

Incorporation of a tetrameric M2e antigen enhances cross-protective efficacy of an inactivated H5N1 avian influenza vaccine

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Abstract. Avian influenza (AI) viruses continuously undergo genetic mutations that reduce vaccine efficacy and allow outbreaks to persist. The ectodomain of the M2 protein (M2e) is highly conserved among AI viruses, making it a promising universal vaccine candidate, although its small size limits immunogenicity. This study evaluated a tetrameric M2e antigen (4×M2e51) to enhance vaccine performance. Initial attempts to fuse 4×M2e51 with *Salmonella pullorum* flagellin were successful at the DNA and expression levels, but the protein was unstable, and research continued with 4×M2e51 alone. Three inactivated H5N1 oil-emulsion vaccines containing mineral oil as adjuvant were prepared: one containing the M101 isolate, one with the M166 isolate, and one combining M101 with 4×M2e51. M101 (A/Ck/West_Java/M101/2016) and M166 (A/Quail/West_Java/M166/2019) belong to clade 2.3.2.1g and share 95.72% hemagglutinin-1 protein similarity, with evidence of immune evasion. Separate groups of SPF chickens were vaccinated at 28 days of age and challenged with M166 three weeks later. Results showed that hemagglutination inhibition assays detected significant antibody titers only in chickens vaccinated with the homologous M166 strain before challenge. Challenge outcomes demonstrated that supplementation of the M101 vaccine with 4×M2e51 conferred complete protection (100% survival) against heterologous M166 challenge at both full and one-fifth antigen doses, comparable to the homologous vaccine, whereas the M101-only vaccine provided partial (90%) or poor (20%) protection. All unvaccinated controls died (100% mortality). These findings demonstrate, for the first time in field-relevant inactivated vaccines, that incorporating M2e enhances cross-protection, even when the vaccine antigen was not homologous to the challenge virus.

1. Introduction

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Highly pathogenic avian influenza (HPAI) caused by H5N1 remains one of the most persistent transboundary animal diseases affecting both poultry production and public health. Since its emergence in Asia in the late 1990s, H5N1 has evolved into multiple genetic clades and continues to circulate in various regions worldwide. In Indonesia, avian influenza (AI) has become endemic in poultry populations and remains a continuing threat due to its zoonotic potential, as human infections continue to occur globally [1-2]. Despite substantial progress in biosecurity and surveillance, avian influenza remains endemic in large parts of Indonesia, sustained by dense poultry populations, live bird markets, and viral genetic diversification [3-4].

Vaccination has been the cornerstone of Indonesia's long-term strategy for avian influenza control since its nationwide implementation in 2004. The policy was established to mitigate economic losses in the poultry sector and reduce zoonotic risk rather than to achieve complete virus eradication. Under this strategy, vaccination is applied as part of an integrated approach combining enhanced biosecurity and surveillance [5]. Inactivated oil-emulsion vaccines prepared from local isolates have been widely used across commercial poultry systems. However, despite the proven effectiveness of vaccination in preventing infection and reducing viral shedding, outbreaks continue to occur due to antigenic drift and the emergence of antigenically distinct subclades that escape vaccine-induced immunity [6-7]. In endemic settings, this continual viral evolution requires periodic antigen updates to ensure that vaccine seed strains remain antigenically matched with circulating field viruses. These challenges underscore the limitations of strain-specific vaccines in an endemic setting and highlight the need for broader, more cross-protective formulations capable of maintaining efficacy against antigenically drifted viruses [8-9].

To overcome the limitations of strain-specific hemagglutinin-based vaccines, increasing attention has been directed toward the conserved matrix 2 protein (M2) of influenza A viruses. The extracellular domain of M2 (M2e), composed of 23 amino acids, is highly conserved among avian and mammalian influenza A subtypes, making it an attractive target for universal vaccine development [10-13]. Antibodies against M2e do not neutralize viral entry but can mediate cross-protective immunity through Fc-dependent mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), complement activation, and phagocytosis of infected cells [12, 14-15]. These responses can limit viral replication and disease severity even in the absence of complete antigenic matching. However, the natural abundance of M2e on the virion surface is extremely low, and its small size results in poor immunogenicity when administered alone. To enhance immune recognition, various strategies have been developed, including fusion to carrier proteins [16], conjugation to virus-like particles [17], multimerization of M2e repeats [18], or linkage to bacterial flagellin to stimulate innate receptors such as toll-like receptor 5 [19]. Among these approaches, tandem or multimeric M2e constructs have shown particular promise by increasing antigenic density and improving the magnitude and breadth of immune responses across divergent influenza strains [12].

