Research Article

A Bivalent Inactivated Vaccine from Indonesian Local Isolates Confers Protection against Newcastle Disease and Induces Immune Responses to Infectious Bronchitis

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Abstract | Newcastle disease (ND) and infectious bronchitis (IB) are highly contagious viral diseases that result in significant economic detriment to the poultry sector. Vaccination remains the most effective strategy to control these infections, particularly in endemic regions such as Indonesia. This study aimed to develop and evaluate an inactivated bivalent vaccine combining ND and IB viruses isolated from local outbreaks. Molecular identification confirmed that the ND isolate belonged to genotype VIIh, while the IB isolate clustered with the Massachusetts-like (GI-1) lineage, both of which represent predominant circulating strains in Indonesia. The viruses were propagated in embryonated chicken eggs, inactivated with formalin, and formulated with Montanide adjuvant. Vaccine quality was assessed through sterility, safety, stability, and potency tests. The formulation remained stable at 4°C for 30 days with no physicochemical changes and no evidence of bacterial contamination. Safety evaluation in chickens demonstrated no adverse systemic or local reactions, even at twice the dosage. Efficacy testing demonstrated strong protective effects: vaccinated chickens showed high survival rate following challenge with virulent ND virus, whereas the unvaccinated group suffered considerable morbidity and mortality. Vaccinated chickens had markedly elevated antibody titers and neutralization indices against IB in comparison to controls ($p<0.05$). Immunogenicity against IB was demonstrated by significantly elevated antibody titers and neutralization indices, in the absence of an IB challenge experiment. These findings indicate that the formulated vaccine induces immune responses and provides effective protection against ND and induces strong humoral immune responses against IB. The bivalent formulation offers practical advantages by reducing vaccination frequency and improving farm efficiency. This study emphasizes the potential of locally derived bivalent vaccines as a strategic instrument for poultry disease management in Indonesia.

Keywords | Bivalent vaccine, Infectious bronchitis, Newcastle disease, Oil-adjuvanted inactivated vaccine, Poultry disease control, Virus

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INTRODUCTION

Newcastle disease (ND) is a significant viral illness in poultry that may result in considerable losses in chicken farming operations. ND leads to major economic losses and is present in nearly all poultry businesses (Wang *et al*., 2024). The disease is characterized by abnormalities in the respiratory tract, digestive system, and central nervous system (Mao *et al*., 2022). The clinical signs of ND are contingent upon the viral strain, host species, host age, environmental factors, and the immunological state of the chickens, resulting in a wide spectrum of disease severity ranging from subclinical infection to highly lethal disease (Kadhim *et al*., 2024).

Based on virulence, ND viruses are classified into three strains: velogenic, mesogenic, and lentogenic. Another categorization is predicated on clinical symptoms, delineating five ND virus pathotypes: Viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic or respiratory, and asymptomatic types (Zhang *et al*., 2023). In Indonesia, ND is commonly known as tetelo disease, and in Bali, it is referred to as *gerubug*. The disease can appear as either acute or chronic and affects all varieties of poultry, especially chickens, including both commercial and native breeds. ND has remained endemic for nearly a century and continues to pose a serious threat to the poultry industry in Indonesia due to its endemic nature (Dharmayanti *et al*., 2024). Newcastle disease is induced by the Avian Paramyxovirus type-1 (APMV-1) (Triosanti *et al*., 2018).

Infectious bronchitis (IB) is a transmissible and acute respiratory disease in chickens caused by the infectious bronchitis virus (IBV). The illness attacks the upper respiratory tract and urogenital tract in chickens. The disease imposes substantial economic losses on the poultry industry due to respiratory disease in young chickens and reduced egg production and shell quality in laying hens, while certain virus strains, may also causes damage to the kidney, resulting in high mortality (Maletić *et al*., 2025).

The IBV belongs to the family coronaviridae, characterized by a positive single-stranded RNA genome around 27.6 Kb in length. The viral genome encodes information for four structural proteins, namely spike glycoprotein (S), membrane glycoprotein (M), small membrane or envelope glycoprotein (E), and nucleocapsid (N). The spike glycoprotein is linked to viral neutralization, serotype specificity, and cellular attachment, and it is separated during post-translational modification into the N-terminal S1 and C-terminal S2 subunits. Antigenic variation among IBV serotypes is primarily associated with sequence diversity in the S1 subunit, which contains hypervariable regions (HVRs) responsible for serotype differentiation (Kim *et al*., 2025). In Indonesia, infectious bronchitis virus (IBV) remains widely distributed, particularly across Java, with reported prevalence rates of approximately 40–60% (Setiawaty *et al*., 2019). Although IB vaccination is routinely implemented in breeder, layer, and broiler flocks primarily using Massachusetts (Mass)-based vaccines and, in some cases, Connecticut (Conn) serotypes field outbreaks continue to be reported, suggesting limitations in protection against circulating variants.

Currently, there is no effective treatment for infections induced by the ND or IB viruses. The primary strategy to prevent the emergence of ND and IB is vaccination and the enhancement of biosecurity measures (Al-Eitan *et al*., 2025). In Indonesia, prevention programs against ND have been intensively implemented, using both active and inactivated vaccines. The objective of vaccination is to develop specific protective immunity to address field cases. The use of either singular or combined active and inactivated vaccines has been extensively adopted in poultry farming. Due to the rising prevalence of concurrent viral infections in layer farms, combination vaccinations are currently used to enhance the efficacy of vaccine administration (Dharmayanti, *et al*., 2024). Bivalent vaccines provide the benefits of rapid prevention against several contagious viral infections, a labor efficient methodology, and reduced production costs.

