The study of heterosubtypic neutralising antibody responses against H5/H7 influenza viruses in human subjects using a comparative serology approach

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INTRODUCTION

The continuous rapid evolution of influenza H5 and H7 viruses driven by error-prone replication and increasingly by immune pressure significantly influences the sensitivity of available serological assays. As substitution rates are significantly higher in influenza HA and NA genes compared with internal genes, retroviral and lentiviral pseudotypes bearing HA and NA envelope glycoproteins devolved from the rest of the virus are ideal tools to monitor the effects of viral evolution on serological outcomes.

More specifically, pseudotype based assays have been shown to be efficient for the measurement of broadly-neutralizing antibodies directed against the HA2 stalk of influenza making them ideal serological tools for the study of cross-reactive responses against multiple influenza subtypes with pandemic potential, such as the H5N1 and H7N1 used in the current study (1,2).

AIM

In order to study the functional heterosubtypic neutralizing antibody responses in human populations we have constructed pseudotypes bearing haemagglutinin from high pathogenicity (HPAI) H5 and H7 strains and integrated these as serological antigens into a multiplex neutralization assay. The development of a multiplex platform allowing the measurement of neutralizing antibodies against two (in this study) or more viruses with pandemic potential in human sera has considerable time, cost and standardization implications for future serological studies.

MATERIALS AND METHODS

Viruses

Lentiviral pseudotypes with HA glycoproteins derived from the clade 1 H5N1 HPAI virus A/Viet Nam/1194/04 and the HPAI H7N1 virus A/chicken/Italy/13474/99 have been described previously (2).

Pseudotype viruses were produced by co-transfection of 293T/17 cells with the respective HA plasmid, the HIV gag-pol plasmid p8.91 (3), and either the reporter plasmid pCSFLW (expressing firefly luciferase) (4) or pCSRLW (expressing renilla luciferase) (5) using Fugene-6 transfection reagent (Roche, UK).

Sera

Hyper-immune sheep sera SH454 raised against NIBRG-14 (H5N1 HA), and 02/294 raised against A/chicken/Italy/13474/99 (H7N1 HA) were kindly provided by NIBSC .

68 serum samples obtained from the Italian population from 1992 to 2007 found previously to be sero-protected against A/Viet Nam/1194/04 as determined by the SRH assay (6).

HA pseudotype-based neutralization assay

Serum samples were heat inactivated at 56˚C for 30min, two-fold serially diluted in culture medium and mixed with pseudotype virus (500,000 RLU luciferase input) at a 1:1 v/v ratio.

After incubation at 37˚C for 1 hr, 1x10^4 293T/17 cells were added to each well of a white 96-well flat-bottomed tissue culture plate. Firefly and renilla relative light units (RLU) were evaluated 48 hr later by luminometry using the Dual-Glo assay system (Promega, UK). IC_{50} neutralizing antibody titres < 1:40 are considered negative.

SRH assays

Agarose immunoplates were carried out with sheep erythrocytes 10% (v/v of assay buffer) sensitized by 2000 Haemagglutinin Units (HU)/mL of inactivated A/Vietnam/1194/2004 (H5N1) whole virus, and with the addition of 5% of guinea-pig complement.

Before testing, serum samples were heat inactivated at 56˚C for 30 min. Each test run included negative and positive samples: hyperimmune sheep antiserum A/Vietnam/1194/04 (07/148 provided by NIBSC) was used as a positive control.

The diameters of the haemolytic areas were measured using a Transidyne Calibrating Viewer (Transidyne General Corporation, Ann Arbor, MI).

Haemolysis Area <4 mm^2 was considered negative, between 4 and 25 mm^2 was considered positive but not protective and >25 mm^2 was considered protective.

