

# A sensitive retroviral pseudotype assay for influenza H5N1-neutralizing antibodies

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**Background** The World Health Organisation (WHO) recommended the development of simple, safe, sensitive and specific neutralization assays for avian influenza antibodies. We have used retroviral pseudotypes bearing influenza H5 hemagglutinin (HA) as safe, surrogate viruses for influenza neutralization assays which can be carried out at Biosafety Level 2.

**Results** Using our assay, sera from patients who had recovered from infection with influenza H5N1, and sera from animals experimentally immunized or infected with H5 tested positive for the presence of neutralizing antibodies to H5N1. Pseudotype neutralizing antibody titers were compared with titers obtained by

hemagglutinin inhibition (HI) assays and microneutralization (MN) assays using live virus, and showed a high degree of correlation, sensitivity and specificity.

**Conclusions** The pseudotype neutralization assay is as sensitive as horse erythrocyte HI and MN for the detection of antibodies to H5N1. It is safer, and can be applied in a high-throughput format for human and animal surveillance and for the evaluation of vaccines.

**Key words** H5N1, influenza, pseudotypes, serology, viral neutralization.

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## Introduction

Human infections with avian influenza H5N1 virus were first observed during large-scale poultry outbreaks in Hong Kong in 1997. Since its re-emergence in Asia in 2003, 306 laboratory-confirmed human H5N1 cases have been reported from Asia, Europe and Africa of whom 185 have died (World Health Organisation, WHO, May 16, 2007).

The influenza virus surface glycoprotein hemagglutinin (HA) is the most important antigenic determinant for virus-neutralizing antibodies generated during natural infection or elicited by immunization. Hemagglutination inhibition (HI) assays are employed for the detection of antibody in serum, and HI titers correlate with protection from influenza in humans. The WHO has called for research into improved assays for influenza given that HI tests, using turkey erythrocytes have been found to be relatively insensitive for measuring responses to avian H5N1 virus in humans.<sup>1</sup> Since this report was published, however,

significant increases in sensitivity have been observed using horse erythrocytes.<sup>2</sup> Neutralization assays allow for more sensitive detection of H5 antibodies, but these are laborious and require Biosafety Level 3 laboratory facilities or higher which are not always available at the front line of an outbreak, especially in resource-limited regions.

To make neutralization assays more widely applicable, there are two realistic options for rapid development; to use reverse genetics to engineer a safer, attenuated virus by deletion of the polybasic cleavage site in HA as is done for the development of inactivated vaccines for pandemic influenza,<sup>3,4</sup> or the construction of viral pseudotypes bearing the influenza HA glycoproteins as surrogate viruses for use in neutralization assays. The first option has its inherent problems, namely the issue of possible reversion to the wild-type virus via genetic reassortment. The level of attenuation of such mutants may differ between strains/isolates and careful risk assessment is required.<sup>3</sup> With the pseudotype system, however, only the HA from influenza is required, with no possibility of recombination or virus

escape. These particles undergo abortive replication and do not give rise to replication-competent progeny. Retroviral and lentiviral pseudotypes have been used in lieu of replication-competent virus to study neutralizing antibody responses to viral infection.<sup>5–7</sup> These pseudotypes encode reporter genes and bear foreign viral envelopes of interest.<sup>8</sup> The transfer of marker genes to target cells depends on the function of the envelope protein; therefore, the titer of neutralizing antibodies against the envelope can be measured by a reduction in marker gene transfer.

The aim of this study was to establish a widely applicable safe assay, with a high level of sensitivity and specificity, and with adaptation to micro-quantities of serum samples, compared with hemagglutinin inhibition (HI) assays and microneutralization (MN) tests for H5N1 influenza viruses. We have previously constructed murine leukaemia virus (MLV) and human immunodeficiency virus (HIV) pseudotypes that express the SARS coronavirus (SARS-CoV) spike glycoprotein and used these to develop a safe *in vitro* neutralization assay that was shown to be both sensitive and specific for SARS-CoV-neutralizing antibodies.<sup>5</sup> We adopted a similar approach here for H5N1.

