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Short communication

Differences in the detection of highly pathogenic avian influenza H5N1 virus in feather samples from 4-week-old and 24-week-old infected Pekin ducks (*Anas platyrhynchos* var. *domestica*)

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ABSTRACT

Previous studies have reported the detection of H5N1 HPAI virus in feathers from ducks naturally and experimentally infected and suggested that feather calami (FC) could be used as diagnostic samples for the early detection of H5N1 HPAI infections. Ducks are readily infected with H5N1 HPAI viruses although the development of clinical signs and deaths were reported as age-related with younger birds being more susceptible. The correlation between age and virus localisation in FC of infected ducks has not been studied to date.

In the present study juvenile (4-week-old) and adult (24-week-old) Pekin ducks (*Anas platyrhynchos* var. *domestica*) were infected experimentally with a clade 2.2 H5N1 HPAI virus (A/duck/Nigeria/1071-23/2007). Tracheal (Tr) and cloacal (Cl) swabs and FC were collected at 3, 5, 7 and 10 days post infection and tested by RRT-PCR and a double antibody sandwich-ELISA (DAS-ELISA) developed in house.

Virus was detected in swabs and FC of challenged ducks with a higher rate of detection in juvenile ducks. In this age group virus was detected over a longer period of time in FC compared to swabs.

Our study showed that FC samples collected from young ducks are a valid diagnostic specimen for H5N1 HPAI virus detection. The DAS-ELISA on FC proved to be a suitable alternative diagnostic test when molecular and/or virus isolation techniques are not available therefore it could be useful in the diagnosis of H5N1 HPAI infections in under-resourced countries.

1. Introduction

Previous studies have reported the detection of H5N1 highly pathogenic avian influenza (HPAI) virus in feathers collected from both naturally and experimentally infected birds (Perkins and Swayne, 2001; Yamamoto et al., 2007, 2009) and suggested that feather calami (FC) could be used as additional diagnostic samples for the early detection of H5N1 HPAI infections in endemic areas (Slomka et al., 2012).

The presence of H5N1 HPAI virus in feathers has also been reported in asymptomatic ducks, which represent one of the domestic reservoirs of this virus (Hulse-Post et al., 2005; Sturm-Ramirez et al., 2005; Pantin-Jackwood et al., 2007). Moreover, it has been reported that duck

Abbreviations: LPAI, low pathogenic avian influenza; HPAI, highly pathogenic avian influenza; EID_{50}, 50% egg infective dose.

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feathers were positive by RRT-PCR and virus isolation (VI) for a prolonged period of time compared to swabs (Yamamoto et al., 2008) and that VI rate and RRT-PCR sensitivity increased when feathers were included as additional specimens (Slomka et al., 2012). The age-related clinical susceptibility in domestic ducks infected with H5N1 HPAI virus has been established (Pantin-Jackwood et al., 2007; Londt et al., 2010). Londt et al. (2010) suggested the existence of age related differences in H5N1 HPAI virus colonisation of duck feathers based on immunohistochemistry (IHC) data. However to date studies on the correlation between age and the virus detection in feathers of infected and asymptomatic ducks are not available.

To further investigate the potential use of feathers as diagnostic samples and to generate information on the presence of H5N1 HPAI virus in duck feathers of different ages, feather calami (FC) and tracheal (Tr) and cloacal (Cl) swabs from young and adult ducks infected experimentally with an H5N1 HPAI virus were collected and analysed for virus detection. Samples were tested using RRT-PCR and DAS-ELISA and data obtained were compared using statistical analysis.

