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RESEARCH ARTICLE

The Efficacy of bacterial Sialidase *Pasteurella multocida* against Influenza A Subtype H9N2 Virus *in-vitro* and *in-vivo*

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ABSTRACT

This study aimed to analyze the efficacy of NanB sialidase from *Pasteurella multocida* in suppressing Influenza A H9N2 infection both in vitro and in vivo. NanB sialidase experiments for viral suppression were carried out in vitro on MDCK cells at dosages of 0, 32, 64, 129, and 258 mU/mL. On the other hand, 14-day-old commercial laying hens were split up into five groups for the in vivo test: virus control (KV), negative control (KNV), and groups that received sialidase once (P1), twice (P2), and three times (P3) on various days at a concentration of 129 mU/mL intranasally. After the H9N2 virus was administered to groups KV, P1, P2, and P3 on day 4, the blood samples were collected to measure antibody titters against the virus. Clinical symptoms, body weight, antibody titters, the amount of viral replication in the lungs, and Toll-like receptor gene expression were all observed after the infection. NanB sialidase has proven effective in inhibiting H9N2 viruses, with an optimal dose of 129 mU/mL in vitro. P3, which was administered once daily for three consecutive days, was the group that performed best in terms of NanB sialidase's ability to inhibit AI H9N2 virus infection. The anti-avian influenza efficacy of NanB sialidase was also seen from its effect on TLR7 expression in the P3 treatment group, which was not significantly different compared to the KNV group. The results indicated that NanB sialidase, with the appropriate dosage and frequency, can effectively suppress avian influenza virus infection.

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INTRODUCTION

Bird flu, or avian influenza (AI), is a contagious infectious disease brought on by avian influenza viruses that are members of the Orthomyxoviridae family's influenza A species (Simancas-Racines *et al.,* 2023). These viruses are single-stranded RNA viruses consisting of 8 segments. Their envelope is negatively polarized and has a diameter of 200–300 nm x 50–120 nm, giving the appearance of being spherical or filamentous. Due to its genetic makeup, avian influenza have a significant capacity for mutation (Carter and Iqbal, 2024).

Based on their virulence, the AI viruses are separated into two groups: highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). The sort of cells or organs that the AI virus targets is determined by the amino acid composition at the cleavage site, which separates these two groups (Beerens *et al.,* 2020). In Indonesia, the HPAI subtype H5N1 and the LPAI subtype H9N2 are two AI virus subtypes that continue to spread and threaten public health (Lestari *et al*., 2020).

The H5N1 subtype outbreak was initially documented in 2003, infecting various poultry species, i.e. broiler chickens, layers, ducks, and geese, with a very high morbidity and mortality rate of 80–100% (Karo-

karo *et al*., 2019). Clinical signs of infected poultry usually include subcutaneous petechiae on the legs and thighs, blueness of the comb and wattles, diarrhea, nasal discharge, and hypersalivation, which frequently results in abrupt death (Lean *et al.,* 2022). In contrast to HPAI H5N1 infection symptoms, the LPAI subtype H9N2 virus tends to cause reproductive organ problems in poultry, leading to a characteristic decrease in egg production (Seiler *et al.,* 2018). In Indonesia, the first H9N2 outbreak was reported in 2015 after West Javan ducks tested positive for the virus (Tarigan and Sumarningsih, 2016).

Not all AI viruses are zoonotic agents that pose a significant threat to human health. In 1997, Hong Kong reported the first human cases of AI subtype H5N1, with 18 cases six of which were fatal recorded. According to data from the World Health Organization (WHO), there were 861 incidents of H5N1 subtype AI infection in humans globally between 2003 and 2020, resulting in a total of 455 deaths, including 168 occurred in Indonesia (Karo-karo *et al.,* 2019). Despite being categorized as LPAI in general, H9N2 viruses have also been known to infect humans in a number of nations, including China, Bangladesh, Egypt, Oman, and Pakistan (Peacock *et al.,* 2019).

The health threat posed by AI virus infections can be mitigated by implementing vaccination programs aimed at inducing antibodies to reduce symptoms caused by AI virus infections (Niqueux *et al.,* 2023). Various studies on vaccine development have been conducted to prevent infections by multiple subtypes of AI viruses. However, immunization is frequently unsuccessful because of the virus's easily mutable nature and the lack of homology between the vaccine's and the infected viruses (An *et al.,* 2022).

Studies have been developed to prevent AI virus infections, including bacterial-origin sialidase. As a virus receptor, sialidase breaks down sialic acid, in contrast to traditional antivirals that work after the virus enters the cell. By hydrolyzing the sialic acid receptor (Neu5Ac), which serves as a point of entry for a variety of respiratory viruses, sialidase stops the virus from infecting host cells (Nicholls *et al*., 2013; Marjuki *et al*., 2014). Research on *Clostridium perfringens* type A mentioned that their sialidase can prevent birds from being infected with AI from other sick birds (Kurnia *et al.,* 2023). One sialidase that has been created in humans is DAS181, which is derived from the apathogenic bacterium *Actinomyces viscosus* (Belser *et al.,* 2007).

