Pseudotype viruses - applications and troubleshooting

Wednesday, 02 October 2013
The O2, London, United Kingdom

Pseudotype viruses are rapidly establishing themselves as important research and diagnostic tools of basic and clinical scientists facilitating the detailed study of individual viral genes, host cell receptors and highly pathogenic viruses, circumventing the need for high-level biosafety containment. The switching of surface envelope proteins expressed on the surface of these pseudotypes enables them to be used as surrogate viruses in neutralization/antiviral screening assays and for the study of cell–virus receptor interactions. This meeting encompasses the many diverse applications of pseudotype technologies from a practical, translational and public health perspective. This event has CPD accreditation.

Meeting Chair: *Dr Nigel Temperton*, Senior Lecturer, University of Kent, UK

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Who Should Attend

- Public and animal health scientists.
- Individuals establishing diagnostic assays for viruses.
- Epidemiologists, virologists, immunologists, R&D, pre-clinical vaccinologists.

9:00 – 9:45 **Registration**

9:45 – 10:00 **Introduction by the Chairs**: *Dr Nigel Temperton*, Medway School of Pharmacy, The Universities of Greenwich and Kent, UK

10:00 – 10:30 **Current progress with serological assays for exotic emerging/re-emerging viruses**
Dr Janet Daly, Nottingham University, UK
Challenges exist in the development of serological assays for (re-)emerging viruses. Work with live virus is often restricted to specialised containment laboratories, thus limiting capacity to perform traditional serological assays such as the plaque reduction neutralisation test (PRNT). Diagnosis by ELISA-based assays using killed virus or purified or recombinant viral proteins offer an alternative. However, ELISA-based assays are often less specific than PRNT. Sample volume may be limited, for example where cerebrospinal fluid samples are required to confirm a viral cause of encephalitis. Pseudotype virus neutralisation assays offer the potential to address many of these issues.

10:30 – 11:00 **Virus pseudotypes and pandemic preparedness**
Dr Nigel Temperton, Senior Lecturer, University of Kent, UK
The availability of *in-vitro* cell culture based assays that can be readily employed for the efficacy testing of vaccines, antivirals and therapeutic antibodies are key components for effective pandemic preparedness. The exploitation of retroviral vectors pseudotyped with foreign heterologous envelope glycoproteins for the development of such assays will be discussed with particular empahasis on emerging influenza viruses.

11:00 – 11:30 **Speakers' photo then mid-morning break and trade show**
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11:30 – 12:00 **Retroviral pseudotypes for equine influenza virus serology**
Dr Simon Scott, Lecturer in Molecular Biology, University of Kent, UK
Standard assays for influenza serology present certain practical issues; interlaboratory variability, complex protocols and sometimes requirement to work in high biological containment. To address this, retroviruses pseudotyped with the haemagglutinin (HA) glycoprotein have been successfully employed in antibody neutralization assays. Recently, we generated the first equine influenza HA pseudotypes. This necessitated co-transfection with a specific endoprotease plasmid to cleave HA to produce infectious particles. PVs were then used in antibody neutralization assays to distinguish between vaccinated and non-vaccinated equines. Sera were screened in parallel by a standard single radial haemolysis assay. A 65% correlation was demonstrated between the two assays.

12:00 – 12:30 **Interrogating the antibody response to rabies and lyssaviruses using retroviral pseudotyping**
Dr Edward Wright, Westminster University, UK
Rabies virus is responsible for ~70,000 human deaths a year even though highly efficacious vaccines are available. A reliable figure for the number of deaths due to related lyssavirus species is unknown. Within the lyssavirus genus there are 11 species that can be classified into phylogroups, based primarily on their antibody cross neutralisation profile. As the lyssavirus glycoprotein is the major target of a neutralising antibody response a pseudotype-based neutralisation assay was developed to

elucidate the importance of known glycoprotein epitopes in virus neutralisation. The assay has also proved useful for sero-epidemiological studies and testing existing and novel antivirals.

12:30 – 13:30 **Lunch and trade show**

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13:30 – 14:00 **Dissecting serological responses to ruminant viruses using retrovirus pseudotypes**

Dr David Griffiths, Principal Research Scientist, Moredun Research Institute, Pentlands Science Park, UK

Retroviral pseudotypes are valuable tools for investigating serological responses to viruses, particularly in cases where a permissive culture system is not available. An example is jaagsiekte sheep retrovirus (JSRV), which causes ovine pulmonary adenocarcinoma, a fatal lung cancer of sheep. Until recently, it was thought that infected sheep cannot produce an adaptive immune response to JSRV and this has hindered the generation of vaccines and diagnostics for controlling the disease. Using pseudotypes, we have shown that some infected sheep elicit neutralising antibodies to JSRV, opening up new opportunities for the development of control strategies for this important veterinary pathogen.

14:00 – 14:30 **Quantifying the infectivity & neutralisation of companion animal viruses using retroviral pseudotypes**

Professor Brian Willett, University of Glasgow, Scotland, UK

The study of viral diseases of many species is hindered by the paucity of reagents with which viral infectivity may be measured. The ability to generate retroviral pseudotypes bearing envelope glycoproteins from diverse viral genera offers a unique opportunity to investigate hitherto intractable questions in viral pathogenesis. Pseudotypes bearing FIV and FeLV Envs have been used to elucidate viral receptors, to quantify neutralising antibodies and to stage clinical disease, while pseudotypes bearing lyssaviral glycoproteins have facilitated the detection of neutralising antibodies in sera from wild carnivores. Retroviral pseudotypes offer a novel means to broaden our understanding of viral pathogenesis.

