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Recombinant hemagglutinin glycoproteins provide insight into binding to host cells by H5 influenza viruses in wild and domestic birds

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Abstract

Clade 2.3.4.4, H5 subtype highly pathogenic avian influenza viruses (HPAIVs) have caused devastating effects across wild and domestic bird populations. We investigated differences in the intensity and distribution of the hemagglutinin (HA) glycoprotein binding of a clade 2.3.4.4 H5 HPAIV compared to a H5 low pathogenic avian influenza virus (LPAIV). Recombinant HA from gene sequences from a HPAIV, A/Northern pintail/Washington/40964/2014(H5N2) and a LPAIV, A/mallard/MN/410/2000(H5N2) were generated and, via protein histochemistry, HA binding in respiratory, intestinal and cloacal bursal tissue was quantified as median area of binding (MAB). Poultry species, shorebirds, ducks and terrestrial birds were used. Differences in MAB were observed between the HPAIV and LPAIV H5 HAs. We demonstrate that clade 2.3.4.4 HPAIV H5

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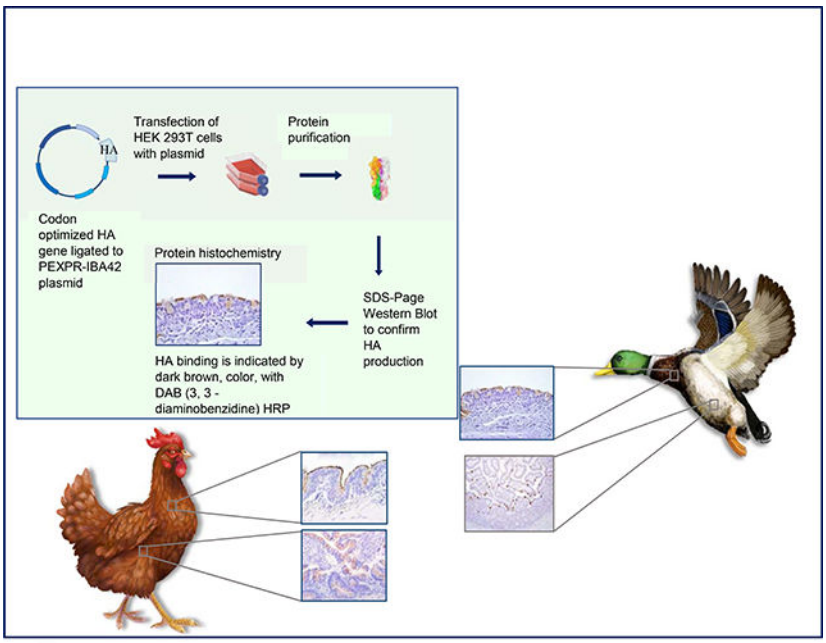
HA has a broader tropism across a variety of bird species compared to the LPAIV H5 HA. These findings support published results from experimental trials, and outcomes of natural disease outbreaks with these viruses.

Graphical Abstract



Avian influenza virus Hemagglutinin (HA) glycoprotein tissue binding is visible by dark brown staining using 3,3-diaminobenzimidine (DAB) HRP with protein histochemistry. Extensive, HA binding is observed in the ciliated epithelial cells of the trachea and the goblet cells of the ceca of both mallards and chickens.

Keywords

highly pathogenic avian influenza virus; low pathogenic avian influenza virus; H5N2; recombinant protein; hemagglutinin; tropism; waterfowl; poultry; terrestrial birds

Introduction

Highly pathogenic avian influenza viruses (HPAIV) continue to pose a significant threat to the global poultry industry. Yearly, millions of dollars are allocated to the surveillance and prevention of outbreaks and pandemics(Swayne et al., 2013). Low pathogenic avian influenza viruses (LPAIV) circulate in wild bird reservoirs, mainly of the orders *Anseriformes* and *Charadriiformes,* with asymptomatic to mild clinical disease and decreased body weight (Franca et al., 2012; Franca and Brown, 2014; Kuiken, 2013). Typically, HPAIV do not establish reservoirs in wild birds (Spackman, 2009). When LPAIVs of subtype H5 and H7 circulate in poultry, mutations can occur in the HA gene to result in HPAIV(Suarez, 2016). In 1997, an outbreak of HPAIV caused high mortality in poultry, wild birds, and humans in Guangdong, China. The ancestor of this virus was traced to a

virus located in Guangdong, China in 1996. Since then, this lineage of viruses, now referred to as the Goose/Guangdong lineage (Gs/GD) has expanded and caused numerous epidemics across continents (Webster et al., 2006). Viruses of the Gs/GD lineage are unique, because some of the viruses from this group are known to cause mortality in wild birds (Lee et al., 2017). These viruses have evolved into clades 0–9 and several other subclades, including clade 2.3.4.4 viruses, which gained entry into the United States in 2014, presumably through movement of wild birds (Berhane et al., 2016; Bevins et al., 2016b; Ip et al., 2015; Lee et al., 2018). Clade 2.3.4.4 viruses have further been classified into 4 groups, A-D, of which group A has been identified in the United States, Canada, China, Korea, Japan, and Europe (Lee et al., 2017). These viruses are documented to have undergone reassortment in wild birds in Alaska, prior to the H5 clade 2.3.4.4 outbreaks in the United States (Hill et al., 2017). Furthermore, reassortment of clade 2.3.4.4.H5N8 viruses from intercontinental spread, with LPAIV in North America has resulted in the generation of H5N8, H5N2 and H5N1 reassortant viruses, which are collectively referred to as H5Nx viruses (Bertran et al., 2017a; Hill et al., 2017; Krauss et al., 2016; Lee et al., 2017). The resulting epidemic caused by HPAIV H5N2 viruses led to the culling of roughly 49 million chickens and turkeys across the United States. This outbreak resulted in death in waterfowl species, and some raptors, including Cooper’s hawk, Bald eagle, Peregrine falcon and Gyrfalcon (Ip et al., 2016; Ip et al., 2015).

Surveillance studies on the prevalence of avian influenza viruses (AIV) in wild birds in North America and Northern Europe have found that ducks, mainly mallards, gulls, and shorebirds consistently have increased prevalence of infection with LPAIV compared to other terrestrial and aquatic birds. (Alexander, 2000; Brown et al., 2006; Ellstrom et al., 2008; Munster et al., 2007; Wallensten et al., 2007). Studies on the dynamics of influenza virus infection in these hosts, suggest that these birds have differences in susceptibility to infection, which is dependent on factors such as prior exposure, host susceptibility, and contact with the viruses when they arrive at different sites of migration (Bevins et al., 2016a; Brown et al., 2012; Lewis et al., 2015; Maxted et al., 2012). Furthermore, differences in the routes of shedding between species of birds have also been well established from experimental studies in mallards, redhead ducks, and laughing gulls (Costa et al., 2011). In one study, it was found that mallard ducks preferentially shed virus through the cloacal route while wood ducks, redhead ducks and laughing gulls had predominant shedding via the oropharyngeal route (Costa et al., 2011). Additionally, it has been demonstrated that in mallards experimental infections with Gs/GD viruses have resulted in higher virus shedding through the oropharyngeal route compared to the cloacal route (DeJesus et al., 2016). These findings demonstrate that there are variations in the routes of viral shedding, even among species of the same order, and may be reflective of differences in host receptor binding and infectivity of influenza virus in these species.

A major determinant for influenza virus infection, replication, and shedding is the attachment of the Hemagglutinin (HA) glycoprotein to host cell receptors (García-Sastre, 2010). The receptor binding site of the HA plays a major role in host specificity, and single amino acid substitutions have been reported to alter binding to host cells (Glaser et al., 2005; Naeve et al., 1984). Moreover, amino acid substitutions in or near the receptor binding site

can modulate the ability of the HA to bind to different glycans *in vitro* (Obadan et al., 2019; Santos et al., 2019).

Tropism studies focus on the ability of a virus to infect specific cells, tissues or organs (Klopfleisch et al., 2006; Laudert et al., 1993; Naeve et al., 1984; Pantin-Jackwood and Swayne, 2009a; Swayne and Pantin-Jackwood, 2006). Influenza virus strains that are well-adapted to humans generally have a preference to sialic acid that are linked through an α2,6-linkage to cell-surface-associated glycan chains, thereby leading to a preference for binding to the cells of the upper respiratory tract in humans. Whereas, avian-adapted viruses generally have a preference for α2,3-linked sialic acids (Kumlin et al., 2008; Matrosovich et al., 2015; Pillai and Lee, 2010; Skehel and Wiley, 2000).

Binding of the HA onto host cells is the first step of the viral infection cycle. Thus, the binding properties of HA can have a large impact on tropism and on disease outcomes in infected hosts. In avian and mammalian tissues, virus and viral protein binding has been previously studied using virus and protein histochemistry to aid in understanding of the pathogenesis and host range of respiratory viruses such as influenza viruses (Ambepitiya Wickramasinghe et al., 2015; Eriksson et al., 2018; Eriksson et al., 2019; Harrison, 2015; Leyson et al., 2016; van Riel et al., 2007; Wickramasinghe and Verheije, 2015). To facilitate understanding of the complexity of tissue tropism across different bird species to highly pathogenic and low pathogenic influenza viruses, recombinant HA glycoproteins were produced using HPAIV and LPAIV H5 gene sequences of viruses originally isolated from a Northern pintail and a Mallard duck, respectively. These recombinant proteins were used for protein histochemistry, wherein HA protein binding is observed as dark brown staining in tissues, which was quantified as median area of binding (MAB). The aim of this study was to provide baseline information of HPAIV and LPAIV H5 HA tissue attachment across bird species.The particular HPAIV and LPAIV HAs were selected to evaluate the differences in HA protein binding between the index virus of the clade 2.3.4.4a and a LPAIV of the same subtype, which was previously used in studies involving waterfowl and poultry species. Birds from the order *Galliformes, Anseriformes, Acciptriformes,Charadriiformes* and *Columbiformes* were selected to better understand differences in susceptibility to infection and pathobiology of H5 AIV. Particularly the wild waterfowl and raptor species were selected because the HPAIV caused death in birds from these taxonomical orders.

