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Short communication

Development and evaluation of an N9-specific enzyme-linked immunosorbent assay to detect antibodies in duck and chicken sera

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A B S T R A C T

A serological test for detecting N9-specific antibodies may be useful as a DIVA strategy to differentiate vaccinated from infected animals or simply for direct serological detection of infection with N9-subtype virus. The method currently recommended for the detection of antibodies against neuraminidase is neuraminidase inhibition (NI), which is a laborious method using toxic chemicals and has low sensitivity. The present study describes the development and validation of an N9-specific ELISA.

Data obtained with this N9 ELISA were compared to those obtained with nucleoprotein-based ELISA, haemagglutination inhibition test using homologous antigen and NI assay. 785 sera from ducks and chickens were used, from flocks previously determined to be AI negative or from experimentally infected or immunized flocks. Sensitivity and specificity were evaluated, and a ROC curve and kappa values, which provide a comparison between methods, were calculated. The results obtained in this study indicate that the N9 based-ELISA is effective in detecting N9-specific antibodies with high specificity and with better sensitivity than the recommended NI method; using data from 177 common sera tested with N9 ELISA and NI assay both compared to NP-based ELISA, their specificity were evaluated at 93.6% and 91.5% respectively, and sensitivity at 90.8% and 39.2% respectively.

Avian Influenza A viruses (AIV) belong to the *Orthomyxoviridae* family and subtypes have been identified in AIV based on the antigenic properties of the 16 haemagglutinin (HA) (H1 to 16) and 9 neuraminidase (NA) (N1 to 9) surface glycoproteins. Each subtype is then defined by a combination of one haemagglutinin (H1 to 16) and one neuraminidase (N1 to 9) (Alexander, 2000; Fouchier et al., 2007). These two proteins are the major surface integral membrane glycoproteins of influenza viruses: HA binds to sialic acid and NA catalyzes the removal of terminal sialic acid residues from oligosaccharide chains (Colman, 1994). NA may be important in virulence and the early stage of infection by promoting virus entry or in facilitating release of progeny virions from the surface of infected cells (Matrosovich et al., 2004; Su et al., 2009).

Generic tests based on the detection of antibodies against the conserved AIV nucleoprotein (NP) are routinely used as screening tests (Brown et al., 2009). Further to these preliminary tests, haemagglutination inhibition (HI) tests allowing the detection of specific HA subtype antibodies are used as the gold standard either for diagnostic purpose to detect infection by a given HA subtype or for assessment of humoral immunity following vaccination using a given HA subtype; although a recent paper demonstrated that for sera from ducks infected or immunized with LP AIV belonging to the H5 subtype of the Eurasian lineage, an H5-based ELISA was more sensitive (Schmitz et al., 2013). In addition to HA identification, a serological test for detecting N9-specific antibodies may be useful in two ways: (i) it could be simply used for direct serological detection of infection in poultry, other captive birds or wild birds with N9-subtype AIV or (ii) in a country which considers AI vaccination to prevent/control AI outbreaks in poultry, a N9-based ELISA test could be used for DIVA strategy to differentiate vaccinated from infected birds either by using a vaccine strain with a N9 subtype different from the NA subtype displayed in the AIV circulating in poultry or the reverse, i.e. by using a vaccine strain with a NA subtype different from the N9 subtype displayed in the AIV circulating in poultry (Avellaneda et al., 2009; World Organization for Animal Health, 2013). N9 viruses are indeed commonly present in both wild birds and poultry, in association with many HA subtypes. The most recent influenza cases concern human and poultry infections with an H7N9 LPAIV which emerged in eastern continental China during the winter of 2013 (Chen et al., 2013; Gao et al., 2013; Li et al., 2014). According to the most recent OMS data available dated

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from the end of June, the number of cases was 450, by the end of summer 2014 the total number of human cases seem to exceed 450. The cases were reported in (i) 15 provinces including the autonomous province of Xingjiang and the capital of Mainland China – including 11 provinces also reporting outbreaks in poultry – and (ii) Hong Kong SAR, Macao and Taiwan and (iii) Malaysia (the cases in the latter four having been contracted in one of the former provinces) (http://www.chp.gov.hk/en/content/599/34480.html; http://www.cidrap.umn.edu/sites/default/files/public/downloads/topics/cidrap_h7n9_update_080414.pdf; http://www.who.int/influenza/human_animal_interface/influenza_h7n9/riskassessment_h7n9_27june14.pdf?ua=1). By the end of June the overall mortality was around 37% (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/riskassessment_h7n9_27june14.pdf?ua=1). Forty three outbreaks of low pathogenic avian influenza (H7N9) have been officially notified involving pigeons, chickens and ducks present essentially in live bird markets and/or environment samples collected in the same area, located in twelve provinces of eastern, southern China, (http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review/viewsummary?reportid=13225). Natural infections with this H7N9 virus in poultry are asymptomatic or induce few signs that are difficult to detect; however they may induce a humoral response that can be detected serologically.

Domestic ducks play a major epidemiological role in AI, since they are often associated with higher prevalence of subclinical AI infection even in some case of infection with AIV that are highly pathogenic in gallinaceous poultry. In addition, China produces about 5.5 billion chickens for consummation a year and 830 million ducks for consummation a year (number of heads) (FAO data: http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/QA/E), the latter corresponding to 70% of the world's population of ducks, often as free-range productions which are therefore in frequent contact with wild birds. Improved detection of H7N9 AIV infection in domestic anseriformes would therefore be beneficial in preventing the spread of those viruses to terrestrial poultry.