14:30 – 15:00 **Afternoon Tea/Coffee and trade show**

15:00– 16:00 **Question and Answer Session**

Delegates will be asked to submit questions to a panel of experts. Questions can be submitted before the event or on the day

16:00 - 16:30 **Use of HIV Pseudovirions to investigate susceptibility to Antiretrovirals**

Katherine Sutherland, Microbiology Services, Colindale Public Health England, UK

Research using HIV pseudovirions can be carried out at Containment Level 2 making research quicker, safer and cheaper. We use pseudovirions based on the HIV Gag-Pol expression vector, p8.9NSX, which encodes the HIV structural proteins and enzymes necessary for viral replication. Patient-derived HIV genes can be inserted into p8.9NSX+ separately or in combination. Pseudovirions comprise a non-HIV envelope glycoprotein enabling infection of common cell types and an HIV-based genome encoding the luciferase reporter gene to enable quantification of infection. We use pseudovirions to investigate susceptibility to different antiretrovirals, determine viral replicative fitness as well as viral structure using electron microscopy.

16:30 - 17:00 **Retroviral pseudotypes for investigating the humoral immune response to diverse hepatitis C virus strains**

Dr Alexander Tarr, Senior Research Fellow, School of Life Sciences, University of Nottingham, UK

Hepatitis C virus (HCV) causes a chronic infection that can result in cirrhosis and liver cancer. Entry of HCV into host cells is mediated by virus glycoproteins E1 and E2. Although entry is an attractive target for therapeutic intervention and vaccine design, the extreme diversity (>30% difference in nucleotide sequence between isolates) exhibited by these proteins presents a significant challenge. We have investigated entry of retroviral pseudotypes possessing genetically diverse HCV glycoproteins to identify broadly neutralizing anti-E1/E2 antibodies that target conserved epitopes. This strategy has revealed key entry steps that can be targeted for therapeutic intervention.

17:00 **Chair's Summary and Close of Meeting**

Keywords: Retroviral pseudotype, virus serology, neutralization assays, pandemic preparedness, virus tropism,zoonotic emerging viruses, serological assays,Rabies virus, lyssaviruses, pseudotypes, vaccines, antivirals, sheep, retrovirus, jaagsiekte, ruminant, serology, Feline, retrovirus, rabies, FIV, FeLV, HIV, Retrovirus, Antiretrovirals, susceptiblity, resistance, sheep retrovirus jaagsiekte ruminant serology, Influenza, pseudotypes, pandemic preparedness, sero-surveillance, neutralizing antibodies, influenza, equine, endoprotease, neutralization, SRH

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About the Chair

Nigel Temperton obtained his BSc in Microbiology and Genetics from UCL in 1990 and an MSc in Applied Molecular Biology of Infectious Diseases (1992), PhD in Molecular Parasitology (1999) and DLSHTM (2000) from LSHTM. After his PhD, Nigel returned to UCL as a post-doctoral scientist at the Centre for Virology. In 2003 Nigel transferred to the MRC/UCL Centre for Medical Molecular Virology initially as a senior post-doctoral scientist and subsequently Principal Investigator, funded by the MRC and industry. He is currently a Senior Lecturer at the Medway School of Pharmacy and Principal Scientist at the Viral Pseudotype Unit.

About the Speakers

Janet Daly gained a PhD for studies on the antigenic and genetic variation of equine influenza viruses at the Animal Health Trust (AHT) in Newmarket. She subsequently worked on human influenza projects at the National Institute for Biological Standards and Control and GlaxoSmithKline. She returned to the AHT to lead the equine influenza surveillance and control programme there for several years before moving to the University of Liverpool to work with Japanese encephalitis virus. She joined the University of Nottingham's School of Veterinary Medicine and Science in 2009 where her main research interests are zoonotic RNA viruses.

Simon Scott began his research career as a DNA virologist, working in Cambridge and Newmarket on the molecular biology of animal herpesviruses. Following an EU fellowship in Amsterdam studying human papillomavirus oncology, he spent over a decade undertaking research in the field of cancer gene therapy using DNA and RNA virus delivery vectors, in both the UK and USA. After joining the University of Kent he established the Viral Pseudotype Unit with Dr Nigel Temperton. The focus of his pseudotype worksince has been on neglected influenza viruses and more recently emerging RNA viruses from other virus families (e.g. flaviviruses, bunyaviruses).

Edward Wright is a Principal Scientist at the Viral Pseudotype Unit labs in central London. After completing his BSc in Virology at the University of Edinburgh, Edward successfully studied for a PhD in Molecular Virology from the University of Cambridge. He subsequently obtained a position at the Medical Research Council/Uganda Virus Research Institute Research Unit on AIDS. This was followed by a period as Research Fellow at University College London where he furthered the understanding of highly pathogenic viruses such as rabies/lyssaviruses and HIV, primarily using pseudotypes as the tools for these studies. As a Lecturer in Medical Microbiology at the University of Westminster, Edward's research continues into the pathogenicity and antigenicity of viral zoonoses.

David Griffiths graduated from the University of Manchester in 1992 with a B.Sc. in Biochemistry. He gained a Ph.D. in Virology from the University of London in 1996 for his studies on the potential role of retroviruses in human rheumatic disease. Following a post-doctoral position at the Chester Beatty Laboratories London, in 1998 he was awarded an Arthritis Research Council Postdoctoral Fellowship at University College London. In 2003, Dr Griffiths moved to the Moredun Research Institute as a Principal Research Scientist to study the pathogenesis of ovine pulmonary adenocarcinoma and the development of control methods for this disease.

Brian Willett graduated from the University of Strathclyde with a BSc (Hons) in Biochemistry and Pharmacology, and a PhD in Immunology. He joined the University of Glasgow in 1989, working with Prof. Oswald Jarrett on the immunopathology of feline leukaemia virus infection. Subsequent studies on the interaction between feline immunodeficiency virus and the cat immune system led to the characterization of the virus-receptor interaction. Current research interests include intrinsic and adaptive immunity to felid retroviruses, viral vaccine development, and novel approaches to the measurement of humoral immunity in wild felids and other animals.