Results

Protein histochemistry for both HPAIV and LPAIV recombinant H5 HA's was performed on a total of 13 species of birds from the orders *Charadriiformes* (Ring-billed gull *(Larus delawarensis),* Laughing gull *(Leucophaeus atricilla),* Sanderling *(Calidris alba),* Short billed dowitcher *(Limnodromus griseus)); Anseriformes* (Mallard duck *(Anas platyrhynchos),* Pekin duck *(Anas platyrhynchos domesticus),* Commercial Muscovy duck *(Cairina moschata),* Canada goose (*Branta canadensis)); Galliformes* (Broiler chicken *(Gallus gallus domesticus),* SPF leghorn layer chicken (*Gallus gallus domesticus*), turkey *(Meleagris gallopavo*)); *Accipitriformes* (Cooper's hawk *(Accipiter cooperii),* Bald eagle *(Haliaeetus leucocephalus),* Golden eagle *(Aquila chrysaetos)* and *Columbiformes* (domestic pigeon *(Columba livia domestica)).* Tissues evaluated included trachea, lung, duodenum/jejunum,

ileum/ceca, and cloacal bursa. HA distribution and intensity of binding were expressed as Median Area of Binding (MAB) score; HPAIV results are presented in Table 1 and Figures 1–3, and LPAIV HA findings are presented in Table 2 and Figures 4–6.

Highly pathogenic avian influenza recombinant HA protein binding

Charadriiformes—Protein histochemistry was performed on tissues from Ring-billed gulls, Laughing gull, Sanderling and Short billed dowitcher. These findings are summarized in Table 1, which shows the HPAIV MAB and distribution in the respiratory and intestinal tract across evaluated avian species.

The HPAIV H5 HA bound to ciliated epithelial cells and goblet cells of the respiratory tract of *Charadriiformes.* All birds of this taxonomical order had low MAB in the trachea, except the Laughing gull, which had no detectable HA binding to this tissue (Table 1). In the lung, very high MAB scores were observed only in the secondary and tertiary bronchi (parabronchi) of the Ring-billed gulls, while high MAB scores were seen in the parabronchi of the lung of Laughing gulls. Sanderling and Short billed dowitcher had low and moderate HA binding in the lung, respectively (Figure 1).

In the intestine, most members of this order had low HPAIV H5 HA MAB scores in the duodenum and jejunum (upper intestine) except the Short billed dowitcher, which lacked small intestinal HA binding. The Laughing gull had very high MAB scores in the enterocytes and goblet cells of ileum and ceca, while the Ring bill gull had no detectable HA binding in ileum and ceca. Other birds in this order (Sanderling and Short billed dowitcher) had low to moderate MAB in ileum/ceca (Figure 1.10). The cloacal bursa was only evaluated in the Laughing gull and very high MAB scores were detected in bursal epithelial cells. Due to small sample size of birds in this taxonomical order, statistical analyses were not performed.

Anseriformes—Protein histochemistry was performed on tissues from Mallards, Pekin ducks, Commercial Muscovy ducks and Canada geese. These findings are summarized in Table 1 and Figures 1 and 3, which show the HPAIV MAB and distribution in the respiratory and intestinal tract across evaluated avian species.

In the trachea, variation in HA binding was observed, with low to very high MAB scores. HA binding was detected in ciliated epithelial cells and goblet cells of Muscovy ducks, Pekin ducks and Canada geese; however, the Mallards had low tracheal MAB scores. Statistically significant differences were not noted in the tracheal HA binding of birds of this taxonomical order using the Kruskal-Wallis test. In the lung, birds of this order had very high MAB scores, with HA bound to ciliated epithelial and goblet cells (Figure 1.2, 1.5), and differences among species were not statistically significant. Furthermore, the overall respiratory tract MAB score for Canada geese was derived by adding the MAB scores for trachea and lung. Generally, there were no significant differences in the overall HPAIV HA MAB scores in the respiratory tract between species of this taxonomical order.

In the duodenum and jejunum, a statistically significant difference was noted in HPAIV H5 HA binding (Kruskal-Wallis test, p=0.039). Using Dunn's post-test, the Canada geese had

significantly higher MAB scores compared to Pekin ducks. While most birds had negligible HA binding in the duodenum and jejunum, the Canada geese had very high MAB scores, with HA biding to the cytoplasm of goblet cells and enterocytes (Figure 1.9). In the ileum and ceca, the Mallards had very high MAB scores with binding to enterocytes and goblet cells, while low to moderate scores were detected in Muscovy ducks, Pekin ducks and Canada geese. Statistically significant differences were not noted in the lower intestine among species of *Anseriformes.* However, when the small and lower intestinal scores were combined, a statistically significant difference was observed between the Muscovy and Mallard ducks (Kruskal-Wallis test p=0.015), with Mallards having statistically higher combined MAB scores compared to Muscovy ducks. No significant pairwise differences were observed among the other species of this taxonomical order. Differences were seen in the location of HPAIV HA binding in bursa, which bound to bursal follicular epithelial cells in Mallards, compared to follicular epithelial cells and lymphocytes within the medullary region of bursal follicles in Canada geese (data not shown).

Galliformes—Protein histochemistry was performed on tissues from broiler chickens, SPF layer chickens and commercial turkeys. These findings are summarized in Table 1 and Figures 2 and 3, which shows the HPAIV MAB and distribution in the respiratory and intestinal tract across evaluated avian species.

Turkeys had high to very high MAB scores, with binding to ciliated epithelial cells and goblet cells of the trachea and lung compared to the broiler chickens and SPF layer which had lower MAB (Figure 2); however, differences in HA binding in trachea and lung among birds of this group were not statistically significant. In contrast, when the overall values of HA binding to respiratory tract tissues (trachea and lung) were combined, a statistically significant difference was seen, with turkeys having higher MAB scores compared to broilers (Kruskal-Wallis test, p=0.04).

In the intestinal tract, the duodenum of SPF layers had very high MAB scores, with attachment to enterocytes of the duodenum and jejunum. Other birds in this taxon had no HA binding to low binding. A statistically significant difference in binding was seen, with SPF layers having significantly greater HPAIV HA binding to duodenum and jejunum compared to broiler chickens (p=0.043). In the ileum and ceca, high to very high MAB scores, with binding to enterocytes and goblet cells was detected in SPF layers and turkeys. The combined surface area of HPAIV H5 HA attachment to the intestinal tract (small intestine and lower intestine) showed significant differences in HA binding among birds of this taxon (p=0.04), with both SPF layers and turkeys having significantly greater surface area of HPAIV HA binding compared to broiler chickens, which had no binding. In the cloacal bursa, only the turkeys had HPAIV HA binding to bursal follicular epithelial cells. There were no statistically significant differences in HA binding to cloacal bursa among species of this taxonomic group.

Accipitriformes—Protein histochemistry was performed on tissues from a Cooper’s hawk, Bald eagle and Golden eagle. These findings are summarized in Table 1 and Figure 3. Birds of this order had low MAB in the trachea, with HA attachment to the ciliated epithelial cells and goblet cells. In the lung, the Cooper’s hawk and Golden eagle had very high MAB,

however, no detectable HA binding was noted in the lung of the Bald eagle. In the intestine, the Bald eagle had moderate MAB score, with HA binding to enterocytes and goblet cells of the duodenum and jejunum. In the ileum and ceca, low levels of HA binding to enterocytes and goblet cells were noted in Cooper's hawk and Bald eagle. Statistical analyses were not performed for birds in this group due to the small sample sizes.

Columbiformes—The pigeon had low MAB scores, in the trachea, lung, ileum and ceca.

Low pathogenic avian influenza recombinant HA protein binding

Charadriiformes—Protein histochemistry using LPAIV H5 HA protein was performed on tissues from Ring-billed gulls, Laughing gull, Sanderling and Short billed dowitcher. These findings are summarized in Table 2.

All birds in this taxonomical order had no LPAIV HA binding in the trachea. In the lung, low levels of HA attachment to epithelial cells was seen in Ring-billed gull, Sanderling, and Short billed dowitcher, while the Laughing gull had no HA binding. In the small and lower intestines, MAB scores were low, with HA binding to enterocytes and goblet cells only detected in the Sanderling and Short-billed dowitcher. HA binding in cloacal bursa was only assessed in Laughing gulls, and no HA binding was present. Due to the small sample size, statistical analyses were not performed on birds in this group.

Anseriformes—Protein histochemistry using the LPAIV H5 HA was performed on tissues from Mallards, Pekin ducks, Commercial Muscovy ducks and Canada geese. These findings are summarized in Table 2 and Figures 4 and 6.

In the respiratory tract, Mallards had moderate to very high LPAIV HA MAB binding scores to epithelial cells compared to low MAB detected in Muscovy ducks, Pekin ducks and Canada geese. A statistically significant difference in HA binding to tracheal epithelial cells was noted between Mallards and Muscovy ducks (Kruskal-Wallis test, p=0.032, Dunn's test). When values for respiratory tract tissues were combined (trachea and lung), a significant difference in the MAB scores was evident, with Mallards having significantly higher MAB compared to Muscovy ducks (Kruskal-Wallis, p=0.02, Dunn's test).

