Multiplex Evaluation of Influenza Neutralizing Antibodies with Potential Applicability to In-Field Serological Studies

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ABSTRACT

The increased number of outbreaks of H5 and H7 LPAI and HPAI viruses in poultry has major public and animal health implications. The continuous rapid evolution of these subtypes and the emergence of new variants influence the ability to undertake effective surveillance. Retroviral pseudotypes bearing influenza haemagglutinin (HA) and neuraminidase (NA) envelope glycoproteins represent a flexible platform for sensitive, readily standardized influenza serological assays. We describe a multiplex assay for the study of neutralizing antibodies that are directed against both influenza H5 and H7 HA. This assay permits the measurement of neutralizing antibody responses against two antigenically distinct HAs in the same serum/plasma sample thus increasing the amount and quality of serological data that can be acquired from valuable sera. Sera obtained from chickens vaccinated with a monovalent H5N2 vaccine, chickens vaccinated with a bivalent H7N1/H5N9 vaccine, or turkeys naturally infected with an H7N3 virus were evaluated in this assay and the results correlated strongly with data obtained by HI assay. We propose that this robust assay may have practical utility for in-field sero-surveillance and vaccine studies in resource-limited regions worldwide.

MATERIALS AND METHODS

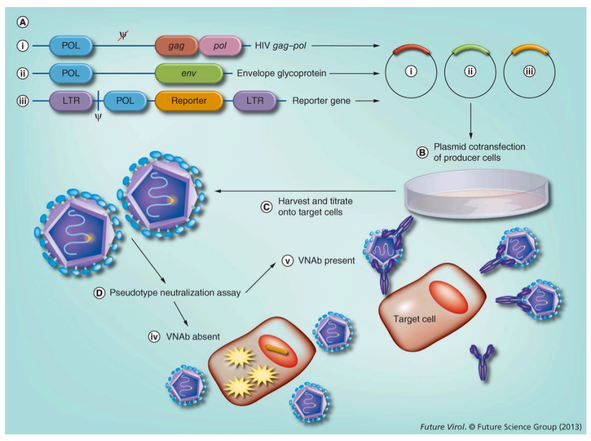
Serum Samples: Sera obtained from chickens vaccinated with a monovalent H5N2 vaccine, chickens vaccinated with a bivalent H7N1/H5N9 vaccine, or turkeys naturally infected with an H7N3 virus were provided by the FAO, OIE, and National Reference Laboratory for Newcastle Disease and Avian influenza.

Inhibition of Haemagglutination (HI) assay: All avian sera employed in the study were tested by HI at FAO, OIE, and National Reference Laboratory for Newcastle Disease and Avian influenza, with different reference antigens routinely used for avian influenza surveillance in Italy, namely, H5N2 (A/turkey/Italy/1980), H7N3 (A/turkey/Italy/9289/V02), H7N1 (A/Africa starling/England/983/1979), H5N9 (A/chicken/Italy/22A/1998), and H7N1 (A/chicken/Italy/1067/1999).

Firefly Luciferase and Renilla Luciferase H5/H7 Pseudotypes: Lentiviral vector (carrying the luciferase reporter gene, pCSFLW) pseudotyped with HA envelope glycoproteins derived from the HPAI H5N1 viruses (clade 1 A/Vietnam/1194/2004 and clade 2.1.3.2 A/Indonesia/5/2005) and the HPAI H7N1 virus (A/chicken/Italy/13474/1999) were produced as described previously, except that the neuraminidase activity was provided by a cognate NA plasmid in lieu of exogenous bacterial NA addition. In parallel, using the same transfection protocol and the same batch of HEK 293T/17 producer cells, HPAI H7 pseudotypes (A/chicken/Italy/13474/1999) carrying the Renilla luciferase gene (pCRLFW), were generated.

