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Comparative Hemagglutination of Avian Influenza A/H5N1 Viruses by Erythrocytes from Ostrich, Emu, Japanese Quail, Chicken and Horse

Kazuhide Adachi[1], Ekowati Handharyani[2], Saaya Ueno[1], Yasuhiro Tsukamoto[1]*

[1]Laboratory of Animal Hygiene, Department of Agricultural and Life Science, Faculty of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan
[2]Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, Indonesia
Email: *ytsuka@kpu.ac.jp

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Abstract

Influenza A viruses initiate infection when the envelope glycoprotein hemagglutinin (HA) binds sialic-acid-containing receptors on host cells and mediates membrane fusion. HA-mediated cross-linking of erythrocytes forms the basis of the hemagglutination (HA) and hemagglutination-inhibition (HI) assays that are widely used to quantify virus and virus-specific antibodies. The choice of erythrocyte species significantly affects these assays, but data for large ratites are limited. This study compared the hemagglutination characteristics of two highly pathogenic avian influenza A (H5N1) isolates, A/H5N1/Bogor 2 and A/H5N1/Lawang, using erythrocytes from ostrich, emu, quail, chicken and horse. Blood was collected from clinically healthy adult animals (n = 5 per species) (wing vein for chickens and quails; jugular vein for ostriches, emus and horses). Washed erythrocytes were examined by bright-field microscopy and used in a microtiter HA assay in which serial two-fold dilutions of erythrocytes were mixed with a constant dose of virus (10^5 $TCID_{50}$ per well). Hemagglutination patterns were recorded up to 300 min and expressed as the highest erythrocyte dilution showing complete agglutination. Ostrich and emu erythrocytes were markedly larger than those of chicken and quail, whereas horse erythrocytes were small and anucleate. All five species supported hemagglutination by both A(H5N1) isolates. For A/H5N1/Bogor 2, horse erythrocytes showed delayed agglutination and a subsequent decline in HA titer, whereas avian erythrocytes yielded high, stable titers. For A/H5N1/Lawang, HA titers were more homogeneous and largely time-independent across species. Overall, differences between virus strains were greater than those between erythrocyte species, suggesting that viral HA properties dominate over gross erythrocyte morphology in determining hemagglutination and that the optimal erythrocyte species for HA and HI testing of H5N1 viruses may vary among strains.

Keywords

Avian Influenza, H5N1, Hemagglutinin, Hemagglutination Assay, Erythrocytes

1. Introduction

Influenza A viruses are enveloped, negative-sense RNA viruses in the family Orthomyxoviridae. Their major surface glycoprotein, hemagglutinin (HA), is responsible for binding terminal sialic-acid residues on host cell glycoconjugates and for driving low-pH-triggered membrane fusion, thereby initiating infection and strongly influencing host range and antigenicity [1] [2]. The term "hemagglutinin" is not confined to influenza A viruses. Several unrelated virus families encode attachment proteins that agglutinate erythrocytes *in vitro*. Examples include the hemagglutinin-neuraminidase glycoproteins of paramyxoviruses, adenovirus fiber proteins, the hemagglutinin-esterase glycoproteins of certain coronaviruses, and the $\sigma1$ attachment protein of mammalian orthoreoviruses [3]. These proteins act as lectins that cross-link sialylated receptors on erythrocytes, leading to macroscopic hemagglutination.

The microtiter hemagglutination (HA) assay exploits this property to detect and semi-quantify hemagglutinating agents, particularly influenza A viruses [4]. In the standard format, serial dilutions of virus are mixed with a fixed concentration of erythrocytes; the highest virus dilution that still prevents erythrocyte settling is taken as the HA titer, and the reciprocal of this dilution is used as a rough measure of virus concentration. The related hemagglutination-inhibition (HI) assay measures the ability of antibodies or other inhibitors to block HA and is widely used for serodiagnosis, antigenic characterization and evaluation of influenza vaccines in both humans and animals [5].

The choice of erythrocyte species is a critical determinant of HA and HI sensitivity and specificity. Chicken and turkey red blood cells (RBCs) are most commonly used, but goose, guinea pig, horse, pigeon, human and other erythrocytes are also employed [6] [7]. The distribution of $\alpha2,3$- and $\alpha2,6$-linked sialic acids on erythrocyte surfaces differs among species and correlates with their ability to be agglutinated by particular influenza A viruses [6] [8]. Several studies have shown that avian influenza viruses, including H5N1, typically prefer $\alpha2,3$-linked sialic acids and may display distinct binding preferences for goose, chicken, guinea pig or horse RBCs [6] [7] [9].