NanB sialidase from *Pasteurella multocida* has also been effectively isolated and investigated for its anti-avian influenza properties. In vitro testing on chicken and rabbit red blood cells has demonstrated that this sialidase has anti-AI abilities. To demonstrate its capacity to inhibit AI infection in high-level species, NanB sialidase's anti-influenza properties still require additional in vitro testing employing MDCK cells and animal models such as chickens. According to the prior research, NanB sialidase has the capacity to hydrolyze not only the sialic acid Neu5Ac2-6Gal prominent in mammalian cells, but also the sialic acid Neu5Ac2-3Gal dominant in bird respiratory cells, which serve as an entry point for AI viruses (Mizan *et al.,* 2000). This study aimed to analyze the ability of NanB sialidase from *P. multocida* to suppress H9N2 AI virus infection, both in vitro and in vivo.

MATERIALS AND METHODS

Ethical declaration: The Animal Ethics Commission of the Faculty of Veterinary Medicine and Biomedical Sciences at IPB University reviewed and approved the experimental protocols, with approval number 096/KEH/SKE/VIII/2023.

The NanB Sialidase: The *Pasteurella multocida* bacteria that contained the NanB sialidase gene was taken from an archival isolate that was acquired from PT. Medika Satwa Laboratoris. The infectious agent was isolated from layer chickens that died from fowl cholera illness in Sukabumi Regency, West Java, Indonesia. The isolation, identification, purification of the pathogen, and synthesis of NanB sialidase were carried out sequentially in a laboratory operating at Biosafety Level 2 (BSL2).

Pasteurella multocida was preserved in freeze-dried ampoules for further cultivation on blood agar medium and underwent an incubation period of 18 hours at 37°C. To evaluate the purity of the isolates, numerous macroscopic, microscopic, and biochemical tests were also carried out (Sunartatie *et al.,* 2024). Subsequently, the isolated colonies were cultivated on brain heart infusion broth and subjected to a 3-hour incubation period by agitation with a water bath shaker at 34°C. The bacterial subcultures later transferred to a larger batch of 1000 mL brain heart infusion broth and agitated using a water bath shaker until they reached an optical density of 0.96 at a wavelength of 540 nm. To generate NanB sialidase-expressing *Pasteurella multocida* cells, they were centrifuged for 45 minutes at 4°C using a force of 4000×g. A number of purification methods were used to separate sialidase from bacterial cells, starting with the chloroform method, followed by anion exchange chromatography with Q-sepharose (Sigma-Aldrich, USA) and finally affinity chromatography with g N-(p-aminophenyl) oxamic acid–agarose (Sigma-Aldrich, USA) (Nugroho *et al.,* 2022). At each stage of purification, sialidase activity was quantified using the neuraminidase assay kit (Sigma-Aldrich, USA), following the appropriate protocols (Kurnia *et al.,* 2022), and the protein count was conducted using the Bradford method.

Cell Culture and Viral Adaptation: Madin-Darby canine kidney (MDCK) cells (ATCC, CCL34) used in this study were cultivated in Dulbecco's modified Eagle's medium (DMEM) high glucose (Sigma, UK), supplemented with 10% (v/v) of fetal bovine serum (Gibco, UK) and 1% penicillin/streptomycin in 25cm^2 flask (Thermo Scientific, USA) at 37°C and 5% CO_2 atmosphere. Cell concentration and viability were measured based on the crystal violet staining method for seeded and grown to 6×10^6 cells/mL for the growth evaluation of suspension cells in the flask. MDCK cells were grown onto 96-well plates and cultured overnight at 37°C and 5% CO_2 (Nugroho *et al.,* 2024).

The subtype H9N2 virus strain influenza A/chicken/Indonesia/MSL0123/2023 (H9N2) belonged to clade h9.4.2.5 (accession number OR243721) and was first cultivated in chicken embryonated eggs (Putra *et al.,* 2024).

The virus from allantois fluid was adapted to the suspension MDCK cells through two viral virus passages. Microplates were observed for cytopathic effect (CPE), and after adaptation, the infectious titer of the final seed virus was determined by TCID50 (Wu *et al*., 2020). The modified seed virus was stored at -80°C in 1 mL aliquots.

Inhibition Test of H9N2 Virus Infection by NanB Sialidase on MDCK Cells: This study used qualitative and quantitative methods to conduct the inhibition test of H9N2 virus infection by sialidase on MDCK cells. Qualitative testing was performed using the Indirect Fluorescent Antibody Technique (IFAT), while quantitative testing was carried out by counting the copy number of the H9N2 HA gene through RT-qPCR.

The inhibition test began by removing the MDCK cell media monolayer, followed by the addition of various doses of sialidase (0 mU/mL, 32 mU/mL, 64 mU/mL, 129 mU/mL, and 258 mU/mL), each 100µL, into the wells and incubated for 1 hour at 37°C and 5% CO_2. Afterwards, the sialidase was removed, and the adapted virus was added, 25 µL each containing 5×10^2 $TCID_{50}$. After incubating for 1 hour, the virus was washed, and Dulbecco's media added to the MDCK monolayer. Incubation was carried out for 96 hours. The MDCK cells post H9N2 inoculation were divided into two lanes. The first was harvested for subsequent RNA extraction and RT-qPCR, and the second was fixed for IFAT.

