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Are microneutralization and hemagglutination inhibition assays comparable? Serological results from influenza experimentally infected mallard ducks

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SUMMARY

The hemagglutination inhibition (HI) assay is commonly used to assess the humoral immune response against influenza A viruses (IAV). However, the microneutralization (MN) assay has been reported to have higher sensitivity when testing sera from humans and other species. Our objective was to determine the agreement between MN and HI assays and compare the proportion of positive samples detected by both methods in sera of mallards primary infected with the A/mallard/MN/Sg-000169/2007(H3N8) virus and subsequently inoculated with homosubtypic or heterosubtypic IAV. Overall, we found poor to fair agreement (PABAK= 0.03 – 0.35) between MN and HI assays in serum samples collected 2 weeks after H3N8 inoculation; the observed agreement increased to moderate or substantial in samples collected 4 to 5 weeks post-inoculation (WPI) (PABAK=0.52 – 0.75). The MN assay detected a higher proportion of positive samples compared to HI assays in serum samples collected 2 WPI ($P=0.01$). This difference was not observed in samples collected 4 WPI. Also, a boosting effect in MN and HI titers was observed when birds were subsequently inoculated with IAV within the same H3 clade. This effect was not observed when birds were challenged with viruses that belong to a different HA clade.

In summary, the agreement between assays varies depending on the post-infection sample collection time-point and the similarity between the antigens used for the assays. Additionally, subsequent exposure of ducks to homosubtypic or heterosubtypic strains might affect the observed agreement.

RESUMEN

La prueba de inhibición de la hemaglutinación (HI) se usa rutinariamente para evaluar la respuesta inmune humoral contra los virus de influenza aviar (IAV), sin embargo, se ha reportado que la prueba de microneutralización (MN) tiene una mayor sensibilidad cuando muestras de suero de humanos y otras especies son analizadas. Este estudio tuvo como objetivo determinar la concordancia entre las pruebas de MN y HI en sueros de patos mallares desafiados con el virus A/

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mallard/MN/Sg-000169/2007(H3N8) y re-inoculados con IAV homosubtipicos o heterosubtipicos. Además, se comparó la proporción de muestras positivas detectadas por ambos métodos. En general, se observó un nivel de concordancia pobre a razonable ($PABAK = 0.03 - 0.35$) entre las pruebas de MN y HI en muestras de suero colectadas 2 semanas post-inoculación (WPI) del virus H3N8; esta concordancia se incrementó a moderada o sustancial en muestras colectadas 4 a 5 WPI ($PABAK = 0.52 - 0.75$). Una mayor proporción de muestras colectadas 2 WPI fueron positivas a MN en comparación con HI ($P = 0.01$), estas diferencias no fueron observadas en muestras colectadas 4 WPI. Adicionalmente, se observó un incremento en los títulos de anticuerpos cuando las aves fueron re-inoculadas con IAV pertenecientes al clado H3 de HA. Este efecto no fue observado en las patos re-inoculados con IAV pertenecientes a un clado distinto.

En resumen, la concordancia entre ensayos de HA y HI varía según el momento de recolección de la muestra y la similitud entre los antígenos utilizados para los ensayos. Además, la re-inoculación de patos con una cepa homosubtípica or heterosubtípica podría afectar el nivel de concordancia observada.

Keywords

Hemagglutination inhibition; microneutralization; mallards; serology; Influenza A virus

Introduction

Influenza A viruses (IAV) are classified based on serological relatedness and the properties of two surface proteins, hemagglutinin (HA) and neuraminidase (NA) (19). Currently, 16 subtypes of HA and 9 subtypes of NA have been described in birds (1, 13). Ducks, especially mallards, are considered the primary reservoirs for most IAV subtypes and play a major role in the epidemiology of these viruses (13). Various serological methods have been used for assessing immunity in serum samples of wild birds during surveillance and experimental studies, and new methods are being developed (2, 3, 6, 28). These methods include screening assays, such as blocking enzyme-linked immunoabsorbent assays (ELISAs), that measure antibody responses to conserved internal proteins, and subtype-specific tests such as the hemagglutination-inhibition assay (HI) (6, 20). HI is a straightforward technique that relies on the ability of antibodies to prevent binding of HA to the sialic acids on red blood cells (RBCs) (14). The microneutralization (MN) assay assesses the ability of antibodies to prevent infection of mammalian cells in vitro (22, 29). HI assays have been routinely used to evaluate humoral immunity against IAV and their subtyping due to its simple and inexpensive nature compared to MN (15). However, lack of sensitivity was reported when duck serum samples were analyzed, resulting in underestimated IAV antibody prevalence in these species (5). The MN assay has been shown to be more sensitive when testing serum samples from humans and other species due to its ability to detect immunoglobulins with non-HI activity (3). Given the benefits of the MN assay, our objective was to determine the correspondence between the results of HI and MN assays when duck serum samples were tested. For comparison, serum samples from mallards experimentally infected with H3N8 IAV and subsequently inoculated with homo- or heterosubtypic IAV were tested by both methods (8, 18).