Highly pathogenic avian influenza (HPAI) A/H5N1 viruses have caused repeated outbreaks in domestic poultry and wild birds worldwide and have sporadically infected humans with high case-fatality rates [10] [11]. International standards for diagnostic tests and vaccines emphasize virus isolation in embryonated eggs or cell culture followed by HA and HI assays for subtype identification and serology [12]. Because H5N1 viruses infect a wide range of avian and mammalian hosts [3] [10] [13], there is a need to understand how different erythrocyte species reflect the interaction between viral HA and host receptors.

Vaccination is considered an extremely effective measure for the prevention of influenza virus infections [14]-[16]. However, during a pandemic it may be difficult to provide sufficient vaccine rapidly enough, and antiviral drugs such as neuraminidase inhibitors are often used as additional control measures [17]. In poultry, vaccines against H5N1 are used in some countries but remain controversial because of concerns about incomplete protection, antigenic drift and interference with surveillance [12] [18] [19].

In parallel with virological and epidemiological work, we have been developing antibody-based strategies that directly target influenza virus HA. Using ostriches (*Struthio camelus*) as large-scale antibody producers, we previously generated neutralizing immunoglobulin Y (IgY) against highly pathogenic A/H5N1 virus by immunizing female ostriches with a full-length glycosylated recombinant H5 glycoprotein [20]. This simple method yielded approximately 200 g of anti-H5 IgY per bird per year, and the antibodies showed strong binding to H5N1 virus and H5 proteins and efficiently neutralized viral infection *in vitro* [20]. We further demonstrated that ostrich egg-derived IgY induced by swine or seasonal influenza vaccines could neutralize pandemic influenza A/H1N1/2009 virus, inhibit HA-mediated hemagglutination and block virus-induced cytopathic effects in MDCK cells [21] [22]. In addition, we showed that ostrich-derived antibodies incorporated into filters or other materials can prevent A/H5N1 virus infection under experimental conditions [23], and that passive immunotherapy with antiserum effectively protects chickens against H5N1 challenge [24]. These findings support the concept that blocking the interaction between viral HA and sialylated host receptors can prevent infection and form the basis for prophylaxis or therapy using HA-targeted antibodies.

From this perspective, understanding in detail how HA from avian influenza viruses interacts with erythrocytes from different animal species is important not only for assay optimization but also for developing antibody-based approaches to infection prevention and treatment. Clarifying the determinants of hemagglutination may help link HA-receptor interactions, HA inhibition by antibodies and *in vitro* neutralization with *in vivo* protection.

Large ratites such as ostriches and emus (*Dromaius novaehollandiae*) are increasingly farmed and are known to be susceptible to avian influenza [10] [11], yet their erythrocytes are rarely used in HA/HI assays. Avian erythrocytes are nucleated and typically ellipsoidal, whereas mammalian erythrocytes are smaller, discoid and anucleate. These structural differences, together with species-specific receptor patterns, could theoretically influence hemagglutination, but it is unclear whether cell size or nucleation per se substantially affects HA titers compared with virus-specific HA properties.

In this study, we conducted a comparative analysis of erythrocytes from ostrich, emu, quail, chicken and horse. Our objectives were to 1) document interspecies differences in erythrocyte morphology; 2) evaluate the ability of these erythrocytes to support hemagglutination by two HPAI A/H5N1 isolates, A/H5N1/Bogor 2 and A/H5N1/Lawang; and 3) characterize the time course of hemagglutination titers for each virus-host combination. We hypothesized that differences between virus strains would exert a stronger influence on HA kinetics than gross differences in erythrocyte size or nucleation, and that the optimal erythrocyte species for HA and HI testing of H5N1 might therefore vary between strains.

2. Materials and Methods

2.1. Viruses

Two avian influenza A/H5N1 field isolates designated A/H5N1/Bogor 2 and A/H5N1/Lawang were used. Both had previously been characterized as highly pathogenic on the basis of molecular criteria and *in vivo* pathogenicity tests performed by the original diagnostic laboratory [24]. Virus stocks were propagated in the allantoic cavity of 10-day-old specific-pathogen-free embryonated chicken eggs under biosafety level-3 conditions, according to standard procedures for avian influenza virus [12]. Eggs were inoculated with diluted virus in the allantoic cavity, incubated for 48 h at 37˚C and then chilled at 4˚C. Allantoic fluid was harvested aseptically, clarified by low-speed centrifugation and stored at −80˚C as single-use aliquots. Influenza virus growth was confirmed by a standard HA assay using 0.5% chicken erythrocytes [4].