The ability of influenza HA to assemble on the envelopes of unrelated viruses was first reported for pseudotypes of vesicular stomatitis virus.<sup>9</sup> Retroviral vectors with H7 HA have been analyzed<sup>10</sup> and pseudotypes that bear H5N1 influenza virus HA glycoproteins have recently been described.<sup>11–14</sup> We describe the construction of retroviral and lentiviral pseudotypes bearing the HA from an influenza H5N1 virus isolated from a Vietnamese patient (A/Viet Nam/1194/2004(H5N1)).<sup>15</sup> This HA has an intact polybasic cleavage site RERRRKKR as found in the HAs of highly pathogenic avian influenza viruses (HPAI) which can be cleaved by ubiquitous host proteases resulting in lethal systemic infection.<sup>16</sup> Characteristic of an avian influenza virus, the HA has glutamine at position 226 and glycine at position 228 (human viruses have leucine at 226 and serine at 228), which form a narrow receptor binding pocket that preferentially binds to host cell receptors containing sialyloligosaccharides (SA) terminated by *N*-acetyl sialic acid linked to galactose with an  $\alpha$ 2,3 linkage (the major form in the avian trachea and intestine). Using this HA pseudotype as a surrogate virus, we have established a safe, sensitive and specific assay for neutralizing antibodies to H5N1.

## Materials and methods

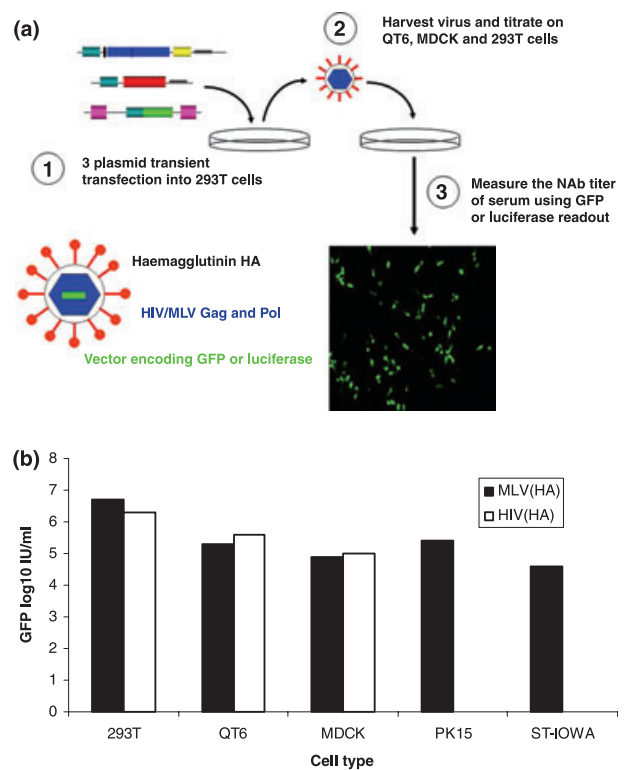
### Plasmids and cell lines

Plasmid pI.18/VN1194 HA was constructed at NIBSC. The full-length HA ORF from A/Vietnam/1194/04 was amplified by PCR and cloned into the expression vector pI.18. This backbone plasmid is a pUC-based plasmid incorporat-

ing promoter and Intron A elements from human cytomegalovirus. The MLV and HIV gag/pol constructs, and the green fluorescent protein (GFP) reporter constructs have been described previously.<sup>17,18</sup> The luciferase (Luc) reporter construct MLV-Luc has been described.<sup>19</sup> Vesicular stomatitis virus envelope protein (VSV-G) expression vector pMDG has been described previously.<sup>20</sup> All cell lines were cultured in Dulbecco's modified eagle medium (DMEM) with Glutamax and high glucose (Gibco, Paisley, Scotland, UK), supplemented with 10% fetal calf serum and penicillin/streptomycin, except for 293T cells (15% fetal calf serum).

### Viral vector production and infection of target cells

Confluent plates of 293T cells were split 1:4 the day before transfection. Each plate of 293T cells was transfected with 1  $\mu$ g gag/pol construct, 1.5  $\mu$ g of GFP or Luc reporter construct, and 1.5  $\mu$ g HA- or VSV-G-expressing construct by using the Fugene-6 transfection reagent (Figure 1a). At 24 h post-transfection, 1 U of exogenous neuraminidase (Sigma, St. Louis, MO, USA) was added to induce the release of HA-pseudotyped particles from the surface of the



**Figure 1.** H5N1 HA retroviral pseudotypes. (a) MLV(HA) and HIV(HA) pseudotype construction and neutralization assay for Influenza A/H5N1. (b) Titters of MLV(HA) and HIV(HA) pseudotypes on various cell lines. HIV(HA) was not tested on porcine cells.

producer cells.<sup>21</sup> Supernatant was harvested 48 and 72 h post-transfection, filtered through 0.45- $\mu$ m filters, and stored at  $-80^{\circ}\text{C}$ . MLV vector titers were measured on human 293T, quail QT6, canine MDCK, porcine PK15 and ST-IOWA cells and are presented as infectious units (IU) per milliliter. HIV vector titers were measured on 293T, QT6 and MDCK cells. Briefly, cells were infected with vector, and GFP or Luc titers were determined 72 h later by flow cytometry or by Luc assay. Titers were expressed as IU/ml for GFP and RLU/ml for Luc.