In the intestine, LPAIV HA binding was detected in Mallards and Canada geese, while Pekin ducks and Muscovy ducks had no binding. A statistically significant difference in LPAIV HA binding to duodenum and jejunum among species was found (Kruskal-Wallis test, p=0.039), however, no significant differences were noted after pairwise comparison using the Dunn's test. In the ileum and ceca, statistically significant differences in median HA binding was also found (Kruskal-Wallis test, p=0.015), and using the Dunn's post-test, Mallard ducks had significantly higher MAB compared to the Muscovy ducks. When the areas of HA intestinal HA binding were combined for each species (small intestine and lower intestine), a statistically significant difference was noted (Kruskal-Wallis, p=0.04), however, no pairwise differences were observed. Only Canada geese and Mallards had low LPAIV HA binding detected in cloacal bursa epithelial cells. A statistically significant difference was noted in the bursal MAB among birds of this taxon, with Canada geese having a significantly higher MAB compared to Pekin ducks (Kruskal-Wallis, p=0.03).

Galliformes—Protein histochemistry using the LPAIV HA was performed on tissues from broiler chickens, SPF layer chickens, and commercial turkeys. These findings are summarized in Table 2 and Figures 5 and 6.

In the respiratory tract, turkeys had the highest MAB to ciliated epithelial cells. A significant difference in LPAIV HA MAB to the trachea was determined using the Kruskal-Wallis test (p=0.027), with turkeys having significantly higher MAB scores compared to broiler chickens. In the lung, only the turkey had very high MAB scores with binding to epithelial cells of the parabronchi. However, no statistically significant differences were noted among LPAIV H5 HA MAB scores in lungs among birds of this taxon. Moreover, no significant differences were found when LPAIV HA MAB scores in the trachea and lung were combined.

In the intestine, only turkeys had LPAIV HA binding detected, with low MAB scores. Statistically significant differences in the MAB to duodenum and jejunum (Kruskal-Wallis test, p=0.039), as well as to ileum and ceca (Kruskal-Wallis test, p=0.012) were found, with turkeys having significantly higher LPAIV H5 HA MAB scores compared to SPF layer chickens. With combined values for the small intestine and lower intestine, statistically significant differences were found (Kruskal-Wallis test, p=0.04), with turkeys having significantly greater LPAIV HA binding compared to broilers. Only SPF layers and turkeys had LPAIV HA attachment to bursal epithelial cells, with low MAB scores. No significant differences in LPAIV HA binding to cloacal bursa were found among *Galliformes.*

Accipitriformes—Protein histochemistry using the LPAIV HA was performed on tissues from Cooper's hawk, Bald eagle and Golden eagle. These findings are summarized in Table 2 and Figure 6.

No discernible LPAIV H5 HA binding was present in the trachea of the Cooper's hawk and only low levels of HA binding was seen in the trachea of the Bald eagle. A small area of HA binding was noted in the tertiary bronchi of the lung of the Bald eagle and Cooper's hawk, which correlated with a low MAB score. LPAIV HA binding to the intestine of these species was overall low. Statistical analyses were not performed for this taxonomic order due to small sample size.

Columbiformes—Only the respiratory tract was evaluated. The pigeon had low MAB scores to epithelial cells in trachea and tertiary bronchi of the lung.

Discussion

Wild aquatic birds are important reservoirs of LPAIV, and to date, 16 of the 18 HA subtypes have been documented from wild birds (Maxted et al., 2012; Stallknecht and Shane, 1988; Stallknecht, 2003). Commercial poultry have been infected with numerous influenza virus HA subtypes including H5 LPAIV which can acquire mutations in the HA to become highly pathogenic (Pantin-Jackwood and Swayne, 2009a). Such HPAIV pose threats to wild bird populations, the commercial poultry industry, pet birds and potentially human health. While there has been previous research on influenza virus tropism in tissues of humans and poultry

species, (Ambepitiya Wickramasinghe et al., 2015; Höfle et al., 2012; Klopfleisch et al., 2006; Nicholls et al., 2007; Slemons and Swayne, 1995) few studies have focused on evaluating influenza virus HA tissue tropism combining both wild and domestic bird species(Ambepitiya Wickramasinghe et al., 2015; Bingham et al., 2009; Bröjer et al., 2015; Chaise et al., 2014; Kuiken, 2013; Kumlin et al., 2008; Pillai and Lee, 2010; Slemons and Swayne, 1995; Wickramasinghe et al., 2011). Additionally, there is a dearth of knowledge of influenza virus HA tissue tropism in shorebirds, and raptor species. Here, LPAIV and HPAIV H5 HA tissue tropism is described in 13 avian species, with specific focus on the respiratory, intestinal tract and cloacal bursa to aid in understanding H5 influenza virus pathobiology and transmission in these species.

For HPAIV, tropism to the respiratory tract was noted mostly in members of *Anseriformes* (ducks and geese), *Charadriiformes* (gulls and shorebirds), *Galliformes* (chickens and turkeys), and *Accipitriformes* (eagles and hawks). Of all evaluated species, Mallard ducks had the highest overall HPAIV MAB score. This is possibly due to the high level of adaptation of this viral HA to mallard ducks. Mallards are the wild predecessor of Pekin ducks which are domesticated, and similar distribution of predominance of α2,3-linked sialic acid receptors in the respiratory and intestinal tract of Mallard ducks and Pekin ducks have been revealed (Franca et al., 2013; Kimble et al., 2010; Kuchipudi et al., 2009). Studies have shown that Mallards have high infectivity and transmissibility of HPAIV, when infected with H5N2 viruses of clade 2.3.4.4 and clade 2.2 (DeJesus et al., 2016; Pantin-Jackwood et al., 2016). Using mallards, and a clade 2.3.4.4 A/Northern pintail/Washington/40964/2014 virus, staining for H5N2 HPAIV viral antigen was abundant in the trachea and lung. In that study, moderate to high titers of virus was shed via the oropharyngeal route, which agrees with our HPAIV H5 HA binding results. However, in that study, no positive cells were detected in the intestine and the use of quantitative real-time RT-PCR demonstrated that lower viral titers were shed through the cloacal route (Pantin-Jackwood et al., 2016).

Canada geese had the second highest overall HPAIV HA MAB scores compared to all evaluated birds. This finding may partially explain the susceptibility of Canada geeseto HPAIV infection of this clade and subtypeduring the HPAIV H5N2 outbreak in North America(Lee et al., 2018). Experimental trials involving Canada geese have demonstrated high susceptibility of this species to HPAIV infection (Pasick et al., 2007). Immunohistochemistry detected HPAIV antigen distribution in cells of the lung, and moderate to numerous immunopositive cells in the intestine of geese (Nemeth et al., 2013; Pasick et al., 2007).

Using the HPAIV HA, the Muscovy duck had the highest MAB scores in the respiratory tract. While Pekin and Mallard ducks are more commonly encountered in North America, Muscovy ducks contribute with a large percentage of reared ducks in Asia and South America (Kameshpandian et al., 2018). It has been shown that Muscovy ducks respond differently to infection with HPAIV compared to other domestic ducks such as Pekins and Mallards, and consequently, Muscovy ducks succumbed to experimental infection earlier than other types of domestic ducks (Cagle et al., 2011; Guionie et al., 2010; Pantin-Jackwood et al., 2013; Pantin-Jackwood and Suarez, 2013; Phuong et al., 2011). Moreover, Muscovy ducks were reported to shed high levels of the virus via the oropharyngeal routes

(Pantin-Jackwood et al., 2013), this can be related to high levels of viral binding, as elucidated by HA binding in this present study. Conversely, high levels of HPAIV HA binding was not seen in the intestinal tract of Muscovy ducks in our study. When Phuong du *et al.* evaluated the levels of H5N1 HPAIV virus titers in tissues of Muscovy ducks that died after infection, the birds had variable levels of virus in the intestine, ranging from absent to high, however, the titers in the lung and trachea were higher and more consistent (Phuong et al., 2011). Another study investigated the virus titers shed by Muscovy ducks via the cloacal and oropharyngeal routes, using clade 2.3.4.4 viruses that were isolated in Japan and a clade 2.2 virus of French origin, respectively (Guionie et al., 2010). Higher titers of virus were found in the trachea and oropharyngeal route, compared to the intestinal tract and cloacal route of shedding, which supports the findings of this present study (Uchida et al., 2019). Differences in HPAIV H5 HA binding could be due expression of lower levels of α2,3-linked sialic acid receptors in the intestinal tract of Muscovy ducks compared to the respiratory tract. To the authors' knowledge, sialic acid receptor distribution in tissues of Muscovy ducks has not yet been described.

Pekin ducks are the most common commercial duck species reared worldwide and have been shown experimentally to be very susceptible to H5 HPAIV (Cagle et al., 2011; Löndt et al., 2010; Pantin-Jackwood et al., 2013; Pantin-Jackwood et al., 2017; Pantin-Jackwood et al., 2012; Rodenburg et al., 2005). The sialic acid repertoire in the respiratory tract and intestinal tract also demonstrates predominance of the avian α2,3-linked sialic acid receptor, similar to Mallards (Kimble et al., 2010; Kuchipudi et al., 2009). Here, Pekin ducks had very high MAB scores in the respiratory tract and moderate scores in the intestine, which correlates with the high expression of α2,3-linked sialic acid receptor facilitating virus binding (Kimble et al., 2010; Kuchipudi et al., 2009). Moreover, it has been described that experimentally infected Pekin ducks shed high titers of virus via the oropharyngeal route (Pantin-Jackwood et al., 2013). Additionally, using quantitative real-time RT-PCR (qRRT-PCR), there were high titers of virus in the lung (Pantin-Jackwood et al., 2013), which supports our finding of very high MAB scores in the lung of these birds. Interestingly, despite the high level of expression of the avian receptor in the intestine and moderate levels of HA binding seen in the present study, only low titers of virus were shed via the cloacal route experimentally (Pantin-Jackwood et al., 2013). This disparity could be due to the role of the innate immunity of Pekin ducks, the mucosal microenvironment and microbiome of the intestinal tract. .

