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## Optimization and evaluation of an influenza A (H5) pseudotyped lentiviral particle-based serological assay

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### ABSTRACT

**Background:** Novel serological methods provide alternative options for sero-diagnosis, sero-epidemiology and for determining evidence of naturally acquired or vaccine induced immunity. Micro-neutralization tests are currently the gold standard for serological studies of highly pathogenic avian influenza in mammalian species but require handling live virus in a biosafety level (BSL) 3 environment. We previously reported the use of H5 pseudotyped lentiviral particles (H5pp) as an alternative to micro-neutralization tests in a BSL-2 setting (Nefkens et al., 2007).

**Objective:** To optimize and evaluate this newly developed H5pp assay on relevant clinical specimens.

**Study design:** We optimise and evaluate the performance of the H5pp assay using well-characterized sera from humans with confirmed H5N1 disease or controls.

**Results:** The H5pp assay is a reliable serological method for the detection and quantification of neutralizing antibody to H5-viruses.

**Conclusion:** H5pp provide a reliable and safe alternative for sero-diagnosis and sero-epidemiology of H5N1 infections in a BSL-2 setting.

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### 1. Background

Assessment of the sero-prevalence to potentially pandemic highly pathogenic avian influenza (HPAI) H5N1 viruses is important for evaluation of its potential to transmit to humans.<sup>1</sup> Such tests need to be reliable, safe and amenable to high throughput implementation. At present, the haemagglutination inhibition (HI) and the micro-neutralization (MN) tests are available for this purpose.<sup>2</sup> The HI test is useful in serological studies of mammalian viruses in mammalian species or of avian viruses infecting avian species. However, the conventional HI test is not sufficiently sensitive or specific for detecting antibody responses of humans infected with

avian influenza viruses although an adaptation using horse erythrocytes does make the test more useful.<sup>3,4</sup> MN, which remains the “gold standard” serological test for HPAI H5N1 at present, requires access to the live virus and to biosafety level (BSL)-3 facilities. While reverse genetics can be used to generate H5-viruses that are safe enough to be used in BSL-2 conditions, such viruses require intensive safety evaluation for non-pathogenicity in chickens and ferrets before release for use in a BSL-2 setting.<sup>5</sup> This limits its widespread applicability of MN tests for those without access to BSL-3 facilities. Recently we introduced a BSL-2 H5 haemagglutinin pseudotyped lentiviral particle (H5pp) based assay.<sup>6</sup> These pseudoparticles can be produced using synthetic genes based on virus haemagglutinin (HA) sequence data, even when there is no access to live virus or viral cDNA. Pseudotyping with HA only allows us to detect the functional neutralizing antibody response to the H5 without contribution from cross-reacting neuraminidase antisera. While others have also evaluated H5pp serological assays, they have not described an analysis of the parameters that affect assay performance.<sup>7,8</sup> Moreover, their test validation has been based on small numbers of sera.

### 2. Objectives

Here we optimise H5pp assay, assess its reproducibility and define its performance characteristics using sera from patients with virologically confirmed H5N1 disease.

**Abbreviations:** HPAI, highly pathogenic avian influenza; MN, micro-neutralization; BSL, biosafety level; H5, haemagglutinin of H5N1 HPAI virus; H5pp, H5 pseudotyped viral particles; HI, haemagglutination inhibition assay; RT-PCR, reverse transcription polymerase chain reaction; HA, haemagglutinin; MDCK, Madin–Darby canine kidney; DMEM, Dulbecco modified Eagle medium; RLU, relative luminescence unit; ROC curve, receiver operating characteristic curve; CI, confidence interval; AUC, area under curve; SE, standard error; “c”, closest-to-(0,1) criterion; “J”, Youden index; PPV, positive predictive value; NPV, negative predictive value; NA, neuraminidase.

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### 3. Study design

#### 3.1. Serum samples

Controls sera were from healthy Hong Kong residents (106 children, 102 adults and 118 persons >60 years of age) with no known exposure to H5N1 influenza. The “positive” test panel was 41 sera from 10 patients with RT-PCR confirmed H5N1 disease collected during the outbreak in 2003/2004 in South Vietnam from days 7 to 784 after disease onset at the Hospital for Tropical Disease of Ho Chi Minh City, Vietnam. The individual clinical details of these patients have been previously described in references.<sup>9–11</sup> Furthermore, [Supplementary Material Table S1](#) gives correspondence of patient ID number within the cited references to the sera tested in this evaluation. A pool of avian influenza H5 vaccinated chicken sera (MN titre 640) was used to optimize viral input and assess the reproducibility of the assay. A set of 15 subtyping polyclonal hyperimmunized chicken antisera provided by Office International des Epizooties (OIE) Reference Laboratory (Veterinary Laboratory Agency, Surrey, UK) was used for testing subtype specificity.

