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## INTRODUCTION

Influenza A viruses are classified using the antigenic and phylogenetic characteristics of the envelope glycoproteins haemagglutinin (HA) and neuraminidase (NA). Currently 17 different haemagglutinin subtypes are recognized and are divided into two lineages: Group 1 and Group 2 (Figure 1). In humans influenza A viruses that harbour on their envelope haemagglutinin of H1 and H3 subtypes usually circulate, cause seasonal epidemics and are included in the seasonal vaccine. However viruses with other HA subtypes (eg H5, H7, H9) have been shown to infect humans and if these viruses acquire efficient human-to-human transmission they can potentially cause pandemics.

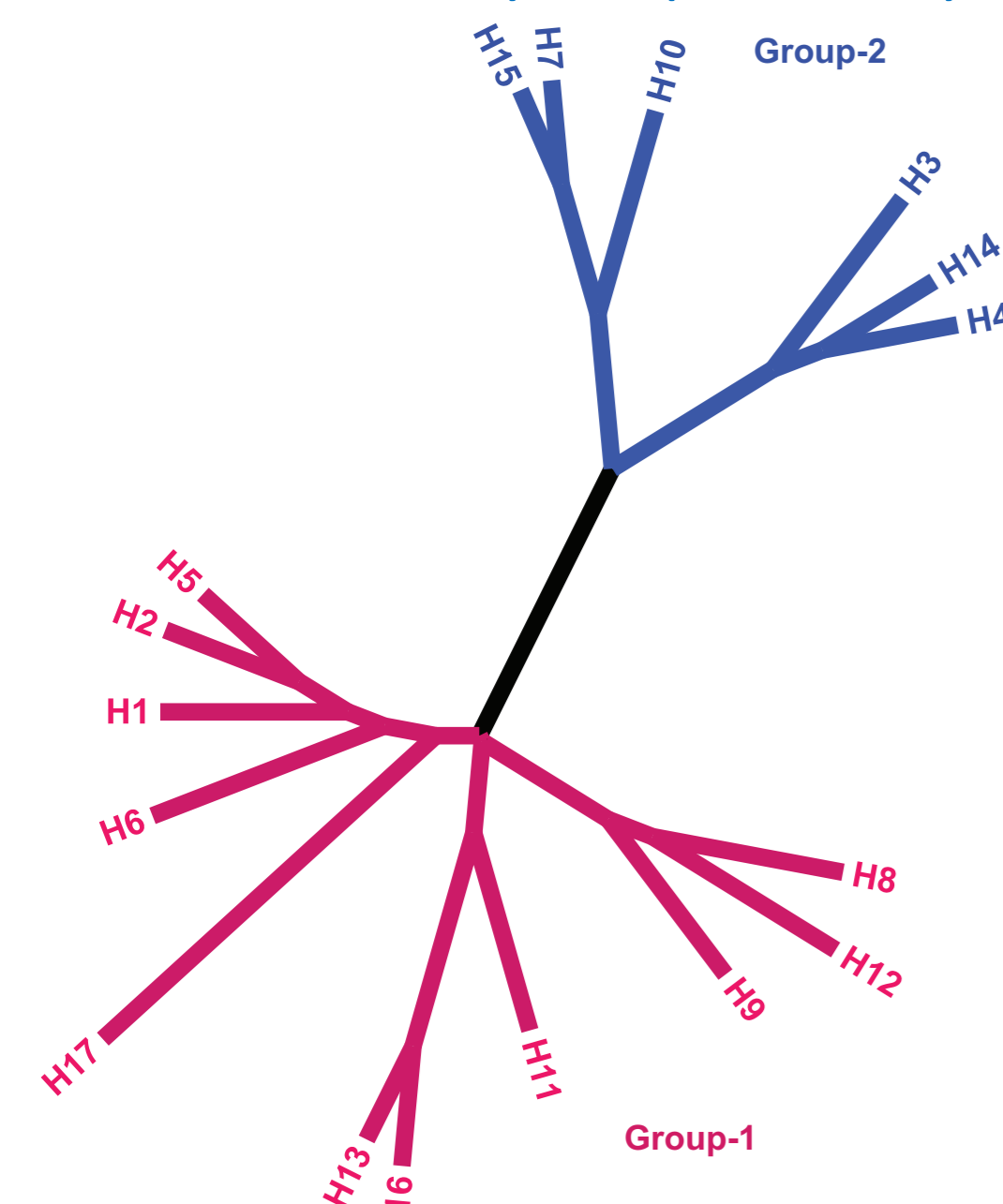


Figure 1: Phylogenetic relationship of influenza A haemagglutinin subtypes

The study of heterosubtypic antibody responses directed against influenza A haemagglutinins in human populations is an important facet of pandemic preparedness. For this purpose it is important to study whether seasonal vaccination can elicit and/or boost a cross-neutralizing antibody response.

Classical serological assays, such as haemagglutination inhibition and microneutralization, have demonstrated low sensitivity for the detection of cross-neutralizing antibodies, especially those directed against epitopes in the haemagglutinin HA2 stalk region. Here we use pseudotype particle neutralization assays performed using representatives of Group 2 influenza viruses to detect the presence of heterosubtypic neutralizing antibody against Group 2 viruses before and after seasonal vaccination.

## MATERIALS AND METHODS

### Sera

Sera from 18-60 year old people (13) and from elderly (9) collected before and after seasonal vaccination with A/Wisconsin/67/2005 (H3N2), A/Solomon Island/3/2006 (H1N1) and B/Malaysia/2506/2004 were used for this study.