Katherine Sutherland is currently studying for a PhD at Public Health England in Colindale, London and University College London. Her PhD has involved the investigation of susceptibility of HIV viruses to protease inhibitors using pseudovirus based assays. Her work has been presented at national and international conferences.

Alexander Tarr graduated with a PhD in molecular virology from the University of Nottingham. His postdoctoral research focussed on the entry pathway of hepatitis C virus (HCV) and characterizing the specificity of the antibody response during chronic HCV infections. This collaborative research revealed common entry pathways for HCV, and subsequently identified monoclonal antibodies that potently neutralize entry of genetically diverse HCV strains. His current research examines the interplay between innate and adaptive immunity in chronic viral infections, especially the contribution of soluble pattern recognition receptors to virus neutralization.

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POSTER PRESENTATIONS

USE OF LENTIVIRAL PSEUDOTYPES TO EVALUATE ARENAVIRUS ANTIVIRALS

Amy L. Moore, Dima N. Gharaibeh, Natasha R. Cerruti, Dennis E. Hruby, Sean M. Amberg
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Arenaviruses are a family of rodent-borne, enveloped RNA viruses. Several viruses in this family cause hemorrhagic fever in Africa and South America and are classified as Category A pathogens. One of the most prominent arenavirus-associated diseases is Lassa fever, endemic in West Africa; Lassa infects up to 500,000 people annually and results in mortality for ~20% of hospitalized patients. Research using these pathogens is restricted to the highest level (BSL-4) of biological containment. Arenavirus entry has been studied using pseudotypes that incorporate the envelope glycoprotein (GP), a ~500 amino acid precursor that is post-translationally processed into three subunits. Using lentiviral pseudotypes as a surrogate platform to identify arenavirus entry inhibitors, several small molecule inhibitors have been identified with potent activity against the hemorrhagic arenaviruses. An optimized version of one of these inhibitors, a derivative of benzimidazole, is currently undergoing preclinical development as a potential arenavirus antiviral. The spectrum of activity is being evaluated using lentiviral pseudotypes incorporating GP from several different strains of Lassa (representing all four known lineages), as well as many other arenaviruses from both Old World and New World lineages. Evaluation of the spectrum of inhibitor sensitivity across this panel has informed subsequent site-directed mutagenesis to enhance the understanding of the contributions of specific residues within the GP2 subunit to overall sensitivity to the preclinical lead candidate. Remarkably, the amino acid previously identified as a determinant of attenuation for the vaccine strain (Candid) of Junín virus, the causative agent of Argentine hemorrhagic fever, was found to play a significant role in antiviral sensitivity.

Characterization of a monoclonal antibody cocktail for human rabies prophylaxis

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Rabies continues to kill many thousands of people throughout the developing world every year. The murine monoclonal antibody (mAb) 62-71-3 was recently identified for its potential application in rabies post-exposure-prophylaxis (PEP). The purpose here was to establish a plant-based production system for a chimeric mouse-human version of mAb 62-71-3, to characterize the recombinant antibody and investigate at a molecular level its interaction with the rabies virus (RV) glycoprotein. The chimeric mAb 62-71-3 was successfully expressed in *Nicotiana benthamiana* and functionality was analyzed by antigen binding ELISA and by neutralization of a panel of lyssavirus pseudotypes. The antibody-antigen interaction was investigated using pseudotype virus expressing mutagenized RV glycoproteins. A critical role for antigenic site I of the glycoprotein, in particular for two specific amino acid residues, was identified.

The construction of chimeric rabies virus glycoproteins rescues Arctic-like rabies pseudovirus production.

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Arctic-like rabies viruses (AL RABV) are a lineage of rabies viruses circulating widely in the Middle East and Asia, with distinct antigenic and genetic characteristics. As with other members of the RABV species they can cause a zoonotic disease that ultimately leads to death of infected patients. RABV glycoprotein (G) lentiviral pseudovirus (PV) has been shown to be highly sensitive and specific when used as a surrogate for live virus in neutralisation assays. However, using wildtype AL RABV glycoprotein failed to generate any infectious PV. Therefore, we sought to determine if it was possible to increase the biological titre of AL RABV PV through the construction of chimeras using vesicular stomatitis virus (VSV) G and the G from the RABV isolate CVS-11. Initial studies were undertaken to generate a chimeric G by splicing the ecto- and transmembrane domains of four AL RABV G strains with the cytoplasmic domains of VSV or CVS-11 G. PV were produced expressing wildtype or chimeric G, revealing the use of chimeric AL RABV with VSV G but not CVS-11 lead to an increase in PV titres. The production of a robust AL RABV PV will allow the efficacy of current animal vaccines to be tested against this subset of RABV.

USE OF COMPUTATIONAL BIOLOGY TO STUDY THE HEMAGGLUTININ PROTEIN OF ITALIAN AVIAN H7N1 VIRUSES

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Influenza virus A is a zoonotic agent with a significant impact on both public health and poultry industry. It has a marked genetic and antigenic variation due to the high error rate of the low-fidelity RNA polymerase and to the capacity for the genome segments to be exchanged via reassortment events. Nowadays the development of effective vaccines, able to confer a greater cross protection thus reducing the risk of emergence of viral mutants, is probably the most effective strategy to control the spread of these viruses. There are 17 different HA (H1 to H17) and 10 different NA antigens (N1 to N10); however, only subtypes H5 and H7 have generated highly pathogenic avian influenza strains (HPAI). Between 1999 and 2001 Italy was affected by four consecutive epidemic waves of avian influenza caused by viruses of the H7N1 subtype. The first one was caused by a low pathogenicity avian influenza (LPAI) virus of the H7N1 subtype, which subsequently mutated into a highly pathogenic avian influenza (HPAI) virus; following the eradication of the HPAI virus, the LPAI virus re-emerged. Phylogenetic analysis of nucleotide and amino acid sequences is the starting point to understand the origin and evolutionary dynamics of the viral pathogens; the three-dimensional protein structure, allowing to display the spatial coordinates of amino acid residues, highlights the precise localization of the receptor site, the antigenic sites described in literature and the location of specific amino acids. The amino acid sequences of the HA proteins of the H7N1 HPAI and LPAI viruses were analyzed by intra group alignments and compared to sequences of the solved 3D structures available on protein data bank (http://www.rcsb.org/pdb). An homology modelling approach was applied to obtain the structure of hemagglutinin homotrimers for the H7 strains, to localize the position of the differences between HPAI and LPAI and to determine the electrostatic potential. Six amino acid residues have been identified as peculiar of highly pathogenic

