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Potential of Neuraminidase from *Pasteurella multocida* for Inhibiting Avian Influenza Virus Subtype H9N2 Replication *In Ovo*

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ABSTRACT

In recent decades, neuraminidase/sialidase-based antivirals have been produced to suppress respiratory viral infections, including avian influenza, which relies on sialic acid as the entry point for viruses into cells. While neuraminidase has been extensively studied as an antiviral agent, numerous neuraminidases still have not been evaluated for their antiviral activities. Among these is NanB neuraminidase derived from *Pasteurella multocida*, which has received limited research attention. This study aimed to assess the potential of NanB neuraminidase in inhibiting H9N2 avian influenza virus infection *in ovo*. The research commenced with the molecular re-identification of the H9N2 A/Layer/Indonesia/WestJava-04/17 virus isolate, followed by determining the EID50 through Rapid HA test results. The toxicity of NanB neuraminidase was assessed by administering various doses to embryonated chicken eggs (ECE). The antiviral activity of NanB neuraminidase on ECE was evaluated through challenge tests, including treatment before, during, and after the challenge. The assessment involved monitoring the time of embryo death, virus titer through HA test, and viral copy number via RT-qPCR. The results indicated that the H9N2 virus titers capable of infecting 50% of ECE amounted to 108.83 EID_{50}/mL. A dose of 0.258 U/mL of NanB neuraminidase was found to be toxic, leading to embryo mortality after 48 hours of incubation at 37 °C, while a non-toxic dose was determined to be 0.129 U/mL. The post-challenge treatment group exhibited the most significant reduction in virus titer in ECE. Notably, NanB neuraminidase derived from *P. multocida* demonstrated the ability to inhibit H9N2 avian influenza virus infection in the ovo model, with the optimal dosage of 0.129 U/mL. The observed decrease in virus titers in the hemagglutination assay and viral copy number assays suggests that NanB neuraminidase holds promise as a potential antiviral candidate for therapeutic approach.

Keywords: antiviral; H9N2; in ovo; NanB neuraminidase; Pasteurella multocida

INTRODUCTION

Avian influenza (AI), or bird flu, is an acute viral RNA-enveloped infection caused by the Orthomyxoviridae family and Influenza A genus that can infect poultry and mammals (Rehman *et al.*, 2021). The highly pathogenic avian influenza virus subtypes H5N1 and H7N9 can directly infect humans from poultry (Shoimah *et al.*, 2019; Philippon *et al.*, 2020). H5N1 initially occurred in Hong Kong in 1997, and as of July 2019, 462 people had died out of 881 infected with a 52.4% CFR (Philippon *et al.*, 2020). According to WHO data, Indonesia has the most AI virus infections in Southeast Asia (WHO, 2020).

Another subtype of avian influenza virus, H9N2 can also harm animals and people. The H9N2 virus infects poultry reproductive organs, decreasing egg production, and costing layer chicken farms (Ismail *et al.*, 2018). H9N2's capacity to evolve and repeated human infections have sparked concerns about a new pandemic (Pusch & Suarez, 2018). According to WHO, China and Yangquan reported 2 human H9N2 subtype infections in 2022. From 1999 to 2022, 72 human H9N2 subtype infections occurred worldwide, including two deaths (WHO, 2022). According to Peacock *et al.* (2019), antigenic drift and antigenic shift mutations modify the pathophysiology of H9N2, making it potentially zoonotic.

Neuraminidase, also known as neuraminidase is an enzyme that plays a crucial role in the life cycle of certain viruses, including influenza viruses. One of the most well-known antiviral treatments targeting neuraminidase is a class of drugs called neuraminidase inhibitors. These inhibitors work by blocking the activity of neuraminidase, preventing the release of newly formed viral particles from infected cells and limiting the spread of the virus within the body (Heida *et al.*, 2021). Recent research has developed an antiviral treatment that targets neuraminidase, one of which is DAS181 (Fludase®). Unlike neuraminidase inhibitors, which inhibit the activity of the viral neuraminidase, DAS181 is designed to cleave sialic acid receptors on the surface of respiratory epithelial cells, thereby preventing viral attachment and entry into the cells. It has been primarily studied as a potential treatment for respiratory viral infections, including influenza, parainfluenza, and respiratory syncytial virus (RSV) (Vanderstocken *et al.*, 2020).