Among *Charadriiformes,* Laughing gulls had very high HPAIV MAB scores in the ileum, ceca and cloacal bursa, which indicate these sites can be regions of viral attachment of H5 HPAIV in this species. One study found that Laughing gulls were highly susceptible to H5N1 HPAIV and had prolonged viral shedding through the cloaca (Brown et al., 2006) which is supportive of our findings. Mallards were also examined in that study, and the shedding time was found to be 1 day from cloaca, which does not completely support the high levels of MAB found in the mallard intestine of the present study. Additionally, examination of the distribution of sialic acid receptors showed that Laughing gulls had strong expression of α2,3-linked sialic acid in the respiratory and intestinal tracts, which correlates with HA binding to these tissues in our study (Franca et al., 2013).

In shorebirds, intestinal tropism of H5 HPAIV HA may suggest foraging activity as a route of infection. Examination of the sialic acid receptor distribution in the Sanderling demonstrated high expression of α2,3-linked sialic acid in the respiratory and intestinal tracts as well as α2,6-linked sialic acid in the respiratory tract (Franca et al., 2013). The Short billed dowitcher and Sanderling have not been reported to be infected with HPAIV in nature.

Among gallinaceous poultry species, turkeys and SPF layer chickens had higher overall HPAIV HA MAB scores in the respiratory tract compared to broilers. The strong tropism of the H5 HPAIV HA to the respiratory tract of SPF layers and turkeys aids in explaining the susceptibility of commercial turkeys and layer chickens to clade 2.3.4.4 of H5 HPAIV during the 2014 outbreak, which caused the culling of millions of poultry in several poultry farms in the Midwest region of the U.S (Arruda et al., 2016). Turkeys specifically, were found to have high levels of virus shed via the respiratory and cloacal routes experimentally (Spackman et al., 2016). These findings could be explained by an increased tropism of this H5 HA to the respiratory and intestinal epithelium of turkeys. It has been previously noted that some clade 2.3.4.4 viruses, are better adapted to poultry species, such as the White Leghorn chicken (DeJesus et al., 2016; Swayne and Slemons, 2008).. Additionally, turkeys were among the first group of birds to become infected during the introduction of the index virus to North America (Bertran et al., 2017b; Lee et al., 2017). Furthermore, it has been demonstrated, that turkeys had both α2,3-linked and α2,6-linked sialic acid receptors, in the trachea, lung and large intestine (Kimble et al., 2010). Moreover, several studies have shown that viruses of waterfowl origin are more efficient for transmission in turkeys compared to domestic chickens (Pillai et al., 2010b). The results in this study, however, do not explain the finding that broilers and turkeys had a similar mean infectious dose and mean death time, compared to turkeys in another study (Bertran et al., 2016). While the present study cannot prove infection or replication of the virus, an extensive surface area of HA binding has been detected in these tissues, which is a major prerequisite for infection. The combination of high levels of influenza receptors in the respiratory tract, and increased HA tropism to tissues of turkeys reinforces that these viruses pose a formidable risk to turkey flocks.

In *Accipitriformes,* moderate HPAIV MAB scores in the intestinal tract of the Bald eagle, may support feeding activity in raptors as a route of infection of these viruses, as was proposed in the 2014 outbreak where gyrfalcons died after consuming an infected American wigeon(Ip et al., 2015). In addition, low to moderate MAB scores in the respiratory tract of the Golden eagle, Cooper's hawk and Bald eagle also suggest inhalation as a possible route of infection for these species, during consumption of infected carcasses, or if in contact with other infected birds at aviaries or wildlife rehabilitation centers.

For the LPAIV, evaluation of H5 LPAIV HA binding revealed significantly higher HA binding to the respiratory tract of Mallards and turkeys. As previously mentioned, Mallards are the natural host species for LPAIV influenza viruses and are an important species in the ecology and transmission of LPAIVs. In studies conducted by Franca *et al.,* high levels of α2,3-linked sialic acid receptors were present in the respiratory tract, lower intestine and cloacal bursa of Mallards. (Franca et al., 2013; Franca et al., 2012). The presence of high levels of sialic acid receptors in tissues of Mallards can be linked to greater amounts of

LPAIV HA binding, infection and viral replication leading to shedding of large quantities of virus via both the oropharyngeal and cloacal routes (Franca et al., 2013; Franca et al., 2012). However, with experimental infections, Mallards preferentially shed higher titers of H5 LPAIV via the cloacal route, but in one study, small numbers of cells positive for LPAIV antigen were also detected in the respiratory tract (Franca et al., 2012).

LPAIV HA binding in Pekin ducks was low in the respiratory tract, and there was generally no binding in the intestinal tract, suggesting strong preference of this LPAIV H5 HA to the natural host it was isolated from, the Mallard. In reference to the distribution of sialic acid receptors in Pekin ducks, predominantly α2,3 sialic acid receptors were present in the small and lower intestine of Pekin ducks (Kuchipudi et al., 2009). Thus, it was expected that there would be similar HA binding to what was seen in the wild counterpart, the Mallard (Kuchipudi et al., 2009). Additionally, in another study it was found that Pekin ducks are highly susceptible to H5 LPAIV infections and shed the virus via the cloacal route (Chaise et al., 2014). This contrast in findings could be related to differences in glycosylation of the host tissues and the glycosylation of the H5 HAs used in those studies compared to the present study (Imai and Kawaoka, 2012; Matrosovich et al., 1999). At this time, there is limited knowledge of the variations of the glycosylation of different tissues of avian species. In humans, diet and genetics have been suggested to play a key role in the expression and distribution of intestinal glycans (Kavanaugh et al., 2015; Lowe and Marth, 2003). Furthermore, differences in glycosylation may explain the low levels of LPAIV H5 HA binding in Muscovy duck tissues (Pantin-Jackwood et al., 2007; Pantin-Jackwood and Swayne, 2009b; Spackman et al., 2010; Spackman et al., 2009; Spackman et al., 2007). In the present study, Canada geese had very low and sometimes no binding of LPAIV HA in the respiratory and intestinal tissues. In one study, evaluating the role of poultry in the spread of novel LPAIV H7 viruses, it was found that Muscovy ducks are able to become infected and shed virus via the oropharyngeal and cloacal route. It should be noted, however, that the virus used in that study was of H7 subtype, compared to the H5 HA used in the present study. (Pantin-Jackwood et al., 2014)

Among shorebirds, most birds had negligible LPAIV HA binding in tissues, except for the Ring-billed gull, which had low binding in the lung. In a recent study, 1,346 Ring-billed gulls were used to evaluate the preferred route of influenza viral shedding. It was found, that regardless of the age of bird, the predominant route of viral shedding in Ring-billed gulls was the oropharyngeal, as opposed to the cloacal route (Froberg et al., 2019). However, it should be noted that some of the viruses used in that study, were highly adapted to gulls, and therefore, comparisons cannot be made as the HA used in the present study is adapted to mallards.

Among *Galliformes,* turkeys had the highest LPAIV HA MAB scores in the respiratory and intestinal tracts. Tropism of LPAIV to the ileum and ceca of turkeys supports the finding by Spackman *et al.* of LPAIV infection and cloacal shedding and the high susceptibility of turkeys to infection with these viruses compared to chickens and ducks as reported by Pillai *et al.* (Pillai et al., 2010a; Spackman et al., 2010). Birds of the order *Accipitriformes* and *Columbiformes* had only low LPAIV binding in all tissues, which correlates with the

relatively rare occurrence of LPAIV detection from these birds, especially pigeons (Bosco-Lauth et al., 2019).

While the glycan diversity in the host and the 3-dimensional conformation of the HA molecule can influence HA binding in vivo, it is important to note that HA binding is not the only determinant factor of AIV infection. Other important factors such as the immune response of the host, the ability of the virus to escape the innate immune response, the presence of host cellular proteases and the ability of these enzymes to cleave the HA proteins, as well as mutations in the PB2 and PA viral proteins can affect the ability of the virus to enter and replicate in tissues. Moreover, the activity of the neuraminidase to release newly assembled virions and the ability of the virus to disseminate within the vasculature to multiple organs can also influence viral tissue tropism (Kuiken et al., 2006; Lion et al., 2018; Uchida et al., 2019).

Conclusions

Our findings demonstrate significant differences in the HA binding between highly pathogenic and low pathogenic avian influenza H5 HA across avian species. Furthermore, differences in tissue binding among LPAIV and HPAIV HA were seen, with the HPAIV H5 HA having abundant binding to respiratory and intestinal tract tissues of several species of wild waterfowl and poultry, such as Mallards, Canada geese, SPF chicken layers and turkeys. Tissue binding to the trachea and ceca with the LPAIV H5 HA was predominantly seen in Mallards and turkeys. Overall, most of these findings are supportive of previously published results from experimental trials, as well as outcomes of natural disease outbreaks with H5 LPAIV and HPAIV involving wild and poultry bird species.