viruses. Four substitutions are located in the HA1 portion of the protein and two in the HA2 stem; particularly interesting are 3 amino acid residues positioned in two structural elements that form the receptor binding domain (RBD), i.e. two residues are located in loop 130, the 3rd residue flanks the 220 loop. LPAI viruses were grouped into two cluster and 5 amino acid signatures proved to belong to the LPAI-I group. H7N1 viruses show a different distribution of additional glycosylation sites (AGS); most HPAI viruses have AGS in position 123, LPAI-I in 149 and LPAI can have either or none of them. Studying the electrostatic potential of the virus (without considering the potential glycosylation), we highlighted a different charge distribution among the HPAI and the two populations of LPAI viruses. Considering that virions possess about 900 HA monomers, an increased positive charge may enhance cellular receptor binding by increasing attraction with a negatively charged cell surface. The hypothetical different binding avidity of representative LPAI and HPAI H7N1 viruses could be determined using a cell binding assay, as suggested by Hensley et al., 2009 (1). To assess whether all these mutations can represent an advantage to the HPAI viruses in their ability to escape antibody responses, haemagglutination inhibition (HAI) and Pseudotyped microneutralization test will be performed. Furthermore, VLP with STD-NMR and glycan arrays could be very helpful.in order to investigate the link between viral glycoproteins and host receptors.

Development and use of LPAI and HPAI H7 avian influenza pseudotypes for serological studies

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HPAI and LPAI H5 and H7 influenza viruses have been disastrous for the economies of affected countries reliant on poultry production. In a situation mirroring H5, H7 strains show adaptation to humans and therefore pose a serious public health concern as evidenced by the recent H7N9 outbreak in China. Applying knowledge acquired from the study of H5 virus evolution and spread to the development of sensitive serological methods will improve our ability to understand and respond to the emergence of other HPAI and LPAI viruses with pandemic potential. We describe the production of pseudotypes bearing envelope glycoproteins of LPAI and HPAI H7 avian influenza for use as tools in pseudotype-based (pp-NT) neutralization assays. A crucial feature of H7 pseudotype production is efficient intracellular cleavage of haemagglutinin. We show that the LPAI strain A/chicken/Italy/1082/1999 possesses a monobasic cleavage site and requires TMPRSS2 to effect cleavage of the HA. The HPAI strain A/Pakistan/34668/95 possesses a sub-optimal furin cleavage site resulting in low yields. In order to circumvent this we have used site-directed mutagenesis to replace the polybasic cleavage site with a monobasic site that can subsequently be cleaved (during production) via the co-transfection of the TMPRSS2 protease. Sensitive pp-NT assays were then carried out on post-vaccination sera using these new surrogate viruses. These studies will impact on the development of new antibody assays for the recently emerged H7N9 virus.

The study of heterosubtypic neutralising antibody responses against H5N1 influenza viruses in human subjects using a comparative serology approach

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Background: The continuous rapid genetic and antigenic evolution of H5 subtype influenza viruses has major implications for the sensitivity of serologic assays and can limit the efficacy of prepandemic human vaccines and the ability to undertake effective serosurveillance in susceptible populations. Materials and Methods: A panel of serum samples collected from the Italian population between 1992 and 2007 and previously found to be positive for antibodies against H5N1 as determined by serial radial hemolysis (SRH) were evaluated using pseudotype-based neutralisation assays (PPN) in combination with haemagglutination-inhibition (HI) assays using a clade 1 and a clade 2 H5N1 serologic antigen. Results: Sixty-eight human sera were evaluated for their ability to neutralize A/Vietnam/1194/04 H5 and A/chicken/Italy/13474/99 H7 pseudotypes. IC50 neutralizing antibody titres against the Group 1 A/Viet Nam/1194/04 H5 pseudotypes ranged from < 40 (cut-off) to 1:5120-10240. Seven of 68 (10.3%) sera were found to be negative against A/Vietnam/1194/04 pseudotypes. IC50 neutralizing antibody titres against the Group 2 A/chicken/Italy/13474/99 H7 pseudotype ranged from < 40 (cut-off) to 1:160-320. Forty-seven of 68 (69.1%) sera were found to be negative against A/chicken/Italy/13474/99 H7. Conclusions: From the results obtained it can be concluded that the pseudotype assay can efficiently measure cross-reactive antibody responses that are not detected by the HI assay. It is postulated that these responses are directed against epitopes on the HA2 stalk. All three serologic assays (PPN, SRH, HI) measure antibodies with different (functional) overlapping specificities, contributing to a comprehensive analysis of humoral immunity to influenza viruses.

