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Review
Future Virology

Current progress with serological assays for exotic emerging/re-emerging viruses

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Recent decades have witnessed an unprecedented rise in the outbreak occurrence of infectious and primarily zoonotic viruses. Contributing factors to this phenomenon include heightened global connectivity via air travel and international trade links, as well as man-made environmental alterations, such as deforestation and climate change, which all serve to bring humans into closer contact with animal reservoirs and alter the habitat of vectors, thus facilitating the transmission of viruses between species. Serological assays are integral to tracking the epidemiological spread of a virus and evaluating mass vaccination programs by quantifying neutralizing antibody responses raised against antigenic epitopes on the viral surface. However, conventional serological tests are somewhat marred by equipment and reagent costs, the necessity for high-containment laboratories for studying many emerging viruses, and interlaboratory variability, among other issues. This review details 'next-generation' assays aimed at addressing some of the persistent problems with viral serology, focusing on how manipulating the genomes of RNA viruses can produce attenuated or chimeric viruses that can be exploited as surrogate viruses in neutralization assays. Despite the undoubted promise of such novel serological platforms, it must be remembered that these assays have to withstand rigorous validation and standardization measures before they can play an integral role in curtailing the severity of future emerging virus outbreaks.

In the mid-20th century, there was a widespread belief that infectious diseases could be conquered. This belief has been challenged in recent decades by an unprecedented increase in the emergence or resurgence of many pathogens. The definition of an 'emerging infectious disease' is widely accepted as being a newly recognized disease or an existing disease that is rapidly increasing in incidence or extending in geographic or host range [1]. A collation of international efforts identified that approximately 75% of emerging pathogens are zoonotic and that emerging viruses are one of the disease agents most likely to be transmitted to humans via an animal reservoir [2]. Indeed, RNA viruses are held accountable for many outbreaks because they can be asymptomatically maintained in a reservoir host species and frequently mutate during replication due to the low fidelity of the virus-encoded RNA polymerases [3].

The dramatic rise in the emergence and resurgence of exotic viruses cannot be explained entirely by genetic recombination and mutation; modern environmental factors have facilitated their zoonotic transmission. Encroachment into previously remote rural areas, as a result of deforestation and urban expansion, brings people and livestock into closer proximity to viral reservoir hosts, increasing the risk of viral transfer between species. This was the case for initial henipavirus outbreaks throughout southeast Asia and the surrounding areas, where land use and intensive animal husbandry practices within the vicinity of pteropid bats exposed workers to Hendra and Nipah viruses via transmission through equine and swine intermediary amplifying hosts in Australia in 1998 and Malaysia in 1999, respectively [4,5]. Increased global connectivity, air travel and international trade exacerbate the issue by circumventing geographical barriers to the spread of exotic viruses. It is commonly hypothesized that the introduction of West Nile virus (WNV) to New York in 1999 was due to air travel either transporting infected passengers or mosquito vectors from a region where the flavivirus is endemic. This is also a probable cause of the recent introduction of the novel Middle East respiratory syndrome coronavirus into the UK

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after its initial detection in Saudi Arabia [6,7]. Climate change is further altering the habitats of both reservoir hosts and disease-carrying vectors, resulting in adapting ranges and, in turn, extending the potential regions for zoonotic transmission of exotic viruses [8].

Another consideration that has recently challenged conventional thought regarding the emergence and re-emergence of exotic viruses is the notion of 'emerging diagnosis'. Human seroprevalence studies of Ebola and Lassa viruses, which cause lethal, systemic hemorrhagic fevers, indicate widespread exposure of the pathogens throughout areas of sub-Saharan Africa [9,10]. Furthermore, analyses of the evolutionary histories of Ebola and Lassa viruses [11,12] and putative evidence of the development of human genetic resistance against Lassa virus in Nigeria [13] suggest that these viruses have been circulating for longer than had been realized. This has led some to believe that the apparent 'boom' in emerging pathogens may actually be a consequence of the development of improved and novel diagnostic methods unveiling the true extent of the circulation of viruses that have previously been overlooked [14].

Regardless of the causes, the increased emergence or resurgence of exotic viruses highlights the vital importance of maintaining and developing high standards of diagnostic surveillance. Serological assays that detect antibodies, usually against the antigenic surface glycoproteins of many pathogenic viruses, are integral diagnostic tools. They are readily applicable throughout the process of emerging viral outbreaks and offer distinct benefits when compared with alternative diagnostic methods. The ability to measure antibodies to a particular virus enables the epidemiological spread of an emergent disease to be traced even after the symptomatic stages of infection. Identification of antibodies in the absence of the pathogen is advantageous when viremia is transient and attempting to detect viral RNA or quantify viral load by means of reverse transcription PCR in blood or tissue samples could result in a falsely negative result when testing for acute viral infections. Where vaccines are available, assessment and serosurveillance of mass vaccination programs and antiviral treatment schedules can also be achieved using serological assays.