Harvested MDCK cells were obtained by centrifugation at 1500xg for 5 minutes. The extraction of RNA was conducted independently for further analysis using the ReliaPrep RNA miniprep kit (Promega, USA). The acquired RNA concentration was measured using the QuantiFluor® RNA System kit (Promega, USA) following the instructions provided by the manufacturer. RNA concentration was quantified using a Quantus™ Fluorometer, and the RNA was kept at a temperature of −70°C, including an RNase inhibitor. A quantitative RT-PCR was performed to quantify the viral genome using the SensiFAST™ SYBR® Lo-Roc One-step Kit (Bioline, USA). The reaction was conducted using a 20 µl total volume of a mixture consisting of a one-step mix, RNase inhibitor, RT enzyme, and primers that targeted a highly conserved section of the HA gene of the H9N2 influenza A (Table 1). Nucleotide confirmed by DNA sequencing was utilized to determine the viral copy number by serially diluting the sample to a 10-fold concentration. A standard curve was generated using 1 to 1×10^6 copies per reaction for the absolute quantitative real-time PCR.

The IFAT procedure began by removing the cell media after the post-virus inoculation incubation process, followed by washing with PBS, 300µL per well, then drying and adding 150µL of cold acetone (80%) for fixation and incubating for 30 minutes at 4°C. The acetone was removed, and the cells were dried at room temperature. H9 antiserum was added to each well, 50µL (1:20), followed by incubation at 37°C for 30 minutes and drying. Then, it was washed with PBS six times, 300µL each. FITC-conjugated anti-chicken IgG (1:200) was added to each well and incubated at 37°C for 30 minutes. The conjugate was removed, washed four times with PBS, dried, and read under a fluorescent microscope.

Evaluation of the Anti-Avian Influenza Capability of NanB Sialidase on Commercial Layer Chickens: The experiments were conducted on 30 fourteen-day-old commercial layer strain Hy-line brown chickens. Prior to the trial, the hens spent a week acclimating to the Animal Research Facilities. They were divided into a completely randomized design of five treatment groups with six replications. The hens divided into the following groups: KNV, which received no treatment; KV, which received 0.03mL of PBS once on the first day of treatment; P1, which received 0.03mL of NanB sialidase once on the first day of treatment; P2, which received 0.03mL of NanB sialidase twice on the first and second days of treatment; and P3, which received 0.03mL of NanB sialidase once on the first, second, and third days of treatment.

The animal model in this investigation received an intranasal dose of 0.03 mL of NanB sialidase at a concentration of 129 mU/mL, which is equivalent to 0.00387 U of NanB sialidase, in each nostril. The H9N2 AI virus was administered intranasally to chickens in the KV, P1, P2, and P3 groups at a dose of 50µL 106 EID50 on the fourth day of the trial. Blood samples were then obtained to use the hemagglutination (HA) test to measure antibody titers against the H9N2 virus. To track symptoms, observations were made every day after the challenge. In 2 and 4 dpi (days post-inoculation), tracheal and cloacal swabs were obtained for viral shedding examination by RT-qPCR. On the ninth day of the treatment, all the chickens were weighed, blood samples were taken for post-challenge antibody titers, and they were terminated for lung organ retrieval. In order to measure the viral copy number of the avian AI H9N2 virus and the gene expression of Toll-like receptors (TLRs), specifically TLR3 and TLR7, total RNA extraction was then carried out on a portion of each tracheal swab and the lung organs using the Total RNA Mini Kit (Geneaid, Taiwan) for the subsequent RT-qPCR.

The Calculation of the Viral Copy Number of the H9N2 HA Gene and Toll-like Receptor Gene Expression: The RT-qPCR was undertaken for converting RNA into cDNA using the ReverTraAce cDNA synthesis kit (Toyobo, Japan). The RT-qPCR process was carried out using the SensiFAST™ SYBR Lo-ROX Kit (Bioline, Taunton) with specific primers (Table 1). The viral copy number calculation was analyzed by absolute quantification. The standard curve was obtained using dilutions by a factor of 10 against the positive control of H9N2 virus RNA, which ranged from 1 to 1×10^6 copies/reaction. The amplification result's cycle threshold (Ct) values were converted into viral copy numbers based on calculating the slope and intercept values from the obtained standard curve (Nugroho *et al.,* 2022).

Table 1: Primers encode gene expression for viral copy numbers (H9 gene) and toll-like receptors (TLRs).