The study of heterosubtypic antibody responses against influenza A viruses elicited by seasonal vaccination using a pseudotype neutralisation assay

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Background: The study of heterosubtypic antibody responses directed against influenza A haemagglutinins in human populations is an important facet of pandemic preparedness. The evaluation of the ability of vaccines to increase heterosubtypic antibody responses to confer broad protection against different influenza subtypes is one approach to this. Classic serological assays, such as haemagglutination inhibition and microneutralisation, have demonstrated low sensitivity for the detection of cross-neutralising antibodies, especially those directed against epitopes in the haemagglutinin HA2 stalk region. For this reason there is a need for new assay formulations that are able to detect and quantify these heterosubtypic antibody responses. Influenza pseudotypes represent safe tools to study the neutralising antibody response since they are replication-defective viruses and they harbour on their envelope only the haemagglutinin that is the major target of this response. Materials and Methods: We have generated a panel of group 2 influenza A pseudotypes (H3 A/Udorn/307/1972, H4 A/duck/Czechoslovakia/1956, H7 A/chicken/Italy/1082/1999, H10 A/chicken/Germany/N49, H14 A/mallard/Astrakhan/263/1982, H15 A/shearwater/West Australia/2576/1979) and we have used them as surrogate antigens in neutralisation assays to study the presence and magnitude of heterosubtypic neutralising antibody responses in human sera collected before and after the 2007-2008 seasonal influenza vaccination. Results: In the human sera tested, neutralising antibody responses are detected

against not only human influenza viruses, but also against influenza pseudotypes harbouring avian haemagglutinins belonging to group 2 viruses. After seasonal vaccination, the pseudotype neutralisation assays detect variation in the neutralising antibody titres against avian influenza pseudotypes. Conclusions: The increased sensitivity of the pseudotype neutralisation assay performed using a panel of influenza A pseudotypes permits the detection of heterosubtypic antibody responses before and after seasonal influenza vaccination. This has implications for the development of pandemic preparedness plans at the population level.

The use of equine influenza pseudotyped lentiviruses for serological screening

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Background: The H3N8 subtype of equine influenza virus continues to produce outbreaks worldwide, with resultant economic and agricultural impact. The surface hemagglutinin (HA) glycoprotein elicits neutralizing antibody responses following a natural infection or vaccination. Unfortunately, the standard assays used for influenza serology present certain practical issues, such as interlaboratory variability, complex protocols, and the necessity to handle certain virus strains in high biological containment facilities. To address this, avian and human influenza HA pseudotyped retroviruses have been used successfully in antibody neutralization assays. In this study, we have, for the first time, generated equine influenza pseudotyped lentiviruses to detect HA-directed neutralizing antibodies in equine sample sera. Materials and Methods: Stocks of H3N8 A/equine/Sussex/89 were grown in 10-day-old embryonated eggs, with viral RNA extracted and HA amplified using RT-PCR and custom primers. The product was cloned into the pI.18 expression vector. Pseudotyped viruses were then produced through co-transfection of HEK293T cells with plasmids expressing the HA, HIV gag-pol, and firefly luciferase reporter genes and harvesting of virus from the supernatant. To produce infective pseudotype particles, it was necessary to also cotransfect a plasmid encoding the TMPRSS2 endoprotease to cleave the HA. High-titer pseudotype virus (PV) was then used in PV antibody neutralization assays (PVNAs); a luminometer was used to quantify PV infectivity (luciferase expression). Sera from vaccinated (n = 21) and influenza-naïve (n = 2) equines were tested. These samples were also screened using the single radial hemolysis (SRH) assay. Correlation and significance were determined by Pearson analysis using GraphPad Prism software. Results: Pseudotype viruses were generated successfully, with a titer of 1 x 109 relative luminescence units per mL. The TMPRSS2 protease was essential for this process, with no significant PV generation in the absence of the protease expression plasmid. The PVs thus produced were used in PVNAs to distinguish all test sera from vaccinated versus nonvaccinated equines. All vaccinated equines exhibited PVNA antibody titers of IC50 >1100 (positive control IC50: >40,000; negative sera: <80). Antibody was detected by SRH in 18/20 of the vaccinated equines (range: 61-207 mm2). There was a 65% correlation between results from the 2 assays (r = 0.65, *P* = .002). Conclusion: H3N8 equine influenza pseudotyped lentiviruses have been generated for the first time. These were successfully used to detect anti-HA neutralizing antibodies in serum samples. We were also able to use the pseudotype virus neutralization assay to distinguish sera from vaccinated versus influenza-naïve (by SRH) equines. The correlation between PVNA and SRH assays was good (65%), with the pseudotype assay exhibiting slightly more sensitivity. Future work will require more extensive testing of the PVNA using a larger number of serum samples to assess sensitivity/specificity and inter/intralaboratory variability and to define a protective antibody titer.

Chicken IFITM3 restricts influenza viruses and lyssaviruses *in vitro*

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Background: The interferon inducible transmembrane protein 3 (IFITM3) is an effector protein of the innate immune system, which confers potent, cell intrinsic resistance to infection by diverse enveloped and non-enveloped viruses both *in vitro* and *in vivo*, including influenza viruses, West Nile virus, dengue virus and reovirus. IFITM3 prevents cytosolic entry of these viruses by blocking complete virus fusion with cell endosome membranes. *Materials and methods:* Assessment of avian IFITM3 was initially carried out in a stable over-expression system using HA tagged proteins in human A549 cells. The stable cell lines were established using lentiviruses produced by a triple plasmid transfection in HEK293T cells. Endogenous expression was subsequently assessed by qRT-PCR in a range of chicken tissues and viral restriction tested by flow cytometry in chicken DF-1 fibroblast cells. *Results:* Although the IFITM locus, which includes *IFITM1, 2, 3 and 5*, is present in mammalian species, this locus has not been unambiguously identified or functionally characterised in avian species. Here we show that the IFITM locus exists in chickens and is syntenic with the IFITM locus in mammals. Using lentiviruses expressing IFITM genes we obtained human cell lines stabling expressing the IFITM proteins with a C-terminal HA tag. Using this model we show that the chicken IFITM3 protein restricts cell infection by influenza A viruses and lyssaviruses to a similar level as its human orthologue. Furthermore, we show that chicken IFITM3 is inducible in chicken DF-1 cells by quantitative RT-PCR and that knock-down of constitutive expression in chicken fibroblasts results in enhanced infection by influenza A virus. Chicken *IFITM2* and *3* are constitutively expressed in all tissues examined, whereas *IFITM1* is only expressed in the bursa of Fabricius, gastrointestinal tract, caecal tonsil and trachea. *Conclusions:* Despite being highly divergent at the amino acid level, IFITM3 proteins of birds and mammals can restrict replication of viruses that are able to infect different host species, suggesting IFITM proteins may provide a crucial barrier for zoonotic infections.