Several commercial vaccines against infectious bronchitis (IB) and Newcastle disease (ND) currently used in Indonesia are based on the Massachusetts (Mass) and LaSota genotypes, which were originally derived from strains circulating in Europe and the United States (Ike *et al*., 2021). Although these vaccines have contributed substantially to disease control, increasing evidence indicates limited cross-protection against locally circulating field variants, particularly heterologous non-Mass IBV lineages and genotype VII NDV. Molecular surveillance studies in Indonesia have demonstrated considerable genetic diversity among circulating IBV strains, including QX-like (GI-19) and Mass-like lineages, which remain epidemiologically relevant in several poultry production systems (Wibowo *et al*., 2019). Similarly, NDV isolates are predominantly classified as genotype VII, which shows only partial antigenic similarity to the LaSota-based genotype II vaccine strains (Putri *et al*., 2017).

This genetic and antigenic divergence has been associated with suboptimal vaccine performance in the field, as evidenced by reports of IB and ND outbreaks in well-vaccinated flocks. Antigenic mismatch between vaccine and field strains may compromise virus neutralization, allowing continued virus circulation despite routine vaccination programs (Franzo *et al*., 2025). Therefore, the development of vaccines based on locally circulating

viral isolates is essential to improve homologous antigenic matching and enhance vaccine effectiveness under Indonesian field conditions. In this study, a locally isolated Mass-like infectious bronchitis virus (IBV) strain was selected to represent field-relevant viruses circulating in Indonesia and to serve as an epidemiologically appropriate component of a bivalent inactivated Newcastle disease–infectious bronchitis (ND–IB) vaccine. This study was conducted to formulate a combination inactivated vaccine consisting of ND and IB from Indonesian local viruses, emulsified with an oil-based adjuvant. The vaccine formulation was evaluated for its physicochemical stability and immunogenic performance, with antibody responses to assess its potential protective efficacy against the target diseases.

MATERIALS AND METHODS

Experimental chickens

A total of 150 commercial laying hens, aged 4 weeks of the Hy-Line breed, were included in this study, with 50 hens allocated for safety testing of the formulated vaccines. Both local injection site reactions and systemic responses were monitored. The chickens were confined in battery cages with a wire mesh flooring. The laying hens were maintained in experimental cages and had access to feed and water ad libitum during the vaccination assessment period. All challenge experiments were conducted under BSL-2 containment.

Virus isolate seeds identification by PCR and sequencing

The ND (NDV/Chicken/Central Java/178/2019) virus utilized in this study was isolated from ventriculus and C. tonsil of confirmed molecular and clinical case Newcastle Disease infection in Central Java poultry. The IB (IBV/Chicken/West Java/071/2015) virus for master seed was isolated from tracheal and oviduct organ that obtained from a confirmed clinical case outbreak of infectious bronchitis in West Java. The isolate was molecularly characterized and phylogenetically classified as a Massachusetts-like (GI-1) strain prior to vaccine preparation to confirm its lineage identity and relevance. The virus seed vaccine was preserved in a freeze-dried state inside an ampoule at -20 °C. This research performed molecular identification as a quality control procedure for virus seed vaccine preparation. RNA was purified from allantoic-containing virus by Viral Nucleic Acid Extraction Kit II (Geneaid), following the manufacturer's instructions. The fusion gene was detected to verify ND isolate using PCR primers (F) 5′-ATGGGCTCCAGACCTTC ACCA-3′ and (R) 5′-CTGCCACTGCTAGTTGTGATAATCC-3′ (Radwan *et al*., 2013; Saputri *et al*., 2021). The target Spike gene was used for the detection of IB using PCR primers (F) 5′-AGGAATGGTAAGTTRCTRGTWAGAG-3′ and (R) 5′-GCGCRGTACCRTTRAYAAART ARGC-3′ (Fujisawa *et al*., 2019). The MyTaq™ OneStep RT-PCR kit (Bioline®, Taunton) was used to test the ND and IB isolates. The following thermal profile was used to amplify the genes: incubated at 48°C for 20 minutes, then 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds, 52°C for 10 seconds, and 72°C for 2 minutes. The final procedure of the amplification was carried out at 72°C for 10 minutes (Putra *et al*., 2024). Electrophoresis was used to visualize the amplified samples. The standard size marker is 100 bp (Geneaid, Taipei, Taiwan). The desired band was purified for sequencing by First BASE Laboratories Sdn Bhd, Malaysia. Determination of the nucleotide and amino acid sequences for the F gene (NDV) and S1 gene (IBV) from a recently isolated strain using Bioedit v.7 and alignment with ClustalW. MEGA 7 was used to construct the phylogenetic tree of contemporary viruses using the neighbor-joining method with 1,000 alignment repeats (Nugroho *et al*., 2021).