Despite promising results from M2e-based vaccine studies in mammalian models, research on their application in poultry has remained limited, particularly within conventional inactivated vaccine platforms that are most widely used in endemic countries. The incorporation of conserved antigens such as M2e into existing inactivated vaccines could provide a practical and cost-effective approach to enhance cross-protection without altering current production infrastructure or regulatory frameworks. This strategy is especially relevant for Indonesia, where antigenic drift frequently reduces vaccine efficacy and vaccine updates require substantial time and resources. To address this gap, a tetrameric M2e construct (4×M2e51) was developed to enhance antigenic density and immunogenicity, and its efficacy was evaluated in combination with an inactivated H5N1 vaccine containing local field isolates. Specifically, the research aimed to determine

whether inclusion of the tetrameric M2e antigen could enhance protection against heterologous challenge and maintain efficacy under conditions of antigenic mismatch. These findings underscore the importance of developing vaccines with broader antigenic coverage to maintain protection in endemic environments.

2. Materials and Methods

2.1 Vaccine antigens

Three inactivated H5N1 vaccine formulations were prepared as oil-emulsion vaccines containing mineral oil as adjuvant. The first vaccine consisted of the M101 isolate (A/Ck/West_Java/M101/2016), the second of the M166 isolate (A/Quail/West_Java/M166/2019), and the third combined the M101 isolate with the recombinant tetrameric M2e protein (4×M2e51). Both M101 and M166 are local isolates belonging to clade 2.3.2.1g and share 95.72% amino acid similarity in the HA1 region, with genetic evidence of immune escape mutations. Each vaccine dose contained 10^7 EID₅₀ of inactivated virus per 0.5 mL, and the 4×M2e51 antigen was incorporated at a concentration of 30 µg per dose [19–20]. Virus inactivation was performed using 0.1% formalin, followed by confirmation of complete inactivation through three serial passages in embryonated chicken eggs. The inactivated antigens were emulsified with mineral oil as adjuvant to form a stable water-in-oil emulsion prior to animal vaccination [21].

2.2 Recombinant protein design and expression

The gene encoding four tandem repeats of the M2e peptide (4×M2e51) was synthesized based on the consensus M2e sequence of avian influenza A viruses and optimized for expression in *Escherichia coli* BL21(DE3). The sequence was cloned into the pET-SUMO vector to generate an N-terminal fusion with the small ubiquitin-like modifier (SUMO) tag. A construct pET-SUMO-fla-4×M2e51 was initially designed to fuse the tetrameric M2e with *Salmonella pullorum* flagellin to enhance innate immune stimulation.

For laboratory-scale expression, a 2.5 L bench-top fermenter was used to culture the recombinant *E. coli* in chemically defined medium (Thermo Fisher Scientific). The culture was maintained at 37 °C under controlled conditions of 30% dissolved oxygen and pH 7 with agitation gradually increased to a maximum of 1200 rpm. Cells were grown until the optical density (OD₆₀₀) reached 30–50, at which point protein expression was induced with 0.75–0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Following incubation, cells were harvested by centrifugation at 5,000 × g for 20 minutes and stored at -20 °C until purification.

2.3 Protein purification and characterization

Cell pellets obtained after fermentation were resuspended in phosphate-buffered saline (PBS, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. Bacterial cells were disrupted using a sonicator at 80–90% amplitude with 0.6 s on / 0.4 s off pulse cycles for a total of 15 minutes, while keeping the sample on ice to prevent overheating. The lysate was clarified by centrifugation at 10,000 × g for 30 minutes at 4 °C, and the supernatant was subjected to purification using a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column connected to an FPLC (Fast Protein Liquid Chromatography) system. The His-tagged SUMO-4×M2e51 protein was eluted with 250 mM imidazole in PBS buffer.

Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to assess purity and molecular size. Protein identity was confirmed by dot-blot assay using an influenza A M2 polyclonal antibody (Thermo Fisher Scientific). The purified protein was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Prior to vaccine formulation, the purified protein solution was sterilized through a 0.22 µm syringe filter (Millipore) to ensure sterility and stored at -20 °C until use.