Sheep in Wolves Clothing: Filovirus Reagent Production Using Virus-Like Particles

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Background: With the ever increasing levels of international travel, legal and illegal trade including natural outbreaks combined with the potential for deliberate release, the need to quickly and efficiently identify filovirus infection is of critical public health importance. Specialised high-containment laboratories (CL4) that are required to work safely with such agents are time consuming and heavily utilised. Alternative strategies which facilitate simple diagnostis outside of containment bring added value and efficiency savings. Our work focuses on the development of differential immuno-assays for identification and characterisation of *filovirus* sub-types. Here we describe a strategy for producing monoclonal antibodies (mAb) to HG-4 agents in CL2 facilities using recombinant virus-like particles (VLPs). Results: Baculovirus derived *Ebola* and *Marburg* recombinant VLPs were produced and shown to exhibit a similar morphology to wild-type virus, and produced a filo-specific immune response in mice. Arrays of polyclonal

and mAbs were identified that displayed species-specificity, or cross-reactivity in the assays used. Conclusions: We have successfully shown that the baculovirus expression system can be used to produce *Ebola* and *Marburg* VLPs, and that they are immunogenic in mice, producing specific and cross-reactive Abs to different filoviruses. These reagents provide the basic building blocks for developing differential and broad spectrum immunological detection assays, and potential therapeutics.

PSEUDOTYPED NIPAH VIRUS BASED NEUTRALISATION ASSAY

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Bats have gained increased recognition as a reservoir host for zoonotic viruses, such as the henipaviruses. With evidence of henipavirus infection on the African continent, there is a need to develop robust assays to screen local wildlife and human populations against these viruses. Some success has been achieved using Luminex-based assays but these assays still require gold standard live virus serum neutralisation testing at biosafety level 4 for confirmation. With access to this confirmatory test hampered by limited resource settings, this study aimed to produce non-replicative lentivirus-based pseudotyped Nipah virus expressing a luciferase reporter gene for use in a, more accessible, biosafety level 2 neutralisation assay. This assay was used to detect neutralising antibodies against henipaviruses in human serum samples from Sub-Saharan Africa with the aim of confirming previous Luminex-based assay testing results. The testing of human serum samples however, demonstrated unexpected neutralisation levels against the pseudotyped Nipah virus produced here. A selection of small-scale experiments were conducted in an attempt to identify possible causes of this observation. Main findings indicated that the heat treatment of samples had the biggest impact on repeated neutralisation results. Further work is required to assess the use of this pseudotyped Nipah virus assay to confirm Luminex results.

Haemagglutinin activation by human transmembrane protease serine 2 or by human airway trypsin-like protease is necessary for the production of high titre influenza A virus pseudotypes that can be employed for the evaluation of pandemic potential

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Background: The monomer of influenza haemagglutinin (HA) is synthesized as a single polypeptide precursor that during maturation is cleaved by proteases into two active subunits. Recent studies have demonstrated that the human transmembrane protease serine 2 (TMPRSS2) and the human airway trypsin-like protease (HAT) can activate, by cleavage, the HA of human seasonal influenza viruses. As a model of activation of all influenza subtypes (human H1, H2, H3 viruses and other representative viruses from group 1 and group 2 avian subtypes), we have investigated the use of human TMPRSS2 and HAT to produce high titre influenza HA lentiviral pseudotypes. Such pseudotypes represent powerful and safe tools to study viral entry mechanisms and zoonotic potential. Materials and Methods: Influenza pseudotype particles are obtained by cotransfecting human embryonic kidney HEK293T/17 cells using plasmids coding for the influenza HA, HIV gag-pol and a retroviral vector incorporating firefly luciferase. To investigate the role of these proteases in the HA activation necessary for infective particle generation, a plasmid expressing TMPRSS2 or HAT was cotransfected during pseudotype production. The HA lentiviral pseudotypes produced were used to investigate cell entry potential via transduction of target cell lines and as surrogate antigens in neutralization assays. Results: Influenza pseudotype particles produced by cotransfection of these proteases can transduce HEK293T/17 cells with high efficiency compared with the pseudotypes produced in the absence of proteases. The high titre of these influenza pseudotypes permits their use as surrogate antigens in neutralization assays and subtype-specific sera can readily neutralize them. Conclusions: TMPRSS2 and/or HAT can activate, in vitro, both the HA of human seasonal influenza and also other avian HA influenza strains in a pseudotype particle production system. Furthermore, this panel of influenza pseudotypes can be used in neutralization assays to study heterosubtypic antibody responses and pandemic potential.

The application of pseudotype assays in detecting virus neutralising antibodies to African Lyssaviruses.

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Conventional methods of detecting virus-neutralising antibodies (VNAb) to rabies and other lyssaviruses are technically demanding and expensive. In this study we optimised a gammaretroviral pseudotype assay for measuring VNAb titres to the four lyssaviruses present on the African continent; rabies virus, Lagos bat virus (LBV), Mokola virus (MOKV) and Duvenhage virus (DUVV). We compared rabies VNAb titres measured using our pseudotype assay with the 'gold standard' fluorescent antibody virus neutralisation assay (FAVN). A strong correlation was seen between our pseudotype assay and the FAVN (r =0.77). No cross-neutralisation was observed with either of the phylogroup II lyssaviruses (LBV and MOKV), but a strong correlation between rabies and DUVV VNAb titres was observed (r = 0.79). Pseudotype assays are a useful tool for serosurveillance and our assay has been used for multiple applications including monitoring vaccination response in dogs, detecting VNAb in wildlife from areas of Tanzania classified as "rabies-free" and to screen a variety of wildlife species from Russia prior to reintroduction of the Amur leopard. Lyssavirus pseudotypes are also an important tool for investigating viral entry mechanisms and preliminary findings suggest DUVV may utilise an additional cellular receptor as a selection of cell lines are susceptible to DUVV but resistant to infection with the other lyssavirus pseudotypes.

