monoclonal antibodies (mAbs). Several neutralizing or cross-neutralizing antibodies targeting G or F proteins have been isolated, which have shown ideal animal protection [5].

3.3 Pseudovirus (PV)

Pseudoviruses (PVs) are recombinant viruses with backbones and surface proteins derived from various viruses. Genes in PVs are modified to eliminate expression of native surface proteins, but retain functional aspects of the virus, making them valuable research tools. Because of their high

pathogenicity and infectiousness, some viruses must be handled under biosafety level-3 conditions. Additional plasmids are then added to the virus code to express alternative surface proteins, resulting in PVs that can infect susceptible hosts but replicate intracellularly for only one round. Compared with native viruses , PVs, which can be produced naturally during infection or artificially in the laboratory for research purposes, contain fragments of host cell DNA without the nucleic acid components of the infectious virus associated with them [26].

The modified genetic material of PV prevents the particle from producing its own viral surface proteins unless additional plasmids or stable cell lines expressing these proteins are available to PV. Once inside a susceptible cell, PV is capable of replicating only once, which is comparable to the native virus which often replicates multiple times. In addition, PV lacks the virulent components of the native virus , virtually eliminating the possibility that these virus particles could cause active infection in an exposed individual. This unique property of PV allows it to be safely replicated in biosafety level -2 (BSL-2) laboratories, which typically work with agents that pose a moderate health hazard to humans. Although this virus is much safer to handle than the native virus , the structural conformation of the PV surface proteins is very similar to that of the native virus [4] .

The similarity in the structure of these surface proteins allows PV to remain effective in its ability to enter cells. In addition to these advantages, the PV system is scalable and can be rapidly manufactured. Therefore, PV can be safely manipulated in biosafety level 2 facilities to study the internalization of highly infectious viruses and other aspects of viral infection, such as cellular tropism and/or cellular receptor identification, without the need for high-level containment measures [10]. Since viral surface proteins play a critical role in cell entry, the conformational structure of PV surface proteins closely resembles that of native viral proteins. However, PV has a lower virulence compared to native viruses, allowing it to be safely handled in BSL-2 laboratories. Therefore, PV is widely used to study cellular tropism, receptor recognition, drug discovery, and for the development and evaluation of antibodies and vaccines [27].