To ensure that all erythrocyte species were tested against the same infectious virus dose, the infectious titers of the allantoic fluid stocks were determined in Madin-Darby canine kidney (MDCK) cells as 50% tissue culture infectious doses ($TCID_{50}$). Confluent MDCK monolayers in 96-well plates were inoculated with serial 10-fold dilutions of virus, incubated at 35˚C - 37˚C in infection medium and examined daily for virus-induced cytopathic effect (CPE) for 3 days, following standard procedures for influenza virus titration [24] [25]. $TCID_{50}$ values were calculated according to the method of Reed and Muench [26] [27]. For the hemagglutination experiments, virus suspensions were diluted in phosphate-buffered saline (PBS) so that each test well contained 10^5 $TCID_{50}$ of infectious virus. The same virus suspension (10^5 $TCID_{50}$ per well) was used for all erythrocyte species, allowing direct comparison of hemagglutination patterns among different animal red blood cells.

2.2. Animals and Blood Collection

Clinically healthy adults of each species—ostrich (*Struthio camelus*), emu (*Dromaius novaehollandiae*), Japanese quail (*Coturnix japonica*), domestic chicken (*Gallus*

gallus domesticus) and horse (*Equus caballus*)—were sampled. For each species, blood samples were collected from five independent individuals (n = 5 per species). Chickens and quails were bled from the wing (brachial) vein, whereas ostriches, emus and horses were bled from the jugular vein using sterile needles and syringes. Approximately 2 - 10 mL of blood per animal was collected into tubes containing heparin or EDTA as anticoagulant.

Animals were handled by experienced veterinarians or animal-care staff. All procedures complied with institutional and national regulations on animal experimentation and were approved by the Animal Experiment Committee of Kyoto Prefectural University (approval number KPU190410R).

2.3. Preparation of Erythrocyte Suspensions

Whole blood was centrifuged at 800 ×g for 10 min at 4˚C. Plasma and buffy coat were removed, and packed erythrocytes were washed three times with cold PBS (pH 7.4), following standard protocols used for avian influenza HA/HI testing [4] [12]. After each wash, cells were resuspended 1:10 in PBS, gently mixed and centrifuged again. The final packed erythrocytes were resuspended in PBS to obtain a working suspension of 1% (v/v), prepared at the same nominal concentration for all species. Suspensions were stored at 4˚C and used within one week; samples showing hemolysis were discarded.

2.4. Microscopic Observation of Erythrocytes

For morphological comparison, a small drop of each erythrocyte suspension was placed on a glass slide, covered with a coverslip and observed under bright-field microscopy without staining. Images were captured with a digital camera at identical magnification for all species. A stage micrometer was photographed under the same conditions for scale calibration. Representative images are shown in **Figure 1**.

2.5. Hemagglutination Assay

Hemagglutination assays were performed in V-bottom 96-well microtiter plates according to established influenza protocols, with minor modifications [4] [6]. Briefly, 25 μL of PBS was dispensed into each well in columns 2 - 12. For each erythrocyte species, a two-fold serial dilution of the erythrocyte suspension (e.g. from 1:8 to $1:2^{12}$) was prepared across the row by transferring 25 μL from one well to the next and mixing thoroughly, resulting in a decreasing erythrocyte concentration with a constant volume. Column 1 served as the cell control (erythrocytes without virus).

Virus stocks were thawed on ice and diluted in PBS so that each well received 25 μL of virus suspension containing 10^5 TCID_{50}. Then 25 μL of virus suspension was added to each well of columns 2 - 12, including virus controls without erythrocytes. Plates were gently tapped to mix and incubated at room temperature (approximately 25˚C) on a level surface.