2.4 Vaccine preparation

Three inactivated H5N1 oil-emulsion vaccines with mineral oil as adjuvant were prepared: M101 isolate (10^7 EID₅₀ per dose), M166 isolate (10^7 EID₅₀ per dose), and M101 (10^7 EID₅₀ per dose) combined with ~30 µg of purified 4×M2e51 protein. Virus propagation was performed in 10-day-old embryonated chicken eggs, and allantoic fluids containing the virus were harvested and inactivated using formalin following standard procedures for avian influenza vaccine production. Complete inactivation was confirmed by three serial passages in embryonated eggs without hemagglutination activity [21].

For vaccine formulation, the inactivated viral antigen and 4×M2e51 protein were mixed in the aqueous phase prior to emulsification. The emulsion was prepared as a water-in-oil (W/O) formulation containing mineral oil as adjuvant, using standard homogenization procedures to obtain a stable emulsion. Each vaccine batch was tested for sterility according to the standard requirements for sterile biological products [21]. The final vaccine dose was 0.5 mL, administered via intramuscular injection.

2.5 Animal trial

Specific-pathogen-free (SPF) White Leghorn chickens, four weeks old, were used in this study. Chickens were maintained under biosafety level 3 (BSL-3) containment with ad libitum access to feed and water throughout the experiment. A total of 55 chickens were randomly allocated into seven groups (n = 10 per group, except for the one-fifth dose and positive control groups, which contained 5 each): (1) M166 full dose (10^7 EID₅₀/dose), (2) M101 full dose (10^7 EID₅₀/dose), (3) M101 + 4×M2e51 full dose (10^7 EID₅₀ + 30 µg 4×M2e51/dose), (4) M101 one-fifth dose, (5) M101 + 4×M2e51 one-fifth dose, (6) positive control (unvaccinated, challenged), and (7) negative control receiving phosphate-buffered saline (PBS) as placebo (unvaccinated, unchallenged). Vaccines were administered once via intramuscular injection into the thigh muscle at a volume of 0.5 mL per chicken.

Blood samples were collected from all chickens via the wing vein immediately before vaccination (day 0) and three weeks post-vaccination (day 21, prior to challenge) for serological analysis. Sera were separated and stored at -20 °C until testing. Serological analysis was performed by hemagglutination inhibition (HI) assay using the M166 antigen according to standard procedures [21]. HI titers were expressed as log₂ values. Statistical analysis of serological data was performed after log₂ transformation of HI titers. Group comparisons were conducted using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, with significance determined at $p < 0.05$.

Three weeks after vaccination (day 21 post-vaccination), chickens were challenged intramuscularly with 0.5 mL of the M166 strain at a dose of 50 CLD₅₀ per chicken [22]. Following challenge, chickens were observed daily for 14 days for clinical signs, including ruffled feathers, lethargy, respiratory distress, decreased feed intake, collapse, and pale or bluish discoloration of the comb. Clinical signs and mortality were recorded to evaluate the level of protection provided by vaccination. The animal trial was performed in a BSL-3 animal facility as part of regulated vaccine efficacy and challenge testing. All animal

experiments were conducted in accordance with institutional guidelines for the care and use of laboratory animals and complied with relevant animal welfare regulations. All efforts were made to minimize the number of animals used and to reduce animal suffering throughout the study.

3. Results

3.1 Protein expression of 4×M2e51

Two recombinant constructs containing four tandem repeats of the M2e peptide (4×M2e51) were generated in *Escherichia coli*: a flagellin-fused construct (fla-4×M2e51) and a non-flagellin construct (4×M2e51). Both proteins were expressed from the pET-SUMO vector, which introduces a small ubiquitin-like modifier (SUMO) tag at the N-terminus to enhance solubility, promote correct folding, and protect nascent proteins from proteolytic degradation [23]. Mechanical lysis by sonication was used instead of chemical extraction reagents to improve protein yield and recover inclusion body-associated fractions.