### Sera

Hyper-immune sheep sera raised against recombinant HA from different influenza subtypes, H3N2 (S1: A/England/427/88 and S2: A/Sichuan/2/87), H5N1 (S3: A/Viet Nam/1194/2004 and S4: NIBRG-14), H7N1 (S5: A/Chick/Italy/13474/99) and H9N2 (S6: A/HK/1073/99) were prepared at NIBSC. Serum from ferrets infected with the influenza H5N1 viruses A/Hong Kong/489/97 (FA), A/Hong Kong/213/03 (FB), A/Viet Nam/1194/04 (F1-F4) and NIBRG-14 (F5-F6) were prepared at NIBSC. Serum from ferrets infected with the influenza B viruses B/Egypt/144/05 (F7A) and B/Florida/7/05 (F7B) were prepared at NIBSC.

Avian sera raised against the H5 viruses, H5N1 (C1: A/Chicken/Scot/59(Wilson) and C2: A/TKY/Turkey/05), H5N2 (C3: A/Ost/Den/72420/96), H5N3 (C4: A/Tern/South Africa/61), H5N7 (C5: AV1356/03) and H5N9 (C6: A/TKY/ONT/7732/66) were prepared at the VLA. Anti-

sera against these viruses were produced in specific pathogen-free white leghorn chickens by intra-muscular inoculation with viruses (inactivated by beta-propiolactone if HPAI virus) grown in embryonated eggs and challenged 3 weeks later with the same virus. Two weeks post-challenge, the birds were bled and homologous HI titers were determined (Table 3). From five patients who had recovered from influenza H5N1 during the 2004 and 2005 outbreaks in Viet Nam, heparine-anticoagulated plasma was obtained 8–27 weeks after illness onset (median 11 weeks). Serum from a patient that had recovered from infection with influenza H5N1 during the 1997 outbreak in Hong Kong was kindly provided by JSM Peiris. Human sera with HI titers against A/Wyoming/3/2003(H3N2) and A/Panama/2007/1999(H3N2) ranging from <8 (negative) to 1024 (panel 1, Table 1), and control sera from healthy UK residents (panel 2, Table 1) were provided by the HPA.

### Viruses

Reactivity using MN and HI (horse) was tested against the A/Vietnam/1194/2004/NIBRG14 (H5N1) vaccine candidate reference strain. The NIBRG14 virus contains the HA (with the polybasic cleavage site removed) and NA of the wild-type antigen donor strain and the internal genes of A/Puerto Rico/8/1934(H1N1) and was obtained from the stocks at NIBSC (Potters Bar, UK). The virus stock for all experiments was propagated in the allantoic cavity of 9- to 11-day-old embryonated hens' eggs at

**Table 1.** Comparison of serological assays for the detection of antibody responses to H5 in human serum samples

Serum ID	MLV(HA)	HI (horse)	MN	HI (CL26)	MN(CL26)
VN1/CL1(H5N1)	1600–3200	1024	529–552	640	894–912
VN2/CL26(H5N1)	1600	64–128	51–54	10	112–195
VN3/CL36(H5N1)	1600–3200	1024	196–235	640	316–427
VN4/CL114(H5N1)	800–1600	128	63–69	20	126–138
VN5/CL115(H5N1)	400–800	256	46–48	40	34–68
HK97(H5N1)	400–800	128	80 (VN1203)		
Panel 1 (n = 50)					
Age 1–4 (n = 10)	<50	<8	<20		
Age 5–10 (n = 10)	<50	<8	<20		
Age 11–17 (n = 2)	<50	<8	<20		
Age 18–24 (n = 5)	<50	<8	<20		
Age 25–34 (n = 3)	<50	<8	<20		
Age 35–44 (n = 5)	<50	<8	<20		
Age 45–54 (n = 5)	<50	<8	<20		
Age 55–64 (n = 5)	<50	<8	<20		
Age 65–74 (n = 5)	<50	<8	<20		
Panel 2 (n = 50)	<50	ND	ND		

All HI and MN assays performed with NIBRG-14 virus except for those where the virus used is shown in brackets. HI assays were performed using horse erythrocytes except for those against A/Vietnam/CL26/2004 virus which were performed with guinea-pig erythrocytes. MLV(HA) titers were obtained using the GFP reporter.