Nonetheless, conventional assays for serological diagnosis of emerging and re-emerging exotic viruses present various challenges, depending on the particular disease outbreak. For instance, due to the severe pathogenicity and lack of effective vaccines or treatments for many viruses in families such as the *Arenaviridae* and *Bunyaviridae*, handling of virus for clinical, diagnostic or research-based purposes is often limited to biosafety level 4 laboratories. This limits the capacity for widespread and rapid serological diagnosis in many resource-poor, endemic areas. In addition, knowledge of protective immune correlates, such as serum neutralizing antibody levels, will need to be determined following the emergence of a novel virus such as Schmallenberg virus [15]. Vaccine efficacy testing can be hindered until protective thresholds are known. High levels of cross-reactivity between viruses of the same family can also be a serious drawback for diagnosis using serological assays, which is a particular problem for members of the *Flaviviridae* [16,17].

This review will summarize and consider the advantages and drawbacks of some established serological assays before documenting the latest approaches to the detection of virus-neutralizing antibodies (VNAbs) and outlining where further advances could be made in order to improve the serological diagnosis of viral infections.

Traditional serological assays

There are many different approaches that may be taken to measure virus-specific antibodies, often by harnessing the properties of interaction between virus and antibody, including cell agglutination, precipitation and complement fixation. This section focuses on three methods in common use.

The plaque reduction neutralization test (PRNT) is the traditional assay for the measurement of VNAbs. In this assay, a series of dilutions of the patient sample, such as serum or cerebrospinal fluid, is prepared and mixed with a standardized amount of virus. After allowing time for any antibodies in the sample to bind to the virus, the mixture is added to a confluent monolayer of permissive cells. After a further short incubation period sufficient for virus to have infected the cells, the sample suspension is replaced with a layer of agarose or carboxymethylcellulose to prevent a virus that is released from infected cells spreading to nonadjacent cells. Virus infection is quantified indirectly by observing plaques of virus-induced cytopathic effect (CPE), which is measured in PFU/ml. The concentration of antibody in the patient sample that reduces the formation of plaques by a certain amount (usually 50% [$PRNT_{50}$] or 90% [$PRNT_{90}$]) compared with a virus control mixed with a sample or diluent known to be antibody free enables quantification of the neutralizing antibody present in the sample. Antibodies to

viruses that do not cause discernible CPE can be measured using a variant of the PRNT where the virus is detected using virus-specific antibodies and, usually, fluorescence- or enzyme-labeled secondary antibodies. Infectivity is measured in FFU/ml. A less traditional approach applicable to viruses that neither cause CPE nor form plaques that are measurable in the focus-forming assay, and also allows primary human cells to be used, is flow cytometry [18,19].

A prerequisite for performing the PRNT is the availability of a readily cultured cell line that is permissive to the given virus. As mentioned earlier, the necessity for using infectious virus can bring additional restrictions in terms of expenses associated with high-biosafety laboratories. The six- or 12-well format and as many as 6 days' incubation that are required for plaque visualization restricts assay throughput. Despite its limitations, PRNT remains widely regarded as the gold standard for the detection of VNAbs, particularly for measuring the immune response to vaccination, due to its high sensitivity and specificity [20,21]. PRNT has been used in laboratories worldwide for many years, and as a result, variations in how the test is performed, even for the same virus, have arisen [22], further complicating international standardization.

The hemagglutination inhibition (HI) test is a traditional measure of antibodies to viruses that are able to agglutinate red blood cells, which includes adenoviruses, alphaviruses, bunyaviruses, flaviviruses, influenza, parainfluenza, rabies and rubella. The assay is quite variable, particularly between laboratories, because some of the reagents used are difficult to standardize. For diagnostic purposes, this variability is less of an issue than the limited sensitivity because acute and convalescent sera will usually be tested in parallel in order to detect a rise in antibody titer. Various techniques have been applied to improve the sensitivity of different HI assays, such as bromelain treatment of goose red blood cells for the detection of rabies virus antibodies [23] or Tween® 80 (Sigma-Aldrich, MO, USA) and ether treatment of parainfluenza virus [24]. Unfortunately, these tend to increase the inherent variability of the test. Another factor that further impairs standardization of the HI test is the need, in some instances, to remove nonspecific inhibitors of agglutination [25]. Different species vary in the inhibitors present in sera and failure to neutralize these using the correct pretreatment can lead to false-positive results. The HI assay continues to be widely used to assess the efficacy and effectiveness of influenza vaccines, even though it only detects antibodies that bind to the hemagglutinin molecule but do not necessarily neutralize virus infectivity [26]. However, an advantage of the HI test compared with the PRNT is that it is quick and relatively simple to perform.