RESULTS

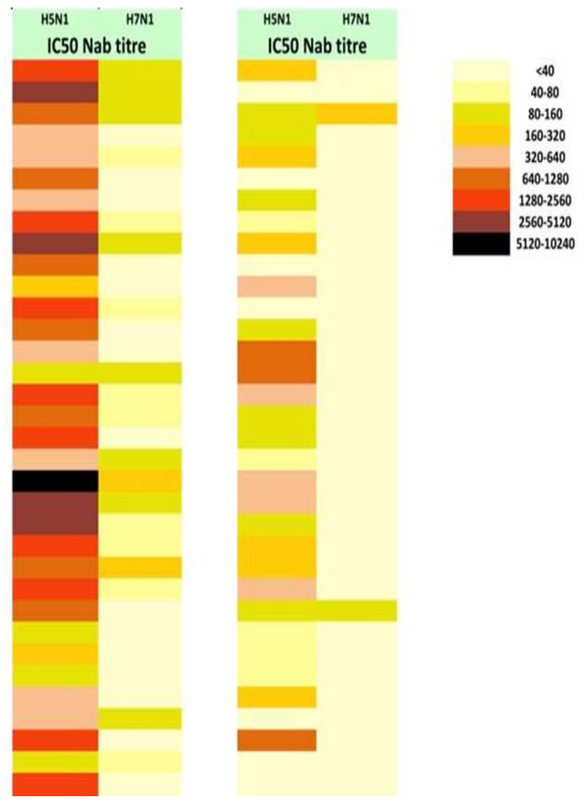

Figure 1: Cross-reactivity antibody profiles of SRH positive human sera against a Group 1 H5 and a Group 2 H7 virus as measured using pseudotype neutralization assays. Antibody response colour chart showing the IC_{50} neutralizing antibody titres of sera that have an SRH titre > 25mm^2 (34 in each column) measured against H5 and H7 pseudotypes. Individual end-point titres (as calculated using GraphPad).

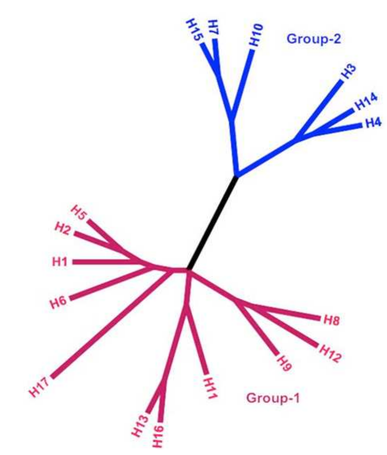

Figure 2: Phylogenetic tree showing the relationship between influenza HA subtypes H1-H17 (MEGA5 Software)

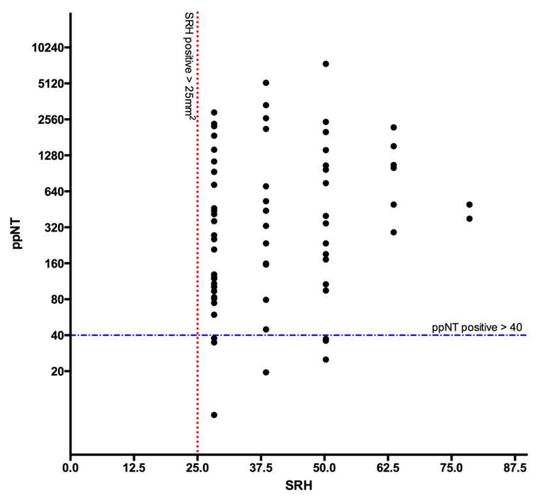

Figure 3: Comparison of SRH assay versus pseudotype assay. SRH titres expressed as diameter of haemolysis (in mm^2) are plotted on the X-axis. SRH titres > 25 mm^2 are considered to be sero-protective and this cut-off is represented on the plot by a vertical red dotted line. IC_{50} pseudotype neutralization titres expressed as the reciprocal of the serum dilution that results in a 50% inhibition of pseudotype virus entry.
PpNT titres > 1:40 are considered to be positive and this cut-off is represented by a horizontal blue dotted line.

SRH assay has been shown to detect, not only responses against HA, but also non-specific antibody responses that are likely to have been elicited against internal proteins.
Titers obtained by pp-NT assay reveal its sensitivity to measure functional responses to both globular head (HA1) and stalk (HA2) of influenza HA.

CONCLUSIONS

- The multiplex assay described has utility for the study of heterosubtypic antibody responses against Group 1 and Group 2 influenza viruses in the same serum sample.
- The pseudotype-based multiplex neutralization assay presented here is both “serum-sparing” and “antigen-sparing” and likely to be more readily standardised than assays using red blood cells.
- This assay has utility for sero-prevalence studies and for the pre-clinical evaluation of vaccines and antivirals against avian viruses with pandemic potential.

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