35–37°C, harvested 3 days post-inoculation and stored at –80°C.

The antigens for the HAI (turkey) on the age-stratified panel (panel 1, Table 1) were A/Wyoming/3/2003(H3N2) and A/Panama/2007/1999(H3N2) using 8HAU.

Also used for HI (turkey): A/Hong Kong/489/1997(H5N1), A/Hong Kong/212/2003(H5N1) at NIBSC, and A/Vietnam/CL26/2004(H5N1) at the Oxford University Clinical Research Unit, Vietnam.

## MLV(HA) and HIV(HA) pseudotype neutralization assay

Serum samples (5 µl) were heat inactivated at 56°C for 30 min, twofold serially diluted in culture medium, and mixed with MLV(HA) or HIV(HA) virions (~1000 IU for GFP, 10 000 RLU for Luc) at a 1:1 v/v ratio. After incubation at 37°C for 1 h,  $1 \times 10^4$  293T cells were added to each well of a 96-well flat-bottomed plate. GFP-positive cells, or relative light units (RLU) for Luc were evaluated 48 h later by fluorescence microscopy, or by luminometry using the Promega Bright-Glo system (Promega, Madison, WI, USA) according to the manufacturer's instructions. IC<sub>90</sub>-neutralizing antibody titers were determined as the highest serum dilution resulting in a 90% reduction of infection (as measured by marker gene transfer) compared with a pseudotype virus only control. Neutralizing antibody titers are presented as titer ranges in triplicate experiments. For GFP, titers <50 are designated negative. For Luc, titers <100 are designated negative. For  $r^2$  correlation plots, the geometric mean titers (GMT) were used.

## HI assay

Hemagglutination inhibition assays with horse erythrocytes were conducted at the Health Protection Agency (Colindale, UK) using methods described previously.<sup>2,22</sup> Briefly, 25 µl of a twofold serial dilution series from 1:8 to 1:1024 of RDE-treated serum samples were incubated for 1 h at room temperature with an equal volume of the NIBRG-14 virus dilution, followed by the addition of 25 µl of the erythrocyte suspension. HI titers less than eight were considered undetectable and were assigned a value of four. For the Viet Nam patient samples tested by HI against A/Viet Nam/CL26 at the Oxford University Clinical Research Unit, guinea pig erythrocytes were used as horse erythrocytes were unavailable.

## Neutralization assay

Neutralising antibody titres were determined at the Health Protection Agency using a MN assay as described elsewhere.<sup>23,24</sup> Highly reactive sheep sera were pre-diluted at 1:50. The antigen used in the assays was NIBRG-14 virus. MN titers less than 20 were considered undetectable and were assigned a value of 10.

## Results

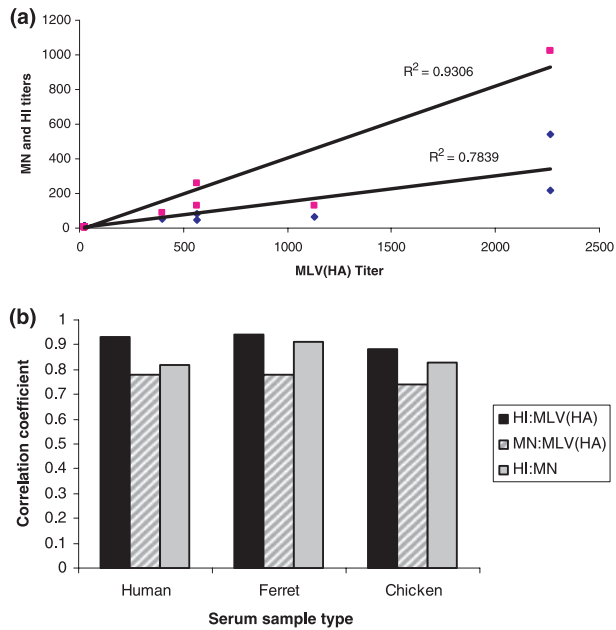
### Production of retroviral particles pseudotyped with influenza A/Vietnam/1194/04 HA