In addition to DAS181, which is a recombinant neuraminidase fusion protein composed of the active domain of *Actinomyces viscosus*, another neuraminidase can be produced from *P. multocida.* Two neuraminidase genes in *P. multocida*, NanH, and NanB, can hydrolyze distinct sialic acids. NanH hydrolyzes Neu5Ac2-3Gal, while NanB hydrolyzes Neu5Ac2-3Gal and Neu5Ac2-6Gal (Nugroho *et al.*, 2022). NanB, a broad-spectrum neuraminidase, can be utilized for producing anti-avian influenza viruses. Therefore, this study aimed to observe NanB neuraminidase from *P. multocida* as an antiviral treatment with AI H9N2 virus *in ovo*.

MATERIALS AND METHODS

Preparation and Molecular Re-Identification of Research Virus

The H9N2 avian influenza virus utilized in this study is an archived isolate obtained from PT. Medika Satwa Laboratoris, Bogor, Indonesia. The specific isolate is identified by the code A/Layer/Indonesia/WestJava-04/17 and has been assigned the GenBank with accession number MG957203. Virus propagation was performed in 10 days old ECE as followed in the previous study (Kurnia *et al.*, 2022a). A molecular examination using reverse transcription polymerase chain reaction (RT-qPCR) was performed to confirm the virus originating from allantois fluid. Geneaid Viral Nucleic Acid Extraction Kit II was used to extract virus RNA from allantois fluid.

Molecular examination using RT-qPCR for the gene encoding HA protein with the forward primer sequence 5'-ATCGGCTGTTAATGGAATGTGTT-3' and reverse 5'-TGGGCGTCTTGAATAGGGTAA-3' was performed according to Nugroho *et al.* (2021). The amplification process consists of 45 cycles. The amplified samples were observed using LongGene Real-Time PCR - Q2000C. The RT-qPCR running result is considered valid if the cycle threshold (Ct) value of H9 on the negative control is >40 and the Ct value of the positive control is <40.

Determining EID50 in Embryonated Chicken Eggs (ECE)

The amount of virus titer that can infect 50% of the used ECE is determined by calculating the embryo infectious dose 50 (EID50). Before calculating the EID50, the allantois fluid resulting from virus propagation in sterile PBS is diluted by a factor of 10. Each dilution was then inoculated into 5 ECEs with 0.1 mL/egg and then incubated at 37 ℃ for 4 days. Every 12 hours, the ECE was observed to know the embryo death time. Dead embryos were taken from the incubator and stored at 4 ℃ overnight. A rapid agglutination test was carried out simultaneously. The EID50 value is calculated using the Reed and Muench method (Kurnia *et al.*, 2022a).

Toxicity Test of Nanb Neuraminidase *In Ovo*

The toxicity test of purified NanB neuraminidase was carried out by observing embryo death in a 10-day-old ECE (n=3). The test was carried out with neuraminidase doses of 0.032 U/mL (Group I), 0.064 U/mL (Group II), 0.129 U/mL (Group III), 0.258 U/mL (Group IV), with PBS as the negative control representing 0 U/mL. Each dose of 0.1 mL was inoculated through the chorioallantois membrane and incubated at 37 ℃. The ECEs death was observed every 12 hours for 4 days using light Candler as a parameter to calculate the toxicity of the given neuraminidase (Galluzzi *et al.*, 2018).

Testing the Antiviral Activity of Nanb Neuraminidase *In Ovo*

Observation of the antiviral activity of NanB neuraminidase on ECE (n=5) was divided into three treatment groups, namely embryonated chicken eggs inoculated with the virus (Group V), placebo control group (Group C), and challenge group. The challenge group was divided into three administration groups: K1= Inoculation of the neuraminidase with the virus; K2= Inoculation of the neuraminidase before the virus; and K3= Inoculation of the neuraminidase after the virus. A total of 0.1 mL virus was mixed to 0.1 mL neuraminidase and incubated at room temperature for 30 minutes, then inoculated to each ECE in Group K1. Group K2 was first given 0.1 mL of neuraminidase, incubated at 37 ℃ for 2 hours, then inoculated with 0.1 mL 500 EID50 H9N2 virus, and vice versa with Group K3. The neuraminidase dose used in this test refers to the best dose in the previous study (Nugroho *et al.*, 2022), which was 0.129 U/mL, and PBS pH 7.2 was carried out as a negative control.

Observations of embryo death were carried out every 12 hours for four days after virus inoculation. Embryos that were still alive at 72 hours post-inoculation were stored at 4 ℃ to later harvest allantois fluid and the embryo. Comparison and evaluation of the results were based on the time of embryo death, virus titer, and viral copy number measured by absolute RT-qPCR.