ELISAs were first developed in the early 1970s as a safer alternative to radioimmunoassays. As with the HI assay, ELISA measures antibodies that bind to viral proteins, but these antibodies do not necessarily neutralize virus infectivity. Measuring antibodies to a specific viral protein can allow antibodies raised in response to infection to be differentiated from those raised to a vaccination – the differentiation of infected from vaccinated animals (DIVA) approach. This strategy was used effectively during an outbreak of equine influenza in Australia. Horses were vaccinated with a canarypox-vectored vaccine expressing only the hemagglutinin protein of equine influenza. Use of a blocking ELISA that detects antibodies to the viral nucleoprotein enabled infected animals to be distinguished from vaccinated animals, which do not raise antibodies to nucleoprotein. This allowed vaccination to be implemented (along with movement restrictions) in order to rapidly bring the outbreak under control and enabled the Australian authorities to declare the region free of equine influenza [27].

On the one hand, the 96- or 384-well format of ELISA allows high-throughput screening, and the assay is also rapid. On the other hand, there are often limitations in specificity compared with PRNT. Cross-reactivity might occur with the secondary antibody in the indirect ELISA format. In addition, the use of purified or recombinant proteins can reduce specificity due to nonnative folding and modifications to proteins. As well as replacing radioimmunoassays, the use of ELISAs has largely overtaken the use of the indirect immunofluorescence test (IFTs) for antibody detection. However, IFTs remain useful; an IFT was recently proposed as a potential alternative to ELISA using recombinant hepatitis E virus proteins expressed in bacteria [28]. An IFT was also recently applied as a screening assay for the detection of antibodies to Middle East respiratory syndrome coronavirus [29].

Cross-reactivity of antibodies between closely related viruses is a particular issue for the flaviviruses that cause similar clinical signs and cocirculate in some regions. For example, dengue virus (DENV) often circulates in the same geographical regions as Japanese encephalitis virus (JEV), and there is increasing awareness that WNV is responsible for a proportion of

encephalitis cases in India, a country in which JEV also circulates [30]. It may therefore be necessary to confirm a diagnosis made by ELISA using PRNT. Alternatively, in two widely used IgM antibody-capture ELISA kits, the Panbio® Japanese encephalitis–Dengue IgM Combo ELISA (Alere, Australia) and the Venture Technologies Dengue–JEV modified antigen capture ELISA (Universiti Sains Malaysia, Malaysia), antibodies to JEV and DENV are tested for in parallel and the higher positive value is taken to be the infecting virus, although no distinction is made between the four serotypes of DENV [31]. The need to test a single sample for antibodies to multiple related cocirculating pathogens increases the sample volume required. Cerebrospinal fluid is the sample of choice to verify the cause of viral encephalopathy, as a positive serum sample only demonstrates recent peripheral infection that may be coincidental [31]. Furthermore, JEV infections in India are more frequent in young children who have not yet developed immunity as a result of exposure or vaccination [32]; therefore, cerebrospinal fluid sample volumes are usually limited. The flavivirus ELISA kits described measure IgM antibody as an indicator of recent infection; measurement of IgG requires demonstration of a rise in titer between acute and convalescent samples in order to confirm recent exposure to virus. However, IgM antibodies to flaviviruses can persist for as long as 4 months after infection [33,34].

'Next-generation' assays for measuring neutralizing antibodies

Considerable research focus has been invested in the development of novel serological assays that permit efficient and sensitive quantification of neutralizing antibodies while addressing persistent problems of containment and expense associated with traditional methods. Viruses used in 'next-generation' assays are primarily based on the process of manipulating genes that encode for structural viral proteins in order to attenuate pathogenicity but maintain a serologically equivalent virus.

Genetically modified & recombinant viruses

The genetic recombination of viral genomes has been explored in the antigen detection and serosurveillance of emerging viruses.

An attenuated chimeric virus, comprising the premembrane and envelope proteins of WNV inserted into the yellow fever virus 17D strain genome, was produced as a vaccine candidate for WNV [35], but was also subsequently used as a biosafety level 2 reagent in PRNT studies to assess WNV infection histories following the 1999 outbreak in northeastern USA. As mutations introduced may potentially result in changes in immunodominant epitopes, it is important to compare the performance of attenuated viruses with the wild-type parent virus in serological assays. Compared with wild-type WNV, 96% of results were concordant when evaluating panels of avian and equine sera sampled from the outbreak, with high levels of sensitivity and specificity and comparable neutralizing antibody titers [36].