Materials and Methods

Production of recombinant HA protein

HA gene sequences from the following viruses were obtained from GenBank: Influenza A virus A/Northern pintail/Washington/40964/2014(H5N2), highly pathogenic, Northern Pintail isolate) and Influenza A virus A/mallard/MN/410/2000(H5N2), low pathogenic, mallard isolate). Amino acid positions 18–564 were taken from these sequences and were codon-optimized. A GCN4 trimerization domain and StrepTag were appended to the HA gene sequence at the carboxy terminal end (Leyson et al., 2016; Wickramasinghe et al., 2011).

The HA gene constructs were cloned into the mammalian expression vector PEXPR-IBA42 using restriction enzyme digestion and ligation. Sequential digestion was performed using restriction enzymes Nhel and Bsal and then the digested plasmids were purified using PCR purification kit (GeneJet). The DNA fragments were electrophoresed on agarose gel and desired bands were purified with a gel purification kit (GeneJet). A ligation reaction was performed by combining digested fragments in a 1:3 ratio (vector: insert) with T4 DNA ligase (Fermentas/Thermo Scientific). The ligation mixture was incubated at 4°C overnight and then introduced to chemically competent *E. coli* cells (JM109, Promega), using heat shock method per the manufacturer's protocol. The transformed cell mixture was plated onto

Luria Bertani (LB) agar plates with 100μg/ml Carbenicillin, to select for pure bacterial colonies. An overnight culture of LB broth and 100μg/ml Carbenicillin, was placed in shaking incubator and then used for plasmid purification using Maxiprep kit (Zymo Research), according to the manufacturer's directions.

Purified plasmids were, thereafter, filter sterilized using a 0.22μm syringe filter. The plasmids were sequenced using Sanger sequencing at the Georgia genomics facility.

The expression vectors containing the HA domains were then transiently transfected into human embryo kidney cells (HEK 293T cells). The cell culture media was harvested 5–6 days post transfection and purified using StrepTactin affinity chromatography (IBA Lifesciences). Nanodrop (Thermo Fisher Scientific) was used to quantify proteins produced. SDS-PAGE and Western blot using StrepTactin-HRP (IBA Lifesciences) was performed on cell culture media as well as purified proteins to confirm expression of recombinant HA proteins. (Ambepitiya Wickramasinghe et al., 2015; Leyson et al., 2016; Wickramasinghe et al., 2011)

Construction of tissue microarrays with formalin fixed paraffin embedded tissues

Sections of respiratory tract and intestinal tract were collected from freshly dead, oreuthanized birds. Tissues were fixed in 10% neutral-buffered formalin for no longer than twenty-four hours, and then processed routinely for histopathology. Tissues were examined for autolytic changes macroscopically and microscopically. Some changes that disqualified sampling was discoloration of the intestinal tract, sloughing of the mucosa on gross examination and pale discoloration of internal organs such as heart, liver, spleen. On microscopic examination with routine stains for histopathology, signs such as cell sloughing, and loss of villi, lysis of erythrocytes, and loss of intestinal brush border were evaluated. Any tissues with signs of autolysis were excluded from the study. Tissue microarrays were constructed using an Arraymold (IHC World Life science products and services). Briefly, tissue blocks containing respiratory and digestive tract embedded along the long axis of the tissue(donor blocks) and a 5mm, biopsy punch was used to remove desired tissues from the donor block to be placed into the recipient block paraffin block composed of 5mm empty cores. For the biopsy punches of the trachea and intestine, the specimens were oriented transverse to the arbitrary axis. Each tissue microarray included trachea, lung, small intestine (duodenum and jejunum) and lower intestine (ileum and ceca). The lungs were sectioned sagittally to include parts of primary, secondary and tertiary bronchi and the adjacent air capillaries. The microarray block was then incubated overnight at 37 °C. The tissues were then sectioned routinely at 4μm.

Protein histochemistry

Tissue sections were deparaffinized, then placed in a steamer for 45 minutes in pH 6 Citrate buffer for antigen retrieval. Endogenous peroxidases were blocked using Bloxall (Vector labs) for 10 minutes at room temperature, and then the tissue was incubated in 10% normal goat serum for 30 minutes at room temperature. The HA protein (2–8μg) was pre-complexed with StrepTactin-HRP, on ice and then added to the slides and incubated overnight at 4°C. The following day, 3'3 diaminobenzidine, (DAB) (Vector Labs) was used to detect HA

binding, following manufacturer's recommendations. (Ambepitiya Wickramasinghe et al., 2015; Leyson et al., 2016; Wickramasinghe et al., 2011) The slides were counterstained with hematoxylin (Vector labs), then dehydrated, cover-slipped and examined with an Olympus BX41 light microscope. Images were obtained with Cellsens software. Two negative controls were used, firstly, the virus HA was omitted and StrepTactin-HRP diluted in elution buffer (1:200). The second negative control consisted of StrepTactin-HRP diluted in elution buffer (1:200) and the molar equivalent of Green Fluorescent Protein (GFP).

Data analysis

The binding of the HA was determined by the intensity of the brown staining of the cells in different organs. Cells were identified based on their known morphologic characteristics histologically. An Olympus BX41 light microscope was used to obtain images with Cellsens software. Using direct light microscopy, the level of HA binding in each tissue section was ranked based on visualization of DAB staining as no binding, low binding, moderate binding, high binding and very high levels of binding. The numerical value of median area of binding (MAB) was then determined as follows. Firstly, the entire tissue was evaluated, and 3 images were photographed at 20X magnification. Using Fiji software(Schindelin et al., 2012), a region of interest (ROI)of 56,000 μm^2 was selected for each analysis. The region of interest (ROI) represents the 700 μm x 800 μm rectangular area of the tissue that is selected at random for further analysis by the Fiji software. This included the epithelium as well as supportive cells. The surface area of HA binding was determined from the region of interest by adjusting the threshold to 150 in RGB split images to select only the area correlating to dark brown DAB staining, measured in μm^2. The threshold was adjusted to 150 to remove any background staining or non-specific staining of tissues such as cells in the lamina propria of intestines. The area of HA binding was obtained from three replicates of each tissue and the median of the three values was used to determine the median area of binding (MAB). A scale of HA binding was determined to correlate with the MAB for descriptive purposes, which was quantified from no MAB to very high MAB. This was done for each tissue of the respiratory and intestinal tract, then the overall respiratory and intestinal binding score (combined MAB score) was determined by adding the values, for trachea and lung (respiratory) and small intestine and lower intestine (intestinal). The MAB in tissues and the overall respiratory and intestinal MAB scores were obtained for each species and used for statistical comparisons using Graphpad Prism. The median area of HA binding was ranked as follows: 0–10 μm^2: no binding, >10–500: low, >500–1000 moderate, >1000–1500: high, >1500: very high

Statistical analysis

The Kruskal-Wallis test was employed to determine if there was a statistically significant difference in the medians for each tissue, among different species belonging to the same taxonomic order. The Dunn's post-test was then used to determine if there was a pairwise statistically significant difference in HA binding among bird species. This was done for both the HPAIV H5 HA and the LPAIV H5 HA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of Interest Form

Declarations of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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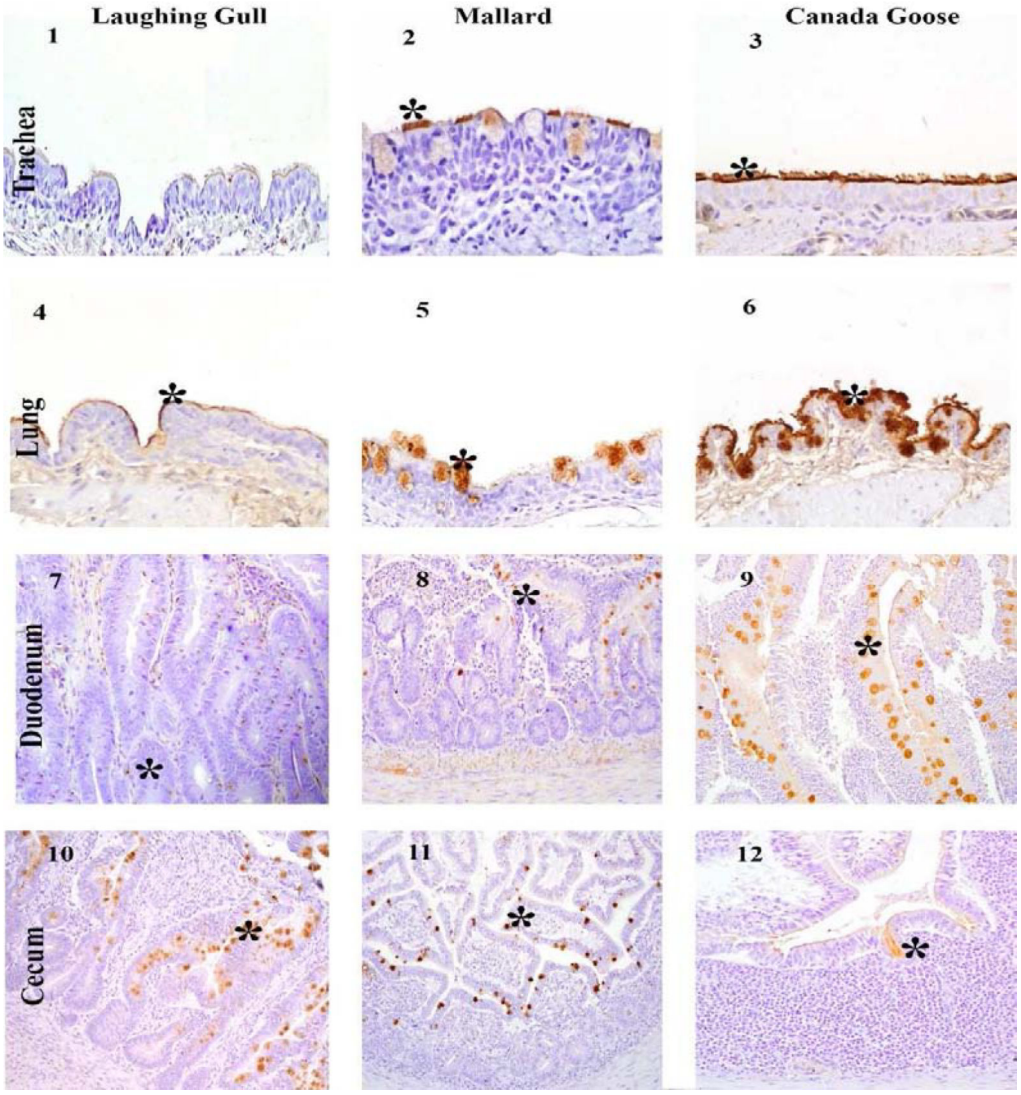