#### 3.2. H5pp assay

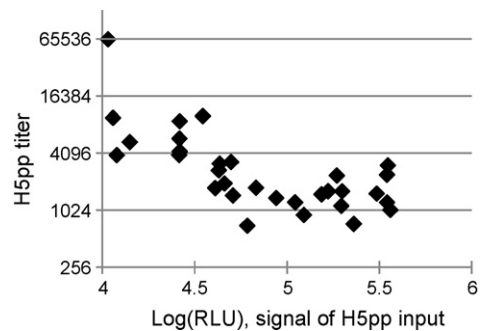
H5pp were produced as described previously using codon optimized H5 gene from A/Cambodia/408008/05 H5N1.<sup>6</sup> Two-fold serial dilutions of heat inactivated (56 °C, 30 min) serum were mixed with an equal volume of H5pp in culture medium (Dulbecco modified Eagle medium (DMEM) supplemented with 2.5% foetal bovine serum (Invitrogen) 1% penicillin–streptomycin (Invitrogen)) corresponding to the dose that generates 10<sup>5</sup> RLU (or otherwise mentioned) in standardized conditions, incubated for 2 h at 37 °C and then added to a preformed monolayer of Madin–Darby canine kidney (MDCK) cells. After 48 h incubation, luminescence was read after addition of Steady-Glo (Promega) luciferase substrate with either Micro-beta (PerkinElmer) or Glomax (Promega) plate readers. The neutralization of infection was detected by measuring the reduction of end-point signal versus controls done in absence of serum or in absence of virus, regarded as equivalent to 0% or 100% neutralization, respectively.

#### 3.3. Micro-neutralization test (MN)

The micro-neutralization test was done as described previously.<sup>12</sup> One hundred tissue culture infectious dose 50 (100 TCID<sub>50</sub>) of A/Vietnam/1194/04 (H5N1) virus was mixed with an equal volume of 2-fold serial dilutions of serum in quadruplicate, incubated for 1 h at 37 °C and the virus antibody mixture was added to a preformed monolayer of MDCK cells. The plates were incubated for 3 days and the cytopathic effect read under an inverted microscope. Virus back titrations were included to confirm whether the challenge dose was as expected.

#### 3.4. Determination of neutralizing titer

In MN assay, titer was defined by the reciprocal highest serum dilution protecting ≥50% of the wells. In H5pp assay, the reciprocal dilution giving 50% (unless otherwise specified) neutralization was computed from the neutralization curve fitted with the Hill equation.<sup>13</sup> This takes into consideration information from all dilutions and does not overly rely on few quadruplicate serum dilutions to determine the titer for a 50% effect as is done using the Reed and Muench method.<sup>14</sup>



**Fig. 1.** Optimization of virus dose in H5 pseudotype assay. Neutralization titers plotted against the input of H5pp expressed as log value of signal in relative luminescence unit (RLU) measured in absence of sera. Values represent the mean of duplicates.

#### 3.5. Determination of diagnostic parameters

A detailed description of the computation and interpretation these parameters (sensitivity, specificity, positive or negative predictive values (PPV or NPV) and accuracy) can be found in references.<sup>15,16</sup>

#### 3.6. Statistical analysis

Bland–Altman analysis was used to evaluate the systematic bias and limits of agreement of H5pp versus MN.<sup>17</sup> The log-transformation of titers was required for a normal distribution, an assumption of the statistical test used, verified using normal (quantile–quantile) plot (data not show). All calculations were done in Excel<sup>TM</sup>.