Large-scale codon re-encoding, or codon optimization, has also recently been employed to attenuate Chikungunya virus as a candidate live-attenuated vaccine [37]. The replicative fitness of this alphavirus has been dramatically reduced through serial passaging in both primate and arthropod cells after reassigning an infectious Chikungunya virus strain with synonymous codon alterations that, importantly, do not confer any coding mutations in the viral proteins.

Such genetic manipulations of emerging viruses could have important implications not only for vaccine creation, but also for use as serological tools for functional antibody detection without the requirement for high levels of containment.

Reporter virus particles

Reporter virus particles (RVPs) are pseudo-infectious virions that encapsidate a subgenomic RNA replicon (SGR) possessing all of the necessary viral nonstructural genes, as well as a reporter gene. Although it can be cloned into the 3′-untranslated region of the replicon, the reporter gene is usually inserted in place of the genomic segments encoding the structural envelope and capsid proteins, which are usually removed from the replicon system. The SGR is then introduced into producer cells along with plasmid DNA expression vectors bearing the relevant structural envelope genes, leading to the production of RVPs in which only one replicon RNA is packaged. These RVPs can be readily used to study the antibody-mediated neutralization of specific viruses in serological assays. Pierson *et al.* constructed WNV RVPs by *trans*-complementation of the luciferase- or GFP-based SGR with plasmids encoding the capsid and premembrane and envelope proteins of the virus [38]. The WNV RVPs were used to study the neutralizing efficiency of the monoclonal antibody 7H2.

RVPs can also be used in serological assays to ascertain neutralizing antibody responses against a particular viral antigenic epitope, following either vaccination or natural infection. One such example is the application of GFP-expressing RVPs of the four DENV serotypes in a novel diagnostic neutralization assay [39]. Results from this correlated strongly with those obtained by PRNT and were serotype specific, overcoming a persistent problem with DENV serology. Using RVPs, assays are robust and reproducible, and issues of being low throughput that are inherent with PRNT-based studies are resolved.

Pseudotype viruses

Pseudotype viruses (PVs) are increasingly being used in serological assays for the diagnosis of viral infection or vaccine seroconversion (Table 1). A pseudotype is a chimeric virion that comprises the structural and enzymatic core of one virus and at least one protein or glycoprotein of another. Retroviruses can be employed as the core for this technology, with lentiviruses and gammaretroviruses such as HIV and murine leukemia virus also providing an ideal pseudotype platform. Rhabdoviruses, such as vesicular stomatitis virus (VSV), and other retroviruses, such as equine infectious anemia virus, are also increasingly used as pseudotype cores. Their RNA genomes are manipulated to encode a quantifiable marker gene, which is packaged by retroviral core proteins. Transduction of the target cells by the pseudotype is dependent on the ability of the envelope protein to engage receptors on the cell surface. If entry is successful, the genome is transferred from virus to cell, resulting in reporter gene integration and expression. Levels of marker protein expressed in infected cells can subsequently be measured, which produces a quantitative readout synonymous with the function of the foreign envelope glycoprotein [40].

PVs are created by simultaneous introduction of the envelope gene, retroviral *gag–pol* genes (responsible for the manufacture and enzymatic processing of the core structural proteins and insertion of the reporter gene into the host chromosome) and the chosen reporter gene into producer cells such as HEK293 T cells, using a three-plasmid cotransfection system, as shown in Figure 1. After transcription and translation of the imported genes by the relevant cellular machinery, an RNA dimer of the reporter gene is packaged into the core; these processes are driven by an upstream promoter and a packaging signal, ψ, respectively. The same packaging signal is omitted from the *gag–pol* construct to prevent replication competence and nullify the potential risk of pathogenic virus proliferation. PV capsids

Table 1. Establishment of the pseudotype platform across several families of emergent RNA viruses.

Virus family	Virus genus and members	Core vector system	Reporter protein	Ref.
Orthomyxoviridae	Influenza A viruses – various subtypes	HIV, MLV	Firefly luciferase, GFP	[42,65–67]
Rhabdoviridae	*Lyssavirus* – rabies, Lagos bat virus, Mokola virus, Duvenhage virus, EBLV-1/-2	HIV, MLV, EIAV	Firefly luciferase, GFP, β-galactosidase	[41,46,47,68]
Coronaviridae	*Coronavirus* – SARS	HIV, VSV	GFP, β-galactosidase	[50,51,69]
Flaviviridae	*Flavivirus* – JEV *Hepacivirus* – HCV	HIV, MLV	GFP, β-galactosidase	[70,71]
Filoviridae	*Filovirus* – Ebola, Marburg	HIV	Luciferase	[52]
Bunyaviridae	*Hantavirus* – Hantaan, Seoul *Orthobunyavirus* – La Crosse	MLV, VSV	GFP, β-galactosidase	[54,72]
Paramyxoviridae	*Henipavirus* – Nipah	VSV	Renilla/firefly luciferase, GFP, SEAP	[48,49]
Togaviridae	*Alphavirus* – Ross River virus, Chikungunya virus	MLV	Luciferase	[73,74]