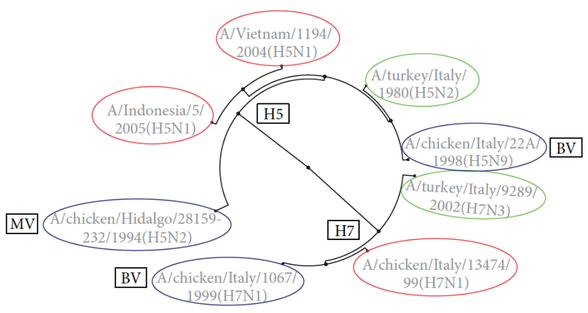
Firefly Luciferase (Monoplex) pp-NT Assay: Serum samples (5 μL) were twofold serially diluted in culture medium (DMEM GlutaMAX supplemented with 15% FBS and 1% Penicillin/Streptomycin) and mixed with pseudotype virus (500k RLU luciferase input) at a 1:1 v/v ratio. After incubation at 37◦C/1h, 1e4 HEK 293T/17 cells were added to each well of a white 96-well flat-bottomed tissue culture plate. For each serum sample, RLUs were normalized and compared with the signal detected in the absence of pseudotype virus (equivalent to 100% neutralization) and the signal of the negative control (equivalent to 0% neutralization). The 50% inhibitory doses (IC50) were determined as the reciprocal of serum dilution resulting in a 50% reduction of a single round of infection (reporter gene mediated signal).

Firefly and Renilla (Multiplex) pp-NT Assay: fixed amounts (corresponding to 500k RLUs estimated by prior pseudotype titration) of both H5 and H7 influenza pseudotypes (one containing the firefly reporter gene and the other the Renilla reporter gene) were added to each well in which twofold serially diluted serum samples (5 μL) were dispensed together with cell culture medium. After 48 hours, the neutralizing antibody responses against each subtype were detected by using the Dual-Glo reagent (Promega).



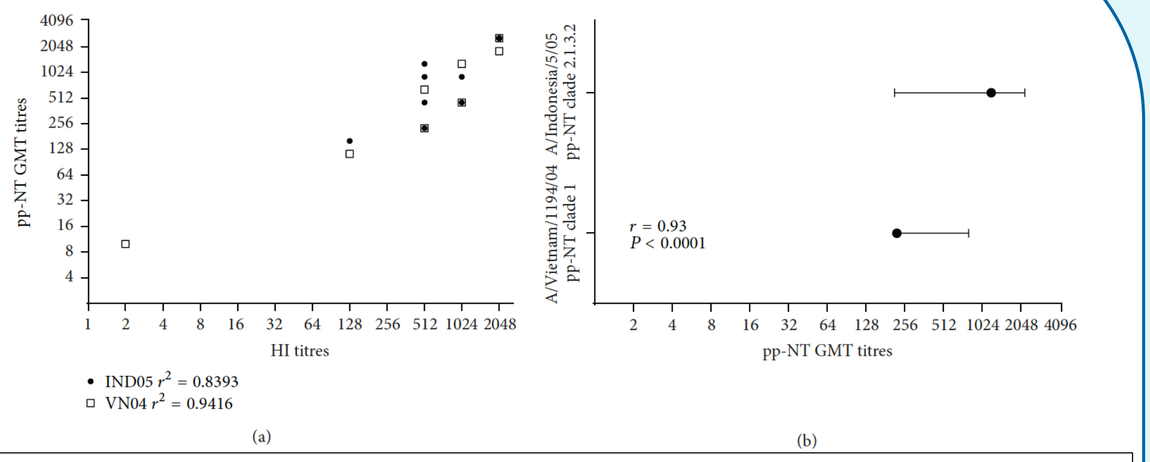
Production and neutralization of pseudotype viruses

1. HIV gag-pol, envelope glycoprotein (HA) and reporter construct transfected into HEK293T/17 producer cells
2. 48 hours post-transfection, supernatant is harvested prior to titration onto susceptible target cells
3. Pseudotypes can be employed as surrogate viruses in virus neutralisation assays (pp-NT)