Methods

Serum samples

A total of 345 paired serum samples from 75 mallards were collected at different time points in three previous studies (8, 17, 18). These studies were conducted at the University of Georgia, Athens, GA, USA. During these experiments, 1-month-old-ducks were inoculated with approximately 10^6 50% embryo infectious doses (EID₅₀) of an H3N8 virus, A/mallard/MN/Sg-000169/2007(H3N8) via the choanal cleft. After inoculation, similar single or multiple challenges occurred ($\sim 10^6$ EID₅₀) with homo- or heterosubtypic IAV at different time intervals and with different doses (10^3 and 10^4 EID₅₀) (Table 1). The strains used for the heterosubtypic inoculations are listed as follows: A/mallard/MN/AI11–4213/2011(H4N5), A/mallard/MN/AI11–4982/2011(H6N2), A/mallard/MN/AI11–4412/2011(H10N7), A/mallard/MN/AI11–3866/2011(H12N5), A/mallard/MN/AI11–4979/2011 (H4N6), and A/blue-winged teal/TX/AI13–1028/2013(H14N5). The subtypes of the viruses used in these studies belong to either Group 1 or 2 of the IAV HAs. The H3, H4, and H14 subtypes belong to the H3 clade and the H10 subtypes belong to the H7 clade of Group 2; while the H6 and H12 subtypes clustered in Group 1. All animals inoculated with the H3N8 virus tested positive by rRT-PCR and virus isolation at 2 days post-inoculation (DPI) in oropharyngeal and cloacal swabs. The patterns of infection after homo- or heterosubtypic challenges were variable (8, 17, 18). The experimental design varied between trials, but all serum samples were collected on 0 and 14 DPI from the brachial or jugular vein. Additional serum samples were collected at intermediate time points between inoculations in the groups that received more than two challenges or had an interval between inoculations longer than 5 months.

Viruses

The second passage of two IAVs of wild bird origin were used as antigens in this study. The A/mallard/MN/Sg-000169/2007(H3N8), which was designated as “H3N8–2007”, corresponds to the inoculum used for the primary challenge of ducks at 1 month of age. The second antigen, designated “H3N8–2010” is a heterologous IAV of the same subtype. Analyses of the amino acid sequences of the A/mallard/MN/Sg-000169/2007(H3N8) (H3N8–2007) and A/mallard/MN/AI10–2593/2010(H3N8) showed a 99.1% amino acid similarity between the HA1 regions of the viruses.

MN assays

The A/mallard/MN/Sg-000169/2007(H3N8) (H3N8–2007) and A/mallard/MN/AI10–2593/2010(H3N8) (H3N8–2010) were used as antigens for the MN assay. Samples were tested by MN in Madin–Darby canine kidney cells as previously described (28). Briefly, serum samples were diluted 1:10 in minimal essential media (MEM; Sigma-Aldrich, St. Louis MO) and heat inactivated at 57 °C for 30 min. Two-fold serial dilutions of sera were prepared in 96-well flat-bottomed plates (1:20–1:640). MEM and a positive chicken serum against the H3N8 virus were used as negative and positive controls, respectively. An equal volume of virus dilution containing 100 tissue culture infective doses (TCID₅₀/25 ul) was added to each well containing sera. Plates were incubated at 37 °C, and the positive titer was recorded as the highest dilution at which no cytopathic effect was visualized at 48–72 hr.

Serum samples with titers 1:20 were considered positive. Due to the differences in timing of the experiments, serum samples from Groups 1–8 were tested with the H3N8–2007 antigen and groups 9–15 were tested with the H3N8–2010 antigen.

HI assays

The A/mallard/MN/Sg-000169/2007(H3N8) (H3N8–2007) and A/mallard/MN/AI10–2593/2010(H3N8) (H3N8–2010) were used as HI antigens to test all serum samples in this study. The HI assays were performed as described elsewhere (15). Briefly, sera were treated with a receptor destroying enzyme (RDE) derived from *Vibrio cholerae* for 18–20 hr at 37 °C followed by heat inactivation at 56 °C for 1 hour. Packed turkey RBCs were used to adsorb non-specific agglutinins before HI testing (27). Two-fold serial dilutions of serum samples (1:16–1:1024) were made in 96-well U-bottom microtiter plates. An equal volume (25 µl) of PBS containing 4 hemagglutinating units (HAU) of the virus was added to each well (1:1) and incubated for 1 hour at 4 °C. Finally, 50 µl of 0.5% turkey RBCs were added to the plate and incubated at 37 °C for 1 hour. All HI tests were conducted in duplicate by using the two H3N8 antigens. The HI titer was defined as the reciprocal of the last dilution of serum that completely inhibited hemagglutination. The cut-off value for determining a sample to be positive was 1:16.