ND and IB antigens production

The ND and IB viral antigens were produced in a BSL-2 laboratory equipped with separate air systems for each virus type. The procedure started by dissolving the freeze-dried viruses (in ampoules) in sterile PBS supplemented with Kanamycin, Penicillin, and Fungizone until a virus titer of 10^4 EID_{50}/mL was attained for both ND and IB viruses according to (Reed and Muench, 1938). A volume of 0.1 mL of the inoculum was injected into the allantoic cavity of 10-days old embryonated SPF chicken eggs. The eggs were then incubated at 37°C for three days. Subsequent to incubation, the eggs were chilled at 4°C for 12 hours prior to the extraction of the allantoic fluid. The harvested fluid was analyzed to confirm the absence of bacterial and fungal contamination and to determine the viral load, measured as EID_{50}/mL. Once the required standards were met, the virus was inactivated by adding 0.2% formalin. The inactivation procedure was carried out for 18 hours using a stirrer at 25 °C to ensure complete inactivation of the virus. To ensure complete inactivation of both NDV and IBV antigens, each formalin-inactivated antigen was subjected to a validation process according to OIE guidelines. After inactivation, 0.2 mL of the treated viral suspension (undiluted antigen) was inoculated into the allantoic cavity of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (five eggs per test) and incubated at 37°C for 5 days. The same procedure was repeated for three consecutive blind passages to confirm the absence of residual live virus. For NDV, the allantoic fluids harvested from each passage were examined by hemagglutination (HA) assay using 1% chicken red blood cells. The absence of HA activity in all passages was interpreted as confirmation of successful inactivation. For

IBV, the allantoic fluids from each passage were treated with 0.2 % trypsin and tested by hemagglutination (HA) assay to assess the presence of replication-competent virus. No hemagglutination activity was observed in any of the passages, confirming the absence of infectious IBV after inactivation. Antigen batches that complied with the criteria were preserved at -20°C, awaiting further processing with an adjuvant.

Mixing of ND and IB viral antigens with adjuvant

The vaccine comprises 15% inactivated ND virus antigen, 15% IB virus antigen, and 70% adjuvant (Montanide ISA 71VG). The inactivated ND and IB antigens were emulsified with an oil-based adjuvant in a sterile tank using an a high-shear mixer (Ultra Turrax T50, IKA) as the mixing device. The emulsification process involved total 10 mixing cycles by several oil-to-aqueous phase addition order, first two cycles by rotor-stator speed 1000 rpm for 30 second, continued with six cycle 5000 rpm for 3 minutes, and 2 minutes 6000 rpm for two cycles. The mixing continued until a perfectly homogeneous mixture of antigen and adjuvant was attained. Homogeneity testing was performed microscopically at 100x magnification by placing a drop of the vaccine mixture into sterile distilled water. Once homogeneity was confirmed, the vaccine underwent sterility, safety, and potency testing.

Sterility testing

The examination is conducted aseptically in a sterile environment within a Class II Biosafety Cabinet. The media used for the test include Thioglycolate broth (TGC) and Soybean Casein Digest broth (SCD). One milliliter of the vaccine product is inoculated into four test tubes, each containing 20 mL of either TGC or SCD media. Two TGC tubes and two SCD tubes are incubated at 22°C for 14 days, while the remaining two TGC tubes and two SCD tubes are cultured at 37°C for the same period. Observations are made on days 3, 7, and 14. The biological product is considered to meet sterility requirements if no bacterial or fungal (mold and yeast) growth is detected in any of the media during the testing period (Indrawati *et al*., 2025).

Safety testing

Vaccine safety testing is carried out for each batch. This test is conducted to guarantee the vaccine's safety. The method involves intramuscular injection of 0.6 mL of the vaccine (a double dose) into 25 SPF chickens aged 28 days. An additional 25 chickens serve as the control group and remain unvaccinated. Observations take place over a duration of 21 days. The vaccine is considered to meet safety requirements if, during the observation period, neither the vaccinated nor control groups of chickens exhibit any clinical symptoms or adverse effects resulting from the vaccination (Parvin *et al*., 2024).

Potency test against ND virus

A total of 25 SPF chickens aged 28 days were administered 0.3 mL of the vaccine by intramuscular injection, while an additional 25 unvaccinated chickens served as the control group. Fourteen days post-vaccination, all chickens from both the vaccinated and control groups were challenged intramuscularly with an ND virus strain containing $10^{4.0}$ CLD_{50}. Observations were conducted over 14 days by monitoring clinical signs of ND in both groups.

Immunogenicity evaluation against IB virus

A total of 25 SPF chickens aged 28 days old were administered 0.3 ml of the vaccine via intramuscular injection. Another 25 unvaccinated chickens were used as the control group. A booster dose of same volume was administered two weeks after the initial vaccination to enhance the humoral immune response, which is commonly required for inactivated IB vaccines. Observations were conducted for a further two weeks. At the end of the observation period, blood samples were collected from both the vaccinated and the unvaccinated control groups. The serum was separated from each blood samples of the groups and then inactivated in a water bath at 56°C for 30 minutes. The inactivated serum was mixed with homologous IB virus that had been diluted tenfold using calcium and magnesium-free PBS. This mixture was incubated either in a 37°C water bath for 60 minutes or stored at 4°C for 18–24 hours. Each combination was inoculated into five embryonated SPF chicken eggs aged 9–11 days by injecting 0.1 mL into the allantoic cavity. The eggs were incubated at 37°C for seven days. Allantoic fluids were tested for residual virus by hemagglutination assay after trypsin activation (20 µg/mL) and the neutralization endpoint for each serum was defined as the highest dilution that completely inhibited HA activity in all five eggs. The Neutralization Index (NI) was calculated using the standard difference-in-log method (Reed and Muench style), expressed here as: NI= $\log_{10}$ (EID_{50} of virus control) – $\log_{10}$ (EID_{50} of virus–serum mixture). The IgG antibody response in serum samples was also assessed using a commercial ELISA kit (ID Screen® Infectious Bronchitis Indirect, IBVARSV2-5P; IDVet, France) according to the manufacturer's instructions.