### Pseudotype particles production and titration

Lentiviral pseudotypes were produced as described previously [4, 5, 6]. Briefly, HIV gag-pol plasmid p8.91, firefly luciferase expressing plasmid pCSFLW, HA expressing plasmid and pCAGGS-TMPRSS2 [3] were co-transfected into human embryonic kidney HEK293T/17 cells using Fugene-6 transfection reagent (Roche, UK). After 24 hours incubation, recombinant neuraminidase from *Clostridium perfringens* (Sigma) was added to facilitate pseudotype exit from the producer cells. 48 hours post-transfection supernatant was harvested, filtered through 0.45µm filters and stored at -80°C. Calculation of the pseudotype titres in relative luminescence units (RLU) per ml was carried out as described by us elsewhere [6, poster P2-493].

### Pseudotype particles neutralization assay

Neutralization titres of each sera were evaluated against A/Udorn/307/1972 H3, A/duck/Czechoslovakia/1956 H4, A/chicken/Italy/1082/1999 H7, A/chicken/Germany/N49 H10, A/mallard/Astrakhan/263/1982 H14, A/shearwater/West Australia/2576/1979 H15 pseudotypes.

Serial dilutions (1:40-1:5120) of pre and post vaccination sera and of positive control sera in white 96-well flat-bottomed tissue culture plates (Nunc) in a final volume of 50µl were performed and 1x10<sup>6</sup> RLU/well of pseudotypes were added to the plate. Following 1 hour incubation at 37°C, 1x10<sup>4</sup> HEK293T/17 cells were added to each well and the plates were incubated for 48 hours at 37°C. Luminescence was evaluated by luminometry using the Bright-Glo assay system (Promega, UK) (Figure 2).

Normalization as percentage of inhibition of pseudotype virus entry (neutralization), and the half maximal inhibitory concentrations (IC<sub>50</sub>) were calculated with Prism version 6 (GraphPad Software) and expressed as reciprocal of the dilutions.