Statistical analyses

Serum samples detected negative at a dilution of 1:20 or 1:16 for MN and HI assays, respectively, were assigned values of 1:10. Titers obtained from both methods were transformed to log₂ values for statistical analyses. Measured agreement between assays was determined by using the Cohen's Kappa coefficient. Kappa values of <0 were considered of poor agreement, 0.01–0.20 slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement, and 0.81–1.0 almost perfect agreement. Due to the presence of the bias and high prevalence, the adjusted kappa statistic (PABAK) was also calculated. The statistical comparison of titers at different time points within groups was performed using a paired Student t-test on the log₂ titers. All statistical tests were conducted using a commercially available software package (Stata version 14.0, StataCorp LP, College Station, TX).

Results

Dynamics of MN and HI antibodies against H3N8 virus

All serum samples collected at 4 weeks-of-age and before inoculation with H3N8 IAV were serologically negative by MN and HI assays. The dynamics of antibodies against the H3N8 viruses detected by both methods were similar after infection. MN titers were higher at 2 WPI as compared to 5 WPI ($P=0.02$), while HI titers were not significantly different when measured at 2 and 5 WPI with both H3N8 antigens ($P>0.05$). Re-inoculation of H3N8-primed ducks after 11 or 15 weeks with the same virus induced a boosting effect in the MN and HI titers; however, this effect was only significant with MN titers ($P<0.05$). Similarly, secondary inoculation with viruses within the H3 clade (H4N5, H4N6, and H14N5) caused a boosting effect on the titers against the H3N8 virus when samples were tested by MN and HI assays; however, the increase was not significantly different ($P>0.05$). Heterosubtypic IAV

re-inoculation with the H6N2, H10N7, and H12N5 subtypes of IAV did not show evidence of a boosting effect on the antibody titers against the H3N8 antigen ($P>0.05$) (Figure 1).

Agreement after single H3N8 inoculation

MN antibodies were detected in 92.5% confidence interval (CI) 95% from 80.0–98.0% of the samples tested 2 WPI when the H3N8–2007 antigen was used. A lower proportion of samples tested positive by MN, 77.1% (CI 95% 60.0–89.6%), when the heterologous H3N8–2010 antigen was used. On the other hand, 62.7% (CI 95% 50.7–73.6%) and 57.3% (CI 95% 45.4–68.7) of the samples analyzed at this time point, were positive by HI when the H3N8–2007 and H3N8–2010 antigens were used, respectively.

Comparison among assays showed improvement of agreement when the homologous antigen was used for both assays. For instance, a fair agreement was observed between MN₂₀₀₇ and HI₂₀₀₇ (PABAK=0.35) as compared to slight agreement between MN₂₀₀₇ and HI₂₀₁₀ (PABAK=0.10). Similarly, slight agreement was observed between MN₂₀₁₀ and both HI_{2007, 2010} assays (PABAK=0.03 and 0.20).

A lower proportion of serum samples positive by MN were detected at 4 to 5 weeks post-H3N8 inoculation ($P=0.04$) as compared to 2 WPI. On the other hand, a higher proportion of samples were positive when tested by the HI assay; however, statistically significant differences were not found ($P>0.05$). Substantial agreement between the MN₂₀₀₇ and both HI_{2007,2010} assays (PABAK=0.75 and 0.60) was observed in samples collected at 5 WPI. Also, the agreement between MN₂₀₁₀ and both HI_{2007,2010} assays was moderate to substantial (PABAK=0.52 and 0.68), respectively. Perfect agreement between assays was observed when samples were tested at 11 and 15 weeks after primary H3N8 inoculation (Table 2).

Agreement after homologous re-infection

The agreements between the MN₂₀₀₇ and both HI_{2007, 2010} were perfect (PABAK=1.00, 1.00) in samples collected after re-inoculation with the homologous H3N8–2007 virus when birds were 15 and 19 weeks old (Table 2).

Agreement after heterologous re-infection

Substantial and perfect agreements between MN and HI_{2007, 2010} assays were observed when birds were re-infected with the H4N5 or H4N6 viruses (PABAK=0.73, 1.00). Perfect agreement between MN₂₀₁₀ and HI_{2007,2010} was observed in birds re-infected with the H14N5 virus (PABAK=1.00, 1.00). Agreement between both assays was variable when birds were re-infected with viruses outside the H3 clade (Table 2).