Although the fla-4×M2e51 protein was successfully expressed, it exhibited instability both during expression and purification. SDS-PAGE analysis (Figure 1a) revealed three distinct bands corresponding to the full-length fla-4×M2e51 (~79 kDa), free flagellin (~70 kDa), and a smaller 4×M2e-containing fragment or cleavage product (~21 kDa). Multimeric M2e constructs and small repetitive or hydrophobic peptides have been reported to migrate anomalously on SDS-PAGE [24], while flagellin fusion proteins are known to undergo partial proteolytic cleavage during expression or purification [25-26]. The ~21 kDa band was not further characterized in this study. Due to the observed instability of the fla-4×M2e51 construct, which resulted in heterogeneous degradation products and raised concerns regarding inconsistent antigen presentation, subsequent analyses focused exclusively on the non-flagellin 4×M2e51 construct.

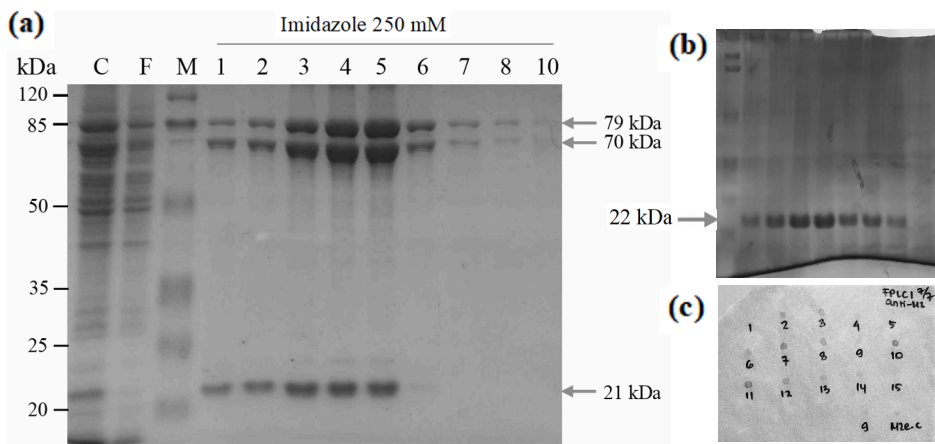


Fig. 1. Expression and characterization of recombinant M2e antigens. (a) SDS-PAGE analysis of the expressed fla-4×M2e51 fusion protein showing three distinct bands corresponding to the full-length SUMO-flagellin-4×M2e51 (~79 kDa), SUMO-flagellin (~70 kDa), and a cleaved 4×M2e51 peptide (~21 kDa). C = cell lysate; F = flow-through; M = molecular marker; lanes 1–10 = eluted fractions with 250 mM imidazole. (b) SDS-PAGE profile of the purified SUMO-4×M2e51 recombinant protein showing a single major band at ~22 kDa, consistent with its theoretical molecular weight, confirming correct expression and purity of the SUMO-tagged construct in *E. coli*. (c) Dot-blot analysis of

purified fractions using anti-M2 monoclonal antibody, verifying the presence of M2e epitopes in the recombinant proteins.

The purified 4×M2e51 protein appeared as a single dominant band of approximately 22 kDa on SDS-PAGE (Figure 1b) consistent with its predicted molecular weight, and was specifically detected by dot blot using an anti-M2 polyclonal antibody, confirming that the M2e epitope remained antigenically intact (Figure 1c). Based on SDS-PAGE densitometric assessment, the purity of the Ni-NTA-purified 4×M2e51 protein was estimated to be greater than 90%. Minor impurity bands were still visible after Ni-NTA purification, likely representing endogenous *E. coli* histidine-rich proteins that co-eluted under the chosen imidazole concentration [27]. Optimization of elution parameters or inclusion of additional purification steps such as ion-exchange or size-exclusion chromatography could further improve purity in future preparations.

3.2 Antibody responses

Serum antibody responses were evaluated by HI assay using the homologous M166 antigen three weeks post-vaccination (day 21). No detectable antibody titers ($HI < 4 \log_2$) were observed in the unvaccinated positive or PBS control groups, confirming the absence of prior exposure. Significant differences ($p < 0.05$) were observed among vaccinated groups when comparing M166 (full dose), M101 (full dose), M101 + 4×M2e51 (full dose), and the negative control (Figure 2).