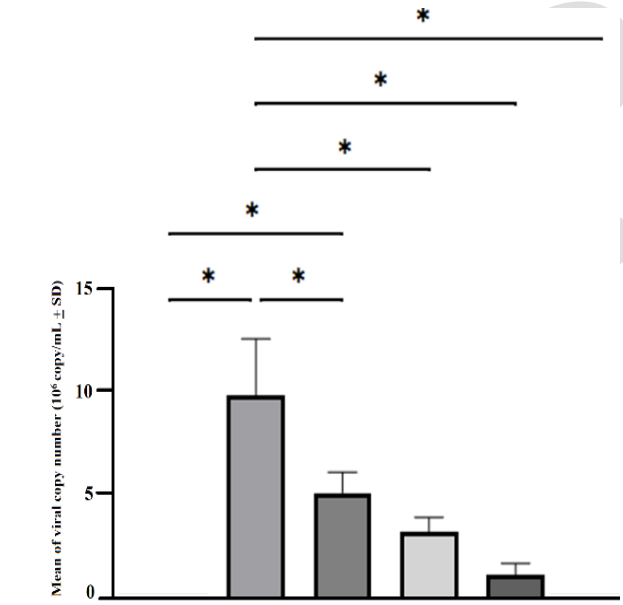
Target genes	Primers	Sequences (5′-3′)	References
H9	H9-F	ATCGGCTGTTAATGGAATGTGTT	(Chaharaein *et al.,* 2009)
	H9-R	TGGGCGTCTTGAATAGGGTAA	
TLR3	TLR3-F	ACAATGGCAGATTGTAGTCACCT	(Kurnia *et al.,* 2022)
	TLR3-R	GCACAATCCTGGTTTCAGTTTAG	
TLR7	TLR7-F	TGTGATGTGGAAGCCTTTGA	(Kurnia *et al.,* 2022)
	TLR7-R	ATTATCTTTGGGCCCCAGTC	
Beta actin	B.act-F	GAGAAATTGTGCATGACATCA	(Wang *et al.,* 2020)
	B.act-R	CCTGATACCTCTCAATGCCA	

TLR gene expression was calculated using the same RT-qPCR method as the viral copy number calculation. Specific primers were used to evaluate the expression of toll-like receptor-encoding genes (Table 1). The relative Ct ratio of the target in the sample to the target Ct in the calibrator/sample control served as the foundation for the gene expression analysis. The results indicate that, compared to reference, gene expression was increased or decreased (Kurnia *et al.,* 2022).

Data Analysis: Quantitative data was analyzed using the SPSS software and GraphPad Prism 9.1.2. Data presented in this study were the results of repeated experiments and were shown as the mean and standard error of the mean or standard deviation (SD). Results were deemed statistically significant when the p-value was less than 0.05 and less than 0.01 following analysis of variance.

RESULTS

The sialidase-induced inhibition test of H9N2 virus infection on MDCK cells revealed that the dosage group receiving 0 U/mL had the highest viral copy number. A significant reduction in the viral copy number was observed at the 32mU/mL, 64mU/mL, and 129mU/mL doses, with varying significant reductions ($P<0.05$) observed. In contrast, the 258mU/mL dose showed the lowest mean value of $0.02 \pm 0.01 \times 10^6$ copy/mL (Fig. 1).



Sialidase(mU/ml)	-	0	32	64	129	258
H9N2 virus	-	+	+	+	+	+
Mean	0	10.00	5.25	3.33	1.25	0.02
SD	0	2.77	1.01	0.71	0.55	0.01

Fig. 1: The mean viral copy number of the H9N2 virus in the supernatant of the MDCK cell culture was incubated for 96 hours.

The IFAT method of qualitative inhibition testing of H9N2 virus infection by sialidase on MDCK cells showed that the most effective dose for blocking virus entry into the cells was 129mU/mL. The IFAT results of all graded doses of sialidase were administered to MDCK cells and then challenged with the H9N2 virus (Fig. 2).

Based on the analysis of chicken body weight, there was a significant difference ($P<0.05$) between one group and another. Following additional testing using multiple comparisons, group P2 was shown to have significant differences ($P<0.05$) from KNV, with respective values of 157.17 ± 10.65 grams and 186.83 ± 15.13 grams. Results from other groups, namely KV, P1, and P3, showed no significant differences ($P>0.05$) with respective means of 166.67 ± 18.26 grams, 167.83 ± 14.11 grams, and 169.00 ± 12.26 grams (Fig. 3A).

The analysis of H9N2 antibody titers tested by the HI method showed no significant differences ($P>0.05$) among treatment groups (Table 2). The study found that following the virus challenge, the antibody titers of groups KNV, KV, P1, and P3 decreased, but no significant differences were observed. Unlike other groups, one group showed increased antibody titers, namely group P2 (Table 2).

Groups P1 and P3 demonstrated substantial differences ($P<0.05$) in the viral copy number of the virus in the lungs when compared to KNV. Although not showing substantial differences, group P2 had the highest mean viral copy number compared to other groups (Fig. 3B).

According to the study, on day four after the challenge, viral shedding in the challenged groups rose in KV and P1. On the other hand, groups P2 and P3 experienced a decrease on day four following the challenge as opposed to day two. On day two after the challenge, group P3 showed the highest viral copy number value, while on day four after the challenge, group KV displayed the highest. Although showing the highest value, on day four post-vaccination, there was a decrease in viral copy number value in group P3 (Table 2).