Data analysis

Quantitative data were analyzed using GraphPad Prism software version 9.1.2 (GraphPad Software, San Diego, CA, USA). Data were presented as means, along with either the standard error of the mean (SEM) or the standard deviation (SD), obtained from repeated independent trials. To determine statistical differences

among groups, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's post-hoc test for multiple comparisons. A p-value less than 0.05 was deemed statistically significant.

RESULTS

Amplification and phylogenetics of ND virus

The ND virus used as a vaccine seed was molecularly verified through PCR targeting the fusion gene, yielding a band at 535 bp (Figure 1A). Based on homology comparison, the ND isolate strain ND/1/78/MSL/17 showed 95.34–95.86% nucleotide similarity with reference isolates from Indonesia available in GenBank, which belong to genotype VII and sub-genotype VIIh. On the other hand, when compared to representative reference isolates of sub-genotype VIIi, the homology level was 90.28% (Table 1). These findings demonstrate that the ND/1/78/MSL/17 isolate is genetically closely related to contemporary NDV strains circulating in Indonesia, thereby supporting its local relevance as a vaccine seed strain. Based on the phylogenetic tree, the ND isolate clustered within the same branch as the reference isolates classified under sub-genotype VIIh (Figure 1B). The most critical part of the target gene is the cleavage site region, specifically at residue positions 112–117, commonly referred to as the F0 position. The ND/1/78/MSL/17 isolate possesses the RRRKRF pattern, indicating its classification within the virulent Newcastle disease virus category.

Amplification and phylogenetics of IB virus

The PCR test findings showed a favourable outcome, indicated by the amplification of the gene at a 670 bp band (Figure 1A). Based on the homology comparison, the IB isolate strain IB/017/MSL/15 showed 99.7% nucleotide similarity with the reference strain Table 2. Massachusetts 41 (accession number DQ830980). This high sequence similarity supports the classification of IB/017/MSL/15 as a Mass-like (GI-1) strain and indicates genetic relatedness to field-relevant IBV lineages circulating in Indonesia. According to the phylogenetic tree, the IB/017/MSL/15 isolate clustered within the same branch as reference isolates belonging to lineage GI-1, in the same lineage as isolates M41, H120, and H52, which are classified as Mass-like strains (Figure 1C).

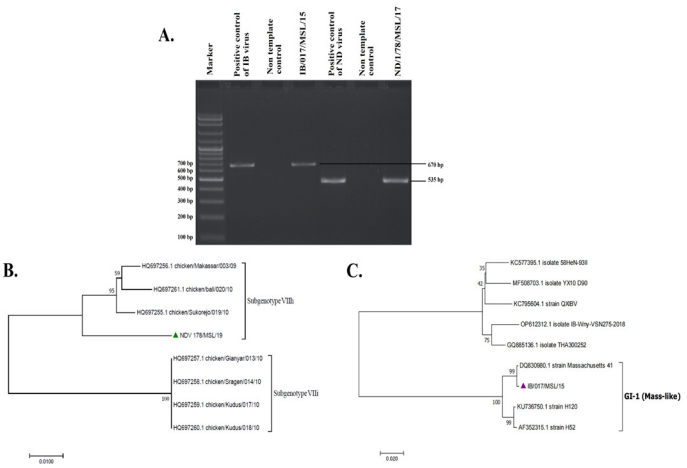

Figure 1: PCR detection and phylogenetic analysis of Newcastle Disease Virus (NDV) and Infectious Bronchitis Virus (IBV). (A) Agarose gel electrophoresis showing the expected amplicons of NDV (535 bp) and IBV (670 bp) alongside positive and negative controls. (B) Phylogenetic tree of NDV isolate ND/178/MSL/17 constructed by the Neighbor-Joining method, clustering within subgenotype VIIh. (C) Phylogenetic tree of IBV isolate IB/017/MSL/15 showing its grouping within genotype GI-1 (Mass-like). Bootstrap values (1,000 replicates) are indicated at branch nodes.

Table 1: Nucleotide sequence homology (%) of the NDV isolate ND/1/78/MSL/17 compared with contemporary Indonesian field isolates and selected reference strains.

Accession number	Strain		1	2	3	4	5	6	7	8
HQ697255	chicken/Sukorejo/019/10	1								
HQ697256	chicken/Makassar/003/09	2	98.56							
HQ697257	chicken/Gianyar/013/10	3	91.34	91.32						
HQ697258	chicken/Sragen/014/10	4	91.34	91.32	100					
HQ697259	chicken/Kudus/017/10	5	91.34	91.32	100	100				
HQ697260	chicken/Kudus/018/10	6	91.34	91.32	100	100	100			
HQ697261	chicken/Bali/020/10	7	98.57	98.56	90.80	90.80	90.80	90.80		
	ND/1/78/MSL/17 (this study)	8	95.86	95.34	90.28	90.28	90.28	90.28	95.35	

Numbers indicate reference strains as follows: (1) chicken/Sukorejo/019/10; (2) chicken/Makassar/003/09; (3) chicken/Gianyar/013/10; (4) chicken/Sragen/014/10; (5) chicken/Kudus/017/10; (6) chicken/Kudus/018/10; (7) chicken/Bali/020/10; (8) ND/1/78/MSL/17 (this study). All Indonesian reference strains were isolated between 2009–2010 from different geographic regions of Indonesia. Homology values were calculated based on aligned nucleotide sequences of the fusion gene. Blank cells indicate self-comparisons.