The M166 full-dose vaccine induced the highest antibody response (mean $6.4 \pm 2.7 \log_2$), consistent with homologous antigenic recognition. The M101 full-dose vaccine produced a lower mean titer ($2.1 \pm 1.7 \log_2$), reflecting reduced cross-reactivity with the drifted M166 antigen. Incorporation of the 4×M2e51 antigen into the M101 formulation improved antibody titers to $4.3 \pm 0.9 \log_2$, demonstrating an enhanced humoral response compared with M101 alone. The reduced antibody response of M101 compared with M166 reflects antigenic drift, while the modest increase observed with 4×M2e51 suggests that inclusion of conserved antigens may help improve the overall immunogenicity of the formulation against drifted strains. HI titers in the one-fifth dose groups were substantially lower, consistent with the reduced antigen input, showing mean values of 0.6 ± 1.3 and $1.2 \pm 1.6 \log_2$ for M101 and M101 + 4×M2e51 one-fifth doses, respectively.

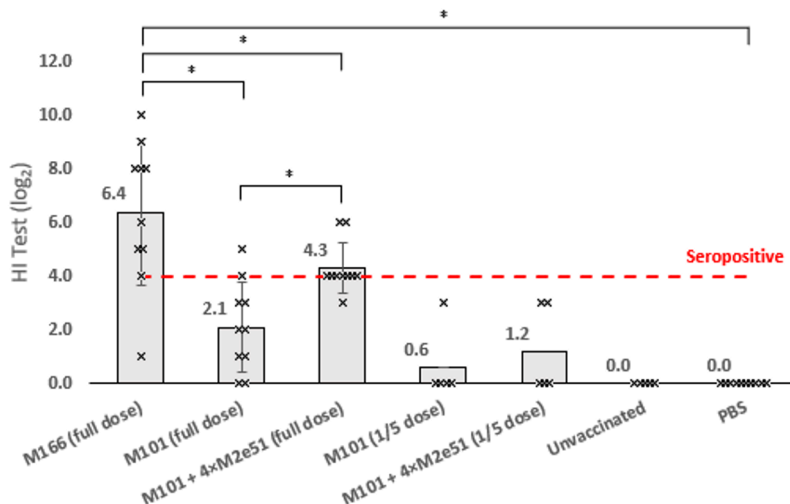


Fig. 2. Serum antibody responses of all groups against antigen M166. Bars represent mean HI titers (\log_2), and individual data points (\times) indicate results from individual chickens. The red dashed line denotes the seropositive threshold ($4 \log_2$). Asterisks (*) above the bars indicate statistically significant differences among groups based on one-way ANOVA ($p < 0.05$).

3.3 Protection against challenge

Protective efficacy was evaluated based on clinical signs and survival following heterologous challenge with the M166 strain three weeks after vaccination. Observed clinical signs included decreased feed intake, lethargy, collapse, and bluish discoloration of the comb. As shown in Figure 3, unvaccinated positive control chickens remained healthy on day 1 and by day 3 all chickens in this group had died, confirming the virulence of the challenge strain. In contrast, chickens in the negative control (PBS), M166 (full dose), M101 + 4×M2e51 (full dose), and M101 + 4×M2e51 (one-fifth dose) groups showed no clinical signs and achieved 100% survival, represented by overlapping lines at 100% in both graphs. In the M101 (full dose) group, only one chicken exhibited clinical symptoms starting on day 4 and died on day 6, resulting in a 90% survival rate, while all other chickens in the group remained healthy. The M101 (one-fifth dose) group exhibited severe illness, with only 20% survival by day 14.

These results demonstrate the level of protection provided by each vaccine against the challenge virus, indicating that inclusion of the conserved 4×M2e51 antigen enhanced vaccine-induced protection. The M101 + 4×M2e51 formulation conferred complete protection against the heterologous virus even at one-fifth of the standard antigen dose, whereas the corresponding vaccine without 4×M2e51 provided only partial protection. This finding highlights the potential of conserved antigens like M2e to strengthen vaccine efficacy against antigenically drifted H5N1 strains, even under reduced antigen dosage.