Table 2: Mean ± SD of HI titers for H9N2 before and after challenge and viral Shedding in different experimental chicken groups at 2nd and 4th dpi.

Group	Antibody Titer Test Using HI Assay		Viral Shedding (10^4 copies/mL)	
	Before challenge	After Challenge	2 dpi	4 dpi
KNV	6.33 ± 0.82a	5.00 ± 1.10a	0.00 ± 0.00a	0.00 ± 0.00a
KV	6.17 ± 0.98a	5.33 ± 1.21a	275.35 ± 30.52a	21402.43 ± 1652.20b
P1	6.17 ± 1.33a	5.17 ± 1.47a	1321.40 ± 90.40b	18585.28 ± 2029.30bc
P2	6.17 ± 1.17a	6.50 ± 2.17a	349.27 ± 25.56a	194.22 ± 54.61a
P3	6.17 ± 1.60a	6.00 ± 0.89a	9504.95 ± 1488.82c	1748.33 ± 358.83a

n=6 samples from each group. Mean values with different letters in the same column differ significantly at $P<0.05$.

The study results showed significant differences ($P<0.05$) in TLR3 expression in groups P3 and KV compared to KNV. However, compared to the standard group KNV, other groups P1 and P2, showed lower results (Fig. 4A). In contrast to TLR3 expression, analysis of TLR7 expression showed significant differences ($P<0.05$) between KNV and groups P1, P2, and KV (Fig. 4B).

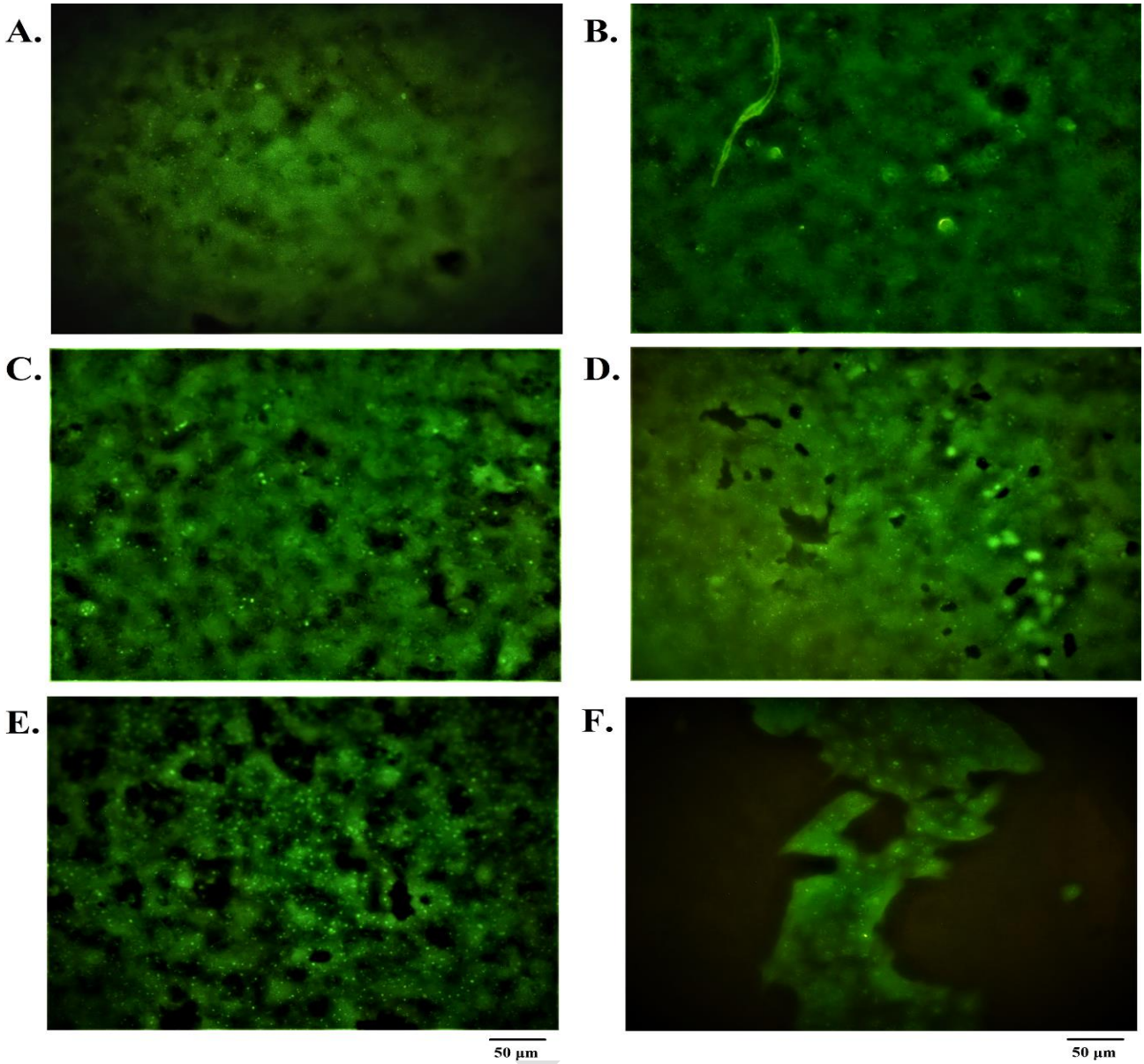
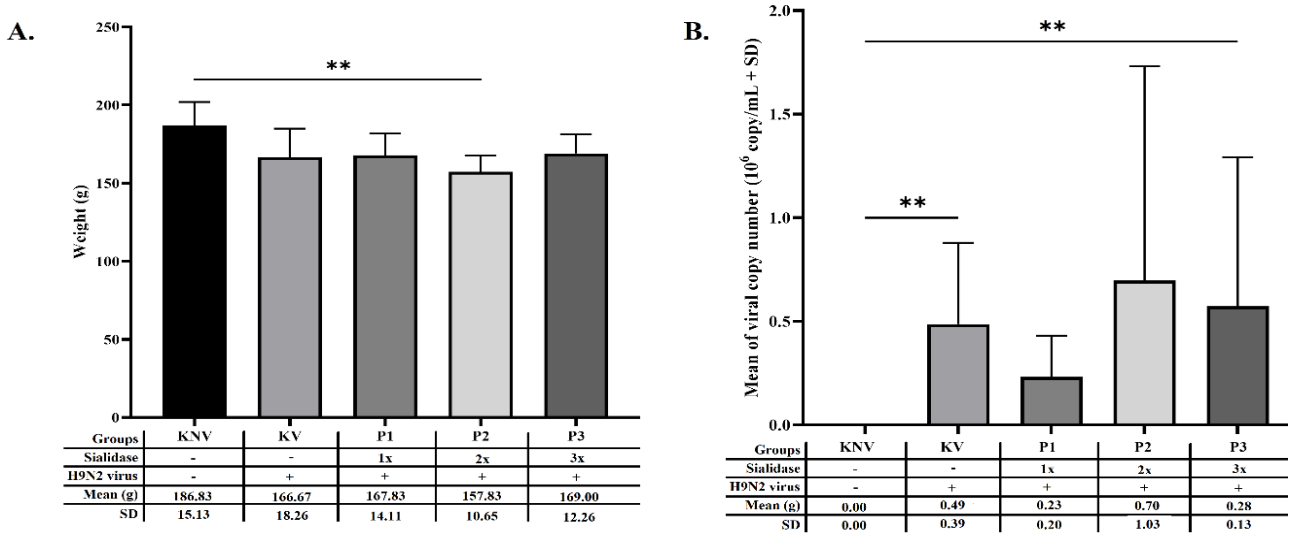