Retroviral (MLV) and lentiviral (HIV) vector particles pseudotyped with Influenza A/Viet Nam/1194/2004 HA were made by cotransfection of an HA-expressing plasmid, pL18/VN1194 HA, with plasmids encoding *gag-pol* and GFP or Luc vector genome in 293T cells (Figure 1a). Culture supernatants were used to infect human 293T cells as a representative human cell line, canine MDCK cells as the preferred cell line for culturing influenza viruses, QT6 quail cells and porcine PK15 and ST-IOWA cells as potential mixing vessels for human and avian influenza viruses.<sup>25,26</sup> VSV-G-pseudotyped particles were used as a positive control and infected all cell lines tested (data not shown). MLV(HA) pseudotypes infected all cell lines tested with the highest titer obtained on 293T cells ( $5 \times 10^6$  IU/ml), while HIV(HA) titers on human, canine and quail cells were broadly equivalent to MLV(HA) titers (Figure 1b). The titer of MLV(HA)-Luc on 293T cells was  $1.6 \times 10^7$  RLU/ml. All neutralization assays were performed using MLV(HA) pseudotypes on 293T cells as the most effective combination.

### Measurement of neutralizing antibodies in human sera

Sera from five patients who had recovered from infection during H5N1 outbreaks in Viet Nam in early 2004 (three patients, VN1-3) and 2005 (two patients, VN4-5) were tested for the presence of neutralizing antibodies using the MLV(HA) pseudotype assay. A broad range of IC<sub>90</sub>-neutralizing antibody titers was observed in these sera (400–3200 for GFP and 800–6400 for Luc) and these were compared with titers obtained by horse HI (against a reassortant containing A/Viet Nam/1194/2004 derived HA (NIBRG-14), guinea pig HI (against A/Viet Nam/CL26/2004), and MN with NIBRG-14 and A/Viet Nam/CL26/2004 MN (Table 1). Titers obtained using the reverse genetic NIBRG-14 virus versus the CL26 virus correlated strongly by HI ( $r^2 = 0.99$ ) and MN ( $r^2 = 0.99$ ).

To test specificity and further validate our assay, a panel of 50 age-stratified human sera with HI titers against Wyo\_2004 and Pan\_2004 H3N2 viruses ranging from <8 (negative) to 1024 was screened by NIBRG-14 MN, NIBRG-14 horse HI and by MLV(HA) assay. All sera tested negative for antibodies against H5 (Table 1 panel 1). No false positive results were observed in the sera from older individuals (age range 55–74) which can present problems in influenza serology.<sup>23</sup> Correlations of NIBRG-14 horse HI and MN assay titers with MLV(HA) titers for the H5 positive sera and the 50 age-stratified sera are shown in Figure 2a. An additional panel of 50 human sera from



**Figure 2.** Comparison of MLV(HA) with HI and MN for the measurement of antibodies to H5N1. (a) Anti-H5 antibody responses in human sera. Scatterplot showing the correlation of geometric mean antibody titers measured by horse HI (pink squares) and MN (blue diamonds) with  $IC_{50}$  geometric mean titers measured by MLV(HA) pseudotype assay with a GFP reporter. HI and MN assays performed against NIBRG-14 virus. Total number of sera assayed is 56 (six positive for antibodies against H5 and 50 H5 negative control sera). Linear trendlines were fitted to the data by using Microsoft Excel 2003. (b) Correlation coefficients of HI(horse):MLV(HA), MN:MLV(HA) and HI(horse):MN for human, ferret and chicken sera. Data plotted are  $r^2$  correlation coefficients determined using Microsoft Excel 2003.

healthy subjects also tested negative by the MLV(HA) assay for neutralizing antibodies against H5 (Table 1, panel 2).

### Comparison of HI and pseudotype neutralization assays to detect antibody to H5N1 in experimental ferret sera

Sera from ferrets infected with Hong Kong and Viet Nam H5N1 viruses were tested by MLV(HA) and NIBRG-14 HI and MN assays for the presence of antibodies against A/Viet Nam/1194/2004. Using the turkey erythrocyte HI, titers ranged from undetectable (<20) to 40, but horse erythrocytes were more sensitive with titers from 64 to 1024 (Table 2). When tested using homologous virus, turkey HI titers for ferret FA (infected with A/HK/489/97 H5N1) and ferret FB (infected with A/HK/213/03 H5N1) were 240 and 640 respectively. For the ferrets infected with the A/Viet Nam/1194/2004 virus, neutralizing antibody titers obtained with MLV(HA) correlated strongly with titers obtained by NIBRG-14 horse HI and MN (Figure 2b). The titer range was 50–3200 for GFP and 100–3200 for Luc.