Serosurveillance of Rift Valley fever virus in Tanzania

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Rift Valley fever virus (RVFV) is an arthropod-borne Phlebovirus which causes severe disease in ruminants and when transmitted to humans can cause a fatal haemorrhagic fever. Outbreaks occur following periods of exceptionally high rainfall, but little RVFV is observed in domestic livestock during inter-epidemic periods. Low level transmissions of RVFV may take place between outbreaks, however as limited serosurveillance has taken place, the likely reservoir hosts are not known. High virus neutralising antibody titres have been observed in many wildlife species, suggesting wildlife may be important in virus maintenance during these inter-epidemic periods. We have developed an indirect ELISA using the recombinant

nucleoprotein of RVFV which can be used to detect RVFV antibodies in diverse species. Using this assay we have measured RVFV antibodies from wild and domestic animals from the Serengeti region of Tanzania, seropositivity was detected in cattle, sheep, goat, buffalo, gazelle and lion, including samples from animals which had not lived through a RVFV epidemic. In addition, we tested over 300 rodents from the same region and did not detect any antibodies to RVFV in any of the specimens. The ELISA provides a diagnostic assay enabling rapid serosurveys to be performed on a wide range of species which will help predict major wildlife reservoirs of RVFV.

Investigating the functionality and antigenicity of chimeric lyssavirus glycoproteins and their neutralisation profiles

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Rabies, the archetypal lyssavirus, is one of the most feared viruses known to man and globally, is the cause of more than 50,000 deaths per year. Alongside rabies virus, numerous related lyssaviruses exist that are also capable of causing fatal clinical disease consistent with that seen following infection with rabies virus. Whilst the human burden of these viruses remains unknown, fatalities have been reported. The lyssavirus glycoprotein is the sole target for virus neutralising antibodies and several amino acid epitopes have been linked to virus neutralisation. Lyssaviruses are genetically and antigenically categorised into phylogroups that indicate the level of protection afforded by current vaccines. It is generally accepted that an antibody response to the currently available rabies vaccines affords protection against all viruses that are categorised into phylogroup I. However, this antibody response does not protect against lyssavirus species within phylogroups II and III. Indeed, experimental data has shown that the antibody repertoire induced by rabies virus vaccines is completely unable to neutralise viruses in these phylogroups. In this study we have generated lentivirus pseudotypes containing chimeric lyssavirus glycoproteins that have had their antigenic sites swapped between phylogroup I and II viruses. Using these, we show alteration in both G protein functionality alongside altered neutralisation profiles using a variety of hyperimmune sera. Here we overview results using these chimeric glycoproteins and suggest areas of the G protein responsible for the development of phylogroup specific neutralising responses.

Towards a Pseudotype assay for West Nile virus.

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West Nile virus (WNV) is a mosquito-borne virus infecting wild birds that causes zoonotic infections in humans, horses and other mammals. WNV infection of humans and horses can be asymptomatic, or it can cause a neuro-invasive disease termed West Nile encephalomyelitis, which can range from fever through to paralysis and sudden death, and occurs with a greater frequency in horses. There are no anti-virals available and vaccines are only available for horses, requiring annual boosters. WNV is currently not present in the UK; however favourable climates that are facilitating the spread of the vector into new regions of Eastern Europe and the Mediterranean combined with expanding global trade further increase the likelihood of WNV reaching the UK. Effective disease monitoring and control requires a rapid, reliable and widely available diagnostic test. The gold standard diagnostic test for WNV (plaque reduction neutralisation test – PRNT) can take up to 14 days to return a definitive result and requires BSL 3 facilities, which make it unsuitable for disease monitoring and control. Pseudotype assays can be carried out at BSL 1 and by combining them with a reporter molecule they can provide a definitive result within in 3-6 days, depending upon the reporter type and assay sensitivity. Pseudotype assays detect virus-specific neutralising antibodies meaning they are less cross-reactive than other rapid assays; they can be used to assess the sero-conversion of vaccinees as well as in the development of new vaccines and virus entry inhibitors. Here, we present our progress towards pseudotyping WNV using a lentiviral and retroviral backbone. The detection of infectious particles derived from 4 constructs encoding the WNV glycoproteins with varying leader sequences compared with a VSV-g control glycoprotein are presented.

Contribution of the *gag* gene to variation in susceptibility to protease inhibitors between different strains of HIV-1

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Background: Resistance to HIV-1 protease inhibitors (PIs) develops by the accumulation of mutations in protease. Mutations in the cleavage sites of Gag, the protease substrate, partially compensate for the loss of fitness conferred by protease mutations. There is evidence that Gag may contribute directly to resistance, independently of compensating for loss of fitness. We have explored whether natural variation in Gag may contribute to differences in susceptibility of wild-type viruses to PIs. Methods: A single-cycle, phenotypic drug susceptibility assay was used to assess the contribution of Gag and protease to the PI susceptibility of six HIV subtype B molecular clones and four subtype B viruses from treatment-naive patients. Unique restriction sites were used for the cloning of full-length *gag*-protease or *gag* and protease separately. Results: Significant differences in PI susceptibility between the viruses were observed with some strains displaying up to 17-fold decrease in PI susceptibility compared to assay reference strain, with variation being seen with respect to six PIs. For two molecular clones, JRFL and YU2, the reduced susceptibly of full-length Gag-protease was conferred solely by the gag gene. In particular, the gag gene of the molecular clones YU2 was responsible for 14-fold, 8-fold and 7-fold reduced susceptibilities to amprenavir, atazanavir and lopinavir, respectively. In the treatment-naïve patient viruses, a 17-fold and 16-fold decrease in atazanavir susceptibility of full-length Gag-protease was observed in two patient viruses. In contrast to molecular clones, this variation was shown to be cause by protease, despite the absence of major protease resistance mutations. Conclusions: Variation in the gag gene of HIV-1 contributes to different susceptibilities to PIs. Full-length patient-derived Gag is not currently included in genotypic or phenotypic drug resistance testing, giving the potential for incomplete results. In addition, significant variation in the susceptibility to atazanavir of full-length Gag-protease derived from treatment-naïve patient viruses merits further investigation, given the role of atazanavir as a first-line PI.