Observation of H9N2 Virus Titer Using Hemagglutination Assay (HA) Test

Observation of H9N2 virus titer was conducted using the HA test with a microtiter plate technique. The HA test was carried out according to operational standards for HA/HI test for AI virus with twofold serial dilutions (Shoimah *et al.*, 2019). The HA titer was determined from the highest antigen dilution that could still perfectly hemagglutinate red blood cells.

Observation of Viral Copy Number of H9N2 Virus HA Gene

The allantois fluid RNA was extracted using the ReliaPrep RNA cell miniprep system (Promega). The concentration of RNA obtained was quantified using the QuantiFluor® RNA System kit (Promega) according to the manufacturing procedure. The pure RNA was then dissolved in 50 µL of RNase-free water and stored at −80 °C. The Quantus™ Fluorometer was used for RNA concentration readings. The RT-qPCR process was performed with the Kapa SYBR® Fast Master Mix Kit (Roche) using specific primers that converted RNA to cDNA using the ReverTra Ace cDNA synthesis kit (Toyobo).

The viral copy number calculation was performed through absolute quantification using forward and reverse primers specific to the HA gene, previously used in the H9N2 virus confirmation test. The standard sample was adjusted to known concentrations to generate external standard curves. This involved creating a series of six points through tenfold serial dilutions at different concentrations of viral cDNA. Additionally, dilutions with 10-fold increments ranging from 1 to 1×10^6 copies/reaction of positive control RNA virus H9N2 were employed to establish a standard curve. The Ct values obtained from the amplification results were then converted into viral copy numbers using the slope and intercept values calculated from the obtained standard curve (Nugroho *et al.*, 2022).

Data Analysis

The data were presented as mean ± SEM. Independent sample Mann-Whitney U tests were utilized to compare the groups, considering normality and homogeneity criteria. A significance level of 0.05 and a confidence level of 95% were employed to determine statistical differences between the two groups. The data were analyzed, and bar graphs were generated using GraphPad Prism 9.1.2 software.

RESULTS

Molecular Re-identification of Viruses

The re-identification of the study virus using RT-qPCR showed that the positive and negative controls yielded valid results following the kit's recommendations, with a Ct value of 29.11 for the positive control and no Ct value for the negative control. Based on the amplification results, the A/Layer/Indonesia/WestJava-04/17 isolate sample with the isolate code 081 had a Ct value of 28.64. The positive RT-qPCR result for the A/Layer/Indonesia/WestJava-04/17 isolate sample indicated that the sample was a H9 virus RNA sample. The amplification curves and the Ct of positive control, negative control, also A/Layer/Indonesia/WestJava-04/17 (isolate 081) can be seen in Figure 1.

Determined EID50 in ECE

The EID50 calculation was performed to determine the amount of virus titer that could infect 50% of the ECE. Inoculation of the virus into the ECE, which was incubated for 4 days at 37 °C, resulted in virus growth in the allantois fluid confirmed by the positive result of the rapid agglutination test in Figure 2 or HA test. The result of the rapid agglutination test is shown in Table 1. Reed and Muench method (Ramakrishnan, 2021) Log10 50% endpoint dilution = log10 of dilution showing a mortality next above 50% - (difference of logarithms x logarithms of dilution factor).

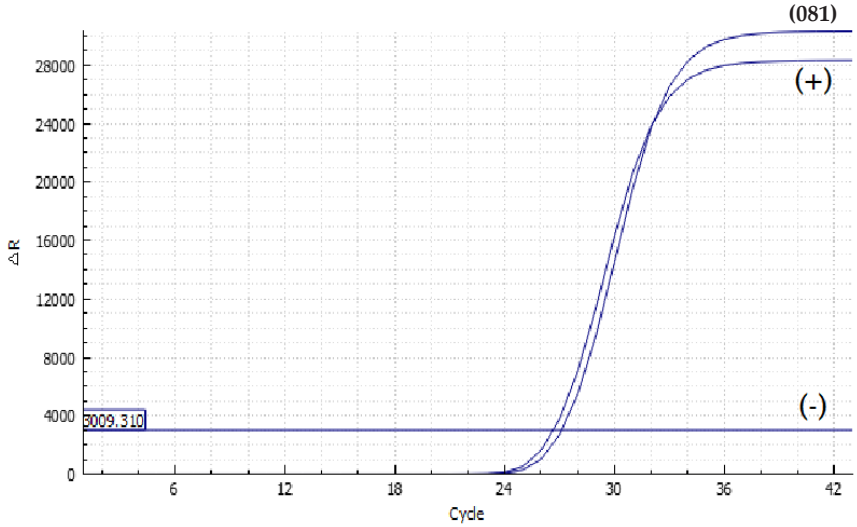

Figure 1. The RT-qPCR amplification curve of the avian influenza (AI) H9 gene. The CT of positive control is 29.11; negative control is 0.00; and isolate 081 is 28.64.