Along with the conventional retroviral vector system, VSV core pseudotyping is also commonly used. Reporter gene flexibility within the plasmid cotransfection method for pseudotype production enables cost customization of the pseudoparticles, maximizing the scope for laboratories with varying budgets to use the pseudotype system.
EBLV: European bat lyssavirus; EIAV: Equine infectious anemia virus; JEV: Japanese encephalitis virus; MLV: Murine leukemia virus; SARS: Severe acute respiratory syndrome; SEAP: Secreted alkaline phosphatase; VSV: Vesicular stomatitis virus.

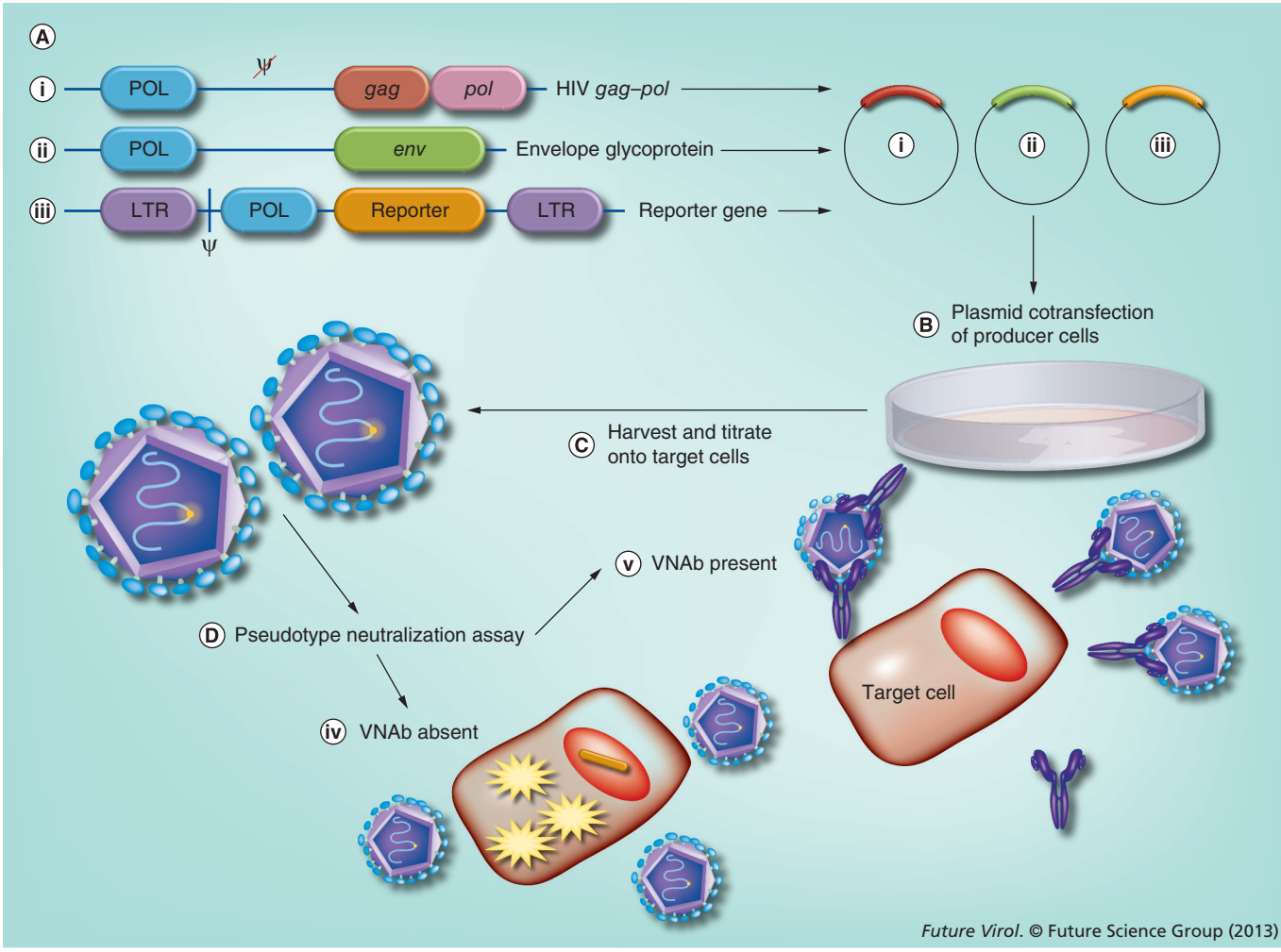

Figure 1. Three-plasmid cotransfection method for pseudotype virus production. (A) Conventional plasmid DNA expression vectors bearing **(i)** the HIV *gag–pol* gene, **(ii)** the envelope glycoprotein from the virus of interest or **(iii)** a reporter gene (e.g., luciferase) are generated. **(B)** All three plasmids are transfected into 'producer' cells (e.g., HEK293 T cells). **(C)** Supernatants are harvested at 48 h post-transfection and produced pseudotype viruses (PVs) are titrated onto target cells expressing receptors recognized by the envelope protein in order to ascertain a relative transduction titer. **(D)** PVs can be subsequently employed as surrogate viruses in pseudotype neutralization assays to quantify VNAb responses. Titrated patient samples are preincubated with a fixed titer of PV before addition to target cells. **(iv)** In the absence of VNAbs, the envelope protein of the virus of interest enables entry of the PV into the target cell and the reporter gene is integrated and expressed. **(v)** Binding of the envelope protein by specific antibodies in the sample blocks entry of the PV into the target cell, thus preventing expression of the reporter gene. As for traditional plaque reduction neutralization tests, the titer of antibody can be expressed as the highest dilution of sample that inhibits expression by 50 or 90%.
LTR: Long tandem repeat; VNAb: Virus-neutralizing antibody.

are subsequently induced by further signals in order to transit to the plasma membrane of the producer cell before they bud extracellularly. The virus envelope bearing the heterologous glycoprotein is formed from the plasma membrane [40]. This process results in a virus-rich supernatant of culture medium, which can be harvested and titrated on the target cell. The reporter gene is flanked by long tandem repeats; these facilitate integration into the target cell genome. Integration is catalyzed by the lentiviral polymerase/integrase, which is packaged as part of the pseudotype virion. The titer of the PV is calculated as a function of reporter gene expression.