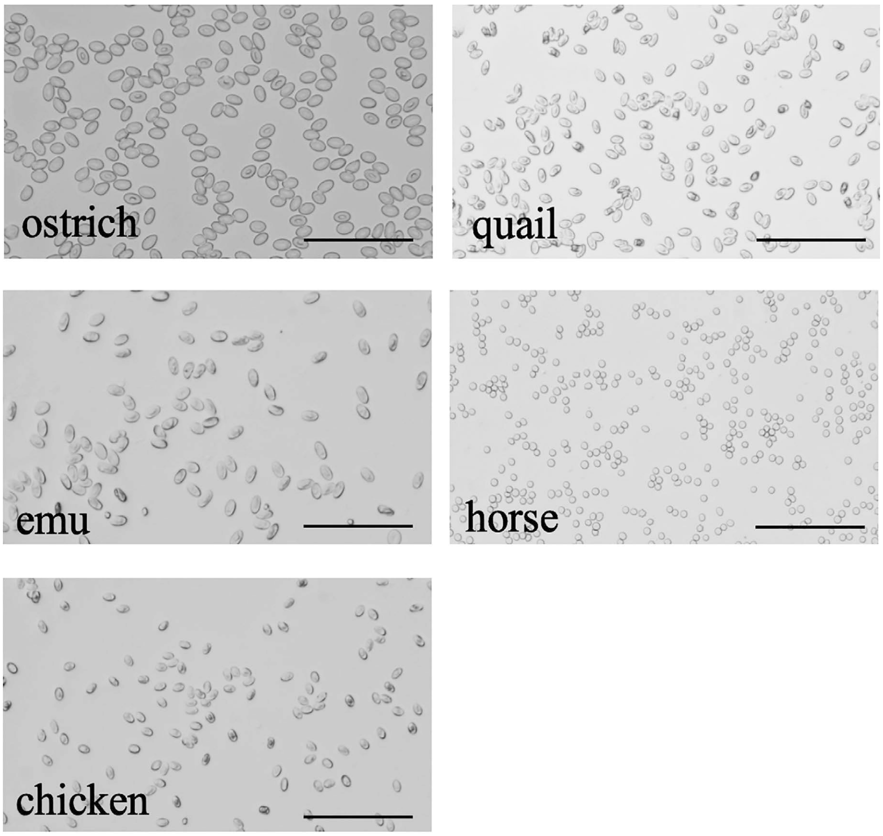

Figure 1. Morphology of erythrocytes from each animal species (unstained, same magnification). Representative bright-field micrographs of washed erythrocytes from ostrich, quail, emu, horse and chicken. Avian erythrocytes are oval and nucleated, whereas mammalian horse erythrocytes are smaller, round and anucleate. A stage-micrometer image (lower right panel) provides scale calibration, illustrating the much larger size of ratite (ostrich and emu) erythrocytes compared with those of chicken, quail and horse. Bars, 100 µm.

Complete hemagglutination was defined as a diffuse reddish layer of erythrocytes covering the bottom of the U-shaped well, whereas a compact red "button" in the center of the well indicated absence of agglutination. This visual end-point is standard in influenza HA and HI assays [4] [5]. This unconventional assay design—titrating erythrocyte concentration rather than virus concentration—was adopted to directly compare the minimal erythrocyte concentration required for stable hemagglutination across different animal species while keeping viral input constant. This approach enables clearer assessment of erythrocyte-dependent differences in HA kinetics and stability, which would be obscured if virus concentration were varied simultaneously.

2.6. Time-Course of Hemagglutination

To examine hemagglutination kinetics, plates were read at 0, 60, 90, 120, 180, 240 and 300 min after mixing virus and erythrocytes. At each time point, the highest erythrocyte dilution (2^n) showing complete hemagglutination was recorded as the HA titer for that virus-erythrocyte combination. Titers were expressed as the reciprocal of the erythrocyte dilution (HA titer = 2^n) and plotted versus time (**Figure 2**).