Table 2: Nucleotide sequence homology (%) of the IBV isolate IB/017/MSL/15 compared with contemporary Indonesian IBV isolates and representative reference strains.

Accession number	Strain		1	2	3	4	5	6	7	8	9
OP612312.1	Isolate IB-Wny-VSN275-2018	1									
KC577395.1	Isolate 58HeN-93II	2	94.4								
GQ885136.1	Isolate THA300252	3	96.5	95.9							
MF508703.1	Isolate YX10_D90	4	94.4	95.9	95.9						
KC795604.1	Strain QXIBV	5	94.0	95.6	95.9	95.5					
DQ830980.1	Strain Massachusetts 41	6	73.6	73.7	73.2	73.2	73.4				
KU736750.1	Strain H120	7	73.6	74.0	73.4	73.5	73.6	97.6			
AF352315.1	Strain H52	8	73.5	73.9	73.3	73.4	73.5	97.4	99.6		
	IB/017/MSL/15 (This study)	9	73.5	73.6	73.1	73.1	73.3	99.7	97.6	97.3	

Numbers indicate reference strains as follows: (1) IB-Wny-VSN275-2018; (2) 58HeN-93II; (3) THA300252; (4) YX10_D90; (5) QXIBV; (6) Massachusetts 41; (7) H120; (8) H52; (9) IB/017/MSL/15 (this study). Nucleotide sequence homologies were calculated based on aligned nucleotide sequences of the partial spike S1 gene. At the time of analysis, limited contemporary Indonesian IBV S1 sequences were available in GenBank; therefore, representative global reference strains were included for lineage assignment and comparative purposes.

Table 3: Physicochemical stability of the inactivated bivalent ND–IB vaccine assessed by pH and viscosity measurements during storage at different temperatures (4°C, 25°C, and 37°C) for 30 days.

Temperature stored	Parameter	Sampling (days)		
		10	20	30
4°C	pH	6.9	6.9	6.8
	Optical appearance	-	-	-
	viscosity (mPa-s)	44.9 ± 0.38	44.5 ± 0.77	44.4 ± 0.86
25°C	pH	6.6	6.4	6.4
	Optical appearance	-	-	-
	viscosity (mPa-s)	42.1 ± 0.25	38.9 ± 0.37	37.7 ± 0.46
37°C	pH	6.6	6.4	6.3
	Optical appearance	-	-	+
	viscosity (mPa-s)	28.3 ± 0.21	28.1 ± 0.23	27.4 ± 0.18

Physicochemical stability was defined based on quantitative measurements of pH and viscosity over time, with minimal variation indicating preservation of emulsion integrity. Viscosity values are expressed in millipascal-seconds (mPa·s), equivalent to centipoise (cP). Optical appearance was evaluated as a supportive qualitative parameter to detect gross phase separation or turbidity. "–" indicates no observable change in color, clarity, or phase separation; "+" indicates the presence of visible phase separation or slight turbidity.

Antigen and vaccine quality assessment

The ND and IB viruses produced in this study had titers of $10^{9.5}$ EID_{50}/mL and $10^{9.9}$ EID_{50}/mL, respectively. Inactivation assays conducted over three passages verified that both viruses were successfully inactivated. This was evidenced by the absence of embryo mortality due to ND infection and no abnormalities in embryos infected with the IB virus up to 96 hours post-inoculation. All vaccine preparations showed no bacterial contamination when tested for sterility using Thioglycolate (TGC) and Brain Heart Infusion (BHI) broth media.

The physicochemical stability of the inactivated bivalent ND–IB vaccine was quantitatively evaluated by monitoring pH and viscosity during storage at 4°C, 25°C, and 37°C for 30 days (Table 3). At 4°C, minimal changes were observed in pH (6.9–6.8) and viscosity (44.9–44.4 mPa·s), indicating preserved emulsion integrity. At 25°C, pH values remained relatively stable (6.6–6.4), while viscosity gradually decreased from 42.1 to 37.7 mPa·s. In contrast, storage at 37°C resulted in a more pronounced reduction in viscosity (28.3–27.4 mPa·s) accompanied by visible turbidity at day 30, suggesting temperature-dependent emulsion destabilization.

Vaccine safety

The safety assessment of the bivalent ND-IB vaccine showed that neither the vaccinated group (n= 25) nor the control group (n= 25) exhibited specific clinical signs of ND or IB infection. Safety observations over 21 days also revealed no abnormalities, either systemic reactions or localized effects at the injection site.