Functional PV particles can be used as 'surrogate viruses' in neutralization assays to ascertain the immune response of neutralizing antibodies raised against the envelope glycoprotein coating the pseudotype. Neutralization is quantified as a decrease in reporter gene expression compared with the original pseudotype transduction titer. This avoids the use of native, pathogenic virus in current serological assays, dramatically widening the scope of laboratories able to effectively diagnose suspected cases of viral infection. Another advantage of the pseudotype neutralization assay (PNA) is the small sample volume required (≤10 µl), as evidenced by a recent study employing PNA

methodology to quantify Lagos bat virus (LBV), Mokola virus (MOKV) and West Caucasian bat virus VNAbs in African fruit bat populations [41].

Pseudotype technology for diagnostic serology has now been explored for many groups of viruses, and several robust and reproducible neutralization assays have been developed. The influenza pseudotype platform is among the best established, with the majority of initial emphasis being placed on subtypes responsible for notorious zoonotic outbreaks in the human population, such as H5N1 highly pathogenic avian influenza [42]. Recent elaborations include the production of lentiviral pseudotypes bearing the heterologous hemagglutinin of highly pathogenic avian H7 strains and representatives of each of the group 2 influenza A virus subtypes, including equine H3 [43–45]. This will facilitate heterosubtypic serosurveillance in regions where resources are limited, which will be highly beneficial for the early anticipation of zoonotic transmission of influenza subtypes with pandemic potential. Large-scale, in-field serodiagnostic studies have also been performed using pseudotypes of rabies and other lyssaviruses [46,47]. Pseudotype neutralization tests provided a specific diagnosis, distinguishing between different lyssavirus infections, and their results correlated strongly with validated serological assays. Indeed in some cases, the PNAs were more sensitive. Other emerging viruses to which pseudoparticle technology has been applied are Nipah, using a VSV vector [48,49], as well as SARS coronavirus [50,51], Marburg and Ebola filoviruses [52,53] and Hantaan and Seoul hantaviruses [54].