Figure 1:

Protein histochemistry using recombinant, HPAIV H5 HA binding in the trachea, lung, duodenum and ceca of wild bird species (Laughing gull, Mallard and Canada goose). HA binding is indicated by dark brown staining with 3’3 diaminobenzidine (DAB) denoted with an asterisk (*). Note very high levels of HA binding to ciliated epithelial cells in the trachea of the Canada goose (3) compared to the Laughing gull (1). High MAB score in the duodenum of the Canada goose (9, 200X magnification). Also note high to very high MAB score in the ceca of the Laughing gull (10, 200X) and Mallard (11, 200X), compared to low to moderate HA binding in Canada goose (12, 400X).

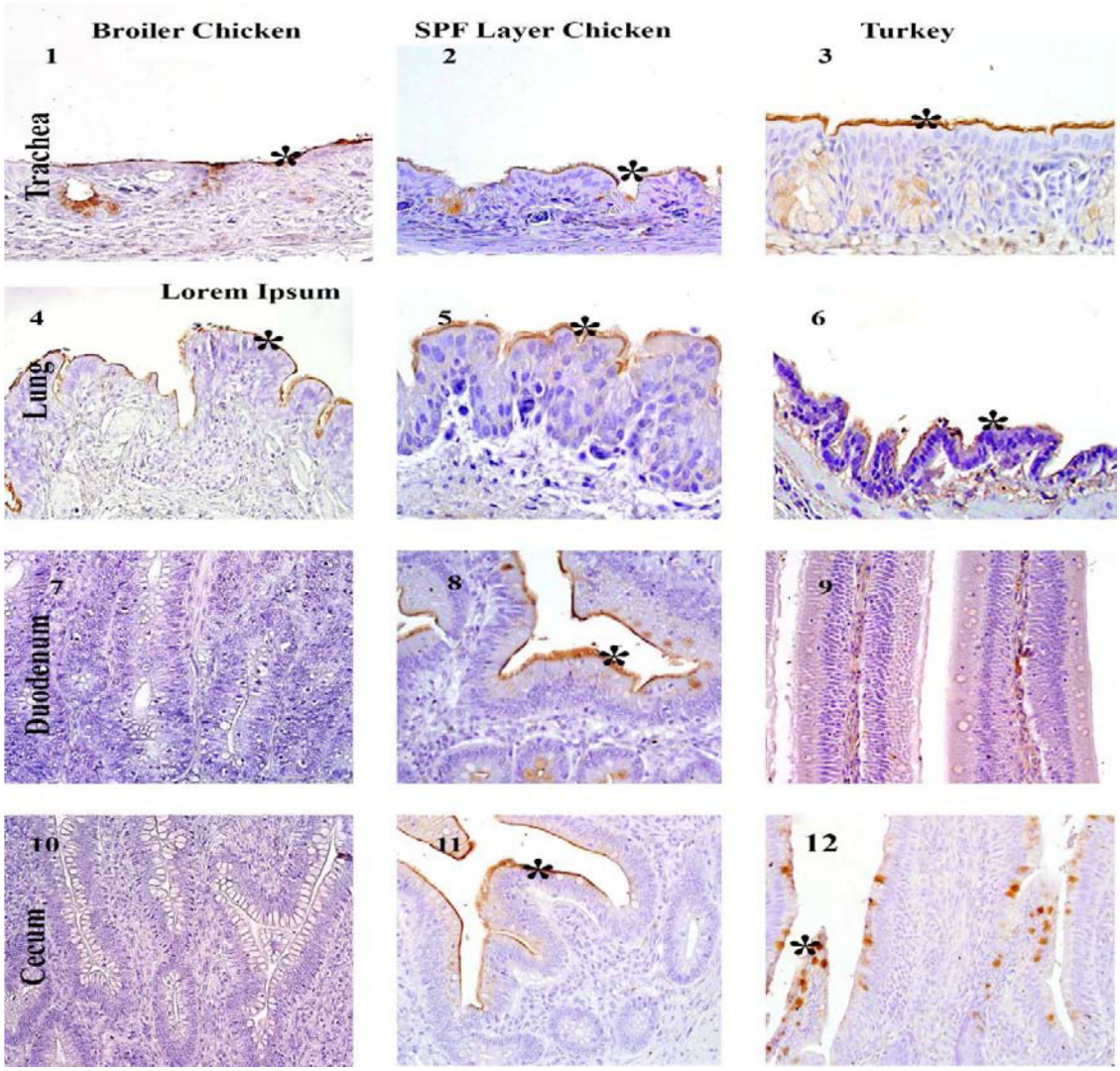

Figure 2:

Protein histochemistry using recombinant, HPAIV H5 HA binding in the trachea, lung, duodenum and ceca of gallinaceous poultry species (broiler chicken, SPF leghorn layer chicken, and turkey). HA binding is indicated by dark brown staining with 3’3 diaminobenzidine (DAB) denoted with an asterisk (*). Note low to high MAB scores in ciliated epithelial cells of the trachea and secondary bronchus of the broiler chicken, SPF layer and turkey (1–6).Very high MAB scores in the duodenum is only present in the layer chicken (11). Note high to very high MAB scores in the ceca of the layer chicken (11) and turkey (12) with binding to enterocytes and goblet cells. Images are at 400X magnification.

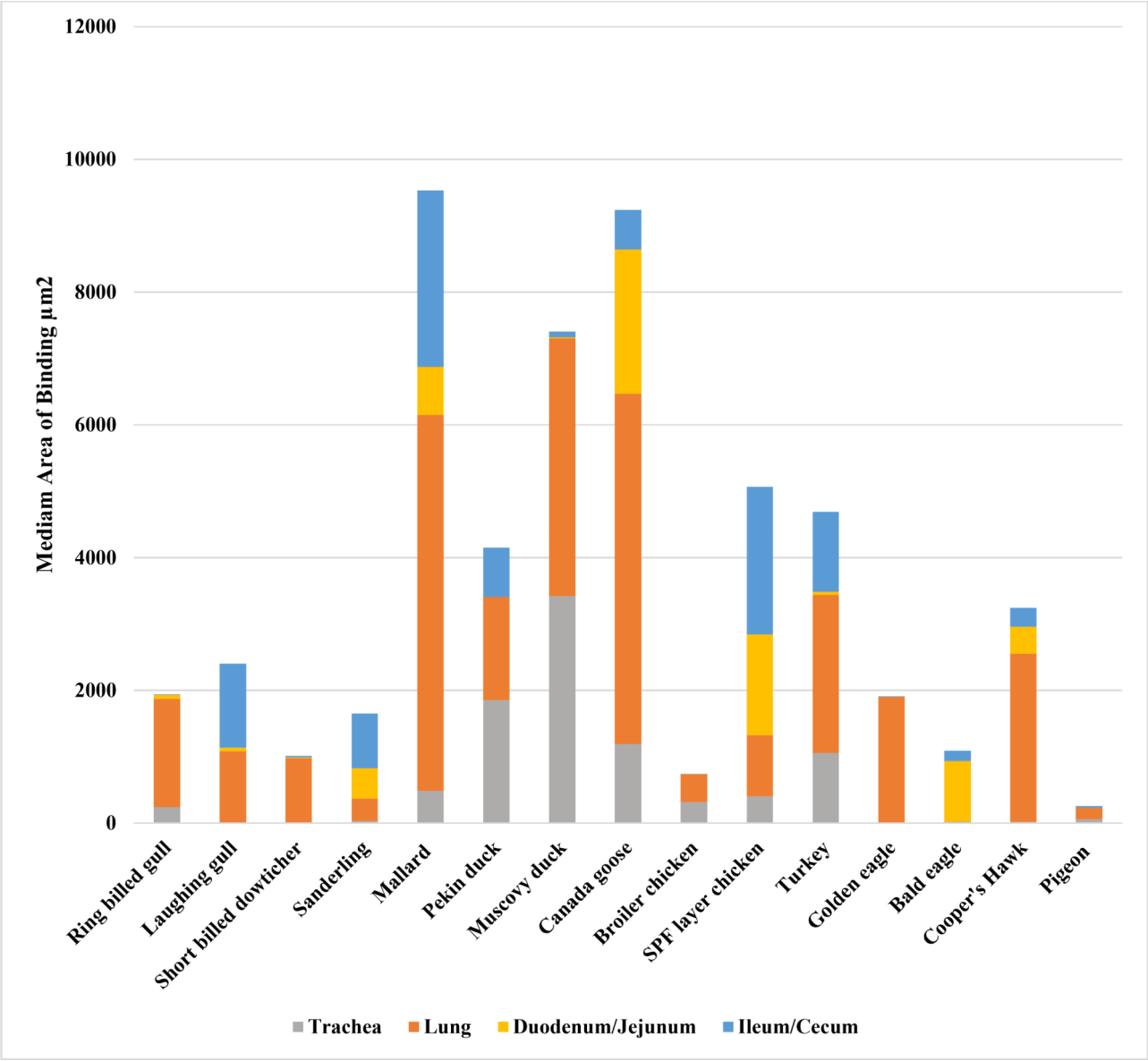

Figure 3:

Stacked column chart illustrating differences in Median Area of Binding (MAB) of HPAIV H5 HA binding in the respiratory tract (trachea and lung) and intestinal tract (small intestine and lower intestine). Note the largest surface area of HA binding in the Mallard duck, with most binding distributed in the respiratory tract. The Muscovy duck had the largest combined surface area of HA binding in the respiratory tract. SPF Layer chickens have the largest overall surface area of HPAIV HA binding in the intestine.