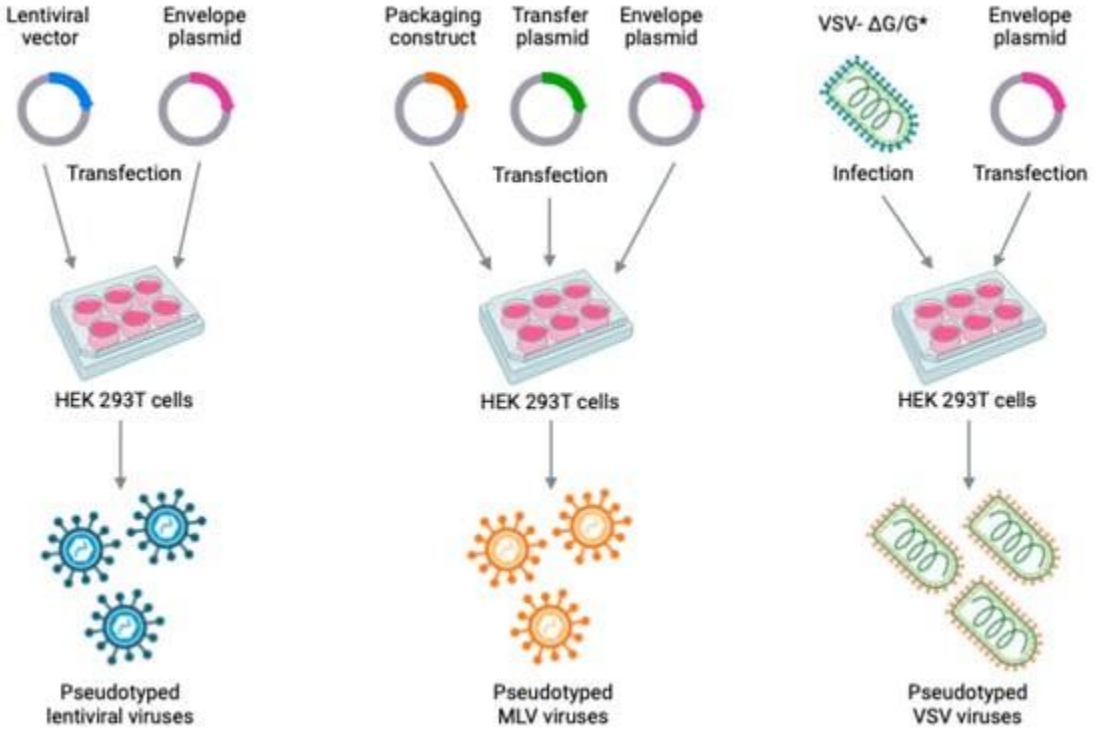

Figure 3. Three different packaging systems for PV production [10]

The assembly of PVs relies on three different packaging systems, namely the *Human Immunodeficiency Virus* (HIV)-based lentivirus packaging system, the *Murine Leukemia Virus* (MLV)-based packaging system, and the *Vesicular Stomatitis Virus* (VSV) packaging system. Additional examples include *Simian Immunodeficiency Virus* (SIV), *Feline Immunodeficiency Virus* (FIV), and *West Nile Virus* (WNV). The construction of these viral platforms has been effectively achieved through reverse genetics, and a detailed molecular understanding of the regulation of the viral replication cycle. Due to their strong operability, low biological risk, easy detection, and high sensitivity characteristics, PVs have been widely used in the study of highly pathogenic viruses, such as SARS, MERS, Ebola, Influenza, Chikungunya, Hanta and Seoul viruses, and especially in newly discovered and highly infectious viruses [28].

The PV platform is capable of producing high-titer NiV PVs with modified HIV backbone vectors and has successfully established *in vitro* and *in vivo infection models* for a range of viruses, including Rabies virus, Ebola virus, Marburg virus, Lassa virus, and Chikungunya virus. Researchers [28] developed a PV-based neutralization assay for *in vitro* and *in vivo analysis* of immune responses stimulated by NiV vaccine candidates. NiV PVs produced [16] by the HIV platform can be effectively neutralized by polyclonal antibodies that specifically target the F and G proteins. The NiV PV assay has all the advantages of recombinant virus assays, namely high throughput, high reproducibility, and high flexibility of virus *strains* [4]. If a VSV packaging system is used to produce PVs, there may be residual VSV viruses mixed with the PVs, complicating the neutralization assay or producing false-positive results. It is recommended that the amount of VSV be minimized. However, excess VSV will interfere with PV-based tests. Treatment of PV with VSV neutralizing antibodies may be considered before use in further trials [11].

3.4 Lentivirus Packaging System on *the Human Immunodeficiency Virus* (HIV) Platform

The HIV PV system is widely used because of its flexibility and comprehensive methodology. The HIV genome consists of structural, regulatory, and helper genes, which are carefully manipulated to construct PVs. To reduce the occurrence of unwanted recombination, different segments of the HIV genome are cloned into various DNA expression vectors. Some unnecessary elements are removed during the cloning process. In addition, mutations in the envelope genes allow the incorporation of envelope proteins from other viruses [12]. Depending on the complexity of the system, HIV PV construction can involve two, three, or four plasmids. Commonly used two-plasmid systems include an expression plasmid and a packaging plasmid, such as pSG3 Δ env or pNL4–3. Three-plasmid systems separate the HIV components into packaging and transfer plasmids, while four-plasmid systems further divide the components to increase safety. The most commonly used transfer and packaging plasmids are psPAX2 and pLenti–GFP [18].

Vectors based on lentiviruses such as HIV have the advantage of being able to transduce both dividing and nondividing cells, although in HSCs progression to at least G1 is still required. This ability is due to the fact that lentiviral PICs can enter the nucleus without disrupting the nuclear membrane. Nuclear translocation is facilitated by the MA and IN proteins together with the accessory protein Vpr. The MA and IN proteins contain nuclear localization signals and remain associated with the PIC. The MA protein has also been shown to bind directly to importin- α , which facilitates entry of the PIC through the nuclear pores. Vpr binds directly to the sporinuclear complex and causes transient herniation of the nuclear membrane [4].