The generation of PVs can pose some challenges. Although most of the PVs generated so far express a single viral envelope protein, expression of two envelope proteins may be a prerequisite for successful generation of a PV, as is the case for HCV [55]. Furthermore, critical processes in the maturation and assembly of the envelope proteins in the wild-type virus may be lost in the generation of a PV. For example, particle formation of lentiviruses occurs at the plasma membrane, whereas for flaviviruses, it occurs at the endoplasmic reticulum. Hsu *et al.* overcame this obstacle by replacing the transmembrane domain of the DENV envelope protein with the transmembrane and cytoplasmic domains of the VSV glycoprotein [55]. Kolokoltsov *et al.* observed that the density of Venezuelan equine encephalitis envelope proteins was reduced in PVs [56]. This may result in the loss of quaternary epitopes created by tightly packed envelope proteins, which may be important VNAb targets [57]. Similarly to genetically modified virus assays, it is therefore important to carefully compare results obtained with assays using PVs with those from traditional assays using wild-type virus. On the other hand, greater maturation of WNV envelope protein has been shown to reduce the sensitivity of RVPs to neutralization due to masking of epitopes that are accessible and recognized by the host immune system during infection [58].

Further advances

Wright *et al.* have explored the potential of multiplexing the PV system as a means for simultaneous screening of VNAb responses to more than one virus in diagnostic and serosurveillance studies [41]. Renilla or firefly luciferase reporter genes were incorporated into pseudotyped particles bearing the envelope proteins for LBV and MOKV, respectively. LBV and MOKV are phylogroup 2 lyssaviruses that are clinically indistinguishable. A dual PNA was performed in which renilla and firefly luciferase expression were quantified. Results from the duplex assay correlated well with those from PNAs using the individual pseudotypes, and overall seroprevalence of the two lyssaviruses within an *Eidolon helvum* bat reservoir was in accordance with previous studies [59,60]. The ability to multiplex the pseudotype platform enables resource-poor laboratories to detect VNAbs for several viruses at once, reducing the necessary reagent and sample volumes. The system could be further multiplexed by use of fluorescent markers such as GFP and RFP for additional PVs. Ultimately, serological assays could be carried out for whole families of emerging viruses, such as henipaviruses, coronaviruses and hantaviruses, which have already been adapted to the pseudotype system.

The flexibility of reporter genes that can be incorporated into the cotransfection stage of pseudoparticle production is a major advantage of the viral pseudotyping approach. The most frequently used reporter genes are GFP, firefly luciferase and renilla luciferase. Switching of these genes has been facilitated by the development of a common HIV plasmid construct, into which each reporter has been subcloned: pCSGW, pCSFLW and pCSRLW, respectively [47,51,61]. Luciferase can be regarded as the 'gold standard' reporter gene for the pseudotype platform, with respect to the assay's readout preparation time and quantitative data analysis, although the cost of luciferase reagent kits and the necessity for specialist detection equipment may restrict its widespread application. Use of a GFP reporter

does not necessitate additional reagents for reading neutralization titers, but nevertheless requires relatively expensive readout equipment, such as a fluorescent microscope. Therefore, the production and expression of cheaper, alternative reporters is of vital importance. β-galactosidase substrates can be used to quantify pseudotype and VNAb titers by the introduction of the *lacZ* gene as a reporter (pCSLZW). The readout can be obtained in a cost-effective fashion, either by counting cells under a light microscope after incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) or by the evaluation of a colorimetric substrate – either *O*-nitrophenyl-β-D-lactopyranoside (ONPG) or chlorophenol red-β-D-galactopyranoside (CPRG) – using an ELISA plate reader or by eye [46]. A further development in the choice of reporter system is the use of secreted alkaline phosphatase, which was recently incorporated and expressed in Nipah virus pseudotypes [62]. In serological assays, secreted alkaline phosphatase is independently secreted into the cell culture supernatant with no need for an additional readout substrate or lysing agent. A colorimetric reaction develops and results are obtained within 24 h of the initial infection, which is more rapid than established pseudotype titration or neutralization protocols. High-throughput screening of antisera could be undertaken and measured in an inexpensive ELISA plate reader, similar to β-galactosidase expression systems.

A rapid-entry assay first described by Kolokoltsov and Davey provides a potential solution to the lengthy incubation required for a read-out to be determined, which can be in the region of 48–72 h [56], for laboratories with sufficient funding to regularly carry out luciferase- and GFP-based pseudotype assays. Virus-encapsulated luciferase bound to a Nef accessory protein is incorporated into PV particles. Upon transduction of the virus into the target cell, the Nef–luciferase complex is released into the cytosol. Luciferin introduction creates a rapid light-emitting reaction that can be quantified using a luminometer. This technique circumvents the incubation period required for assays that are reliant on the integration and expression of a plasmid-derived reporter gene. Using this assay, viral entry signals were detected in as short a period as 15 min with high specificity and low background readings that are comparable with similar serological tests.

Conclusion

The promise of genetic modification of viruses and the production of 'next-generation' serological assays is undeniable. However, they cannot yet compete with the more traditional assays in terms of speed of development. Further advances must now be made with regard to cost, dissemination and speed of acquiring results to maximize the utility of these assay platforms.