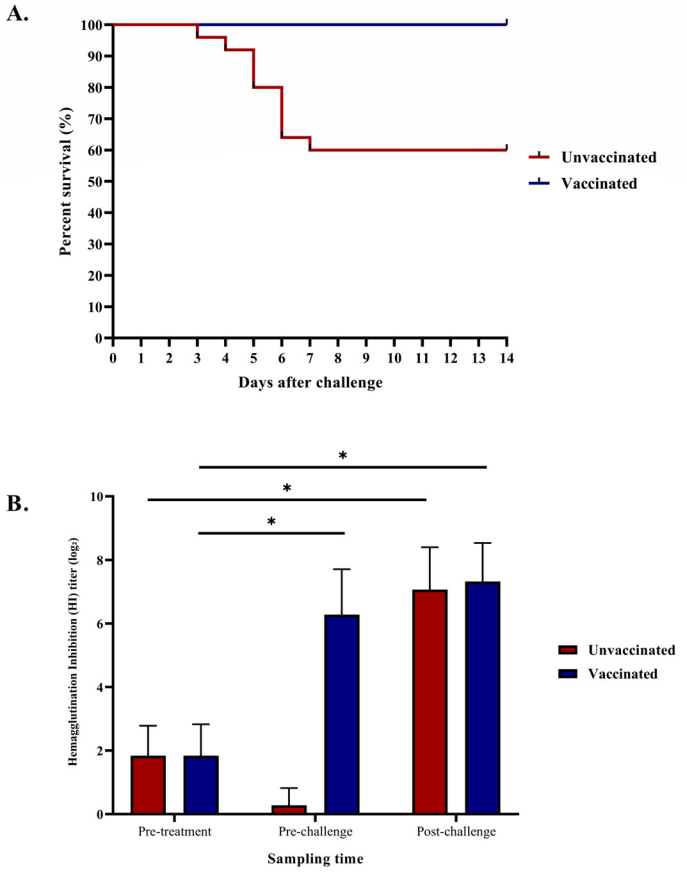

Figure 2: Survival curve of vaccination against Newcastle Disease Virus (NDV). (A) Kaplan–Meier survival curve showing 100% survival in vaccinated chickens, while unvaccinated chickens showed a marked decline in survival following challenge. (B) Hemagglutination Inhibition (HI) titer (log_2) of NDV antibodies at pre-treatment, pre-challenge, and post-challenge sampling times, with significantly higher titers observed in vaccinated chickens compared to unvaccinated controls (*p < 0.05). The geometric mean HI titer of the vaccinated group prior to challenge is indicated in the figure.

Vaccine efficacy against ND virus infection

Following a 14-days observation period post-challenge, only one chicken in the vaccinated group showed mild post-challenge symptoms such as ruffled feathers and diarrhea. However, no deaths occurred in this group throughout the observation period. In contrast, several chickens in the unvaccinated group exhibited clinical signs of ND infection, culminating in a total of 10 mortalities recorded by the end of the observation period (Figure 2A). Figure 2B presents the seroconversion analysis of all chickens, both vaccinated and unvaccinated, at pre-treatment, pre-challenge, and post-challenge stages.

Antibody response to IB virus

The bivalent ND-IB vaccine induced significantly higher antibody titers in the vaccinated group compared to the unvaccinated control group (p < 0.05). The vaccinated group exhibited a significantly higher mean S/P ratio than the unvaccinated group (p < 0.05), indicating enhanced humoral antibody responses following vaccination. In contrast, unvaccinated chickens remained negative with S/P ratios below the cut-off value (0.2), indicating absence of detectable IBV antibodies (Figure 3A). The vaccinated group exhibited a significantly higher mean neutralization index (NI) (4.60 ± 1.14 log_{10}) compared to the unvaccinated group (1.83 ± 0.76 log_{10}; p < 0.05). The distribution of individual NI test results is shown in Figure 3B. The relatively wide standard deviation of the neutralization index reflects individual variability in immune responses among vaccinated chickens, which is commonly observed following inactivated vaccination. Importantly, all vaccinated individuals exhibited neutralization indices higher than those of the unvaccinated controls, indicating the absence of true non-responders.

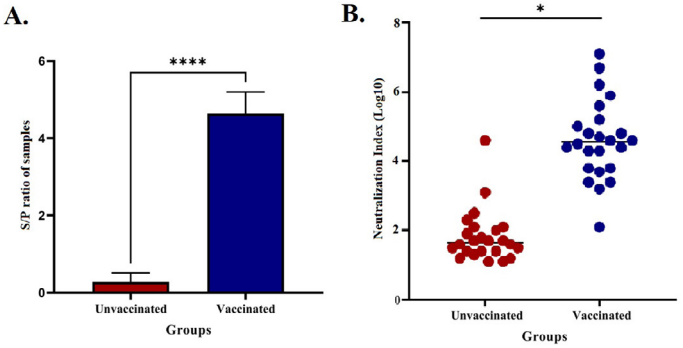

Figure 3: Antibody response in unvaccinated and vaccinated chickens. (A) The comparison of S/P ratios between unvaccinated and vaccinated groups, based on the conversion of ELISA group data to quantitative values according to the IDVet ELISA protocol. (B) Neutralization index (log10) was significantly higher in the vaccinated group compared with the unvaccinated group (*p < 0.05).