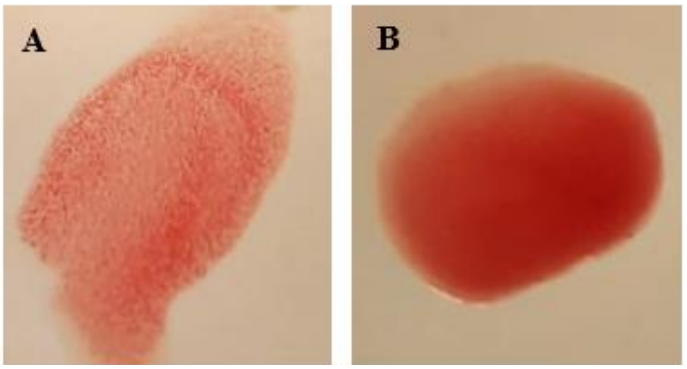

Figure 2. The results of the rapid hemagglutination test on the allantois fluid, [A] positive, with the presence of rough agglutination of red blood cells; [B] negative, without the presence of rough agglutination of red blood cells.

Table 1. The result of EID50 rapid hemagglutination assay (HA) test in embryonated chicken eggs

Dilution	Rapid HA test result in ECE (n=5)				
	ECE 1	ECE 2	ECE 3	ECE 4	ECE 5
10^{-6}	+	+	+	+	+
10^{-7}	-	+	+	+	+
10^{-8}	-	+	-	-	+
10^{-9}	-	+	-	-	-
10^{-10}	-	-	-	-	-

Note: ECE, embryonated chicken eggs (10 days old).

Difference of logarithms = ([(mortality at dilution next above 50%)-50%])/([(mortality next above 50%)-(mortality next below 50%)])

EID50 $= [10^{7 + ((87.5-50)/(87.5-42.8))}]$

$= 10^{7 + (37.5/44.7)}$

$= 107^{+ 0.83}$

$= 10^{7.83}$ / 0.1 mL

$= 108^{.83}$ EID50 /mL

The calculation of the EID50 using the Reed and Muench method showed that the virus titer that can infect 50% of the ECE was 108.83 EID50 /mL. The virus titer obtained from the EID50 calculation was used for virus inoculation in the NanB neuraminidase challenge test.

NanB Neuraminidase Toxicity and Antiviral Activity in ECE

Based on NanB neuraminidase toxicity test results, Group I with 0.258 U/mL dose of neuraminidase caused embryo death in all ECE from day 2 of incubation. However, groups II, III, and IV with doses of neuraminidase 0.129, 0.064, and 0.032 U/mL, respectively, were determined to be non-toxic to ECE.

The antiviral activity was evaluated based on embryo death time, the virus titer was measured using the HA test, and the viral copy number was measured by absolute RT-qPCR. The result showed no embryo death in the 4-day incubation period at 37 °C. However, the HA test showed that Group V has the highest average virus titer of 277.33 ± 125.85 (Figure 3). As was hypothesized, the viral titer reduced in all challenge groups compared to the virus inoculation group. The highest decrease was observed in group K3, with 106.66 reduction of titer virus.

Observation of Viral Copy Number of H9N2 Virus HA Gene

Quantitative evaluation by counting viral copy number through RT-qPCR assay (Figure 4) showed several results, including (1) all samples in the treatment groups were positive for H9N2 with a Ct value <40, (2) a dose of 0.129 U/mL of neuraminidase could inhibit H9N2 virus infection in ECE, (3) there was a difference in the mean viral copy number of H9N2 virus infecting ECE, where the virus group had the highest mean viral copy number of 5.60±10.03 copies/ml compared to the challenge groups (K1, K2, and K3). In addition, a significant difference was shown by the K1 and K3 challenge groups compared to the V group. The administration of neuraminidase in the K3 group was proven to have the best activity in inhibiting the replication of the H9N2 virus with the lowest mean of viral copy number value, which is 1.19±1.51 copies/mL, and the highest reduction in viral copy number of H9N2 virus, which is 4.41.