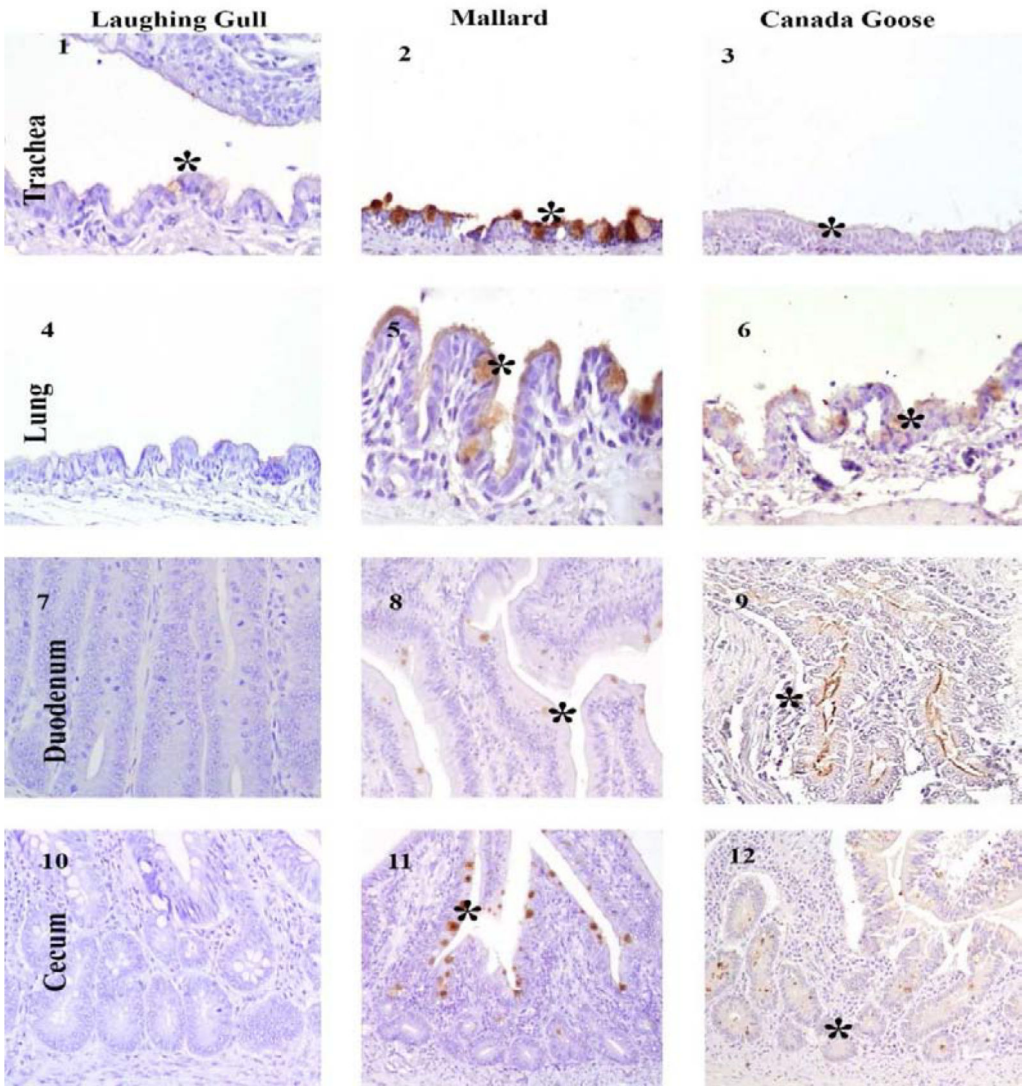

Figure 4:

Protein histochemistry using recombinant LPAIV H5 HA binding in the trachea, lung, duodenum and ceca of wild bird species (Laughing gull, Mallard and Canada goose). HA binding is indicated by dark brown staining with 3’3 diaminobenzidine (DAB) denoted with an asterisk (*). Note moderate to high MAB in the trachea, and lungs of the Mallard (2, 5 respectively,400X magnification)Also note low to moderate HA binding to enterocytes of the duodenum (200X) and ceca of the Mallard duck (8, 11, 200X) and low HA binding to the intestine of the Canada goose (9,200X).

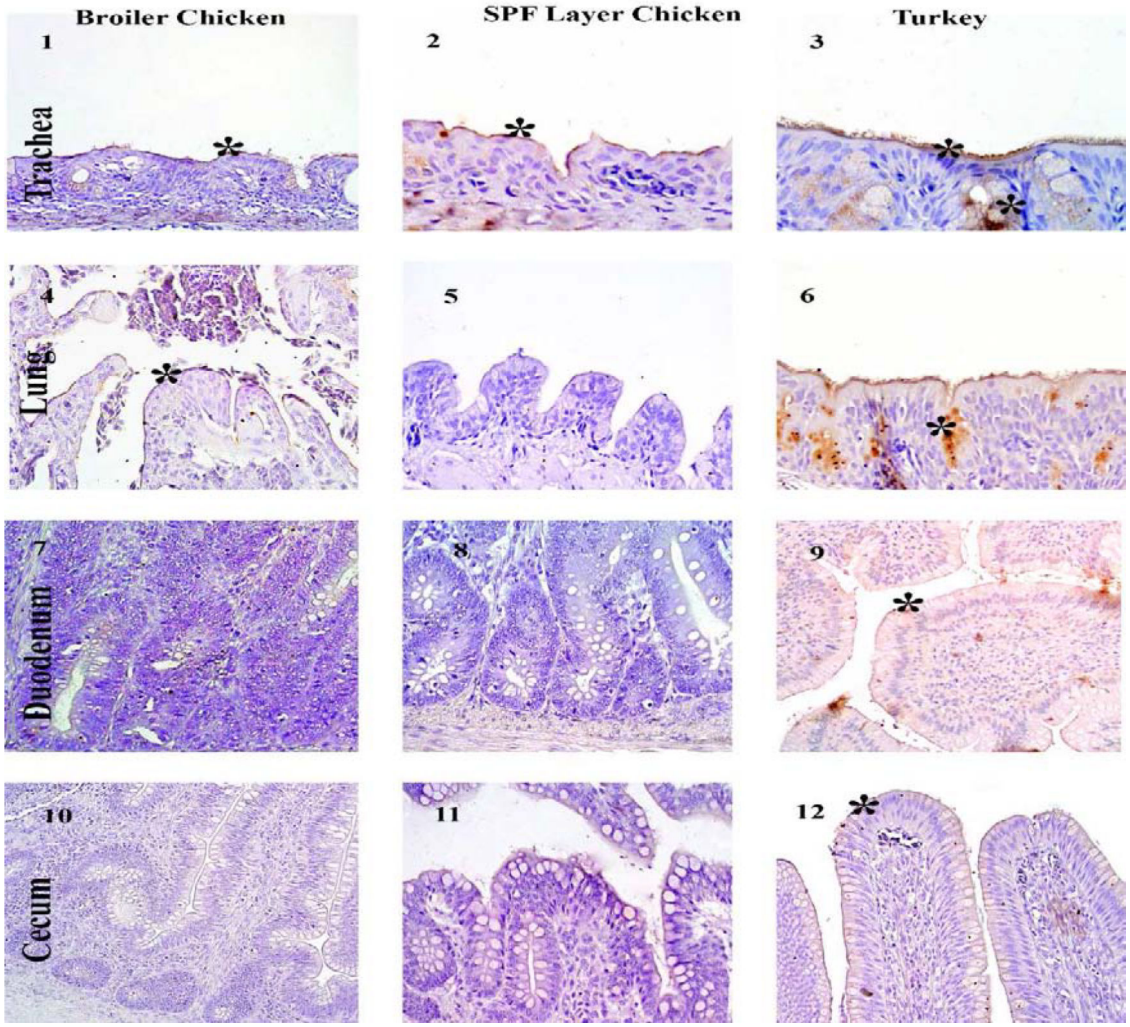

Figure 5:

Protein histochemistry using recombinant LPAIV H5 HA binding in the trachea, lung, duodenum and ceca of gallinaceous poultry species. HA binding is indicated by dark brown staining with 3’3 diaminobenzidine (DAB) denoted with an asterisk (*). Note high MAB in the trachea of the turkey (3), compared to low binding in the broiler chicken (1) and SPF layer chicken (2) Images 1–6 are at 400X magnification. Very high MAB is seen in the secondary bronchus of the turkey (6) Note the absence of LPAIV HA binding to the intestine of broiler chickens (7, 10) and SPF layer chickens (8, 11). Images 7–12 are at 200X magnification.