DISCUSSION

The present study demonstrated the successful development of an inactivated bivalent vaccine combining Newcastle disease (ND) and infectious bronchitis (IB) viruses of Indonesian origin. As demonstrated by the nucleotide homology analysis (Table 1), the NDV isolate used in this study shows close genetic relatedness to contemporary Indonesian field strains, underscoring its relevance to the current epidemiological situation in Indonesia. The molecular identification confirmed that the ND isolate belonged to genotype VIIh, clustering with contemporary Indonesian field strains, while the IB isolate clustered within the Mass-like lineage (GI-1). These findings are consistent with previous reports demonstrating that genotype VII of ND and Massachusetts-like IBV are the predominant circulating strains in Indonesia. The selection of locally circulating strains is essential for enhancing

vaccination effectiveness, particularly for ND, where antigenic divergence between vaccine and field strains can markedly reduce protective performance, while for IB it improves local relevance within established serotypes. By incorporating indigenous isolates, the formulated vaccine is anticipated to improve homologous immune recognition, particularly for ND, while providing a locally relevant immunogenic platform for IB that warrants further evaluation of protective performance under challenge condition. This approach corresponds with the strategy of tailoring vaccines to regional epidemiology, which has been emphasized in recent poultry health management research (Ike *et al*., 2021).

The antigen synthesis and inactivation steps in this study showed consistent outcomes, with viral titers reaching levels sufficient for vaccine formulation. Complete inactivation was verified through serial passages, ensuring safety and eliminating the risk of residual live virus. The physicochemical stability of the vaccine was maintained under refrigeration (4˚C) with no changes in pH, viscosity, or optical appearance for a period of 30 days. These results are comparable to other oil-adjuvanted inactivated vaccines documented in the literature, which typically exhibit significant stability under cold storage (Jaffar *et al*., 2019). In contrast, vaccines stored at higher temperatures showed decreased stability, emphasizing the necessity of preserving cold-chain storage. The reduction in viscosity observed at 25 ˚C and 37 ˚C indicates temperature-dependent thinning of the emulsion phase, which may impair antigen distribution if exposure is prolonged. Although no phase separation was observed up to 30 days at 25 ˚C, this finding reinforces the importance of maintaining storage within 2–8 ˚C to prevent loss of emulsion stability and ensure vaccine efficacy under field conditions. Stability is an essential consideration for field application, especially in tropical regions where cold-chain preservation may be challenging (Erassa *et al*., 2023). Therefore, the observed stability profile augments the practical value of this vaccine for Indonesian poultry farmers.

The sterility and safety tests further confirmed the quality of the formulated vaccine. No bacterial or fungal contamination was observed, demonstrating the efficacy of aseptic manufacturing protocols. Safety evaluations revealed no adverse systemic or localized effects in vaccinated chickens, even when administered at twice the recommended dose. These findings demonstrate that the formulation complies with international safety criteria for poultry vaccinations. The absence of vaccine-induced pathology is particularly important for commercial layer flocks, since vaccine-related stress may adversely affect egg production and overall flock performance (Nielsen *et al*., 2023). Moreover, the use of Montanide as an adjuvant likely contributed to both the safety and immunogenicity profiles, as this adjuvant is recognized for its ability to elicit robust immune responses while maintaining low reactogenicity.

This research provided compelling evidence of the vaccine's effectiveness against the ND virus challenge. Chickens immunized with the bivalent vaccine exhibited high levels of protection, with no mortality and only mild transient clinical signs in one individual. In contrast, the unvaccinated group experienced elevated morbidity and 40% mortality, confirming the pathogenicity of the challenge strain. These results highlight the vaccine's ability to provide robust protection against ND, which remains one of the most devastating diseases in the global poultry industry. The protective outcome correlates with the use of genotype VIIh strains, which match the circulating field isolates. This is consistent with previous studies indicating that homologous vaccines provide superior protection compared to heterologous vaccine strains (Mahamud *et al*., 2022; Sultan *et al*., 2020). In this study, the assessment of protective efficacy was focused on survival rate and clinical protection following NDV challenge. Other important parameters such as morbidity scoring and virus shedding were not evaluated. Future work will include quantitative analysis of virus shedding and transmission potential to further assess the vaccine's ability to reduce viral spread in vaccinated populations

In terms of IB immunogenicity, the vaccine markedly improved antibody responses in the vaccinated group. ELISA results demonstrated that the majority of vaccinated chickens attained elevated antibody titers, whereas the control group remained seronegative or exhibited low titers. Furthermore, the neutralization index (NI) of the vaccinated group was markedly higher compared to the controls, confirming the induction of functional virus-neutralizing antibodies. These results align with prior observations that oil-emulsified inactivated IB vaccines may elicit robust humoral immunity (Hassan *et al*., 2024). The variability observed in IBV neutralization indices reflects individual differences in humoral responsiveness and does not indicate the presence of non-responders, as all vaccinated chickens showed higher responses than controls. Importantly, the use of a Mass-like strain ensured antigenic relevance to a commonly circulating serotype in Indonesia and contributed to the induction of strong humoral immune responses. For infectious bronchitis virus, elevated systemic antibody titers primarily reflect humoral immunogenicity and do not necessarily correlate with protection against respiratory infection or viral shedding. However, protective efficacy against IBV infection cannot be conclusively determined in the absence of a challenge study. Despite IBV possessing multiple serotypes with limited cross-protection, selecting a Mass-like strain remains practical due to its extensive

use and compatibility with existing vaccination programs. However, this compatibility refers to ease of integration rather than a claim of broader cross-protection beyond the Mass lineage.