DISCUSSION

A safety factor is an important aspect that must always be considered in developing new drugs in the health field. Toxicity testing is a crucial step in drug development that will be used by living organisms such as humans and animals. Toxicity testing is an initial pharmacological and toxicological screening test to consider dosage determination, administration time range, and application (Zheng *et al.*, 2020; Darbar *et al.*, 2018). The toxicity testing of the purified NanB neuraminidase aims to determine a safe dosage that does not cause embryo death in the 10-day-old ECE model.

Further toxicity testing of neuraminidase activity is necessary, considering the development target of neuraminidase as an antiviral to inhibit the H9N2 virus in respiratory epithelial cells that are easily infected by high doses of neuraminidase. These results are consistent with the previous study (Nugroho *et al.*, 2022; Kurnia *et al.*, 2022b), which found that the tested toxicity activity of NanB neuraminidase on chicken and rabbit red blood cells and MDCK cells at doses of 0.032, 0.064, and 0.129

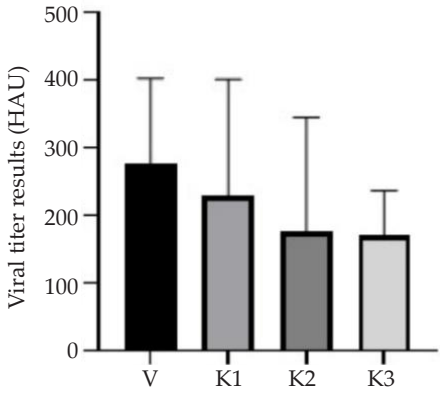

Figure 3. Diagram of virus titer results from hemagglutination assay (HA) on inoculated sample. V= embryonated chicken eggs inoculated with the virus; K1= Inoculation of the neuraminidase with the virus; K2= Inoculation of the neuraminidase before the virus; K3= Inoculation of the neuraminidase after the virus.

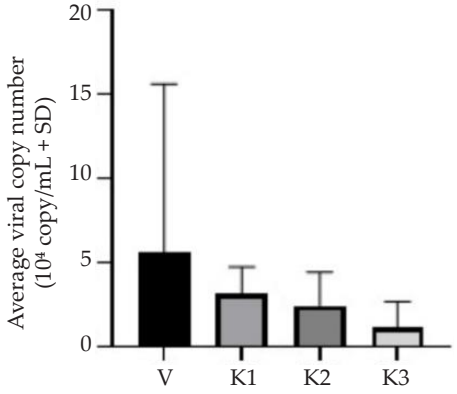

Figure 4. Diagram of the average viral copy number of the H9N2 virus that infects embryonated chicken eggs. Counts were made after 4 days of incubation. V= embryonated chicken eggs inoculated with the virus; K1= Inoculation of the neuraminidase with the virus; K2= Inoculation of the neuraminidase before the virus; K3= Inoculation of the neuraminidase after the virus.

U/mL showed low toxicity levels, although the dose of 0.258 U/mL tended to be toxic to the tested cells. Huo *et al.* (2020) also mentioned that neuraminidase is not toxic to human respiration as a therapeutic target, and there are no side effects related to neuraminidase treatment.

Sialic acid, as a virus receptor on the surface of respiratory epithelium, is an important factor in the process of avian influenza virus infection. Sialic acid can be hydrolyzed and degraded by neuraminidase, which acts as an inhibitory agent that blocks the binding of the host receptor by hydrolyzing it, thus preventing the virus from entering the host cell. In addition, the cleavage of sialic acid causes the failure of the virus budding process, thus preventing the virus from infecting other host cells (Juge *et al.,* 2016). The testing of NanB neuraminidase activity in this study aimed to prove its potential in inhibiting the initial attachment and final infection mechanism of the H9N2 virus. The use of the ECE model in this research has several advantages, including being non-contradictory to ethical or animal protection laws and reducing the use of laboratory animals (Ghoke *et al.,* 2018).

The three challenge groups in this study represent three different approaches: prophylactic in Group K1, virucidal in Group K2, and therapeutic in Group K3. This approach was used to systematically explore the potential of NanB neuraminidase from *P. multocida* bacteria in inhibiting the replication of the H9N2 virus in the ECE model. The absence of embryo death in the NanB neuraminidase toxicity test indicates that the H9N2 virus inoculated in the ECE has not reached the minimum titer that can cause embryo death. This can be caused by the neuraminidase activity that inhibits virus replication, either by preventing the attachment process of the virus with the receptor or by inhibiting the virus budding process.