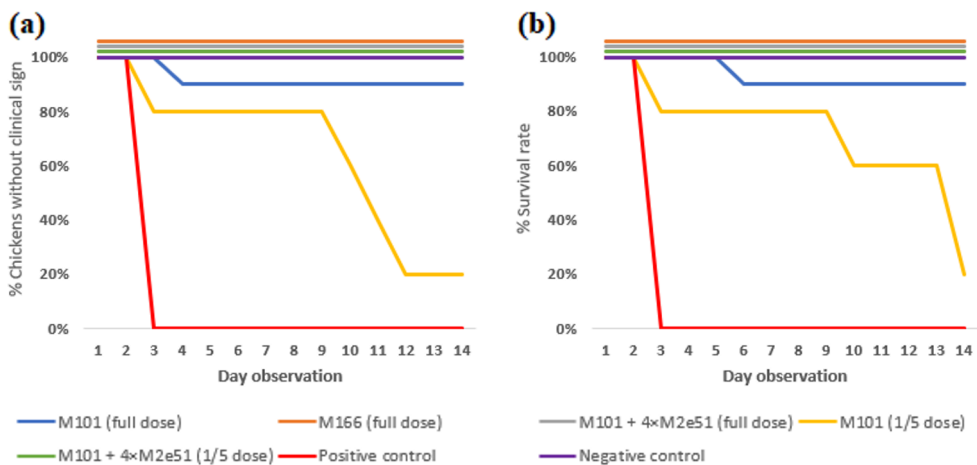


Fig. 3. Clinical signs (a) and survival rate (b) of vaccinated and control groups following challenge with the AI H5N1 M166 isolate. Chickens were monitored daily for 14 days post-challenge. Panel (a) shows the proportion of chickens without clinical signs, while panel (b) shows the cumulative survival rate. Vaccines containing the 4×M2e51 antigen provided complete protection, even at reduced antigen dosage, whereas the M101 vaccine alone showed partial protection.

4. Discussion

This study demonstrates that incorporation of a tetrameric M2e construct (4×M2e51) into an inactivated H5N1 vaccine enhances protection against antigenically drifted avian influenza viruses in chickens. Although the homologous vaccine (M166) elicited the strongest antibody response, the heterologous formulation containing M101 combined with 4×M2e51 achieved comparable protective efficacy, with complete survival following challenge. This finding highlights the functional advantage of supplementing conserved M2e epitopes into conventional HA-based vaccines to broaden immune protection against antigenically drifted variants within the same clade. Such an approach may offer a practical and sustainable strategy to maintain vaccine effectiveness in poultry populations where continual antigenic drift frequently reduces the match between vaccine and circulating field strains.

Recombinant expression of the tetrameric M2e construct (4×M2e51) in *E. coli* using a SUMO fusion tag produced a well-expressed and stable recombinant protein, whereas the flagellin-fused variant exhibited instability during purification. Such degradation is commonly attributed to proteolytic cleavage or conformational strain at the junction between the flagellin carrier and inserted peptide, as reported in previous studies of flagellin fusion systems [25-26]. Due to this instability, subsequent research focused on the use of the 4×M2e51 construct formulated within a mineral oil-emulsion vaccine. This formulation was selected to enhance the immunogenicity of the relatively small recombinant protein (~22 kDa), as mineral oil-based emulsions are known to create a strong depot effect and promote prolonged antigen release, leading to a strong and durable antibody responses [28]. Such adjuvants have been widely used in commercial avian influenza and Newcastle disease vaccines, where they effectively stimulate humoral immunity and protection in poultry [29-30]. Therefore, incorporation of 4×M2e51 into this emulsion-based formulation was expected to improve immune activation and contribute to the enhanced protection observed in vaccinated chickens.

Serological evaluation using the HI test revealed a clear difference between homologous and heterologous vaccine responses, reflecting antigenic drift between the M101 and M166 isolates within clade 2.3.2.1g. The lower HI titers observed in the M101 group compared with M166 confirm the challenge of maintaining vaccine efficacy when antigenic variation arises among circulating field strains. Although the hemagglutination inhibition assay measures antibodies directed against hemagglutinin rather than M2e, chickens vaccinated with M101 + 4×M2e51 exhibited higher HI titers against the heterologous M166 antigen than those vaccinated with M101 alone. This observation suggests that inclusion of the 4×M2e51 antigen may enhance the overall immunogenicity of the vaccine formulation, potentially by providing additional CD4⁺ T-cell help or exerting an intrinsic immunostimulatory effect that supports B-cell responses to co-administered antigens, including HA. In a separate animal experiment using the same vaccination protocol and HI assay against the M166 antigen, similar kinetics of HI antibody induction were observed; however, differences between M101 alone and M101 supplemented with 4×M2e51 were not statistically significant beyond four weeks post-vaccination. This may reflect partial cross-reactive HA immunity within the same subtype, which could mask subtle indirect effects of M2e on HA-specific antibody responses. Comparable indirect enhancement of HA-directed humoral responses has been reported in influenza vaccine constructs combining M2e antigens with HA, indicating synergistic immunogenic effects when M2e is co-delivered with the HA antigen [31-32].