The neuraminidase inhibition (NI) assay is the miniaturized test used for detection of NA subtype specific antibodies (Van Deusen et al., 1983; WHO Global Influenza, 2011). The NI assay is a laborious technique and requires handling toxic chemicals. Its substitution by a more sensitive and specific ELISA test would be useful. The aim of the present paper is to describe the development of a blocking N9-based ELISA test and its validation with a panel of approximately 800 sera essentially from ducks and chicken.

The present in-house blocking ELISA test was developed using a N9-specific mouse monoclonal antibody (reference IdVet 6B1 A12) produced by IdVet, Montpellier, France. The concentrations of the reagents used, such as coating antigen and monoclonal antibody, as well as the serum to be tested, were optimized. The first step of this ELISA is to coat ELISA plates with a N9 antigen. When a whole AIV is used as antigen and not a recombinant N9 protein, the choice of the antigen for coating is important because HA subtype-specific antibodies can block the access of specific NA (N9 presently) antibodies. This happens because HA subtype-specific antibody binding hampers the access of NA (N9 presently)-specific antibodies to NA (N9 presently)-epitopes. Using a very rare subtype such H15 – that has not been reported (to date) in poultry – should reduce the chances of these unwanted HA antibody/antigen interactions when using whole virus as antigen for a NA specific ELISA. For this reason, the N9 ELISA reported in this study was developed using an uncommon subtype H15N9 A/shearwater/WestAustralia/2576/1979. This virus was grown in 9-day-old embryonated chicken eggs, inactivated with formol at 1‰ overnight at 37 °C, purified with 20–50% gradient sucrose and concentrated 17.5×. Microplates were then coated with the inactivated and purified H15N9 antigen diluted to 1/600 in 0.05 M carbonate buffer (pH 9.6) and incubated at 4 °C overnight. After washing with PBS (pH 7.4) and 0.05% Tween 20, plates were blocked with PBS-Tween 20 containing 5% milk and 10% foetal bovine serum for 1 h at 37 °C. After washing, serum samples and controls (both previously diluted to 1/5) were added for 1 h at 37 °C, followed after washing by incubating N9-specific monoclonal antibodies diluted to 1/600 for 1 h at 37 °C. The unbound monoclonal antibodies were removed by washing and then phosphatase labelled goat anti-mouse IgG (H + L) (KPL) diluted to 1/400 was added and incubated for 1 h at 37 °C. After washing, the presence of secondary-conjugated-antibody was revealed by incubating the *p*-nitrophenyl phosphate substrate (*p*NPP) (Uptima) for 30 min at room temperature. The intensity of the colour measured as the optical density (OD) at 405 nm with a spectrophotometer (Dynex Technologies–MRX Revelation), was correlated inversely to the amount of N9-specific antibodies present in the sample to be tested. Results were expressed as a competition percentage (CP) corresponding to the ratio of the sample analyzed to the negative control [S/N%] = 100 × (OD Sample)/(OD Negative control)).

A total of 785 sera were tested. Details of their origin and numbers are provided in Table 1. Status of 521 sera with a known negative AIV were used for establishing the threshold, then 57 reference sera against different AIV subtypes or against pathogens other than AI produced in SPF chickens and ducks were used for checking preliminary specificity. The remaining 207 sera, collected from SPF chickens and N9 ducks experimentally infected or immunized with AIV belonging to the N9 subtype, were used for validation of this N9 ELISA. Most of them were collected from ducks (85.2%), mainly from muscovy ducks. SPF animals were maintained in air-filtered facilities and monitored on a regular basis for ~20 bacterial and viral avian pathogens including avian influenza virus, to check for their negative status. All experimental immunisations were performed in biological safety level BSL2+ (intermediate between BSL level 2 and 3 – this level requires complete change of clothes, double door access, sealed windows, negative airflow, shower before exit) or BSL3 facilities, approved by the French veterinary authority (entitled officer from the French Ministry of Agriculture) according to EU standards; animal experimentation was performed by duly authorized personnel following reception of approval from the Ethical Committee for experimentation, Cometh ANSES/ENVA/UPEC (National Ethical Committee for Animal Experimentation registration no. 16).

All 785 sera were tested with the NP ELISA using a commercial NP-based ELISA test (IDEXX – Elisa IA Multiscreen, recently re-named Influenza A Ab Test). 34 positive sera by NP-ELISA collected from infection/immunization with AIV not belonging to the N9 subtype were removed from the comparative study. Therefore 751 were used for comparison between NP-ELISA and N9-ELISA (Table 1). Sera from experimental infection or immunization ($n = 177$) with AIV belonging to the N9 subtype were also tested with the HI test (using the same antigen inoculated for immunization or the homologous virus, although inactivated, as the one used for infection) and NI assay. The HI test was performed according to the World Organization for Animal Health manual (World Organization for Animal Health, 2012), using four haemagglutinating units of homologous antigen; HI titres ≥16 (or $\log_2$ titre ≥ 4) were scored positive. NI assay was performed as described by Aymard-Henry et al. (1973), except the method was miniaturized and the results were given as OD (Van Deusen et al., 1983). The antigen used was the same as the one used with N9-based ELISA, i.e. the uncommon subtype H15N9 A/shearwater/WestAustralia/2576/1979: as well as N9-ELISA, again as previously described using a very rare subtype such H15 should prevent unspecific reactions. The threshold was determined beforehand using an Inhibition Percentage (IP) (corresponding to [(N − S)/N%] = 100 × (OD negative control − OD sample)/(OD negative control) equivalent to 30% – positive with