**Table 2.** Comparison of serological assays for the detection of antibody responses in ferrets experimentally infected with Hong Kong and Vietnam H5N1 viruses

Serum ID	MLV(HA)	HI (turkey)	HI (horse)	MN
FA (HK489/97)	1600	<20 ( <b>240</b> )	ND	ND
FB (HK/213/03)	200–400	<20 ( <b>640</b> )	ND	ND
F1 (VN1194/04)	1600	40	512–1024	136–139
F2 (VN1194/04)	800–1600	40	512	90–134
F3 (VN1194/04)	800–1600	20	256–512	29–39
F4 (VN1194/04)	1600–3200	40	512–1024	106–107
F5 (NIBRG-14)	100	<20	64	<20
F6 (NIBRG-14)	50–100	<20	64	<20
F7A (B/Egypt/144/05)	<50	ND	<8	<20
F7B (B/Florida/7/05)	<50	ND	<8	<20
Negative ferret sera 1	<50	ND	<8	<20
Negative ferret sera 2	<50	ND	<8	<20

All HI and MN assays were performed against NIBRG-14 except those shown in bold where the homologous virus was used. HI titers were obtained using both turkey and horse erythrocytes. MLV(HA) titers were obtained using the GFP reporter. ND = not done.

Sera from ferrets infected with influenza B viruses (F7A and F7B), and from uninfected ferrets were negative for the presence of antibodies to A/Viet Nam/1194/2004 HA (Table 2).

### Detection of antibody to H5 viruses in white leghorn chickens

Sera from birds that had homologous turkey HI titers greater than 128 were tested for neutralizing antibody responses against A/Viet Nam/1194/2004 HA by MLV(HA), by horse erythrocyte NIBRG-14 HI, and by MN. All chicken sera except serum from the H5N9 immunized chicken (serum C6) were shown to have strong neutralizing antibody titers to A/Viet Nam/1194/2004 (100–3200 for GFP and 100–6400 for Luc) (Table 3). Neutralizing antibody titers obtained using MLV(HA) correlated strongly with titers obtained by horse HI and MN (Figure 2b).

### Comparison of neutralization assays with HI of sera from sheep immunized with recombinant influenza HA

Sera raised in sheep against recombinant HA vaccines derived from H3N2, H5N1, H7N1 and H9N2 viruses were tested for the presence of neutralizing antibodies to H5N1 by pseudotype assay using MLV(HA) particles. Sheep serum S4, prepared against NIBRG-14, and S3 (A/Viet

**Table 3.** Comparison of serological assays for the detection of antibody responses in white leghorn chickens immunized with avian H5 viruses

Serum ID	MLV(HA)	Homologous	HI	MN
		HI (turkey)	(horse)	
C1: H5N1 (1959)	800–1600	<b>128</b>	128–256	191–194
C2: H5N1 (2005)	3200	<b>256</b>	512	253–270
C3: H5N2 (1996)	1600	<b>256</b>	64	61–78
C4: H5N3 (1961)	3200	<b>128</b>	512	497
C5: H5N7 (2003)	800	<b>512</b>	32	55–61
C6: H5N9 (1966)	100	<b>256</b>	32	36
CN: Negative chicken serum	<50	<b>&lt;8</b>	<8	<20

All HI and MN assays were performed against NIBRG-14 except those shown in bold where the homologous virus was used. MLV(HA) titers were obtained using the GFP reporter. Strain designations are given in the Materials and methods section.

**Table 4.** Comparison of serological assays for the detection of antibody responses in sheep immunized with recombinant HA vaccines

Serum ID	MLV(HA)	HI (horse)	MN
S1 (H3N2)	<50	<20	<20
S2 (H3N2)	<50	<20	<20
S3 (H5N1)	51200	8192	13589–17425
S4 (H5N1)	25600–51200	1280 (turkey)	7000
S5 (H7N1)	<50	<8	<20
S5 (H7N1) RED3	ND	1024–2048	467–536
S6 (H9N2)	<50	<8	<20

All HI and MN assays were performed against NIBRG-14. Serum from sheep S5, immunized with an H7 virus was also assayed against the reverse genetics RED3 H7 virus. MLV(HA) titers were obtained using the GFP reporter. Strain designations are given in the Materials and methods section.

