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Lyophilisation of influenza, rabies and Marburg lentiviral pseudotype viruses for the development and distribution of a neutralisation -assay-based diagnostic kit

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Pseudotype viruses (PVs) are chimeric, replication-deficient virions that mimic wild-type virus entry mechanisms and can be safely employed in neutralisation assays, bypassing the need for high biosafety requirements and performing comparably to established serological assays. However, PV supernatant necessitates −80 °C long-term storage and cold-chain maintenance during transport, which limits the scope of dissemination and application throughout resource-limited laboratories. We therefore investigated the effects of lyophilisation on influenza, rabies and Marburg PV stability, with a view to developing a pseudotype virus neutralisation assay (PVNA) based kit suitable for affordable global distribution. Infectivity of each PV was calculated after lyophilisation and immediate reconstitution, as well as subsequent to incubation of freeze-dried pellets at varying temperatures, humidities and timepoints. Integrity of glycoprotein structure following treatment was also assessed by employing lyophilised PVs in downstream PVNAs. In the presence of 0.5 M sucrose–PBS cryoprotectant, each freeze-dried pseudotype was stably stored for 4 weeks at up to 37 °C and could be neutralised to the same potency as unlyophilised PVs when employed in PVNAs. These results confirm the viability of a freeze-dried PVNA-based kit, which could significantly facilitate low-cost serology for a wide portfolio of emerging infectious viruses.

1. Introduction

The impact of emerging and re-emerging viral diseases on global health is becoming increasingly apparent year on year. Influenza (family *Orthomyxoviridae*, genus *Influenzavirus A*, species *Influenza A virus*) remains one of the viruses most likely to cause high morbidity and mortality in human populations, after significant outbreaks of H5N1 and H7N9 subtypes beginning in 1997 and 2013 respectively, and the low pathogenic but highly transmissible 2009 H1N1 pandemic virus (Yuen et al., 1998; WHO, 2010; Gao et al., 2013). This threat persists with the first human cases of H6N1 and H10N8, and the recent discovery of diverse H17N10 and H18N11 subtypes in bat reservoirs (Tong et al., 2013; Wei et al., 2013; To et al., 2014). Similarly, rabies (family *Rhabdoviridae*, genus *Lyssavirus*, species *Rabies virus*) is a globally ubiquitous virus, present on all continents other than Antarctica, and responsible for over 60,000 deaths per year, primarily of children in resource-limited areas of Asia and Africa (WHO, 2013). Once symptoms occur, rabies has a close to 100% case fatality rate, the highest of any viral infection. Indeed, only a handful of people have survived following development of clinical symptoms and most of those had neurological sequelae (Jackson, 2013). Sporadic outbreaks of Marburg virus (family *Filoviridae*, genus *Marburgvirus*, species *Marburg marburgvirus*) in the Democratic Republic of the Congo in 1999–2000, and then in Angola in 2004–2005 (respective mortality rates of 83% and 90%), as well as small Ugandan outbreaks more recently, serve to remind us that spillover events into human populations from unexpected viral sources can create serious public health concerns (Brauburger et al., 2012). Therefore, options for monitoring the spread and curtailing the outbreak severity of pathogenic viruses are vitally important.

Serological assays that can detect and quantify antibody responses raised against antigenic surface glycoproteins enable the evaluation of potential vaccines and antiviral treatments, as well as sero-surveillance to monitor the epidemiological movements of a virus, thus contributing to international public health initiatives. Serology compliments direct virus isolation or reverse

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transcription polymerase chain reaction (RT-PCR) diagnosis, by enabling the identification of an acute viral infection after the temporary viremic stage has passed (Papenburg et al., 2011).

However, conventional serological assays possess drawbacks which detrimentally affect their efficiency. Importantly, most require the use of infectious wild-type virus, necessitating expensive, specialised biosafety level 3 or 4 (BSL-3 or -4) laboratories which are not readily available, especially in resource-limited areas. Haemagglutination inhibition (HI) assays, used routinely for influenza, suffer from variability caused by different erythrocytes and inhibitory factors, as well as low sensitivity. ELISA-based assays do not require the use of wild-type virus, but are also hindered by low sensitivity and cross-reactivity between samples. Furthermore, both HI and ELISA cannot differentiate between virus neutralising and non-neutralising antibody responses (Mather et al., 2013). Virus neutralisation assays, such as plaque reduction neutralisation test (PRNT) and fluorescent antibody virus neutralisation (FAVN) assay, can measure virus neutralising antibody (VNAb) responses with high sensitivity and specificity levels but also require high biosafety for assay preparation, and in some cases are time-consuming and suffer from low-throughput (Cliquet et al., 1998; Mather et al., 2013).

A potential solution to these issues is the utilisation of retroviral pseudotype viruses (PVs). PVs are composed of the structural and enzymatic core of one virus combined with heterologous envelope glycoproteins (Temperton and Wright, 2009). Manipulations to the genomic RNA of the lentiviral core create a replication-defective PV that encapsulates a quantifiable reporter gene. Transduction of a permissible target cell line is dependent upon the ability of the envelope glycoprotein to engage its cellular receptor in a process that mimics wild-type virus entry mechanisms. If this is successful, the reporter gene can be integrated into the host cell genome and subsequently expressed. Resultant levels of reporter protein in transduced cells can be measured, giving a readout equivalent to viral titre. Pseudotype virus neutralisation assays (PVNAs) attain comparable, if not higher, sensitivity and specificity results than many traditional serological assays (Desvaux et al., 2012).