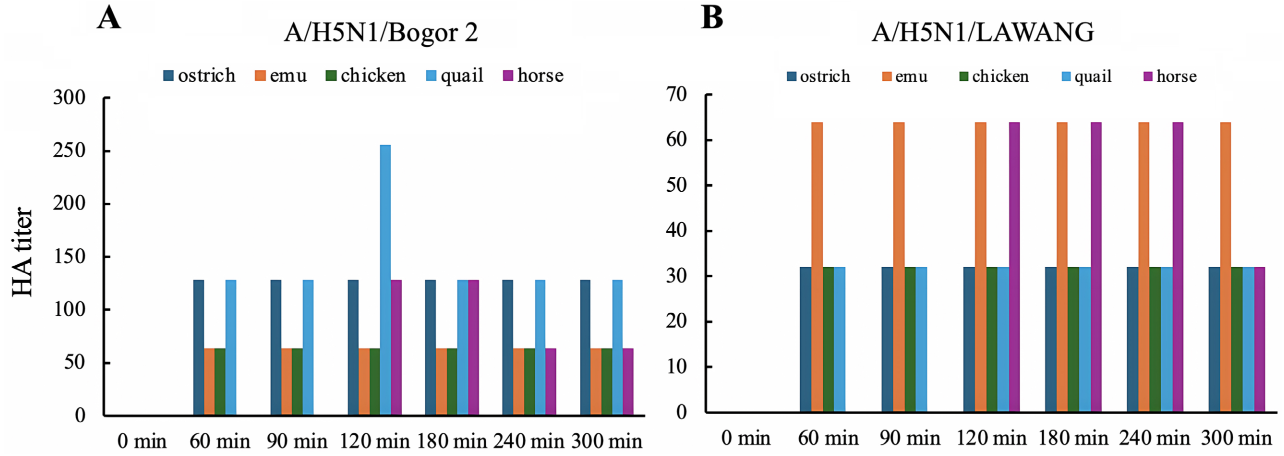

Figure 2. Time-course of hemagglutination titers for avian influenza A(H5N1) viruses using erythrocytes from ostrich, emu, chicken, quail and horse. Hemagglutination (HA) titers, expressed as the reciprocal of the highest erythrocyte dilution showing complete agglutination, were measured at 0, 60, 90, 120, 180, 240 and 300 min after mixing a constant viral dose (10^5 $TCID_{50}$ per well) with serially diluted erythrocyte suspensions. (A) A/H5N1/Bogor 2. (B) A/H5N1/Lawang. For A/H5N1/Bogor 2, horse erythrocytes show delayed and transient hemagglutination compared with avian erythrocytes, whereas ostrich and quail erythrocytes exhibit high and stable titers. For A/H5N1/Lawang, HA titers are more homogeneous and stable over time across all species, indicating that virus strain has a stronger effect on hemagglutination kinetics than erythrocyte species.

3. Results

3.1. Morphology of Erythrocytes

Bright-field microscopy of unstained erythrocytes revealed pronounced interspecies differences (**Figure 1**). Ostrich and emu erythrocytes appeared as very large, elongated ellipsoids with centrally located nuclei and relatively pale cytoplasm. Chicken and quail erythrocytes were smaller but retained the typical oval, nucleated avian morphology, with a higher density of cells in the field of view at the same magnification. In contrast, horse erythrocytes were the smallest among the five species, circular and anucleate, consistent with typical mammalian biconcave discs. Blood samples obtained from five animals per species (n = 5) showed consistent erythrocyte morphology and hemagglutination behavior. No discernible inter-individual variation was observed within any species, and representative images are therefore shown.

The stage micrometer image confirmed that ratite erythrocytes were several times longer than those of horses and approximately two- to three-fold longer than those of chickens and quails. Thus, the experimental panel comprised nucleated avian erythrocytes spanning a wide size range and small anucleate mammalian erythrocytes. Blood used for the hemagglutination experiments was obtained from at least several individuals for each animal species, and all animals yielded qualitatively identical hemagglutination profiles and titers. No clear inter-individual differences were observed in HA patterns under the conditions tested.

3.2. Time-Course of Hemagglutination Titers and Hemagglutination Patterns

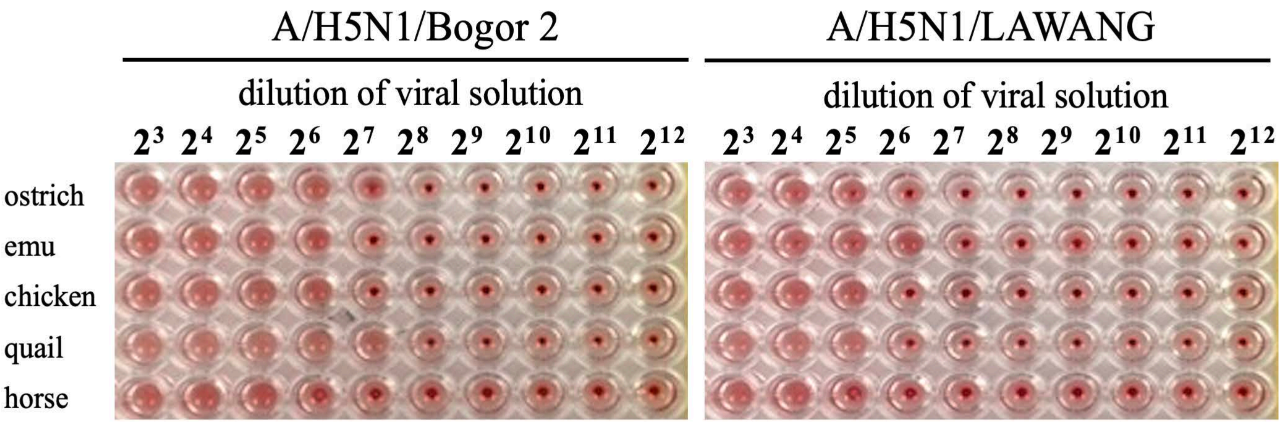

Figure 3. Hemagglutination patterns produced by avian influenza A/H5N1 viruses with erythrocytes from different animal species at 120 min. Microtiter plates showing hemagglutination of serially diluted erythrocyte suspensions from ostrich, emu, chicken, quail and horse at 120 min after addition of A/H5N1/Bogor 2 (left) or A/H5N1/Lawang (right). Each row corresponds to one animal species; columns show two-fold erythrocyte dilutions (2^3 - 2^{12}). A diffuse reddish layer covering the well bottom indicates complete hemagglutination, whereas a compact central red "button" indicates absence of agglutination. All erythrocyte species supported hemagglutination by both H5N1 strains, and the range of dilutions showing HA is consistent with the time-course data presented in Figure 2.