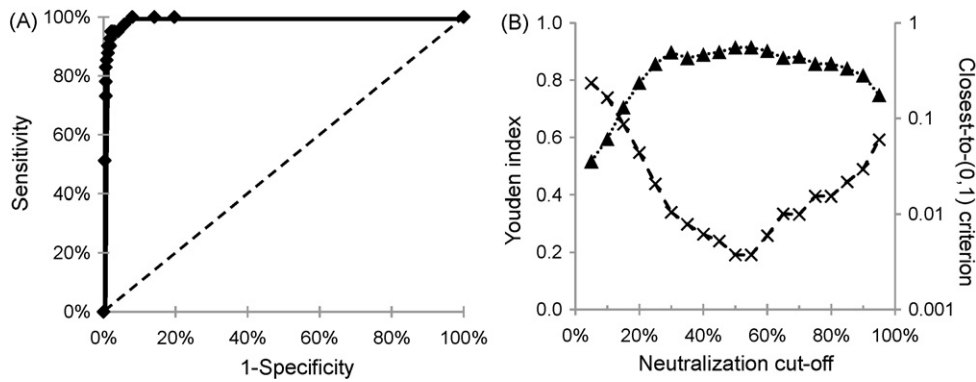
## 4. Results

#### 4.1. Determination of optimal amount of H5pp

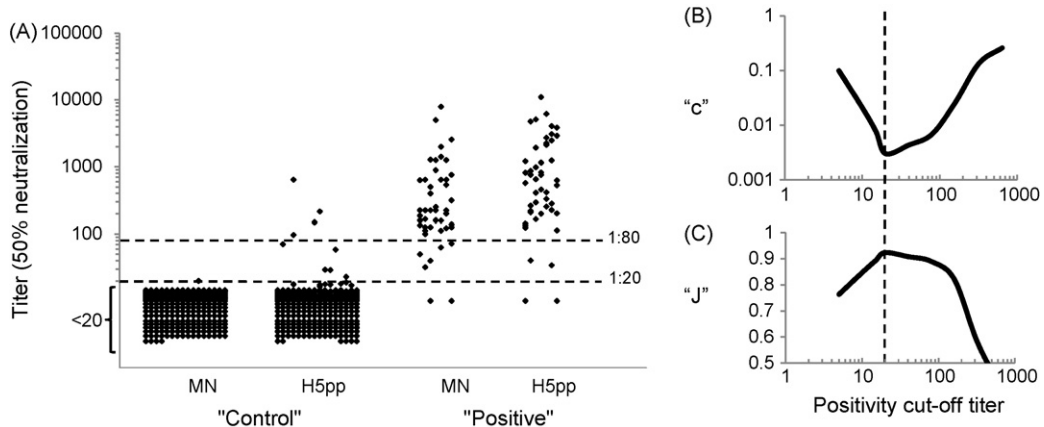
In 33 independent experiments, we measured the titer of a single pooled chicken serum for different batches of H5pp (mean values of duplicate were used for the calculations). We found that once the H5pp input exceeds around 10<sup>5</sup> RLU, the titer is relatively independent of the H5pp concentration used ([Fig. 1](#)).

#### 4.2. Determination of optimal cut-offs

The receiver operating characteristic (ROC) curve was used for determination of optimal cut-off point.<sup>18</sup> It was generated by plotting the sensitivity as a function of 1–specificity ([Fig. 2A](#)).<sup>19</sup> The optimal diagnostic effectiveness was determined using the sensitivity/specificity that gives either the minimal value for  $(1 - \text{sensitivity})^2 + (1 - \text{specificity})^2$ , called the “closest-to-(0,1)” criterion, denoted “c”; or the maximal value for  $(\text{sensitivity} + \text{specificity} - 1)$ , the Youden Index,<sup>20</sup> denoted “J”. We computed ROC curves, “c” and “J” criteria using the panel of human sera by simultaneously varying the positivity threshold (range [10–640]) and the percentage of neutralization (range [5–95%]) used to define the titer. Both criteria identify the same optimal cut-off values corresponding to 50% neutralization ([Fig. 2B](#)) and to positivity threshold of a serum dilution of ≥20 ([Fig. 3](#)). Furthermore, 50% signal reduction is the value for which fitting using the Hill equation is the most robust (inflection point). These ROC curves also indicate that the assay has excellent accuracy as estimated by the area under curve (AUC > 0.99) with a corresponding standard errors (SE) of <0.5%.<sup>21</sup>



**Fig. 2.** Determination of optimal neutralization cut-off for titer calculation in H5pp assay using (A) ROC curve, (B) Youden index “J” (triangle, vertical left axis) and closest-to-(0,1) “c” criterion (cross, vertical right axis).