The highest decrease in titer observed in Group K3, as determined by the HA test, indicates the superior antiviral activity of NanB neuraminidase in preventing virus replication. According to Kausar *et al.* (2018), the determination of virus titer in embryonated allantois fluid solely through the HA test may not be sufficient, and molecular testing such as RT-qPCR is necessary due to its sensitivity to detect small amounts of viral RNA. The results of the HA test align with the viral copy number findings, where group K3 exhibited the lowest gene copy number and the greatest reduction in viral copy number. These test results collectively indicate that NanB neuraminidase exhibits the most potent activity in inhibiting the virus using the ECE model in the challenge test, making it a potential candidate for antiviral therapy.

These results differ from our previous study on MDCK cells, where NanB neuraminidase from *P. multocida* demonstrated potential as a prophylactic agent against avian influenza infection in the respiratory tract (Nugroho *et al.,* 2022). The discrepancies in these findings can be attributed to variations in the methods of virus infection. In this study, the ECEs used predominantly possess Neu5Acα(2,3)-Gal, while MDCK cells predominantly have Neu5Acα(2,6)-Gal. These results suggest that using NanB neuraminidase as an antiviral candidate in poultry is approached from a therapeutic standpoint, whereas in mammals, it is approached from a prophylactic perspective.

To inhibit the binding of viral respiratory pathogens to their primary recognition sites, which are Neu5Ac molecules on surface receptors, it may be plausible to utilize bacterial neuraminidase as an inhibitory agent to cleave Neu5Ac and impede the binding process. However, to achieve this objective, certain properties are required. Firstly, effective neuraminidase should possess broad catalytic activity since the binding of pathogens like the influenza virus relies on the linkage between Sia and galactose. This was initially demonstrated by Rogers and Paulson in 1983 when they desialylated red blood cells and subsequently resialylated them using specific sialyltransferases linked to Neu5Acα(2,6)-Gal or Neu5Acα(2,3)-Gal. Avian or equine viruses agglutinated the former, while human

and swine viruses agglutinated the latter (Roger & Paulson, 1983). Consequently, a prevailing paradigm emerged suggesting that avian viruses primarily bind to Neu5Acα(2,3)-Gal, whereas human and swine viruses favor Neu5Acα(2,6)-Gal binding. These studies indicate that avian viruses would require multiple mutations to facilitate cross-species transmission since ducks and waterfowl predominantly possess α2-3-terminated Sia molecules in their intestines, offering little selection pressure for avian viruses to acquire α2-6 binding ability (Wilks *et al*., 2012).

Using neuraminidase as a prophylactic agent can reduce the severity of infection but may accelerate the virus budding process. This is due to the functional similarity of neuraminidase to the virus's own neuraminidase (Nugroho *et al.,* 2022). Therefore, the discovery of NanB neuraminidase activity as a therapeutic agent can reduce the possibility of infection severity by inhibiting the infection process within cells and virus budding. Additionally, NanB neuraminidase as a therapeutic agent can serve as an alternative treatment strategy within the class of neuraminidase inhibitors to control H9N2. This discovery is expected to reduce reliance on neuraminidase antiviral inhibitors like oseltamivir and zanamivir, which are widely reported to lead to virus resistance (Saadh *et al.,* 2021; Khairullah *et al.,* 2023).

In a previous study, NanB neuraminidase demonstrated effective hydrolysis activity towards both Sia Neu5Acα(2,3)-Gal and Neu5Acα(2,6)-Gal. This indicates that NanB neuraminidase holds significant potential as a therapeutic agent by removing sialic acid receptors, thus impeding the virus budding process within cells (Nugroho *et al.,* 2022). Although this study has shown that NanB neuraminidase is better suited as a therapeutic agent rather than a prophylactic one, further testing on animal models representing the dominance of each Neu5Acα(2,3)-Gal and Neu5Acα(2,6)-Gal receptor is necessary to complement this *in ovo* study. Additionally, investigating the impact of NanB neuraminidase usage as an agent on the potential for virus mutations is also crucial.

CONCLUSION

The NanB neuraminidase derived from *Pasteurella multocida* has effectively inhibited H9N2 avian influenza virus infection in the *in ovo* model. The administration of NanB neuraminidase at a dose of 0.129 U/mL has significantly reduced the HA titer and viral copy number in the treated group following virus inoculation. These findings suggest that NanB neuraminidase holds promise as a potential therapeutic antiviral agent. However, further studies are required to evaluate the efficacy of NanB neuraminidase as an antiviral agent in vivo using animal models.

CONFLICT OF INTEREST

All authors certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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