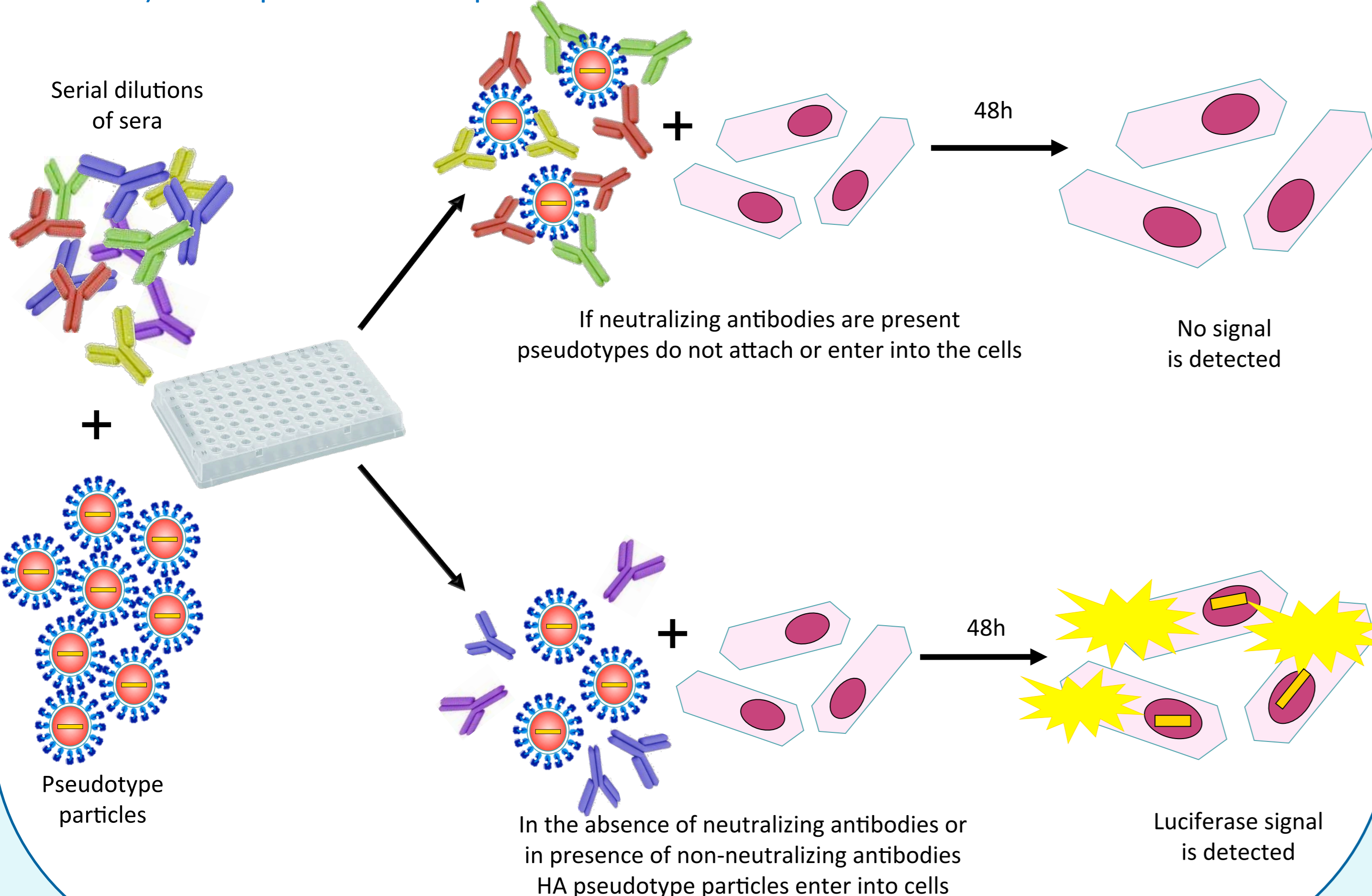


Figure 2: Pseudotype particle neutralization assay

## RESULTS

For each group 2 virus the IC<sub>50</sub> distribution of the sera collected before and after vaccination were reported on Box-and-Whisker plots (Figure 3) for comparison.