Challenge results further demonstrated that inclusion of 4×M2e51 markedly enhanced protective efficacy, achieving complete survival in chickens vaccinated with both full and one-fifth antigen doses of M101 + 4×M2e51, while the corresponding formulation without 4×M2e51 provided incomplete protection at the same dose levels. These findings indicate that inclusion of a conserved antigen such as M2e can improve vaccine potency and

broaden protection under conditions of antigenic mismatch—a particularly relevant advantage for endemic poultry populations where viral evolution is ongoing. The present results may also serve as a preliminary step toward comprehensive potency evaluation through a protective dose 50% (PD₅₀) test, as recommended by the WOAAH standards to ensure vaccine quality and consistency in poultry immunization programs.

Antibodies against the conserved ectodomain of M2 (M2e) are non-neutralizing but can mediate Fc-dependent effector mechanisms such as antibody-dependent cellular cytotoxicity, complement activation, and phagocytosis of infected cells, thereby limiting viral replication and promoting clearance [33-34]. Experimental and review studies in mammalian models have consistently shown that multimeric or nanoparticle-presented M2e antigens can elicit broad cross-protective immunity against divergent influenza A viruses [12, 35]. In poultry, M2e-based vaccine approaches have similarly been shown to enhance immune activation and reduce viral shedding when combined with suitable carriers or adjuvant systems—for instance, fusion of multimeric M2e to HSP70c [36] or formulation with recombinant hemagglutinin nanoparticles [37]. The enhanced protection observed in the present study is therefore consistent with the complementary role of M2e-specific immune responses in compensating for partial antigenic mismatch between vaccine and challenge viruses, while further demonstrating the potential of incorporating tetrameric M2e antigens directly into inactivated H5N1 vaccine platforms for practical field application in endemic regions.

The findings of this study also have practical implications for avian influenza control in Indonesia, where vaccination remains the principal strategy to mitigate disease burden under endemic conditions. Regular vaccine strain updates are costly and time-consuming, while continual antigenic drift frequently reduces the match between vaccine and circulating field viruses. Consequently, there can be a delay between the emergence of new field variants and the availability of updated vaccine formulations. Incorporation of a conserved antigen such as M2e into existing inactivated vaccine platforms offers a practical approach to enhance cross protection within clade variants without requiring major changes to manufacturing processes or regulatory frameworks. By broadening immune protection against drifted strains, this strategy could contribute to more sustainable and practical vaccination programs, in line with WOAAH and FAO recommendations for maintaining vaccine effectiveness in regions with evolving influenza virus diversity.

Some limitations should be acknowledged. The HI assay primarily measures antibodies directed against the hemagglutinin antigen and does not directly detect M2e-specific responses; therefore, complementary immunoassays such as ELISA or functional assays would be required to quantify anti-M2e antibodies and better characterize the immune mechanisms involved. Cellular immune responses and viral shedding were also not evaluated in this study, and their assessment would provide a more comprehensive understanding of the protection induced by M2e-supplemented vaccines. In addition, the relatively small group size used for the one-fifth dose and positive control groups (n = 5) may reduce the statistical power of survival comparisons. Nevertheless, the marked differences observed between vaccine formulations suggest a robust biological effect. Furthermore, optimization of construct design and expression conditions is warranted to improve the stability and yield of flagellin-fused M2e proteins. Previous studies have shown that structural modification of the flagellin D0 domain or inclusion of protease inhibitor cocktails can markedly reduce degradation and improve recovery of flagellin-based recombinant proteins [25-26]. Collectively, these refinements could support more detailed immunogenicity studies and guide the rational design of next-generation influenza vaccines for poultry.

5. Conclusion

In conclusion, this study demonstrates that addition of the conserved tetrameric M2e antigen to an inactivated H5N1 vaccine improved protection against antigenically drifted strains within clade 2.3.2.1g, conferring complete protection against challenge even at reduced antigen concentrations. The results underscore the value of supplementing conserved epitopes such as M2e into existing HA-based vaccines as a practical means to broaden protective efficacy under field-relevant conditions. Together with further optimization of antigen design and immunogenicity assessment, this approach could contribute to the development of more sustainable and adaptable vaccination strategies for avian influenza control in endemic regions such as Indonesia.

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