The evolution of HA titers over 0 - 300 min differed between the two H5N1 isolates (Figure 2). For A/H5N1/Bogor 2, ostrich and quail erythrocytes showed high HA titers already at 60 min that remained essentially stable through 300 min, indicating rapid onset and persistence of hemagglutination. Chicken and emu erythrocytes displayed moderate titers that also changed little over time. In contrast, horse erythrocytes showed a distinct kinetic pattern: titers were low or undetectable at early time points (60 - 90 min), increased markedly by 120 - 180 min and decreased again by 240 - 300 min. This suggests delayed formation and partial loss of stable aggregates with horse erythrocytes for this virus. For A/H5N1/Lawang, HA titers were more homogeneous across erythrocyte species. Ostrich, chicken, quail and horse erythrocytes yielded similar titers from 60 to 300 min, with only minor fluctuations. Emu erythrocytes tended to show slightly higher titers, but without clear temporal trends. Importantly, the delayed rise and subsequent decline in HA seen with horse erythrocytes for A/H5N1/Bogor 2 were not observed with A/H5N1/Lawang. Overall, the time-course data indicate that 1) erythrocytes from all five species are capable of supporting hemagglutination by both A(H5N1) isolates; 2) virus strain has a strong impact on HA kinetics; and 3) horse erythrocytes are particularly sensitive to strain-specific differences, displaying slow and transient agglutination with A/H5N1/Bogor 2 but rapid and stable responses with A/H5N1/Lawang. Representative hemagglutination patterns at 120 min after virus addition are shown in Figure 3. At this time point, clear hemagglutination was observed for both A/H5N1/Bogor 2 and A/H5N1/Lawang with erythrocytes from all five species. At lower erythrocyte dilutions, wells showed a uniform reddish sheet indicative of complete HA, while at higher dilutions distinct red buttons formed, indicating loss of agglutination. With A/H5N1/Bogor 2, ostrich and quail erythrocytes exhibited hemagglutination over a broad range of dilutions, suggesting high apparent HA sensitivity, in agreement with the high and stable titers seen in the time-course analysis. Emu and chicken erythrocytes showed HA over a somewhat narrower range. Horse erythrocytes required relatively higher cell concentrations (*i.e.* lower dilutions) for complete agglutination, consistent with the lower instantaneous titers at 120 min compared with the avian species. With A/H5N1/Lawang, differences among erythrocyte species were less pronounced. Ostrich, emu, chicken, quail and horse erythrocytes all showed comparable ranges of hemagglutination at 120 min, reflecting the more homogeneous and stable HA titers observed over the 0 - 300 min period. Together, the time-course data and the representative plate images provide a coherent picture in which virus strain exerts a stronger influence on hemagglutination behavior than erythrocyte species.

4. Discussion

This study investigated how erythrocytes from five animal species—including two large ratites (ostrich and emu)—interact with two HPAI A/H5N1 viruses in a hemagglutination assay. Two main findings emerge. First, despite marked differences in erythrocyte size and nucleation, all species supported hemagglutination by both H5N1 isolates, and no simple relationship was observed between cell size and HA titer. Second, differences in HA kinetics between virus strains were greater than those between erythrocyte species, suggesting that viral HA properties dominate over gross erythrocyte morphology in determining hemagglutination behavior.

4.1. Erythrocyte Morphology and Hemagglutination

Erythrocytes from ostrich and emu were markedly larger and more elongated than those from chicken and quail, whereas horse erythrocytes were the smallest and lacked nuclei, reflecting typical mammalian morphology. Despite these pronounced interspecies differences in erythrocyte size and nucleation, all erythrocyte types obtained from clinically healthy animals (n = 5 per species) supported hemagglutination by both HPAI A/H5N1 isolates examined in this study. No obvious inter-individual variation in hemagglutination patterns or titers was observed within each species, indicating that the results were reproducible and not driven by individual animal differences.

Notably, erythrocyte size did not correlate in a simple manner with hemagglutination titers. Although ratite erythrocytes possess a much larger surface area than those of chickens, quails or horses, they did not consistently yield higher HA titers. Conversely, horse erythrocytes, despite being small and anucleate, were robustly agglutinated by both viruses. These observations argue against a model in which erythrocyte size or nucleation alone determines HA efficiency. Instead, they support the view that hemagglutination is primarily governed by molecular interactions between viral hemagglutinin and erythrocyte surface receptors.