Fig. 2: Representative results of IFAT (Magnification 40x) showing the inhibition of H9N2 virus infection by sialidase in MDCK cells. Viruses appear as fluorescent dots with varying amounts at all sialidase dose levels. (A) Virus control; (B) dose 0 mU/mL; (C) dose 32 mU/mL; (D) dose 64 mU/mL; (E) dose 129 mU/mL; (F) dose 258 mU/mL.



Groups	KNV	KV	P1	P2	P3
Sialidase	-	-	1x	2x	3x
H9N2 virus	-	+	+	+	+
Mean (g)	186.83	166.67	167.83	157.83	169.00
SD	15.13	18.26	14.11	10.65	12.26

Groups	KNV	KV	P1	P2	P3
Sialidase	-	-	1x	2x	3x
H9N2 virus	-	+	+	+	+
Mean (g)	0.00	0.49	0.23	0.70	0.28
SD	0.00	0.39	0.20	1.03	0.13

Fig. 3: (A) Results of chicken body weight measurements on Termination Day; (B) virus testing results in lung organs using viral copy number with RT-qPCR method.

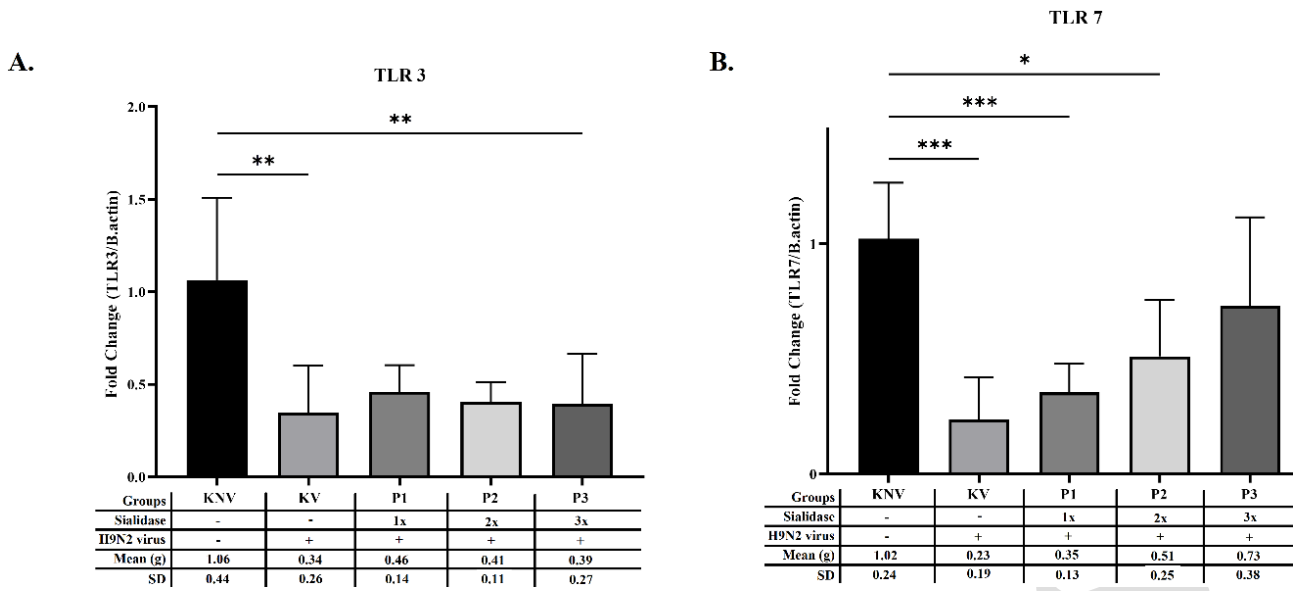


Fig. 4: Results of TLR 3 (A) and TLR 7 (B) gene expression tests using RT-qPCR.

DISCUSSION

Sialic acid is a crucial determinant in avian influenza virus infection, as it serves as a receptor at the beginning and end of the virus replication process in target host cells. Like other avian influenza viruses, H9N2 virus infection starts with the HA protein's cleavage by cellular proteases in the target host cells. This step activates the HA protein into two parts, HA1 and HA2. While HA2 participates in the fusion process between the virus envelope and the target host endosomal membrane, HA1 binds to the sialic acid Neu5Acα(2,6)-Gal or Neu5Acα(2,3)-Gal (Sriwilaijaroen and Suzuki, 2012). Subsequently, the virus replicates inside the host cells until progeny viruses are formed and ready to exit the host cells. Budding is the stage of virus release from the cell. During this stage, the virus's HA protein remains bound to the host cell's sialic acid, requiring the virus's NA protein function to cleave this bond, so the virus can fully detach from the cell. NanB sialidase, developed as an antiviral in this study, aims to inhibit both the initial and final infection stages of the H9N2 virus. Sialidase works by hydrolyzing sialic acid, which stops the virus from infecting a host.

The inhibition of H9N2 infection by sialidase was also examined in vitro using MDCK cells in this work. The results confirm the use of red blood cells as a straightforward indicator for measuring the inhibition of avian influenza virus infection (Nugroho *et al.,* 2022). According to the MDCK cell test outcomes, sialidase at a concentration of 0.129 U/mL could hydrolyze sialic acid and prevent H9N2 virus infection in MDCK cells without harming the cells. Conversely, doses below 0.129 U/mL were less effective in inhibiting H9N2 virus infection, as evidenced by the increased viral copy number observed after 96 hours of incubation.

The decrease in NanB sialidase activity on the third day of incubation suggests that sialic acid hydrolysis in MDCK cells may continue for up to two days. This finding supports the potential value of NanB sialidase from *P. multocida* as a prophylactic agent against avian influenza infection in the lower respiratory tract of humans (Nugroho *et al.,* 2022). This is because avian influenza viruses primarily target alveolar epithelial cells, which exhibit the sialic acid form Neu5Acα(2,6)-Gal. On the other hand, using sialidase as a prophylactic agent would reduce the severity of infection compared to its use as a therapeutic agent. Given the possible severity arising from sialidase's functional resemblance to the virus's neuraminidase, which could speed up the budding process, more research is required before NanB sialidase can be used as a therapeutic treatment.

The success of sialidase as an antiviral in inhibiting Influenza A virus infection has been reported in the development of DAS181. This protein has demonstrated the ability of sialidase to inhibit H1N1 influenza virus infection, both in vitro and in vivo (Triana-Baltzer *et al.,* 2011). In animal studies, DAS181 sialidase has also suppressed H7N9 subtype replication, preventing H7N9 infection-related deaths in mice (Marjuki *et al.,* 2014). Similar investigations are needed to ascertain the efficacy of NanB sialidase from *P. multocida* as an inhibitor of H9N2 subtype infection in animal models.

In addition to the inhibitory effect on infection demonstrated by sialidase activity, using sialidase as an antiviral offers several advantages over conventional antiviral treatments. Triana-Baltzer *et al*. (2011) demonstrated that repeated passages of the H3N2 influenza virus over 30 times with DAS181 inhibition did not lead to genetic mutations resulting in resistance to DAS18. However, one limitation of this study is its failure to demonstrate the effect of administering *P. multocida* NanB sialidase on genetic changes in H9N2 virus inhibited in MDCK cells.