In addition to Vpr, lentiviruses encode a number of other accessory proteins , namely Vif, Vpu, Tat, Rev, and Nef. Only Tat and Rev are absolutely required for viral replication. Tat activates the HIV LTR promoter so that viral RNA is produced more efficiently, and Rev interacts with a region of the RNA known as the Rev response element (RRE) to promote the transport of viral RNA from the nucleus into the cytoplasm. The larger genome size of HIV and other lentiviruses means that large or multiple transgenes can be incorporated into vectors developed from these viruses [4].

HIV lentivirus vectors are more complex than gammaretrovirus vectors. This is due not only to the additional requirements for Tat, Rev, and RRE , but also because the *pol coding region in HIV contains a central PPT sequence (cPPT) that facilitates nuclear import of the viral PIC. In lentivirus vectors, the cis* -acting elements are similar to those in retroviruses, but the additional cPPT and RRE regions are also present upstream of the transgene. The presence of these additional elements increases the risk of homologous recombination events, so the *gag–pro–pol* , *rev* , *tat* , and *env genes* are all provided in *trans* on separate expression constructs to reduce the risk of such events. *Gag –pro–pol* is coexpressed with its RRE. The functions of MA, IN, and *Vpr* are redundant, so the presence of *the gag–pro–pol sequences* encoding MA and IN is sufficient for nuclear import of the viral PIC [11] .

The third generation lentivirus vectors currently in use have a self-inactivating (SIN) design that eliminates the use of Ta . Generation of helper cell lines for lentivirus vectors has proven difficult because some lentivirus proteins are toxic to cells resulting in low vector titers. Lentivirus vectors are usually produced by transient transfection methods, and PV lentivirus vectors with surface glycoproteins from the G protein of vesicular stomatitis virus (VSV-G) have helped improve stability and titers, as well as broaden tropism [29].

Transfection of the plasmid into the host results in the assembly of incomplete HIV PV particles, which are secreted into the extracellular space. During secretion, heterologously expressed envelope proteins are integrated into the viral membrane. Subsequent steps of collection, centrifugation, purification, and concentration yield PV particles suitable for further study [16]. The resulting virus-like particles can mediate certain viral life cycles, but are defective in replication and lack protein-coding genes [10].

Third-generation packaging systems are designed with various safety precautions, namely that the transferred plasmid must be replication-incompetent and become "self-inactive" after integration with the shortened 3'-terminal LTR repeats. However, there is still a potential risk of tumor gene activation due to insertion of the transgene sequence into the host genome through viral transduction. NiV PVs containing NiV-F and NIV-G proteins on the particle surface were developed for *in vivo* infection to mimic infectious NiV entry [10]. A mouse model of NiV PVs containing NiV-F and NiV-G proteins on the particle surface was developed to mimic the in vivo NiV infection process. Balb/c mice were inoculated with PVs via the intrathoracic route, and luminescence detectors reported high flux levels in the spleen and lungs. NiV PVs were registered for *in vivo neutralization assays* that were reported to be less labor-intensive and faster than traditional assays [30].

HIV vectors are an increasingly popular model for developing PVs and chimerics because of their ease of use, stability, and long-term transgene expression. Their ability to transduce cells independently of their division status makes lentiviral vectors ideal for gene therapy of highly differentiated postmitotic cells. HIV packaging systems typically involve partitioning the HIV backbone into separate packaging vector plasmids and gene transfer. These plasmids can then be coexpressed with a viral envelope expression plasmid to produce infectious PVs. Packaging plasmids express the enzymatic proteins Gag and Pol for virion assembly, reverse transcription of viral RNA into double-stranded DNA, and integration into host DNA. Transfer plasmids contain the minimal viral *cis elements* required to package RNA with a transgene cassette driven by a heterologous promoter that can then be reverse transcribed into DNA, integrated, and expressed in target cells [12].