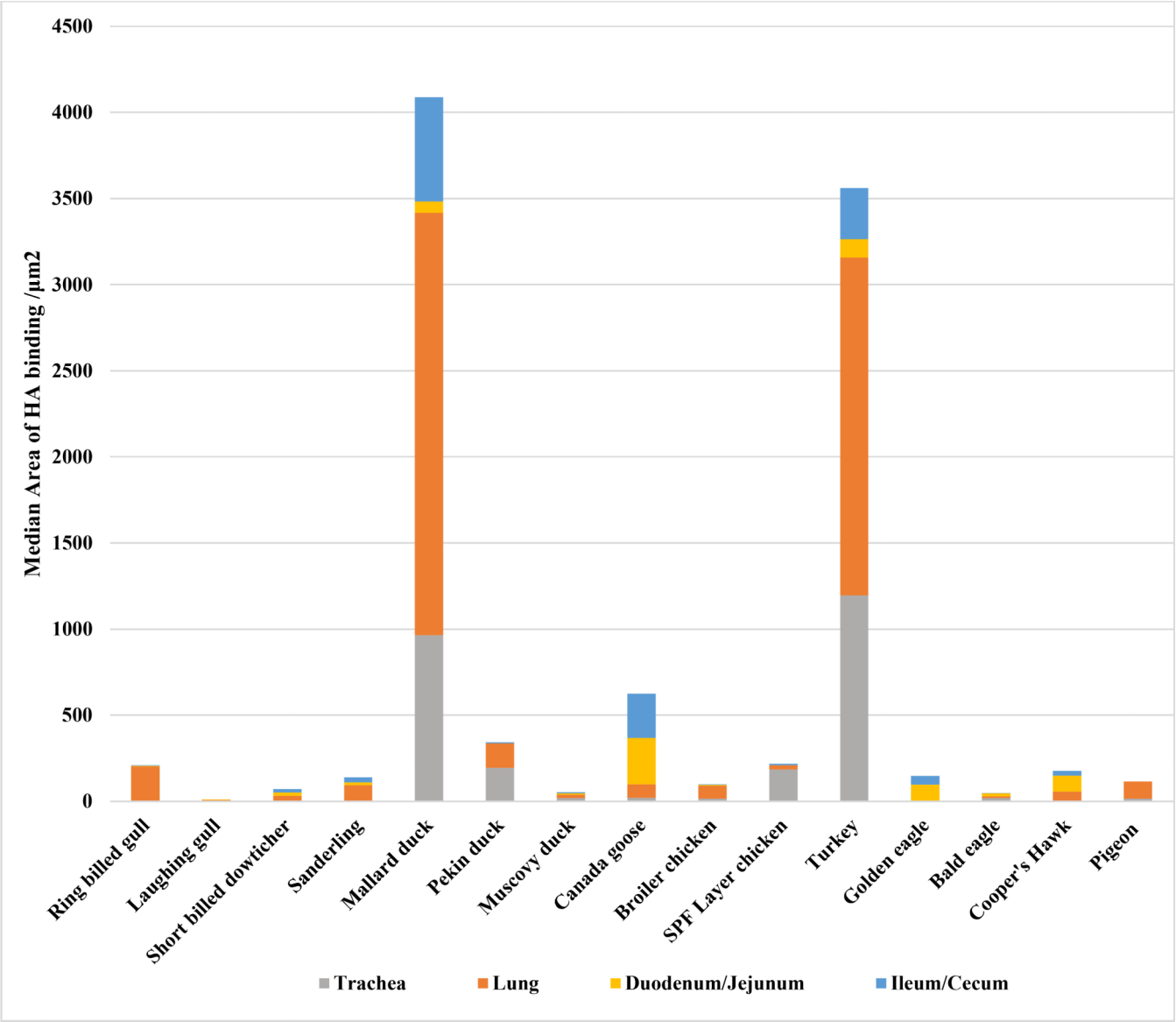

Figure 6:

Stacked column chart illustrating differences in Median Area of Binding (MAB) of LPAIV H5 HA and proportion of HA binding in the respiratory tract (trachea and lung) and intestinal tract (duodenum/jejunum and ileum/cecum). The largest median area of HA binding to the respiratory tract was detected in Mallards and turkeys. In the intestine, the largest area of HA binding was detected in Mallards, followed by Canada geese.

Table 1:

Median area of binding (MAB) distribution of highly pathogenic H5 HA (HPAIV) recombinant protein in the trachea, lung, small intestine, large intestine and cloacal bursa across bird species.

Order	Species	Age	Trachea/ μm^2	Lung/ μm^2	Duodenum/ Jejunum/μm^2	Ileum/ Ceca/μm^2	Bursa/ μm^2
Charadriiformes	**Ring-billed gull** (*Larus delawarensis*) n=2	Ad	238	1633	63.1	4.8	ND
	Laughing gull *(Leucophaeus atricilla)* n=1	Juv	8.6	1070	60.2	1263	2509
	Sanderling *(Calidris alba)* n=1	Ad	34.2	337	457	824	ND
	Short-billed dowitcher *(Limnodromus griseus)* n= 1	Ad	10.3	971	9.8	20.7	ND
Anseriformes	**Mallard duck** (*Anas platyrhynchos*) n=3	Juv	486	5663	725	2657	1595
	Pekin duck (*Anas platyrhynchos domesticus*) n=3	Juv	1854	1552	0.93 *	742	54.8 *
	Muscovy duck *(Cairina moschata)* n=2	Juv	3418	3883	15.0	83.8	164
	Canada goose *(Branta canadensis)* n=2	Ad/Juv	1189	5281	2170 *	596	3532 *
Galliformes	**Broiler Chicken** (*Gallus gallus domesticus*) n=3	Juv	318	418	0.62 *	1.1 *	4.8
	SPF Layer Chicken (*Gallus gallus domesticus*) n=3	Juv	404	920	1519 *	2224 *	4.4
	Turkey (*Meleagris gallopavo*) n=3	Juv	1053	2382	53.9	1199 *	69
Accipitriformes	**Cooper's Hawk** (*Accipiter cooperii*) n=1	Ad	20.4	2536	400	288	ND
	Bald eagle *(Haliaeetus leucocephalus)* n=1	Ad	22.7	2.3	906	155.1	ND
	Golden eagle *(Aquila chrysaetos)* n=1	Ad	ND	1904	ND	7.9	ND
Columbiformes	**Pigeon** (*Columba livia domestica*) n= 1	Juv	57.8	174	ND	24.1	ND

ND: Not done; **n**: Number of birds examined

* Indicates a statistically significant difference between species of birds from the same taxonomical order (Kruskal-Wallis, $p<0.05$)

Ad: Adult

Juv: juvenile

Scale:

0–10 μm^2: No binding

>10–500 μm^2: Low MAB

>500– 1000 μm^2: Moderate MAB

>1000–1500 μm^2: High MAB

>1500 μm^2: Very high MAB

Table 2:

Median area of binding (MAB) scores of low pathogenic H5 HA (LPAIV) recombinant protein in the trachea, lung, small intestine, large intestine and cloacal bursa, across bird species

Order	Species	Age	Trachea/ µm^2	Lung/ µm^2	Duodenum/ Jejunum/µm^2	Ileum/ Ceca/µm^2	Bursa/ µm^2
Charadriiformes	**Ring-billed gull** (*Larus delawarensis*) n=2	Ad	1.2	201	2.9	ND	ND
	Laughing gull *(Leucophaeus atricilla)* n=1	Juv	0.2	7.4	3.1	0.00	1.6
	Sanderling *(Calidris alba)* n=1	Ad	2.3	91.7	16.2	29.4	ND
	Short-billed dowitcher *(Limnodromus griseus)* n= 1	Ad	0.16	31.1	20.6	18.5	ND
Anseriformes	**Mallard duck** (*Anas platyrhynchos*) n=3	Juv	964 *	2450	67.7	606 *	21.6
	Pekin duck (*Anas platyrhynchos domesticus*) n=3	Juv	195	142	0.18	6.4	0.48 *
	Muscovy duck *(Cairina moschata)* n=2	Juv	19.1 *	20.3	7.7	5.9 *	1.2
	Canada goose *(Branta canadensis)* n=2	Juv/Ad	19.8	79.9	267	257	72.8 *
Galliformes	**Broiler chicken** (*Gallus gallus domesticus*) n=3	Juv	14.5 *	76.4	5.1	3.9	9.5
	SPF Layer chicken (*Gallus gallus domesticus*) n=3	Juv	185	23.3	1.4 *	8.8 *	94
	Turkey (*Meleagris gallopavo*) n=3	Juv	1196 *	1961	106 *	299 *	78.1
Accipitriformes	**Cooper's Hawk** (*Accipiter cooperii*) n=1	Ad	1.7	54.5	92.0	29.3	ND
	Bald eagle *(Haliaeetus leucocephalus)* n=1	Ad	18.4	10.3	17.6	2.7	ND
	Golden eagle *(Aquila chrysaetos)* n=1	Ad	ND	2.3	95	50.1	ND
Columbiformes	**Pigeon** (*Columba livia domestica*) n=1	Juv	14.8	100	ND	ND	ND

ND: Not done; **n**: Number of birds examined

* Indicates statistically significant differences between species of birds from the same taxonomical order (Kruskal-Wallis, $p<0.05$)

Ad: Adult, **Juv**: juvenile

Scale:

0–10 µm^2: No binding

>10–500 µm^2: Low MAB

>500– 1000 µm^2: Moderate MAB

>1000–1500 µm^2: High MAB

>1500 µm^2: Very high MAB