Discussion

In this study, we compared the proportion of positive samples detected with MN and HI assays and determined the agreement between them when serum samples of mallards experimentally infected with IAV were tested. Paired serum samples of ducks initially infected with the H3N8 virus and re-inoculated with homo- or heterosubtypic IAV were

tested. Overall, we found slight to fair agreement between MN and HI assays in samples collected two weeks after primary H3N8 infection. This observation was probably attributable to the higher sensitivity of MN assays (3). Variation of the agreement values was also influenced by the similarity between the antigens used for the assays. Consequently, higher PABAK values were observed when samples were tested with the same antigen as compared to lower PABAK values when a heterologous antigen was used for each assay. Agreement between MN and HI increased to moderate or substantial in samples collected 4 to 5 WPI. This result was likely explained by an increase in antibody titers and the number of positive samples detected by HI at this time point.

MN assays are believed to be more sensitive than HI assays because of their ability to detect neutralizing antibodies with no-HI activity such as the antibodies directed against the HA2 stalk and the truncated version of the IgY secreted in ducks (10, 24). Consistent with this, a slightly higher proportion of positive samples was detected by MN as compared to HI at 2 WPI. However, the proportion of positive samples detected by HI increased from 57.0–63.0% at 2 WPI to 71.0–83.0% at 4–5 WPI, potentially due to affinity maturation of antibodies targeting the receptor binding site. These latter HI results were in moderate to substantial agreement with MN and suggest that good sensitivity can be achieved with HI at this time point.

Previous studies that showed a low sensitivity of the HI assay for detection of humoral response against IAV in ducks typically used chicken RBCs (5). Turkey and chicken RBCs express a mix of essentially SA α 2,3Gal and SA α 2,6-Gal receptors (4, 21), and avian influenza viruses preferentially bind to SA α 2,3Gal. Chicken RBCs mainly contain SA α 2,3Gal (4); while opposite results about the relative proportion of SA α 2,3Gal and SA α 2,6Gal linkages on turkey RBCs have been reported (9, 12). Our findings are consistent with another study that reported a high proportion of positive samples detected by HI using turkey RBCs (23). These results suggest a good performance of turkey RBCs for detection of HI antibodies against H3N8 viruses of wild ducks origin. However, more studies that compare the performance of chicken versus turkey red blood cells when duck serum samples are tested are warranted.

Good agreements between MN and HI assays were found in serum samples from ducks infected with just one H3N8 virus until 11 WPI. Re-inoculation of birds with the same virus after 11 or 15 weeks had a boosting effect in MN and HI titers. This pattern was not observed in birds re-inoculated after 5 weeks, probably as a result of rapid clearance of the virus and reduced activation of recall antibody response [25]. The observed agreement between MN and HI assays was variable following challenge of H3N8-primed ducks with a different virus subtype. For instance, substantial to almost perfect agreement between assays was observed when birds were re-inoculated with IAV subtypes within the H3 clade (H4N5, H4N6, and H14N5). However, a slight to substantial agreement was found in serum samples of birds re-inoculated with IAV outside the H3 clade (H6N2, H10N7, and H12N5). An increase in antibodies titers against H3N8 was observed after inoculation of H3N8-primed birds with IAV within the same HA clade (H4 or H14). This result might be explained by the “original antigenic sin” phenomenon (OAS). OAS suggests that if an individual is sequentially exposed to different viruses that are antigenically related, the immune system

can identify the shared epitopes, and progress to a robust immune response against the first virus (7, 25). Conversely, subsequent infections with viruses from a different HA clade (H10, H12 or H6) did not show any boosting effect in the antibodies against H3N8 virus. In agreement with this, previous studies have shown that antigenically different or divergent strains of influenza viruses failed to induce OAS (26).

Inoculation of live viruses has been associated with induction of cross-neutralizing antibodies in some individuals, probably as a result of induction of a subset of B cells secreting antibodies targeting HA antigens common to different IAV subtypes (16, 18). Also, MN assays detect antibodies targeting the NA which have been demonstrated to confer partial protective immunity (11). This characteristic suggests that the higher sensitivity of MN assays as compared to HI may come at the expense of a relative loss of specificity when testing serum samples positive to IAV antibodies. However, more studies that compare sensitivity and specificity between methods by the use of different IAV subtypes as antigens are necessary.