The establishment of safer and more robust alternative diagnostic methods is an important medical requirement because current diagnostic tests for detecting_Serum neutralizing antibodies against NiV mainly use native viruses. Currently, the diagnosis of NiV and HeV mainly relies on reverse transcriptase polymerase chain reaction (RT-PCR) of viral RNA and immunohistochemical analysis of infected tissues [3],[18],[28]. However, safer and simpler diagnostic methods that can be performed outside the very limited BSL-4 facilities are needed. Recently, several studies have reported the advantages of using pseudoviruses (PVs) for virus neutralization testing.

3. 5. Neutralization Test

Pseudoviruses (PVs) are engineered particles that mimic the structure of native viruses but are unable to replicate. PVs have become invaluable tools in a variety of virological and medical research areas. Understanding the mechanism of virus entry, vaccine development, antiviral drug screening, and virus neutralization assays are just a few of their applications. PVs are widely used in virus neutralization assays to evaluate the efficacy of neutralizing antibodies [11]. By measuring the ability of antibodies to block PV entry into target cells, it is possible to assess the potency of the immune response elicited by a vaccine or natural infection. Modified HIV backbone vectors are used to produce NiV PVs and to test by *in vitro* and *in vivo neutralization assays* the protective efficacy of specific antibodies against the virus [29]. Three groups of guinea pigs were immunized with plasmids expressing two NiV outer membrane proteins, the F protein, the G protein, and F and G. Following immunization, serum containing neutralizing antibodies (NAbs), targeting specific antigens (F, G, or F and G) was transferred to the mice, which were then inoculated with NiV PV. This approach enabled the development of an *in vivo imaging mouse model* for NiV PV and allowed data to be obtained on the limited protection by each NAb, compared with complete protection using the F/G NAb.

The NiV PV neutralization test is a test that measures the ability of antibodies to neutralize NiV [28]. This test is used to evaluate vaccines and detect NiV neutralizing antibodies. The following steps are carried out. *Human immunodeficiency virus* (HIV) is used to express NiV glycoprotein and luciferase protein. HIV is incubated with serial dilutions of the test sample. Luminescence is measured after 48 hours. The 50% inhibitory dilution (ID50) is calculated because NiV is a highly pathogenic virus with a high mortality rate, there is no approved vaccine or therapy for NiV, and this test can be performed in a BSL-2 laboratory which is safer and faster than conventional tests that require a BSL-4 laboratory. Therefore, it may be useful to evaluate the neutralization activity of monoclonal and polyclonal antibodies, evaluate the neutralization activity of plasma from NiV-infected hamsters, evaluate the neutralization activity of serum from human patients, and evaluate the performance of new assays for detecting NiV NAb [27].