Previous studies have shown that erythrocytes from different animal species exhibit distinct distributions of α2,3- and α2,6-linked sialic acids, which strongly influence influenza virus binding preferences [6]-[9]. Avian influenza viruses, including H5N1, generally preferentially bind α2,3-linked sialic acids, although individual strains can display variable affinity profiles. Recent glycomic and lectin-binding analyses indicate that avian erythrocytes, including those of ratites such as ostriches and emus, predominantly express α2,3-linked sialic acids, whereas mammalian erythrocytes, including horse erythrocytes, also mainly present α2,3 linkages but with species-specific density and accessibility [28] [29]. These differences in receptor presentation likely exert a greater influence on hemagglutination outcomes than gross morphological features of the erythrocytes.

Taken together, the present findings indicate that, although erythrocyte morphology varies widely among the species examined, hemagglutination by A/H5N1 viruses is more strongly determined by the compatibility between viral HA and erythrocyte sialic-acid receptors than by erythrocyte size or the presence of a nucleus. This conclusion is consistent with previous reports emphasizing receptor specificity as a critical determinant of HA and HI assay performance and provides new comparative data for large ratite species, which have been underrepresented in earlier studies.

4.2. Virus Strain-Specific Effects

The most prominent strain-dependent difference observed in this study involved hemagglutination kinetics with horse erythrocytes. For A/H5N1/Bogor 2, hemagglutination developed slowly and was followed by a gradual decline in HA titer during prolonged incubation, whereas A/H5N1/Lawang produced rapid and stable hemagglutination with horse erythrocytes, comparable to that observed with avian erythrocytes. This finding indicates that virus strain-specific properties, rather than erythrocyte species per se, critically determine the stability and time course of hemagglutination.

Strain-dependent variation in erythrocyte binding and HA/HI titers has been widely reported for avian influenza viruses, including H5N1. Previous studies have shown that the optimal erythrocyte species for HA and HI assays can differ even among viruses of the same subtype, reflecting differences in hemagglutinin receptor-binding affinity and specificity [6]-[9]. Such variability highlights the importance of considering viral strain characteristics when interpreting HA data or selecting erythrocytes for diagnostic and serological assays.

One plausible explanation for the delayed and transient hemagglutination observed with horse erythrocytes for A/H5N1/Bogor 2 is a difference in neuraminidase (NA) activity between the two virus strains. Influenza virus attachment to erythrocytes is governed by a functional balance between HA-mediated binding to sialic-acid receptors and NA-mediated cleavage of these receptors, which facilitates virus release [1] [2] [17]. If A/H5N1/Bogor 2 possesses relatively higher NA activity or a HA-NA balance favoring receptor cleavage, initial HA-mediated aggregation of horse erythrocytes may be followed by NA-driven elution, resulting in destabilization of erythrocyte aggregates and a subsequent decline in HA titer.

This effect may be particularly evident with horse erythrocytes, which predominantly express α2,3-linked sialic acids but may present these receptors at lower density or with different spatial accessibility compared with avian erythrocytes [6] [9]. Under such conditions, NA-mediated receptor removal could disproportionately affect aggregate stability, leading to the observed transient hemagglutination. In contrast, A/H5N1/Lawang may exhibit a different HA-NA functional balance, allowing stable erythrocyte cross-linking to persist over time.

Recent reviews emphasize that subtle differences in HA receptor affinity, HA avidity, and NA catalytic efficiency can substantially influence virus-receptor interactions, host specificity and *in vitro* assay behavior, even among closely related influenza strains [17] [29]. Our observations with horse erythrocytes are consistent with this concept and suggest that hemagglutination kinetics can serve as a sensitive functional readout of strain-specific HA-NA balance.

Taken together, these results demonstrate that virus strain exerts a stronger influence on hemagglutination behavior than erythrocyte species, particularly in mammalian erythrocytes such as those of horses. Accordingly, HA results obtained with a single erythrocyte species should be interpreted with caution, and strain-specific differences should be considered when using HA or HI assays for comparative virological or serological analyses.

4.3. Implications for HA/HI Assays and Antibody-Based Interventions

In practice, HA titers are often used to standardize virus doses in HI and neutralization assays, typically by using a fixed number of HA units per well [4] [5]. If HA titers vary substantially depending on erythrocyte species and virus strain, then the effective amount of virus used in HI tests may also vary, potentially affecting measured antibody titers and complicating comparisons between laboratories or surveillance programs [6]-[8] [12].