Future perspective

Before any serological assays are adopted for high-throughput diagnostic applications, they must undergo rigorous, extensive validation in order to achieve necessary certification and recognition by international public health boards, such as the WHO or the World Organization for Animal Health (OIE). Some measures have been taken to clinically ratify the sensitivity of novel serological tests in comparison to their more established counterparts. For instance, a large panel of avian sera sampled from a partially vaccinated population of Vietnamese ducks and chickens was recently screened for H5N1 influenza using a PNA, as well as an HI test and an H5-specific ELISA, before comparisons were made between the three tests. In this study, the PNA was considered a 'gold standard' reference assay, and the results revealed a comparably low specificity when values close to the positive cutoff value were obtained in the H5-specific ELISA, as well as reinforcing the accurate, sensitive performances of the PNA and HI tests, which correlated very strongly [63].

Optimization of the relevant protocols and reagents to ensure accuracy and reproducibility, as well as the establishment of reference sera of known VNAb titers that can be made available through national and international bodies, such as the European Pharmacopoeia [64], are other factors that are integral to increasing the probability of novel serological assays transcending the divide from proof of concept to significantly influencing public health and reducing the incidence of emerging infectious viruses. These processes are also vital in minimizing interlaboratory variation of 'next-generation' serological assays, enabling their effective use for the purposes of diagnosis and serosurveillance.

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Executive summary

Exotic emerging/re-emerging viruses

- During the second half of the 20th century and onwards, an unprecedented global increase in the emergence of infectious viruses was witnessed.
- Factors generally perceived to contribute to this phenomenon include increased global connectivity and trade, deforestation and climate change, as well as the development of improved viral diagnostics.
- Serological assays are integral for tracking the epidemiology of an emerging virus outbreak, in addition to evaluating the success of mass vaccination or antiviral treatment programs.
- Conventional serological techniques are limited in scope due to high biosafety requirements, and cost of equipment and reagents, as well as cross-reactivity and low sensitivity in some cases.

Traditional serological assays

- The plaque reduction neutralization test is considered the 'gold standard' serological assay for viral infection due to its high sensitivity and specificity, but it requires high biosafety laboratory facilities and is limited in terms of assay throughput.
- Hemagglutination inhibition is a widespread, high-throughput serological approach in comparison with the plaque reduction neutralization test, but it also detects non-neutralizing antibodies and can be subject to interlaboratory variations due to difficulties concerning reagent standardization.
- ELISA is another high-throughput assay with the ability to utilize the differentiation of infected from vaccinated animals (DIVA) approach in vaccine evaluation schemes, but this serological assay can be prone to low sensitivity and high levels of cross-reactivity in evolutionarily similar viruses.

'Next-generation' assays for detecting neutralizing antibodies

- Novel serological techniques often revolve around the ability to attenuate or genetically alter a virus, decreasing its pathogenicity and thus enabling its use as a surrogate reagent in either traditional or 'next-generation' assays.
- Recombinant viruses are usually exploited as attenuated vaccine candidates, but they can also be valuable in serology, being utilized in place of wild-type virus subsequent to genetic augmentations, such as codon deoptimization or envelope glycoprotein gene switching.
- Reporter virus particles encapsidate a subgenomic RNA replicon capable of expressing a reporter gene upon target cell infection, with reporter virus particle production being accomplished by *trans*-complementation of replicon RNA with virus structural genes cloned into plasmid DNA expression vectors.
- Pseudotype viruses are produced via multiplasmid cotransfection systems – they comprise a retroviral core vector displaying foreign envelope glycoproteins and harboring a reporter gene, and are ideal surrogates for pathogenic virus in neutralization assays.

Further advances

- The ability to multiplex the pseudotype system has considerable beneficial implications for increasing assay throughput, reducing volumes of valuable reagents and thus saving on the cost-per-assay burden of this platform.
- Flexibility to choose which pseudotype virus reporter gene is integrated into target cell chromosomes enables the cost customization of the platform – luciferase and GFP reporters have more expensive associated costs, whereas β-galactosidase expression can be quantified very affordably.
- Rapid-entry luciferase assays dramatically reduce the incubation time required for conventional pseudotype neutralization assays, thus providing an ideal solution to throughput dilemmas, as results can be measured within 15 min of assay preparation.
- Despite the considerable research effort on a global scale to overcome the persistent issues with conventional serology and to maximize the utility of novel assays for neutralizing antibody detection, it must be remembered that without withstanding the rigorous clinical validation measures, these 'next-generation' assay platforms will not be recognized by global public health bodies and will be unable to positively influence the severity of future emerging virus outbreaks.

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