Supplemental Digital Content 2

Hemagglutination inhibition assay

Hemagglutination inhibition (HI) antibody titers were determined using a method derived from the WHO Manual on Animal Influenza Diagnosis and Surveillance. Measurements were conducted on thawed frozen serum samples with a standardized and comprehensively validated micro method using two hemagglutination units (2 HAU) of the appropriate antigens and a 0.45% chicken erythrocyte suspension. Non-specific serum inhibitors were removed by heat treatment and receptor-destroying enzymes. Starting with an initial dilution of 1:10, a dilution series (by a factor of 2) was prepared up to an end dilution of 1:10240. The titration end-point was taken as the highest dilution step that showed complete inhibition of hemagglutination. All assays were performed in duplicate. The assay cut-off value was 10 1/dil.

Microneutralization assay

Microneutralization (MN) assay was used to determine the neutralizing antibody titer as previously described (Hehme et al 2004). Thawed frozen serum samples were heat inactivated for 30 minutes at 56°C. A standardized amount of virus was mixed with serial dilutions of serum and incubated to allow binding of the antibodies to the virus. A cell suspension containing a defined amount of Madin-Darby Canine Kidney cells was then added to the mixture of virus and antiserum, and incubated at 37°C for 7 days. After the incubation period, virus replication was visualized by hemagglutination of chicken red blood cells. The 50% neutralization titer of a serum sample was calculated as the geometric mean titer between the highest serum

dilution able to totally neutralize the virus and the next serum dilution where viruses remained detectable. Each serum sample was tested once.

The assay cut-off value was 28 1/dil for the A/H1N1, A/H3N2 and B/Victoria strains. In the course of the annual strain revalidation process for the assay, a run effect was observed for two of the seven runs performed, in which approximately half of the supposedly negative samples tested positive for the B/Yamagata (Hubei-Wujiagang/158/2009) strain. The cause of the run effect could not be determined. The Limit of Blank (LOB) was 49 1/dil. In order to be conservative, the cut-off value for this strain was raised to 57 1/dil.

Neuraminidase inhibition assay

The neuraminidase inhibition (NI) antibody titer was determined using an enzyme linked lectin assay (Ella) as previously described (Hehme et al 2004). In this assay, the bottom of enzyme-linked immunosorbent assay (ELISA) plates was coated with a fetuin substrate. The assay is based on the neuraminidase enzymatic activity which releases N-acetyl neuraminic acid from fetuin substrate. After cleavage of the terminal neuraminic acid, ß-D-galactose-N-acetyl-galactosamin is unmasked. Peroxidase-labelled peanut agglutinin binds specifically to the galactose residues and the enzymatic desialylation can be detected and quantified by a colorimetric reaction using 3.3´5,5´-Tetramethylbenzidin (TMB) as a substrate. The neuraminidase inhibition titer of a serum sample was measured by mixing a standard amount of neuraminidase with serial dilutions of serum, and was set as the reciprocal of the serum dilution that reduced the colorimetric signal resulting from desialylation by 50%. The assay was performed with wild-type whole virus.

The assay cut-off value was 20 1/dil for the A/H1N1, B/Victoria and B/Yamagata strains. As part of the validation process, limits below the classical cut-off value were explored to better support assay specificity. For the A/H3N2 (A/Victoria/361/2011) strain, assay specificity fell short of the target (50–60% instead of ≥80%) with the standard cut-off of 20 1/dil. The LOB equalled 28 1/dil. The cut-off value was increased to 40 1/dil, the first measurable titer above 28 1/dil.

The whole virus antigen used for the enzyme-linked lectin NI assay may have overestimated neuraminidase antibody responses, as anti-HA antibodies may inhibit neuraminidase-mediated activation of the lectin by steric hindrance. Whether such potentially beneficial inhibition would occur *in vivo* is unknown.

Reference

Hehme N, Engelmann H, Kuenzel W, Neumeier E, Saenger R. Immunogenicity of a monovalent, aluminium-adjuvanted influenza whole virus vaccine for pandemic use. *Virus Res* 2004;103:163-171