Nam/1194/2004) were tested by horse HI and MN assay for antibodies to A/Viet Nam/1194/2004 HA. Using the MLV(HA) assay, no cross neutralization of H5 was seen with sera raised against H3, H7 or H9 (Table 4). Sheep S5 (H7N1) was also tested by HI and MN against the RED3 reverse genetics H7 virus (Table 4). All sheep sera had positive single-radial haemolysis (SRH) titers against their respective antigens (data not shown).

## Discussion

We have developed a retroviral pseudotype-based assay that facilitates the accurate determination of neutralizing anti-

body responses to influenza H5N1 without the need to use replication-competent virus. Our assay detected neutralizing antibodies to H5 HA in sera from patients who had recovered from infection with influenza H5N1 during the 1997 outbreak in Hong Kong and the more recent outbreaks in Viet Nam, in sera from ferrets experimentally infected with influenza H5N1 viruses, in white leghorn chickens immunized with various avian H5 viruses and in sheep immunized with recombinant H5 HA (Tables 1–4). All sera found to be positive by HI and/or MN for H5 antibodies were also positive by MLV(HA) making this a highly sensitive assay.

The pseudotype assay described here is specific for the detection of neutralizing antibodies against H5 HA. High titer sera raised in sheep against H3, H7 and H9 viruses failed to neutralize the MLV(HA) pseudotype (Table 4). Sera from ferrets infected with influenza B viruses, and a panel of 50 age-stratified human sera negative for H5 antibodies by NIBRG-14 MN and horse HI were also negative by MLV(HA) assay (Tables 1 and 2).

The sensitivity of the HI assay is largely determined by the type of erythrocytes used and the measurement of HI titers against avian viruses has been significantly improved by use of horse erythrocytes. These contain a high proportion of SA $\alpha$ 2,3Gal linkages. Turkey erythrocytes, however, contain a low proportion of SA $\alpha$ 2,3Gal linkages which is thought to be responsible for the relative insensitivity of HI for the detection of avian H5 viruses.<sup>22</sup> HI titers obtained using horse erythrocytes were found to be at least 12-fold greater than those measured using turkey erythrocytes for the detection of antibodies to A/Viet Nam/1194/2004 in ferret sera (Table 2). In sera from patients who had recovered from infection with H5N1 virus, HI titers obtained with horse erythrocytes were up to sixfold greater than those obtained using guinea pig erythrocytes which also contain a low proportion of SA $\alpha$ 2,3Gal linkages (Table 1).<sup>27</sup> HI using turkey erythrocytes failed to detect antibody in ferrets infected with the NIBRG-14 reverse genetics virus (ferrets F5 and F6) but using a modified horse erythrocyte protocol these were shown to be weakly positive. Neutralizing antibodies were detected in F5 and F6 by MLV(HA) assay, however, albeit at low levels (Table 2).

The pseudotype assay is significantly more sensitive than the turkey erythrocyte HI and at least as sensitive as the horse erythrocyte HI and MN for the detection of antibodies to H5 HA (Tables 1–4 and Figure 2b). Sera from ferrets infected with live influenza A/Viet Nam/1194/2004 that had weak turkey HI titers against this virus have high levels of antibodies as measured with our pseudotype assay. Neutralizing antibody titers determined by the pseudotype assay were shown to be strongly correlated with titers obtained by horse HI and MN for human, ferret and avian sera (Figure 2a and b). When comparing the correlations

between MLV(HA) versus HI ( $r^2 = 0.93$ ), MLV(HA) versus MN ( $r^2 = 0.78$ ) and HI versus MN, however, it should be noted that MN will also measure neutralizing responses against other envelope glycoproteins, namely NA, in contrast to the pseudotype assay and the HI assay which only measure responses against the HA component. This factor may explain why we obtained higher  $r^2$  values for correlations between MLV(HA) and HI assays than between MLV(HA) and MN assays for the human, ferret and chicken data (Figure 2b). We have constructed retroviral pseudotypes bearing both HA and NA glycoproteins and these particles may be useful in the development of assays for the detection of neutralizing antibodies to either glycoprotein (N. J. Temperton, unpublished observations).