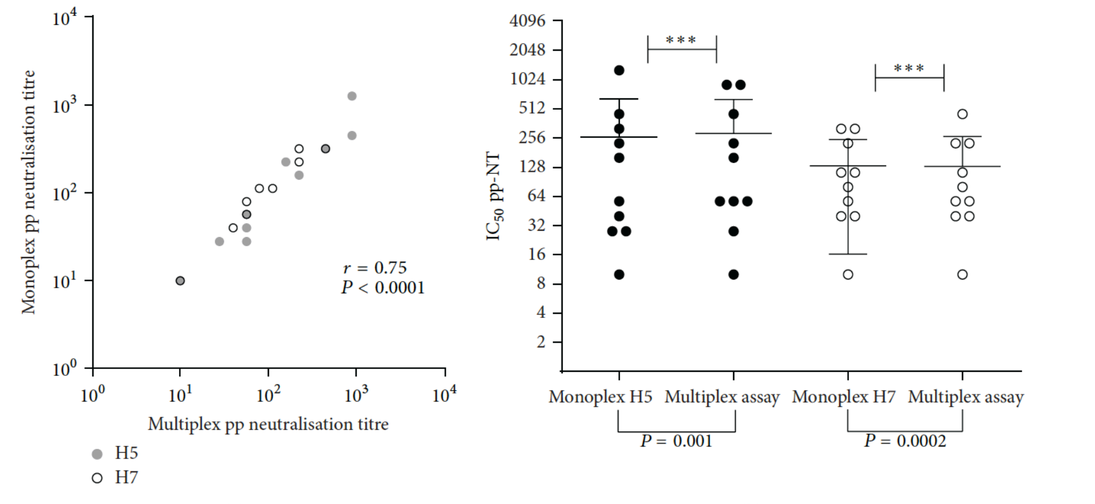


Radial phylogenetic tree showing the relationship between the full-length HA genes of vaccine and serological antigens. MV: monovalent vaccine strain, BV: bivalent vaccine strain. Viruses encircled in blue represent vaccine strains, in red represent pseudotype antigen strains, and in green represent HI antigen strains

RESULTS



Comparison of pp-NT with HI titers. Scatterplots showing the correlation of antibody logarithmic titers measured by pp-NT versus HI. For the pp-NT assay, HPAI H5 from A/Vietnam/1194/2004 (VN04) and A/Indonesia/5/2005 (IND05) were tested. Pearson's correlation analysis was carried out and the coefficient of determination (r^2) for each strain reported on the graph. (b) Paired t-test performed by using GraphPad.



Correlation of monoplex versus multiplex IC50 pseudotype neutralization titres for sera collected from chickens vaccinated with an inactivated bivalent vaccine produced with the AI strains H7N1 (A/ck/Italy/1067/1999, LPAI) and H5N9 (A/ck/Italy/22A/1998, LPAI). Antibody titres between monoplex and multiplex assays correlate when tested with both H5 and H7 pseudotypes. Neutralizing titres against H5 A/Vietnam/1194/2004 (grey dots) and H7 A/chicken/Italy/13474/1999 (empty circles) were determined in separate wells (single) or in the same well (multiplex). Correlation coefficient and P values were calculated using Pearson's correlation. (b) IC50 values for each sera tested by monoplex and multiplex pp-NT assays using H5 A/Vietnam/1194/2004 (firefly luciferase gene) and H7 A/chicken/Italy/13474/1999 (carrying firefly luciferase and Renilla luciferase gene) were calculated and plotted (the wide horizontal bar represents the means of IC50 titres). Results were subsequently analyzed by performing Student's t-test on the paired dataset.

CONCLUSIONS

1. No wild-type virus culture of HPAI H5 or H7 virus is necessary as the serological assay is entirely synthetic.
2. The influenza pseudotype assay is useful as an adjunct to the OiE approved serology assay, HI and the less frequently employed MN (microneutralization).
3. Pseudotype neutralization assays may prove easier to standardize as do not rely on erythrocytes which contribute to the high inter-laboratory variability of HI.
4. Compared to the routinely run HI, this assay is both "serum-sparing" and "antigen-sparing" and "bio-safe" (containment level 1 or 2).
5. Assay is straightforward to scale-up for large sample sizes and in a multiplex format the inter-assay variability is likely to be reduced (single serial serum dilution/batch of cells).
6. Multiplex format can be adapted for different subtype pairs (H5/H7, H1/B, H3/B). Multiplex assay can be adapted to measure antibody responses against two different respiratory virus groups (Influenza/Corona for example with SARS-CoV, MERS-CoV that we have also produced.
7. Pseudotypes are highly stable over time when kept at different storage temperatures and also when subjected to multiple rounds of freeze thawing. They are also amenable to freeze-drying significantly facilitating shipping and deployment in low-resource areas.

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