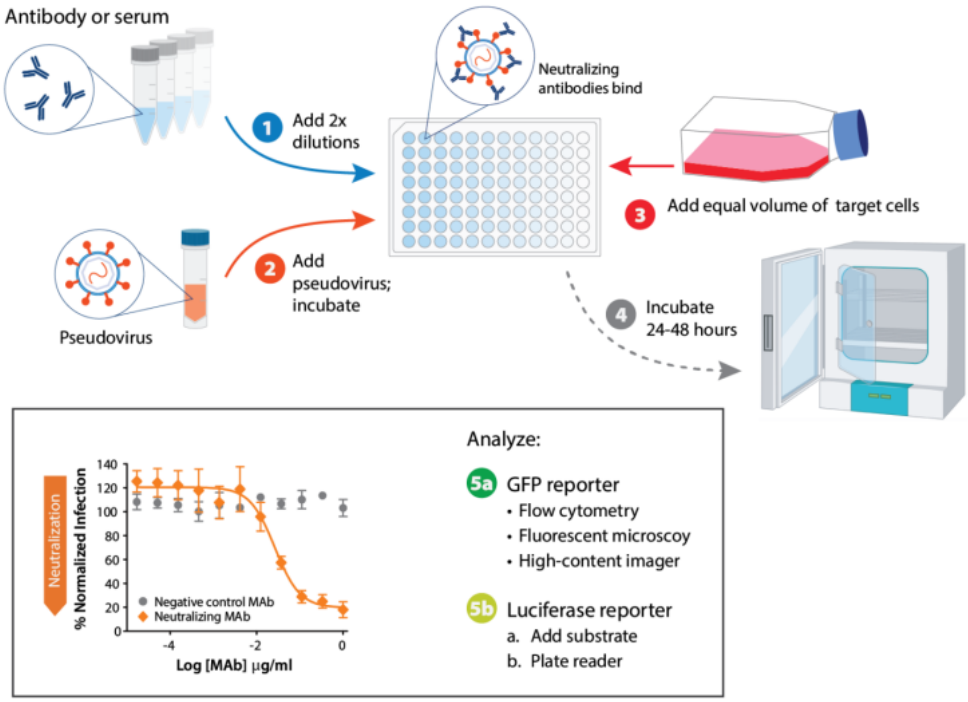

Figure 4. Neutralization Test [4]

In addition to neutralization assays, to avoid handling infectious virus, several surrogate measures for antibody detection have been developed. ELISA assays and multiplex microsphere assays are used to measure NiV-specific antibodies without distinguishing between NAbs and non-NAbs [24]. Ephrin-B2 and ephrin-B3 have been identified as cellular receptors for NiV and He [21],[23],[31]. PVs displaying NiV-F and -G proteins on their particle surfaces can largely mimic infectious NiV in the process of cell entry. Immune responses to candidate prophylactic vaccines targeting this process can be measured by PV-based assays.

The PV platform can serve as a well-controlled model for evaluating the efficacy of antibodies in neutralizing viruses, making it a useful tool for the preclinical phase of vaccine development. However, when generating PVs, it is important to maintain the proteins in a state that is most representative of live virus for use in neutralization assays. This can be achieved by using vector platforms that are structurally similar to native viruses. Although PV envelope proteins can largely mediate virus entry in a manner similar to wild-type viruses, PVs can only replicate for a single round and may not consistently induce pathogenesis as wild-type viruses do. Therefore, results from assays using PVs must be compared and validated against native virus-based assays, which remain the gold standard [32].

For many viruses, NAb, whether acquired through natural infection or vaccination, can effectively inhibit the infectivity of the viral pathogen in the host. Using isolated native virus cultures, neutralizing antibodies can be measured by *in vitro assays* such as *the Plaque Reduction Neutralization Test* (PRNT), *Focus Reduction Neutralization Test* (FRNT), and *Virus Reduction Neutralization* Test (VRNT). Although effective, the use of replication-competent virus in these assays poses significant health risks and requires the use of high containment levels. Studies with influenza virus, Ebola virus, Marburg virus, SARS-CoV-2, and for other viruses as reviewed, have shown that neutralization titers of serum tested against PV correlate well with those obtained when using native virus [33-34]. However, the choice of PV vector affects the neutralization capacity for some pathogens, as in the case of Ebola virus where a comparative study between HIV and VSV-based PV platforms to measure neutralization capacity revealed better agreement between VSV-based PV results and native virus neutralization data [35]. Therefore, if new PVs are to be generated, they should be characterized and compared with native virus assays, to fully verify the suitability of the PV vector system for a particular virus [36].

The use of PV has emerged as a safer alternative for evaluating and measuring viral NAb titers. To measure NAb, serum samples from patients or test animals are serially diluted to various concentrations, mixed with PV, and incubated, allowing antibodies in the serum to interact with PV. Cells expressing receptors for PV are added to the mixture and further incubated. The extent of cell infection is measured, often by measuring the reporter signal produced by PV after successful entry, and the expression of the reporter gene [37].

4. Conclusion

Pseudoviruses (PVs) are invaluable tools in the development of virus neutralization assays. NiV PVs can be produced using 3 packaging vector systems, and serve as a platform for safe PV-based neutralization assays that can be performed in BSL-2 facilities. The nonreplicating nature of PVs eliminates the need for BSL-3 or BSL-4 laboratories, reducing costs and the need for specialized equipment. Their flexibility in accommodating a variety of envelope proteins allows for a wide range of applications, from understanding the biology of known viral pathogens to early characterization of emerging and potentially zoonotic viruses.

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