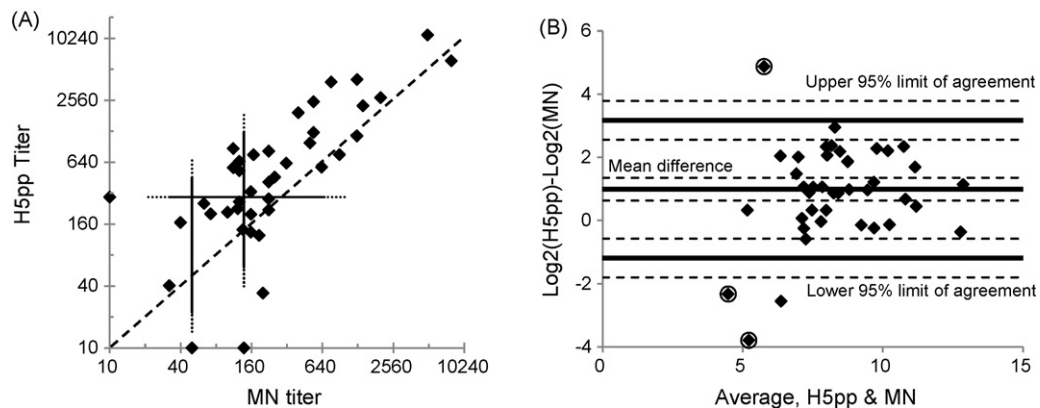
**Fig. 3.** (A) Scatter plot of titers measured by H5pp or MN assay for a panel of 326 “Control” negative and 41 “positive” human sera. Negative (<20) sera were given the value of 10 (first dilution tested 1:20). Dotted lines mark positivity titers of  $\geq 20$  and  $\geq 80$ . (B) Evolution of “c” (B) and “J” (C) parameters for different positivity cut-off. Titer: reciprocal dilution giving 50% neutralization.

4.3. Assay reproducibility

The pooled chicken serum and a single batch of H5pp were used to assess the reproducibility of the assay over 3 batches (tested on 3 different weeks) of 3 plates, each containing 6 replicates. Coefficients of variation (CV%) were  $CV_{intra-plate} < 5\%$ ,  $CV_{inter-plate} < 3\%$  and  $CV_{inter-batch} < 7\%$ .<sup>22</sup> All variances calculated were homogenous according to Cochran test at the level of significance  $\alpha = 0.05$ .

4.4. Agreement of titers measured with H5pp or MN tests in human sera

Titers measured with H5pp are higher than with the MN test (Fig. 4A) suggesting a higher sensitivity in detecting H5 antibody. Using the statistical method developed by Bland and Altman,<sup>17</sup> we found that H5pp titers exceed the MN titers on average by 2-fold (95% CI: 1.5–2.5) ( $p = 0.002$ ).



**Fig. 4.** (A) Plot of titers measured with H5pp versus MN assays. (B) Bland–Altman scatter plot showing the systematic bias and the limits of agreement with 95% CI. Circled data, excluded from analysis (titer <20 in either method), are plotted in (A) against the other method probable range of values in agreement (continuous lines, 95% CI extension in dotted line).

**Table 1**  
Diagnostic parameters of H5pp and MN assays using a comprehensive panel of 244 “control” and 41 “positive” human sera for different positivity titers (reciprocal dilution giving 50% neutralization). AUC: area under curve of the corresponding ROC curve with standard error (SE); in an ideal assay: “J” = 1 and “c” = 0. Positive and negative predictive values are noted PPV and NPV, respectively.

Positivity cut-off	H5pp assay			MN assay			Screening with H5pp and confirming those with H5pp titers $\geq 20$ by MN test		
	$\geq 80$	$\geq 40$	$\geq 20$	$\geq 80$	$\geq 40$	$\geq 20$	$\geq 80^a$	$\geq 40^a$	$\geq 20^a$
Sensitivity	90%	93%	95%	85%	95%	98%	83%	90%	93%
Specificity	99%	98%	97%	100%	100%	100%	100%	100%	100%
PPV	90%	86%	81%	100%	100%	98%	100%	100%	97%
NPV	99%	99%	99%	98%	99%	100%	98%	99%	99%
Accuracy	98%	98%	97%	98%	99%	99%	98%	99%	99%
Closest-to-(0,1), c	0.010	0.006	0.003						
Youden index, J	0.89	0.91	0.92						
AUC (SE)	0.994 (0.36%)	0.995 (0.34%)	0.995 (0.33%)						

<sup>a</sup> Positivity cut-off in confirmatory MN assay.

**Table 2**  
Subtype specificity of H5pp using an OIE panel of reference hyper-immune chicken antisera.

Subtype	Viral strain	H5pp titer
H1N1	A/Turkey/England/250/79	<20
H2N2	A/Singapore/1/57	<20
H3N2	A/Turkey/England/69	<20
H4N6	A/Duck/Czech/56	<20
H5N1	A/Chicken/Scotland/59	1171
H5N3	A/Tern/South Africa/61	1469
H6N8	A/Ostrich/R.S.A./946/98	<20
H7N7	A/England/268/96	<20
H8N4	A/Turkey/Ontario/68	<20
H9N2	A/Turkey/Wisconsin/1/66	<20
H10N9	A/Egyptian goose/S. Africa/238/98	<20
H11N6	A/Duck/England/56	<20
H12N5	A/Duck/Alberta/60/76	<20
H14N6	A/Mallard/Gurjev/244/82	<20
H16N3	A/Gull/Denmark/68110/02	<20

#### 4.5. Performance characteristics of the H5pp and MN assays on human serum panels

Using the optimized H5pp assay parameters, we compared the H5pp assay with MN at different cut-off titres for positivity (Table 1). At the positivity threshold  $\geq 80$  defined by WHO,<sup>1</sup> the performance of the H5pp test compares favourably with MN. At a lower cut-off titer ( $\geq 20$ ), the sensitivity of the H5pp test improves (from 90% to 95%) while the specificity drops (from 99% to 97%).