Serum from ferret FA, infected with the clade 3 influenza A/HK/489/97 virus tested negative by turkey HI when tested against A/Viet Nam/1194/2004 virus, but had a titer of 240 when tested against the homologous virus (HK/489/97). However, with the MLV(HA) assay which is based on Viet Nam/1194/2004 HA, a neutralizing antibody titer of 1600 was obtained (Table 2). Similarly, serum from a patient who had recovered from infection, by a 1997 HK H5N1 virus also had a strong neutralizing antibody titer as measured by MLV(HA) (800 versus a MN titer of 80) (Table 1). Also, sera from chickens that had been immunized with a variety of avian H5 viruses (isolated between 1959 and 2005) showed the presence of high levels of neutralizing antibodies to the recent A/Viet Nam/1194/2004 virus in the MLV(HA) assay (Table 3). Antisera to an avian H5N1 strain isolated in 1959 could neutralize the VN1194 pseudotype at high titer. Only chickens immunized with A/TKY/ONT/7732/66 (H5N9) did not produce high neutralizing antibody titers against the VN1194 HA. These data suggest that the MLV(HA) assay may be detecting cross-neutralizing activity. The significance of such cross-neutralizing activity remains to be evaluated by the use of further panels of human and animal sera, and pseudotyped H5 viruses belonging to different antigenic clades.

To achieve maximum sensitivity in serological assays, the selection of virus isolated from the same influenza outbreak, or the use of an antigenically equivalent strain is required for optimal antigenic match. A competent molecular virology laboratory could produce HA pseudotype virus within 2–3 weeks of the availability of viral RNA. Further studies are underway, making use of a panel of H5 retroviral pseudotypes with HA components derived from H5N1 viruses involved in the recent human and avian outbreaks. Of particular importance would be cross-clade neutralization studies between viruses from the four different clades of H5N1 (1, 1', 2 and 3)<sup>28</sup> as recent studies have shown that there is often poor antigenic cross-reactivity between viruses belonging to the Indonesian (clade 2) and Vietnamese (clade 1) sublineages, an

observation that has implications for the design of vaccines against H5N1.<sup>29,30</sup>

There is a need to study the seroprevalence of influenza H5N1 in countries where outbreaks of H5N1 have occurred in humans and where H5N1 is endemic in the avian population. This is, primarily, to determine the extent of avian-to-human and potentially human-to-human transmission. Influenza A/H5-specific antibody measured by neutralization is currently regarded as the 'gold standard' serological assay for H5N1 infection by the WHO.<sup>1</sup> The neutralization assay is used to detect functional strain-specific antibodies in animal and human sera but as the neutralization assay requires the use of live virus, its use is restricted to those laboratories that have Biosafety Level 3 containment facilities, and for non-attenuated H5N1, this may need to be upgraded to BSL4. Our MLV(HA) assay is at least as sensitive as the live virus-neutralizing antibody assay, and does not require high containment.

The safety, specificity and sensitivity of the pseudotype assay suggest that it may have a role as an alternative or supplement to conventional assays. Our data indicate that for avian H5 viruses it is considerably more sensitive than turkey HI. It is possible that previous serological studies relying solely on turkey HI may have failed to detect low levels of antibody that could readily be detected by a pseudotype based assay as described here. By switching our assay reporter from GFP to Luc our neutralization assay has been adapted for high-throughput usage. This assay is well placed to assess protective neutralizing immunity in sera from experimentally immunized animals (as for the sheep and chickens studied here), and from human clinical trials of pre-pandemic H5N1 vaccines. Further development of  $\beta$ -gal-based pseudotype assays will allow wider application as an ELISA-type assay in laboratories without specialized equipment. In addition, with minor modifications, these assays could be used to screen HA and NA inhibitors.

## Author contribution

N.J. Temperton and R.A. Weiss are the principal investigators on the project. N.J. Temperton prepared the HA pseudotype particles and carried out the pseudotype neutralization assays. K. Hoschler carried out HI and MN assays on sera. D. Major prepared the sheep and ferret sera and carried out HI assays. C. Nicolson constructed the hemagglutinin expression plasmid. R. Manvell prepared the chicken sera and carried out HI assays. M.D. de Jong provided human sera and discussed assay design. V.M. Hien and D.Q. Ha carried out HI and neutralization assays on Vietnam sera. M. Zambon helped to design the study, advised on HI and MN assays, and interpretation of the data. N.J. Temperton, R.A. Weiss and Y. Takeuchi

conceived the study and wrote the article with input from all of the authors.

## Conflict of interest

Health Protection Agency receives funding for work carried out in M. Z. laboratory from a range of vaccine companies.

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