In investigations of free-ranging wild bird populations, interpretation of serologic data obtained by HI and MN assays is often confounded by uncertainty regarding how closely an antigen used in the laboratory compares to the field strains, most of which are never obtained. A substantial to almost perfect agreement was obtained between HI₂₀₀₇ and HI₂₀₁₀ at most time points. However, despite a 99.1% similarity in the HA1 region of the viruses, a lower proportion of positive samples was detected at some time points when a heterologous antigen to the challenge virus was used (H3N8–2010 antigen). Taken together, these results emphasize the utility of HI for field investigations while also illustrating its limitations. Unfortunately, the importance of antigen choice for MN assays could not be directly assessed, as serum samples were tested by MN using only one of the two available antigens.

In summary, our results showed that MN appears to be a more sensitive method than HI for detecting humoral immunity against IAV in mallards. The agreement between assays varies depending on the time-point at which the sample was collected after infection and the similarity between the challenge virus and antigen used for the assay. Also, re-inoculation of ducks with a homosubtypic or heterosubtypic virus might affect the observed agreement between assays.

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Abbreviations:

MN	Microneutralization
IAV	Influenza A viruses
HI	Hemagglutination inhibition

WPI	weeks post-inoculation
HA	hemagglutinin

References

- Alexander DJ A review of avian influenza in different bird species. *Vet Microbiol* 74:3–13. 2000. [PubMed: 10799774]
- Bucukovski J, Latorre-Margalef N, Stallknecht DE, and Miller BL A multiplex label-free approach to avian influenza surveillance and serology. *PLoS one* 10:e0134484 2015. [PubMed: 26241048]
- Gross P, and Davis A Neutralization test in influenza: use in individuals without hemagglutination inhibition antibody. *J Clin Microbiol* 10:382–384. 1979. [PubMed: 489725]
- Ito T, Suzuki Y, Mitnaul L, Vines A, Kida H, and Kawaoka Y Receptor specificity of influenza A viruses correlates with the agglutination of erythrocytes from different animal species. *Virology* 227:493–499. 1997. [PubMed: 9018149]
- Kida H, Yanagawa R, and Matsuoka Y Duck influenza lacking evidence of disease signs and immune response. *Infect Immun* 30:547–553. 1980. [PubMed: 7439994]
- Killian ML Hemagglutination assay for influenza virus In: *Animal Influenza Virus*. Spackman E, ed. Springer pp 3–9. 2014.
- Kim JH, Skountzou I, Compans R, and Jacob J Original antigenic sin responses to influenza viruses. *J Immunol* 183:3294–3301. 2009. [PubMed: 19648276]
- Latorre-Margalef N, Brown JD, Fojtik A, Poulson RL, Carter D, Franca M, and Stallknecht DE Competition between IAV subtypes through heterosubtypic immunity modulates re-infection and antibody dynamics in the mallard reservoir. *PLoS Pathog* 13:e1006419 2017. [PubMed: 28640898]
- Lin YP, Xiong X, Wharton SA, Martin SR, Coombs PJ, Vachieri SG, Christodoulou E, Walker PA, Liu J, and Skehel JJ Evolution of the receptor binding properties of the influenza A (H3N2) hemagglutinin. *Proc Natl Acad Sci U S A* 109:21474–21479. 2012. [PubMed: 23236176]
- Lu BL, Webster RG, and Hinshaw VS Failure to detect hemagglutination-inhibiting antibodies with intact avian influenza virions. *Infect Immun* 38:530–535. 1982. [PubMed: 6292104]
- Marcelin G, DuBois R, Rubrum A, Russell CJ, McElhaney JE, and Webby RJ A contributing role for anti-neuraminidase antibodies on immunity to pandemic H1N1 2009 influenza A virus. *PLoS One* 6:e26335 2011. [PubMed: 22039464]
- Ng TG A comparison of influenza binding to erythrocytes from different animal species In: *Pathology*. The University of Hong Kong p 67 2012.
- Olsen B, Munster VJ, Wallensten A, Waldenström J, Osterhaus AD, and Fouchier RA Global patterns of influenza A virus in wild birds. *Science* 312:384–388. 2006. [PubMed: 16627734]
- Palmer D, Dowdle W, Coleman M, and Schild G Haemagglutination inhibition test In: *Advanced laboratory techniques for influenza diagnosis - Procedural guide*. E. a. W. U.S. Department of Health, ed., Atlanta, GA pp 25–62. 1975.
- Pedersen JC Hemagglutination-inhibition test for avian influenza virus subtype identification and the detection and quantitation of serum antibodies to the avian influenza virus. *Methods Mol Biol* 436:53–66. 2008. [PubMed: 18370041]
- Quan F-S, Compans RW, Nguyen HH, and Kang S-M Induction of heterosubtypic immunity to influenza virus by intranasal immunization. *Journal of virology* 82:1350–1359. 2008. [PubMed: 18032492]
- Segovia KM, Franca MS, Leyson CL, Kapczynski DR, Chrzastek K, Bahnson CS, and Stallknecht DE Heterosubtypic immunity increases infectious dose required to infect Mallard ducks with Influenza A virus. *PLoS One* 13:e0196394 2018. [PubMed: 29698449]
- Segovia KM, Stallknecht DE, Kapczynski DR, Stabler L, Berghaus RD, Fojtik A, Latorre-Margalef N, and Franca MS Adaptive heterosubtypic immunity to low pathogenic avian influenza viruses in experimentally infected mallards. *PLoS One* 12:e0170335 2017. [PubMed: 28107403]
- Shaw M, and Palese P Orthomyxoviruses *Fields Virology*, eds Knipe DM, Howley PM. In. Lippincott Williams & Wilkins, Philadelphia 2013.