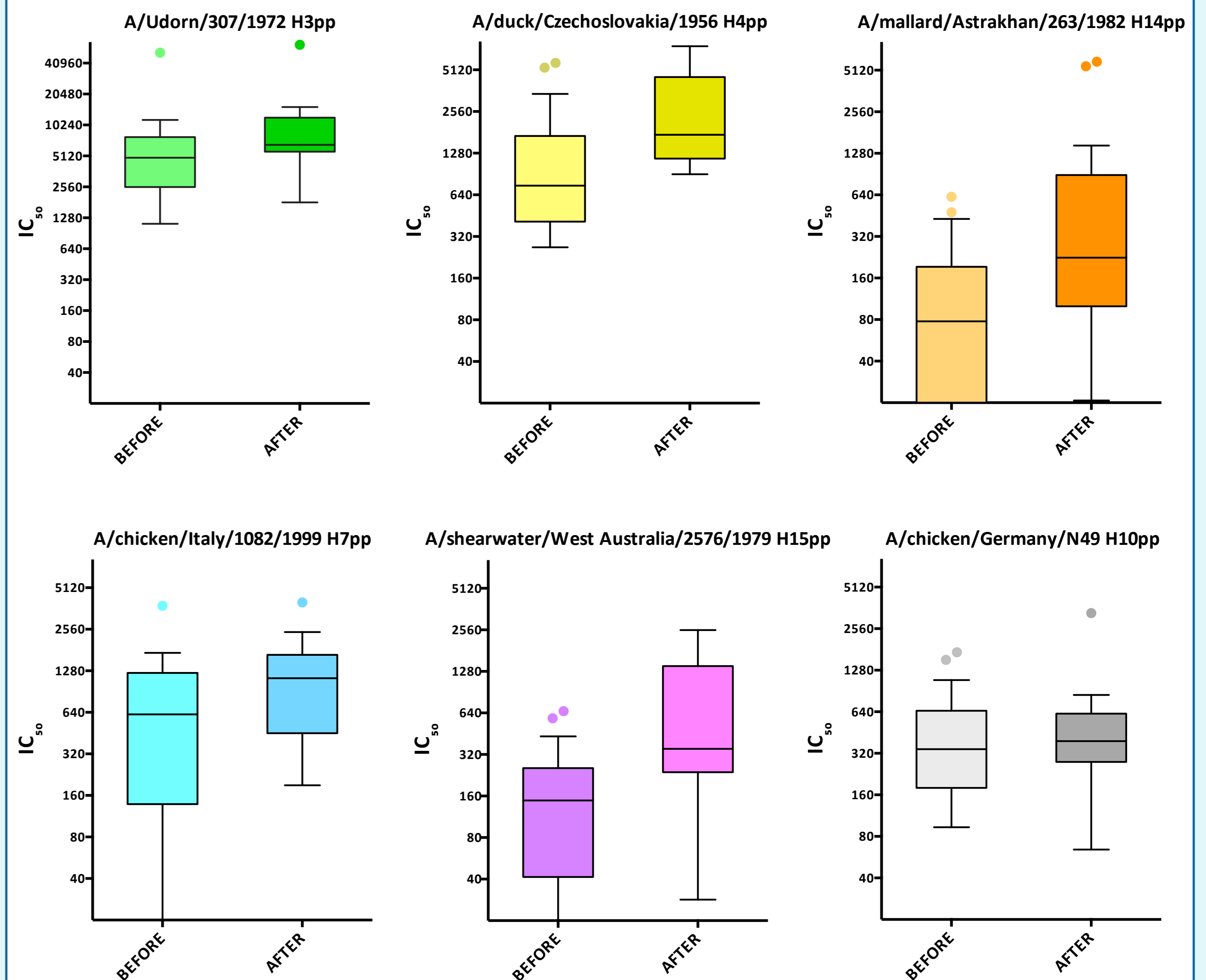


Figure 3. Box-and-Whisker plots showing IC<sub>50</sub> distributions

IC<sub>50</sub> distributions before and after vaccination are different in shape and a shift of IC<sub>50</sub> medians and IC<sub>50</sub> interquartile range is observed. All before and after vaccination IC<sub>50</sub> distributions (with the exception of A/chicken/Germany/N49 H10 distributions) are statistically different (p<0.05) using a non-parametric Wilcoxon matched-pairs signed rank test.

## CONCLUSIONS

In this study we have shown that:

- ❖ The pseudotype particle neutralization assay has increased sensitivity for detecting neutralizing antibody response.
- ❖ Performing pseudotype particle neutralization assays using a panel of influenza A pseudotypes permits the detection of heterosubtypic antibody responses in patients before and after seasonal influenza vaccination.
- ❖ The heterosubtypic antibody response is increased and/or can be elicited after seasonal vaccination.

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