SEQUENCING AND ANALYSIS OF SELECTIVE PRESSURE ACTING ON THE TLRS IN VESPER BATS

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The first step in any immune response is the recognition of an invasive pathogen. In the innate immune this recognition is accomplished through the identification of distinct microbial structures by dedicated receptors known as pattern-recognition receptors (PRRs). The PRRs of the innate immune system recognise specific regions of pathogens known as pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are the best studied of the PRRs, and are a critical component in the mounting of an immune response on exposure to invasive pathogens. Despite the importance of bats in acting as reservoirs for a range of pathogen there have been very few studies on the TLRs in any bat species and, prior to this study, experimentally verified sequence data was available from only two bat species, both members of the Pteropodidae. This study amplified the extracellular (pathogen binding) domain of TLRs 1-9 and analysed these novel sequences for evidence of lineage specific selection affecting either the bat lineage or more specifically the Vespertilionidae lineage. Despite the lack of bat genomic material available in public databases, this study successfully amplified and sequenced partial gene fragments targeting the extracellular LRR regions of TLRs 1-9 in 19 species of vesper bats. There was significant variation observed in the success rates of amplification across the genes and this can be attributed to interspecies variation between different TLRs. Phylogenetic analysis using both Bayesian and maximum likelihood methods supports the phylogeny of previous studies by Teeling and Stadelman. Evidence of positive selective pressure acting on the amplified regions of the TLRs was assessed using the Phylogenetic Analysis by Maximum Likelihood (PAML), Fast Unbiased Bayesien Approximation (FUBAR) and Mixed Effects Model of Evolution (MEME) methods. Sites (codons) under positive selection were identified in TLR's 1-4 and TLR's 6-9 across the range of species analysed. TLR5 did not appear to have any sites under positive selective pressure, although sites were identified under PAML and MEME these methods did not identify any sites in common, this is in contrast to previous studies which have shown significant selection acting on this gene. For the remaining TLR genes the number of sites determined to be under positive selective pressure was between two (TLR4) and 18 (TLR8). Testing for lineage specific selection using PAML and Branch-site REL (BSR) indicated that that TLRs 1 and 7 display evidence of bat specific selective pressure acting on the bat lineage in particular. This suggests that these genes are evolving under diversifying selective pressure at a higher rate in the bat lineage than in other mammals used in the datasets. In addition BSR analysis shows strong positive selection acting on the TLR8 gene within the *Myotis lucifugus* lineage, TLR8 recognizes G-rich oligonucleotides in particular the single stranded RNA common in viral pathogens. It is interesting to note that *M. lucifugus* did not show evidence of diversifying selective pressure in any other TLR, this suggests that the observed difference in susceptibility of European and North American Myotis to White Nose syndrome is not related to the TLR genes. Selective pressure on TLRs 1 and 7 show pressure on bacterial and viral sensitive TLRs to diversify within the bat lineage.

Development of a pseudotype-based neutralisation assay kit for in-field vaccine evaluation and sero-surveillance of highly pathogenic viruses

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Virus neutralisation assays quantitatively detect levels of neutralising antibody response against antigenic surface glycoproteins on many viruses, following vaccination or natural infection. However, high biosafety level requirements and extensive personnel training prevent these tests from broad laboratory application, especially in resource-limited regions. Therefore, development of methods for vaccine evaluation and serosurveillance which can be used in these areas are urgently required. To address these issues lentiviral pseudotype viruses (PVs) have been utilised. PVs are chimeric, replication-deficient particles that mimic the infective mechanisms of their wild-type counterparts. Pseudotype neutralisation assays (PNAs) circumvent the requirement for high biosafety precautions whilst maintaining comparable sensitivity and specificity with existing assays. This study ascertains pseudotype stability through subjection to environmental conditions likely to be encountered in assembly, transport and usage of a PNA-based diagnostic kit. Pseudotypes of clinically-important viruses (e.g. influenza and lyssaviruses) have been used and titres monitored through cumulative freeze-thaw cycles, lyophilisation, and varying temperatures and humidities. Results demonstrated the ability to retain acceptable levels of virus activity following treatments, indicating the potential of PNA-based kits for global distribution and diagnostic application. Such flexible and durable kits could permit accurate in-field vaccine evaluation and serosurveillance for many viruses of endemic and pandemic concern.

Pseudotype and Microneutralization assays, two different approaches for the detection of influenza neutralizing antibodies.

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Hemagglutination inhibition (HI) assay, single radial haemolysis (SRH) assay and virus microneutralization (MN) assay are the serological assays routinely used to assess the immunity against influenza. In recent years a number of new serological assays for the investigation of immunization against influenza have been developed to overcome the limitations of the classical assays. Among these, one of the most promising is the pseudotype neutralization assay (PN). In this work we tested a number of human serum samples against H5N1 and H1N1 influenza strain in order to check the correlation between the results of a standardized assay (MN with two different read-out methods, CPE and ELISA) and the PN assay.