In order to maximise the utility of the pseudotype assay system, multiplexing of PVNAs has been demonstrated which permits simultaneous quantification of VNAb responses against several PVs (each harbouring a different reporter gene i.e. renilla and firefly luciferase, or GFP and RFP) in the same assay, sparing valuable reagents such as serum samples (Wright et al., 2010). The flexibility of reporter genes that can be incorporated into PVs further customises the assay. Luciferase and GFP reporters enable highly quantitative readouts but require expensive reagents and/or equipment. However, infection by PVs that encapsulate *lacZ* (expressing β-galactosidase) or secreted alkaline phosphatase (SEAP) reporter genes can be quantified by adding colorimetric substrates such as ONPG, CPRG or p-nitrophenyl phosphate and measuring colour change with an ELISA plate reader or by eye (Wright et al., 2009; Kaku et al., 2012).

Multiplexing, as well as selecting 'low-cost' reporter genes, considerably reduces the cost-per-assay burden of the pseudotype platform. However, the high expenses associated in optimal transportation and storage can be an inhibitory obstacle in the international distribution of PVNAs. Despite pseudotype studies being conducted on field serum from resource-poor tropical countries, and reports of viruses that circulate in tropical regions being successfully pseudotyped (Wright et al., 2009; Kishishita et al., 2013), there appear to have been no published studies involving the carrying out of pseudotype neutralisation assays in tropical countries, especially in rudimentary laboratories without air-conditioning or access to reliable freezer units.

The aim of this study was to ascertain the viability of lyophilising pseudotype viruses with a view to developing a PVNA-based kit. Pseudotype stability was monitored after subjection to environmental conditions likely experienced in the production, transit and usage of such a kit, especially to tropical countries. PV titres were also assessed subsequent to lyophilisation and immediate reconstitution, as well as incubating freeze-dried pellets at a variety of temperatures and humidities before reconstitution.

2. Materials and methods

2.1. Viruses and cells

The virus isolates pseudotyped in this study were influenza A/H5N1/Vietnam/1194/2004 strain (Genbank accession number ABP51976), rabies virus (RABV) strain Evelyn Rokitniki Abseleth (ERA; UniProtKB/Swiss-Prot code ABN11294) and the Lake Victoria strain of Marburg virus (MARV; Genbank accession number DQ447649). Previously, the influenza HA gene and RABV G gene of these isolates were both sub-cloned into the pI.18 expression vector (Cox et al., 2002). The Marburg GP gene within the pCAGGS expression vector was a kind gift from Graham Simmons (Blood Systems Research Institute, San Francisco, CA, USA).

Human embryonic kidney 293T clone 17 cells (HEK293T/17; ATCC CRL-11268) (Pear et al., 1993) were used for all transfections and as a target cell line for titration and neutralisation assays involving H5 pseudotype virus. Baby hamster kidney 21 cells (BHK-21; ATCC CRL-10) (Stoker and MacPherson, 1964) were used as a target cell line for RABV and MARV pseudotype virus assays. Both cell lines were cultured at 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX (Life Technologies, UK) supplemented with 15% foetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma–Aldrich, UK).

2.2. Serum samples

For use in H5 PVNAs, a sample from a panel of ten sera extracted from chickens vaccinated with an inactivated, monovalent, adjuvanted H5N2 vaccine (A/chicken/Mexico/232/94/CPA strain) was selected. Previous studies have confirmed its seropositivity by HI (a titre of 1:1024 with a homologous H5N2 test antigen) and PVNA, against an H5 A/Vietnam/1194/2004 luciferase PV (Terregino et al., 2010; Molesti et al., 2013). To neutralise RABV pseudotypes, serum was used from a human subject vaccinated on days 0, 7 and 21 with the inactivated Rabipur vaccine (Novartis Vaccines, Germany).

2.3. Production of pseudotype viruses

The generation of all lentiviral pseudotype viruses was performed as detailed previously (Temperton et al., 2007; Wright et al., 2008). 24 h prior to transfection, approximately 4×10^6 HEK293T/17 cells were seeded into sterile 10 cm^3 tissue culture plates (Nunc™ Thermo Scientific, UK). The HIV *gag-pol* plasmid, pCMV-Δ8.91 (Zufferey et al., 1997) and the firefly luciferase reporter construct pCSFLW (Capecchi et al. (2008) based on pHR'SIN-cPPT-SGW outlined in Demaison et al. (2002)) were transfected simultaneously with either the influenza HA, rabies G or Marburg GP expression vectors at a ratio of 1:1.5:1 (core:reporter:envelope) using the Fugene6 lipid-based reagent (Promega, UK). At 24 h post-transfection, the cells were incubated with fresh media. For H5 transfections, exogenous recombinant neuraminidase from *Clostridium perfringens* (Sigma–Aldrich, UK) was also added at this stage. Pseudotype supernatants were harvested at 48 h after transfection and passed through a 0.45 μm pore filter (Millex®, Millipore, Billerica, MA, USA), before being prepared for lyophilisation. Remaining supernatant was aliquoted and stored at −80 °C.