In the 326 control sera, 317 sera were negative in both assays. Nine had H5pp titers  $\geq 20$  (one also had an MN titer of 20) and seven of these were from those over 60 years of age. When a titer of  $\geq 80$  was used as cut-off, 4 sera remained H5pp positive (Fig. 3A). In the positive panel of 41 sera, the H5pp and MN tests yield 4 and 6 false negative sera, respectively at a cut-off of  $\geq 80$ ; while 2 and 1 sera, respectively, remain false negative at a titer of  $\geq 20$ . The latter MN data is comparable to a previous study on naturally infected H5N1 patient.<sup>23</sup>

#### 4.6. Subtype specificity

Using the panel of hyper-immune sera to 14 influenza virus subtypes (H13 and H15 were not available), we confirmed lack of cross-reaction to non-H5 subtypes (Table 2).

### 5. Discussion

We optimized and evaluated a H5 pseudoparticle-based assay as an alternative to the existing MN method for detection of neutralizing antibodies against the potentially pandemic H5 avian influenza.

We established that increasing quantities of H5pp led to progressive reduction of the antibody titer until the latter reaches or exceeds around  $10^5$  RLU equivalents per well, beyond which point the H5pp antibody titer becomes independent of the input H5pp virus. Keeping the H5pp value above  $10^5$  RLU minimises batch-to-batch variations in the antibody titers. It should be noted that the numerical value of luminescence is dependent on the plate reader used (“relative” luminescence unit) as each machine has different signal amplification gain. Therefore, this threshold limit may have to be re-optimized if a different plate reader is used.

We assessed the H5pp assay using a test panel of 326 control sera from the general population in Hong Kong considered as true negative and 41 sera from patients with virologically confirmed H5N1 disease as true positive. An optimal positivity titer threshold of 20 was found to maximise both sensitivity and specificity. However the internationally accepted positivity cut-off titer in the MN assay for H5N1 is defined as 80 to minimise the frequency of false-positive results. We have therefore analysed our data at both cut-off antibody levels. Most “false-positive” sera identified in the H5pp test were those over 60 years of age. It is now recognised that a proportion of elderly persons have neutralizing antibodies to H5N1 virus.<sup>24</sup> In that regard, the current panel of sera for test evaluation is particularly stringent because 36% of the control panel are >60 years of age. At a positive cut-off titer of  $\geq 80$ , the H5pp test compares favourably with the MN test. The specificity and PPV (Table 1) of the H5pp test will be higher in those younger than 60 years of age (i.e. 99% and 97%, respectively, data not shown).

Alternatively, an effective application of the H5pp test would be to use it as a high throughput screening assay at BSL-2 containment with the sera with titers  $\geq 20$  being confirmed in a reference BSL-3 laboratory setting with the MN titer  $\geq 80$ . Such a strategy leads to diagnostic performances that are not markedly inferior to that obtained by screening all the sera in BSL-3 containment using the MN test (Table 1). This approach has major advantages in laboratories with no access to BSL-3, including many developing countries that are in the front-line in confronting HPAI H5N1 infection.

H5pp can be made to carry the virus HA alone or to have both HA and NA. While the latter is technically easier to produce (unpublished data) we have chosen to produce pseudotyped particles that only contain the HA to have the opportunity to eliminate some potential cross-subtype reactivity that may arise between for example the NA of H5N1 and of the seasonal influenza virus H1N1. The strong positive correlation we find between titers obtained with the H5pp (only HA present) and MN (complete virus with HA and NA) tests indicates that the HA plays a dominant role in the neutralizing antibody response.

In conclusion, we have developed and evaluated a high throughput screening assay that can be carried out in BSL-2 containment. By testing a comprehensive set of sera, we have demonstrated

that the H5pp assay faithfully replicates the results with the MN test. Therefore, H5pp assay can be a safer alternative to MN for monitoring the neutralizing antibody levels in applications such as sero-epidemiological investigation of avian influenza or H5N1 related vaccine immunity assessment.

### Conflict of interest

The authors declare no conflict of interest.

### Acknowledgements

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2009.10.009.

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