The combination of ND and IB antigens into a singular formulation offers several practical advantages for poultry health management. Bivalent vaccines diminish the need for multiple vaccinations, thereby lowering labor costs, minimizing handling stress, and enhancing overall farm efficiency (Wang *et al*., 2025). In resource-constrained regions, such as smallholder poultry farms in Indonesia, these advantages are particularly valuable. The reduced frequency of vaccination also decreases the risk of improper handling or administration, which may undermine vaccine efficacy. Furthermore, combined vaccines support integrated disease management strategies by targeting multiple pathogens simultaneously. This is consistent with global trends in poultry vaccine development, where combination products are increasingly adopted to optimize flock protection while minimizing production costs (Ravikumar *et al*., 2022).

This study's findings also highlight the importance of oil-based adjuvants in enhancing vaccine performance. Montanide adjuvants have been reported to prolong antigen release, stimulate both humoral and cell-mediated immunity, and improve antibody persistence (Veenstra *et al*., 2021). The observed antibody levels against IB and the protective responses against ND in this study support these mechanisms. Furthermore, oil-adjuvanted vaccines are recognized for providing a longer period of immunity compared to aqueous formulations. This feature is particularly beneficial for layer flocks, which require extended protection throughout their production cycle. Therefore, the adjuvant system used in this vaccine formulation represents a key factor contributing to its efficacy (Pérez *et al*., 2013).

A primary limitation of this study is the absence of an in vivo challenge experiment for infectious bronchitis virus (IBV). Although robust humoral immune responses were observed following vaccination, the actual level of protection against IBV infection, respiratory lesions, and viral shedding could not be directly evaluated. Previous studies have shown that antibody responses induced by inactivated IB vaccines do not always correlate fully with protective immunity; therefore, the present findings should be interpreted as evidence of strong serological immunogenicity rather than definitive proof of protective efficacy against IBV infection.

In addition, vaccine efficacy was evaluated under controlled experimental conditions, which may not fully reflect the complexity of field environments, where environmental stressors, concurrent infections, and variations in flock management can influence vaccine performance. Furthermore, while the Mass-like IBV strain used in this study elicited strong serological responses against homologous antigens, cross-protection against heterologous IBV serotypes, such as QX or 4/91, was not assessed. Future studies incorporating heterologous challenge experiments under field-relevant conditions will be essential to determine the breadth of protection and confirm the practical applicability of this vaccine across diverse poultry production systems.

From a broader perspective, the development of locally customized vaccinations represents an important step toward strengthening poultry health security in Indonesia. The endemic nature of ND and the widespread prevalence of IB pose continuous threats to poultry productivity and food security. By formulating vaccines derived from indigenous viral isolates, researchers can improve protection levels while reducing reliance on imported vaccines, which may not consistently align with local strains. This approach supports national self-sufficiency in vaccine production and coincides with the goals of sustainable poultry health management. Moreover, using local isolates may provide valuable genetic information for surveillance programs, enabling more responsive control measures against emerging variants.

CONCLUSION

This study successfully developed and evaluated a bivalent inactivated vaccine against ND and IB using Indonesian isolates. The vaccine demonstrated excellent safety, physicochemical stability, and immunogenicity, providing strong protection against ND following challenge and inducing significant serological antibody responses against IB, indicating strong immunogenicity. The integrated formulation offers practical advantages in poultry farming by reducing vaccination frequency and enhancing disease control efficiency. While further field evaluations, IB challenge studies, and cross-protection assessments are required, the current findings support the potential of this bivalent vaccine as a promising tool for ND control and as an immunogenic platform for IB under Indonesian field conditions. Its development contributes not only to improved poultry health but also to the sustainability and resilience of the national poultry industry.

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NOVELTY STATEMENT

This study presents the first development and evaluation of an inactivated bivalent vaccine against Newcastle disease (ND) and infectious bronchitis (IB) using Indonesian local isolates NDV genotype VIIh and IBV Mass-like lineage. The use of a locally circulating NDV genotype VIIh addresses the antigenic mismatch commonly associated with imported genotype II vaccine strains, thereby enhancing homologous antigenic compatibility under endemic conditions. For the IB component, a locally isolated Mass-like strain was selected to ensure field relevance within an established serotype and to evaluate its immunogenic performance in a bivalent inactivated vaccine formulation, without claiming superior cross-protection over existing Mass-based vaccines. Accordingly, the use of indigenous field isolates provides improved antigenic matching for ND and a locally relevant immunogenic platform for IB. The formulation, emulsified with Montanide oil-based adjuvant, demonstrated excellent safety, sterility, and physicochemical stability under cold storage, conferred high survival rates following virulent ND challenge, and induced significantly elevated antibody titers against IB.

AUTHOR'S CONTRIBUTION

Agustin Indrawati, Muhammad Ade Putra, Otto Sahat Martua Silaen, Desak Gede Budi Krisnamurti, Amin Soebandrio, Ryan Septa Kurnia, Christian Marco Hadi Nugroho were involved in conceptualizing the study, designing the experiments, gathering and analyzing the data, as well as drafting the manuscript. Agustin Indrawati and Christian Marco Hadi Nugroho also provided oversight during the research process and contributed to the critical revision of the manuscript. All authors reviewed and approved the final version of the manuscript for submission.

Ethic declaration

All procedures involving animals in this study adhered to ethical standards and received clearance from the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences, IPB University, Indonesia, under approval certificate number 232/KEH/SKE/VII/2024.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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