Our findings suggest that, even within the same H5N1 subtype, the optimal erythrocyte species for HA and HI testing may differ between isolates. For the two viruses examined here, avian erythrocytes from ostrich, emu, quail and chicken all performed well, whereas horse erythrocytes showed strain-dependent kinetics. When designing HI assays for serological surveillance or vaccine evaluation—especially in non-traditional host species—it may therefore be prudent to empirically assess multiple erythrocyte types and select those providing high, stable HA titers with minimal background agglutination. These data also connect naturally with our previous work on ostrich egg-derived IgY antibodies against influenza HA [20]-[24]. We have shown that immunization of ostriches with recombinant H5 HA or with swine/seasonal influenza vaccines can yield large quantities of IgY that strongly bind to H5N1 and pandemic H1N1 viruses, inhibit HA-mediated hemagglutination and neutralize viral infectivity in MDCK cells. The present study reinforces the central role of HA-erythrocyte interactions in viral attachment and provides additional information on how these interactions differ across host species. Such knowledge is valuable for the rational development of HA-targeted interventions, including passive immunization with IgY, mucosal formulations or other antiviral materials designed to block HA and prevent virus adsorption to host cells.

4.4. Limitations and Future Directions

This study has several limitations that should be acknowledged. First, although blood samples were obtained from five independent animals per species (n = 5) and all individuals yielded qualitatively identical hemagglutination patterns and titers, the total number of animals per group remained modest, and formal statistical comparisons were therefore not performed. Future studies with larger sample sizes would be required to rigorously evaluate inter-individual variability and to perform statistical analyses of HA titers across species.

Second, the distribution and density of α2,3- and α2,6-linked sialic acids on erythrocytes from each species were not directly quantified in this study. Although previous reports indicate species-specific differences in sialic-acid linkage patterns [6]-[9] [28] [29], direct biochemical or glycomic analyses, such as lectin-binding assays or mass spectrometry, would be valuable to more precisely correlate erythrocyte receptor composition with observed hemagglutination kinetics.

Third, we did not perform molecular or functional characterization of the hemagglutinin and neuraminidase proteins of the two A/H5N1 isolates beyond existing pathogenicity classification. Detailed sequencing, structural analysis or enzymatic assays of HA and NA could help clarify the mechanistic basis for the strain-dependent differences in hemagglutination behavior observed in this study, particularly the delayed and transient agglutination of horse erythrocytes with A/H5N1/Bogor 2 [1] [2] [17] [29].

Fourth, the hemagglutination assay design employed in this study titrated erythrocyte concentration at a fixed infectious virus dose rather than titrating virus at a fixed erythrocyte concentration. While this approach was intentionally chosen to highlight erythrocyte-dependent differences in the minimal cell concentration required for agglutination, it differs from the conventional HA unit determination used in many diagnostic laboratories [4] [5] [12]. As such, direct numerical comparison of HA titers with those obtained using standard protocols should be made with caution.

Finally, hemagglutination-inhibition (HI) assays were not performed in the present study. Because HI assays remain a reference method for subtype-specific serodiagnosis and vaccine evaluation [5] [8] [12] [18], future work should determine whether the erythrocyte- and strain-dependent HA kinetics described here translate into corresponding differences in HI titers when testing sera from infected or vaccinated animals.

Future studies should therefore aim to expand the range of erythrocyte species examined, include additional avian influenza subtypes and strains with known receptor specificities, and integrate hemagglutination data with molecular analyses of viral glycoproteins and detailed characterization of erythrocyte sialic-acid profiles. Such integrated approaches will be essential for refining HA and HI assay selection and for improving interpretation of serological data in both veterinary and One Health surveillance contexts.

5. Conclusions

Erythrocytes from ostrich, emu, quail, chicken and horse—all readily obtainable veterinary species—were capable of supporting hemagglutination by two HPAI A(H5N1) isolates. Ratite erythrocytes were extremely large and nucleated, whereas horse erythrocytes were small and anucleate, yet hemagglutination titers did not correlate simply with erythrocyte size or nucleation. Instead, virus strain had a stronger influence on HA kinetics than erythrocyte species, particularly evident in the delayed and transient hemagglutination of horse erythrocytes with A/H5N1/Bogor 2 but not with A/H5N1/Lawang.

These findings indicate that viral HA properties—such as receptor affinity and functional balance with neuraminidase—likely dominate over gross erythrocyte morphology in determining hemagglutination outcomes. Consequently, the optimal erythrocyte species for HA and HI testing of A(H5N1) viruses may differ between strains and should be established empirically rather than assumed. Together with previous work showing that ostrich egg-derived IgY targeting HA can neutralize influenza viruses and block hemagglutination, our results support further exploration of HA-host cell interactions as a foundation for both improved serological assays and the development of antibody-based strategies for infection prevention and therapy.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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