In vivo studies showed that all groups challenged with the H9N2 virus exhibited no clinical symptoms. As a result, TLR3 and TLR7 expressions, body weight, antibody titers, virus amount in the lungs, and virus shedding were all noted. After a certain incubation time, when the virus multiplies and throws the body's systems out of balance, clinical symptoms of avian influenza virus infection typically appear. Usually, poultry infected with low pathogenic avian influenza viruses (LPAI) do not show

clinical symptoms. However, conditions including H9N2 virus infection, mild to moderate respiratory disturbances, head swelling, nasal discharge, decreased appetite and drinking, greenish feces, and decreased egg production in mature laying hens may occur (Jonas *et al.,* 2018).

Previous research has indicated that weight loss is one symptom of H9N2 avian influenza virus infection. H9N2-infected broiler chicks lost weight in comparison to uninfected groups (Jonas *et al.,* 2018). In this study, Group P2 had the highest infection rate, as evidenced by their lowest average chicken weight. Conversely, the P3 therapy group had the least severe illness based on average weight loss. The results are suspected to be influenced by a higher infection rate in P2 compared to P3. Based on antibody titer analysis, within five days, antibody titers rapidly increased in group P2, which had the lowest average weight. When an antigen enters the body, antibodies are created in reaction (Yang *et al.,* 2023). Commercial chickens vaccinated from strict parents usually experience a decline in maternal antibodies within 21 days after hatching. However, the decline in H9N2 maternal antibody titers might be postponed in some circumstances, such as when field viruses are present (Liu *et al.,* 2023).

In addition to antibodies, the severe infection experienced by Group P2 was confirmed by the quantity of virus found in the lungs. Group P2 showed the highest presence of H9N2 AI virus in the lungs compared to other groups. The lungs are one of the target organs of avian influenza virus infection. Virus replication in lung cells increases the virus quantity, potentially causing virus shedding if the body's antibodies have not neutralized the replicating virus (Clementi *et al.,* 2021). The increased antibody titer in Group P2 may be attributed to the higher virus quantity in the lungs compared to other groups, which could also affect the lower virus shedding in Group P2 compared to different groups. Sufficient antibody presence can prevent virus shedding.

In contrast to Group P2, the efficacy of sialidase administration was demonstrated in Group P3 across various analyzed parameters. Group P3 showed the most negligible weight loss compared to the KNV control group. In a similar vein, Group P3 had less lung virus than KV control. Group P3 showed increased viral shedding.

TLR3 and TLR7 receptors are crucial for the body's defense against virus infections by recognizing RNA originating from the virus. In AI virus infections, TLR7 is associated with the ssRNA of the virus itself, while TLR3 is associated with dsRNA from infected VAI cells (Hennessy and McKerna, 2021). In this study, the decrease in TLR3 expression could be attributed to the extensive use of TLR3 receptors in lung organs, reducing their expression. The expression of TLR7 in Group P3 showed no significant difference compared to the KNV group. Based on these findings, P3 has the least amount of VAI infection when compared to other groups that were exposed to H9N2 VAI.

In this study, almost all parameters indicate that Group P2 had the most severe H9N2 infection compared to the sialidase-treated groups P1 and P3. Group P2 received sialidase once a day for two consecutive days. Additionally, P2's infection rate was higher than that of the viral control group (KV) in the absence of sialidase treatment. Although the exact cause of these results is not yet known, the findings of the DAS181 study, which involves sialidase from the bacterium *Actinomyces viscosus* in degrading sialic acid, support the results of this study. Compared to smaller dosages, administering a significant amount of sialidase over a certain period may not always hydrolyze the most sialic acid (Triana-Baltzer *et al.,* 2011). On the other hand, hormesis, where a dose-response phenomena implies that low dosages stimulate, could be another reason for the poorest results in P2. In contrast, high doses inhibit effects (Calabrese *et al.,* 2018).

Conclusions: This study concluded that NanB sialidase has proven effective in inhibiting H9N2 subtype avian influenza virus infection, with the optimal dose being 0.129 U/mL both in vitro and in vivo. This was demonstrated by the considerable decrease in the amount of H9N2 viruses entering cells in the IFAT assay and the low virus count in MDCK cells following suppression by NanB sialidase. The best duration of administration was once daily for three consecutive days at 0.00387 U/0.03 mL (converted from a dose of 129 mU/mL). In vivo testing showed that intranasal administration of NanB sialidase from *Pasteurella multocida* in chickens effectively suppressed H9N2 avian influenza virus infection. A drop in antibody titters, which indicates the absence of severe sickness, and the lack of considerable weight loss when compared to the normal control group without infection are indicators of the effectiveness of this dosage and duration. Viral copy number calculations from lung organs showed significantly lower values than the virus-challenged group, accompanied by a decrease in virus shedding four days post-challenge. However, the effect of NanB sialidase on TLR7 expression in the group, which did not differ significantly from that of the normal uninfected group, also demonstrated the enzyme's anti-avian influenza capabilities.

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