2. Materials and methods

Two groups of 16 young (4-week-old) and 16 adult (24-week-old) clinically healthy Pekin ducks (*Anas platyrhynchos* var. *domestica*), originating from an avian influenza negative commercial flock, were oro-nasally infected with 10^7 EID_{50}/0.1 ml of A/duck/Nigeria/1071-23/2007 H5N1 HPAI virus (clade 2.2) [GenBank: EU148428–EU148435]. This virus was isolated from asymptomatic ducks (Cattoli et al., 2009) and proved to be extremely virulent for chickens infected experimentally, causing 100% mortality. Additionally, two groups of 4 young and 4 adult Pekin ducks were used as negative controls. Prior to challenge, all ducks were tested serologically by competitive type A influenza ELISA (IDVet®, Montpellier, France) and haemagglutination inhibition (HI) test (OIE, 2012) using the challenge virus as HI antigen, and virologically by RRT-PCR targeting the matrix (M) gene (Spackman et al., 2002). Clinical signs were monitored twice a day; Tr swabs, Cl swabs and separate pools of 5 non-calcified feather calami (FC) collected from three different regions of the body (wing, breast and tail) were obtained from all challenged ducks on days 3, 5, 7 and 10 post infection (p.i.). Feather calami were extracted from the feather follicle and their vascularised basal part was immediately separated from the external and potentially contaminated part of the feather. Blood samples were collected on day 10 p.i. from all surviving ducks. Additionally, virus detection by M gene RRT-PCR (Spackman et al., 2002) was carried out on lung, brain, thigh and breast muscle of three randomly euthanized ducks per group, on days 3 and 5 p.i. For this reason the sample size progressively decreased during the experiment. All animal experiments were approved by the local Ethics Committee and were conducted in BSL3 containment animal facilities.

Swabs and FC were analysed by M gene RRT-PCR (Spackman et al., 2002), which was chosen as a *gold standard* method for the purpose of this study. Pools of five FC were ground and homogenised with sterile pestles, and suspended in 0.5 ml of sterile phosphate-buffered saline (PBS). Different extraction protocols were compared (data not shown) and the commercial kit High Pure™ RNA (Roche®, Penzberg, Germany) selected as the most appropriate.

For virus detection in FC, an in-house immunoenzymatic method based on a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was developed by modifying a previously described protocol (Lee et al., 1993). Two commercially available monoclonal antibodies specific for type A influenza nucleoprotein (NP) were used. The capture antibody HB 65 (ATCC®, United Kingdom) was diluted 1:300 (v/v) in sodium carbonate buffer (pH 9.6) and 50 μl per well was coated onto 96-wells ELISA plates (MaxiSorp®, Hatfield, United Kingdom). Following an overnight incubation at 4 °C, plates were washed 3 times with 250 μl of 0.05% (v/v) PBS-Tween®20 (Sigma–Aldrich, St. Louis, USA) (PBST), then incubated with 50 μl of 5% (w/v) Bovine Serum Albumin solution (blocking buffer) for 1 h at 37 °C. Plates were washed three times with PBST, 50 μl of feather samples was added in duplicate and plates incubated for 1 h at 37 °C. Washing was repeated as before, 50 μl of horseradish peroxidase (HRP)-conjugated detection antibody (IDVet®, Montpellier, France) diluted 1:3500 (v/v) in PBST containing 1% (w/v) Yeast Extract Powder (LabM®, Heywood, United Kingdom) and 4% (w/v) foetal bovine serum (diluent buffer), was added and incubated for 1 h at 37 °C.

After washing with PBST, colour was developed using 50 μl of 3,3′,5,5′-tetramethylbenzidine (TMB Substrate kit, Pierce, Rockford, USA) chromagen. After incubation at room temperature for 10 min, stop solution (0.5 M H_2SO_4, 50 μl/well) was added to each well and the optical density (OD) was read at 450 nm (OD_{450}).

The assay sensitivity (Se) and specificity (Sp) and the positive/negative cut-off (expressed as OD value) were previously evaluated using 15 different type A influenza viruses (Table 1), 2 avian paramyxoviruses (NDV Ulster 2C strain and APMV-4), 3 avian infectious bronchitis viruses (624I, D274, 793B serotypes) and negative allantoic fluid. Influenza viruses were diluted in diluent buffer and tested at the following concentrations: $10^{3.5}$, $10^{2.5}$, $10^{1.8}$, $10^{1.5}$, $10^{0.8}$ and $10^{0.5}$ EID_{50}/100 μl. Additionally, two avian paramyxoviruses (NDV Ulster 2C strain and APMV-4), three avian infectious bronchitis viruses (624I, D274, 793B serotypes) and one negative allantoic fluid were diluted as before and analysed by DAS-ELISA.

ROC curves (Thrusfield, 2005) were used to determine the cut-off value for the DAS-ELISA and to investigate the assay Se and Sp compared to M gene RRT-PCR. An ANOVA analysis for repeated measurements (West et al., 2007) was applied to RRT-PCR data to investigate presence of significant differences among feathers collected from the three body regions. In addition, the one-tailed exact McNemar's test for paired data was applied to investigate significant differences between FC and swabs in yielding positive virus detection results (Thrusfield, 2005). The existence of an age-related significant difference in feathers and swabs was also statistically investigated by