20. Shriner SA, VanDalen KK, Root JJ, and Sullivan HJ Evaluation and optimization of a commercial blocking ELISA for detecting antibodies to influenza A virus for research and surveillance of mallards. *J Virol Methods* 228:130–134. 2016. [PubMed: 26645952]
21. Stephenson I, Wood JM, Nicholson KG, and Zambon MC Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. *J Med Virol* 70:391–398. 2003. [PubMed: 12767002]
22. Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, Wan H, Murakami A, Yammanuru A, Han T, Cox NJ, Bankston LA, Donis RO, Liddington RC, and Marasco WA Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol* 16:265–273. 2009. [PubMed: 19234466]
23. Thontiravong A, Prakairungnamthip D, Chanvatik S, Nonthabenjawan N, Tunterak W, Tangwangvivat R, Oraveerakul K, and Amonsin A The effect of various erythrocyte species on the detection of avian, swine and canine influenza a viruses isolated in thailand. *Thai J Vet Med* 46:135–142. 2016.
24. Trombetta CM, Perini D, Mather S, Temperton N, and Montomoli E Overview of serological techniques for influenza vaccine evaluation: past, present and future. *Vaccines* 2:707–734. 2014. [PubMed: 26344888]
25. Vatti A, Monsalve DM, Pacheco Y, Chang C, Anaya JM, and Gershwin ME Original antigenic sin: A comprehensive review. *J Autoimmun.* 2017.
26. Virelizier J-L, Allison AC, and Schild GC Antibody responses to antigenic determinants of influenza virus hemagglutinin. *J Exp Med* 140:1571–1578. 1974. [PubMed: 4139234]
27. Webster R, Cox N, and Stohr K WHO manual on animal influenza diagnosis and surveillance. WHO: WHO Global Influenza Programme 4 2002.
28. Wong JK, Wilcox BR, Fojtik A, Poulson RL, and Stallknecht DE Antibodies to Influenza A Viruses in Wintering Snow Geese (*Chen caerulescens*) in Texas. *Avian Dis* 60:337–340. 2016. [PubMed: 27309076]
29. Wulff H, Soeken J, Poland JD, and Chin TD A new micro-neutralization test for antibody determination and typing of parainfluenza and influenza viruses. *Proc Soc Exp Biol Med* 125:1045–1049. 1967. [PubMed: 4292476]

Figure 1. Dynamics of serological response by MN and HI assays.

The graph shows variation of geometric mean antibody titers across time based on HI and MN assays in different experimental groups. Arrows indicate the time points of infections and circles, diamonds, and squares the time-points at which samples were tested by MN and HI assays.

Table 1.
Description of groups of ducks experimentally inoculated with IAV from which sera was analyzed in this study.

All serum samples corresponded to groups of 5 ducks each inoculated with the H3N8 virus at 4 weeks of age. Groups 1, 2, and 3 corresponded to ducks later challenged with the same virus after 5, 11, and 15 weeks, respectively. Groups 4, 5, 6, 7, and 8 were inoculated with heterosubtypic viruses after 5 weeks. Group 9 was challenged with the H4N6xH10N7xH14N5 viruses in 4 week intervals. Groups 10 and 11 were inoculated with the H10N7 and H14N5 viruses after 8 or 12 weeks, respectively. Groups 12, 13, 14, and 15 were challenged with different doses of the H6N2 or H4N6 viruses after 5 weeks.

Group	Description (Intervals between inoculations in weeks)	Age in weeks																	
		4	6	8	9	10	11	12	14	15	16	17	18	19	21				
1	H3N8xH3N8 (5)	<i>i,s</i>	<i>s</i>		<i>i,s</i>		<i>s</i>												
2	H3N8xH3N8 (11)	<i>i,s</i>	<i>s</i>		<i>s</i>		<i>s</i>			<i>i,s</i>		<i>s</i>							
3	H3N8xH3N8 (15)	<i>i,s</i>	<i>s</i>		<i>s</i>		<i>s</i>			<i>s</i>		<i>s</i>		<i>i,s</i>	<i>s</i>				
4	H3N8xH4N6/H6N2 (5)	<i>i,s</i>	<i>s</i>		<i>i,s</i>		<i>s</i>												
5	H3N8xH4N5 (5)	<i>i,s</i>	<i>s</i>		<i>i,s</i>		<i>s</i>												
6	H3N8xH10N7 (5)	<i>i,s</i>	<i>s</i>		<i>i,s</i>		<i>s</i>												
7	H3N8xH6N2 (5)	<i>i,s</i>	<i>s</i>		<i>i,s</i>		<i>s</i>												
8	H3N8xH12N5 (5)	<i>i,s</i>	<i>s</i>		<i>i,s</i>		<i>s</i>												
9	H3N8xH4N6xH10N7xH14N5 (4, 4, 4)	<i>i,s</i>	<i>s</i>	<i>i,s</i>		<i>s</i>		<i>i,s</i>		<i>i,s</i>		<i>s</i>							
10	H3N8xH10N7 (8)	<i>i,s</i>	<i>s</i>			<i>s</i>		<i>i,s</i>											
11	H3N8xH14N5	<i>i,s</i>	<i>s</i>								<i>i,s</i>		<i>s</i>						
12	H3N8xH6N2(10 ³) (5)	<i>i,s</i>	<i>s</i>		<i>i,s</i>		<i>s</i>												
13	H3N8xH6N2(10 ⁴) (5)	<i>i,s</i>	<i>s</i>		<i>i,s</i>		<i>s</i>												
14	H3N8xH4N6(10 ³) (5)	<i>i,s</i>	<i>s</i>		<i>i,s</i>		<i>s</i>												
15	H3N8xH4N6(10 ⁴) (5)	<i>i,s</i>	<i>s</i>		<i>i,s</i>		<i>s</i>												

i= Challenge of ducks with the respective IAV, *s*= Serum sample collected

Table 2.

Proportion of positive samples and agreement between HI and MN assays following exposure to LPAIV in mallards at different time-points.

The table shows the proportion (P) of positive sample by microneutralization (MN) and hemagglutination assays (HI) by the use of the H3N8–2007 or H3N8–2010 antigens. The percentage of agreement and PABAK coefficient with 95% confidence intervals (CI) between MN and HI assays are displayed to the right.

Weeks of age	Virus exposed (weeks post-inoculation)	Proportion of positive samples										HI ₂₀₀₇ vs HI ₂₀₁₀	
		MN ₂₀₀₇	P ₂₀₀₇	MN ₂₀₁₀	P ₂₀₁₀	HI ₂₀₀₇	P ₂₀₀₇	HI ₂₀₁₀	P ₂₀₁₀	Agreement %	PABAK (CI)	Agreement %	PABAK (CI)
4	Naïve	0/37	0.00	0/40	0.00	0/75	0.00	0/75	0.00	100.0	1 (1–1)	100.0	1 (1–1)
6	H3N8 (2)	-	-	27/35	0.77	19/35	0.54	22/35	0.63	51.0	0.03 (-0.31–0.36)	52.5	0.20 (-0.12–0.52)
6	H3N8 (2)	37/40	0.93	-	-	28/40	0.70	21/40	0.53	67.5	0.35 (0.06–0.64)	82.5	0.10 (-0.21–0.40)
8	H3N8 (4)	-	-	18/25	0.72	22/25	0.88	18/25	0.72	76.0	0.52 (0.18–0.85)	84.0	0.68 (0.34–0.97)
9	H3N8 (5)	-	-	-	-	-	-	-	-	-	-	-	-
9	H3N8 (5)	32/40	0.80	-	-	33/40	0.83	30/40	0.83	87.5	0.75 (0.55–0.96)	92.5	0.60 (0.35–0.85)
10	H3N8xH4N6 (2)	-	-	5/5	1	5/5	1	5/5	1	-	-	-	-
	H3N8xH4N6 (10 ³) (2)	-	-	3/5	0.60	4/5	0.80	3/5	0.60	87.7	0.73 (0.38–1.08)	93.3	0.86 (0.61–1.12)
	H3N8xH4N6 (10 ³) (2)	-	-	3/5	0.60	4/5	0.80	2/5	0.40	-	-	-	-
	H3N8xH4N6/H6N2 (2)	5/5	1	-	-	5/5	1	5/5	1	100.0	1 (1–1)	100.0	1 (1–1)
	H3N8xH4N5 (2)	5/5	1	-	-	5/5	1	5/5	1	-	-	-	-
11	H3N8xH3N8 (2)	5/5	1	-	-	4/5	0.80	4/5	0.80	80.0	0.60 (-0.10–1.30)	80.0	0.60 (-0.10–1.30)
	H3N8 (7)	5/5	1	-	-	5/5	1	5/5	1	100.0	1 (1–1)	100.0	1 (1–1)
	H3N8 (7)	5/5	1	-	-	5/5	1	5/5	1	-	-	-	-

Weeks of age	Virus exposed (weeks post-inoculation)	Proportion of positive samples										MN ₂₀₀₇ or 2010 vs HI ₂₀₀₇		MN ₂₀₀₇ or 2010 vs HI ₂₀₁₀		HI ₂₀₀₇ vs HI ₂₀₁₀	
		MN ₂₀₀₇	P ₂₀₀₇	MN ₂₀₁₀	P ₂₀₁₀	HI ₂₀₀₇	P ₂₀₀₇	HI ₂₀₁₀	P ₂₀₁₀	Agreement %	PABAK (CI)	Agreement %	PABAK (CI)	Agreement %	PABAK (CI)	Agreement %	PABAK (CI)
	H3N8xH6N2 (2)	4/5	0.80	-	-	3/5	0.60	2/5	0.40	60.0	0.20 (-0.67-1.06)	60.0	0.20 (-0.65-1.06)	100.0	1 (1-1)		
	H3N8xH6N2 (10 ³) (2)	-	-	3/5	0.60	4/5	0.80	3/5	0.60	70.0	0.40 (-0.16-0.97)	70.0	0.40 (-0.16-0.57)	80.0	0.60 (0.1-1)		
	H3N8xH6N2 (10 ⁴) (2)	-	-	3/5	0.60	4/5	0.80	2/5	0.40								
	H3N8xH10N7 (2)	3/5	0.60	-	-	2/5	0.40	2/5	0.40	40.0	-0.20 (0.65-1.06)	40.0	0.00 (-0.98-0.98)	100.0	1 (1-1)		
	H3N8xH12N5 (2)	3/5	0.60	-	-	1/5	0.20	1/5	0.20	60.0	0.20 (-0.65-1.06)	60.0	0.20 (-0.65-1.06)	100.0	1 (1-1)		
	H3N8xH4N6 (4)	-	-	5/5	1	5/5	1	5/5	1	100.0	1 (1-1)	100.0	1 (1-1)	100.0	1 (1-1)		
12	H3N8 (8)	5/5	1	-	-	5/5	1	5/5	1	100.0	1 (1-1)	100.0	1 (1-1)	100.0	1 (1-1)		
14	H3N8xH4N6xH10N7 (2)	-	-	5/5	1	5/5	1	5/5	1								
	H3N8XH10N7 (2)	-	-	5/5	1	5/5	1	5/5	1	100.0	1 (1-1)	100.0	1 (1-1)	100.0	1 (1-1)		
15	H3N8 (11)	5/5	1	-	-	5/5	1	5/5	1								
	H3N8 (11)	5/5	1	-	-	5/5	1	5/5	1	100.0	1 (1-1)	100.0	1 (1-1)	100.0	1 (1-1)		
16	H3N8xH4N6xH10N7 (2)	-	-	5/5	1	4/5	0.80	4/5	0.80	80.0	0.60 (-0.1-1.30)	80.0	0.60 (-0.1-1.30)	100.0	1 (1-1)		
	H3N8xH10N7 (2)	-	-	5/5	1	3/5	0.60	3/5	0.60	100.0	1 (1-1)	100.0	1 (1-1)	100.0	1 (1-1)		
17	H3N8xH3N8 (2)	5/5	1	-	-	5/5	1	5/5	1								
	H3N8 (11)	5/5	1	-	-	4/5	1	4/5	1	100.0	1 (1-1)	100.0	1 (1-1)	100.0	1 (1-1)		
18	H3xH4xH10xH14 (2)	-	-	5/5	1	5/5	1	5/5	1	100.0	1 (1-1)	100.0	1 (1-1)	100.0	1 (1-1)		
	H3N8xH14N5 (2)	-	-	5/5	1	5/5	1	5/5	1	100.0	1 (1-1)	100.0	1 (1-1)	100.0	1 (1-1)		
19	H3N8 (15)	4/5	0.80	-	-	5/5	1	5/5	1	100.0	1 (1-1)	100.0	1 (1-1)	100.0	1 (1-1)		
21	H3N8xH3N8 (2)	5/5	1	-	-	5/5	1	5/5	1	100.0	1 (1-1)	100.0	1 (1-1)	100.0	1 (1-1)		